

The potential of bacteriophage
therapy in *Acinetobacter* spp.
infections

Alexandra Elisabeth Henein

A thesis submitted in partial fulfilment
of the requirements of
the University of Brighton for the
degree of Doctor of Philosophy.

Volume ONE

March 2009

University of Brighton

Declaration

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to this or any other university for a degree, and does not incorporate any material already submitted for a degree.

31st March 2009

Contents

Abstract	xxiii
List of Tables	xxiv
List of Figures	xxx
Acknowledgements	xxxiv
Abbreviations	xxxviii
Chapter ONE	1
Introduction	1
1.1 Functions and structure of human skin	1
1.1.1 Anatomy of normal skin	1
1.1.2 Host defence of intact human skin	3
1.1.3 Microflora	5
1.2 Burns	6
1.2.1 UK Burns statistics	6
1.2.2 Blond McIndoe Research Foundation	7
1.2.3 Quantification of Burn Injury	7
1.2.3.1 Total body surface area burn	8
1.2.3.2 Classification of burn depth	8
1.2.4 Prognosis	9
1.2.5 Pathophysiology of burn injuries	10
1.2.6 Histological and histochemical aspects of wound healing	11
1.2.6.1 Role of cytokines in wound healing	12
1.2.6.2 Modulation of wound healing	14
1.2.7 Management of burns wound of various depths	15
1.2.8 Burn wound cover and closure	16
1.2.8.1 Amniotic membrane	17

Contents

1.2.8.2 Autologous skin grafts	17
1.2.8.3 Human cadaver allografts and xenografts	18
1.2.8.4 Cultured human dermal keratinocyte sheets	18
1.2.8.5 Keratinocyte cell sheets on supports	19
1.2.8.6 Fibrin-glue and keratinocytes	20
1.2.8.7 Sprayed keratinocyte cell suspension	20
1.2.8.8 Skin substitutes	20
1.2.8.9 Combinations of fibroblasts and keratinocytes	22
1.3 Burn wound infection	23
1.3.1 Sources of burn wound contamination	23
1.3.2 Factors predisposing to microbial proliferation in burns	24
1.3.3 Significance of microorganisms present in the wound	25
1.3.4 Graft failure due to microbial contamination of the wound	26
1.3.5 Bacteria associated with wound infection	27
1.3.6 Burn wound infection management	29
1.3.6.1 Early closure of wounds	29
1.3.6.2 Topical antimicrobial agents	29
1.3.6.3 Regulatory molecules	29
1.3.6.4 Bacterial interference	30
1.3.6.5 Natural agents	30
1.3.6.6 Cytokines	30
1.3.6.7 Antibiotics	31
1.3.7 Pharmacokinetics of antibiotics in burn patients	31
1.4 Antibiotic resistance and resulting clinical problems	32
1.5 <i>Acinetobacter</i> species	32
1.5.1 Morphological, metabolic and cultural characteristics of <i>Acinetobacter</i> spp.	34
1.5.2 Growth temperatures	35
1.5.3 Growth media	35

Contents

1.5.4 Isolation from clinical samples/ Isolation of <i>Acinetobacter</i> spp. from various sources	36
1.5.5 <i>Acinetobacter</i> taxonomy	36
1.5.6 Mechanisms of resistance	39
1.5.6.1 β -lactam resistance	39
1.5.6.2 Imipenem (Carbapenem) resistance	39
1.5.6.3 Aminoglycoside resistance	40
1.5.6.4 Fluoroquinolone resistance	40
1.5.6.5 Phenotypic antimicrobial resistance patterns of <i>Acinetobacter</i> spp. reported by various centres	40
1.5.7 The role of <i>Acinetobacter</i> spp. in human disease	43
1.5.7.1 <i>Acinetobacter</i> spp. in wounds and burns	44
1.6 Alternatives to antibiotics	45
1.6.1 Reasons for a lack of new antibiotics	45
1.6.2 Photodynamic therapy (PDT)	46
1.6.3 Phytomedicines	47
1.6.4 Honey	48
1.6.5 Antibodies	48
1.6.6 Bacterial cell wall hydrolases (BCWH)	48
1.6.7 Antimicrobial peptides (AMP)	49
1.6.8 Bacteriophages	50
1.6.8.1 Phage display	51
1.6.8.2 Bacterial typing and detection	51
1.6.8.3 Phage-delivered medicinal substances	51
1.6.8.3.1 Vaccines	51
1.6.8.3.2 Antimicrobials	52
1.6.8.3.3 Genes	52
1.7 Bacteriophage therapy	52
1.7.1 History of phage therapy	53

1.7.2 Early Clinical application of phage therapy in scientific literature	53
1.7.3 Clinical applications of phage therapy	56
1.7.4 Animal experiments	57
1.7.5 Phage therapy in wounds- animal data	57
1.7.6 Phage therapy in wounds -human data	58
1.7.7 Safety of bacteriophage	61
1.7.8 Cytotoxicity testing	63
1.8 Bacteriophage isolation	63
1.9 Thesis Aim and Objectives	65
1.9.1 Aim	65
1.9.2 Objectives	65
Chapter TWO	67
Materials and Methods	67
2.1. Materials and Equipment	67
2.1.1 Chemicals and Microbiological Media	67
2.1.2 Equipment-Microbiology	68
2.1.3 Chemicals and Materials-Cell culture	69
2.1.3.1 Plasticware	71
2.1.4 Equipment- Cell culture	71
2.1.4.1 Microscopy	72
2.1.5 Molecular biology kits and bacterial identification kits	72
2.2 Bacterial strains and phage material	73
2.2.1 Clinical isolates of <i>Acinetobacter</i> species.	73
2.2.2 <i>Acinetobacter haemolyticus</i> (Leiden strain) and corresponding bacteriophage	74
2.2.3 Microbiological samples obtained from the Pasteur Institute, Paris.	75
2.2.4 Microbiological samples obtained from Laval University, Canada.	77
2.2.5 Long-term storage of <i>Acinetobacter</i> . spp	78
2.2.6 Routine subculture of <i>Acinetobacter</i> . spp	78
2.2.7 Routine culture of overnight suspensions of <i>Acinetobacter</i> spp.	78
2.3 Preparation of agar plates and overlay agar	79
2.3.1 Preparation of TSA plates for viable counts	79

Contents

2.3.2 Preparation of TSA plates for use with soft agar overlays (agar overlay method)	79
2.3.3 Soft agar	79
2.3.5 Selective Media	81
2.3.5.1 Leeds Acinetobacter Medium(LAM) (Jawad et al. 1994)	81
2.3.5.2 Leeds Acinetobacter Medium with varying antibiotic compositions	82
2.3.5.3 Modified Leeds Acinetobacter Medium (MLAM)	82
2.4 Identification methods for bacterial strains	83
2.4.1 Characterisation using microscopy	83
2.4.2 Motility	83
2.4.2 Gram staining	83
2.4.3 Oxidase test (including validation)	83
2.4.4 Catalase test	83
2.5 Viable count methods	84
2.6 Phage screening methods	84
2.6.1 Basic surface spotting method	85
2.6.2 Revised Surface spotting method	85
2.6.3 Soft agar spotting method	86
2.7 Production of crude filtered lysates	86
2.7.1 Liquid Lysate Method	86
2.7.2 Production of purified lysates	87
2.7.2.1 Standard protocol to purify bacteriophage	87
2.8 Phage enumeration method	88
2.8.1 Soft agar overlay method	88
2.9 Optical density measurements	89
2.9.1 Relating Optical density measurements to viable counts at 600nm (cells washed and suspended in PBS)	89
2.9.2 Relating optical density measurements to viable counts at 600nm (overnight suspension)	91

Chapter THREE	93
Identification and characterisation of nine clinical 'Sussex' <i>Acinetobacter</i> spp. isolates	93
3.1 Introduction	93
3.2 Materials and Methods	95
3.2.1. Storage and routine subculture of Sussex strains	95
3.2.2 Routine culture and growth of Sussex isolates	96
3.2.3 Characterisation of Sussex isolates using microscopy	96
3.2.4 Gram staining of Sussex isolates	96
3.2.5 Oxidase test	96
3.2.6 Catalase test	96
3.2.7. Identification of nine clinical Sussex strains using the API20NE system	96
3.2.8 Growth of 9 clinical Sussex isolates at 42 and 44°C	97
3.2.9 Growth curves at 37°C, using Bioscreen	97
3.3 Antibiotic susceptibility of nine Sussex isolates using the BSAC disc diffusion method	98
3.3.1 The British Society for Antimicrobial Chemotherapy (BSAC) Disc Diffusion Method for Antimicrobial Susceptibility Testing	98
3.3.1.1 Establishing ideal inoculum density- method optimisation	99
3.3.1.2 Preparation of inoculum	101
3.4 LAM agar and liquid medium validation experiments	102
3.4.1 Selective media for <i>Acinetobacter</i>	102
3.4.2 Growth characteristics of Sussex isolates on LAM	104
3.4.3 Growth characteristics of Sussex isolates on LAM with varying antibiotic compositions	105
3.4.4 Modifications to LAM	105
3.4.5 Growth characteristics of Sussex strains on MLAM	105
3.4.6 Liquid Leeds <i>Acinetobacter</i> medium validation experiment	106
3.4.7 Growth of clinical <i>Acinetobacter</i> isolates in LAM liquid	106

Contents

3.4.8 Growth of clinical <i>Acinetobacter</i> isolates with artificial background contamination in LAM liquid	108
3.4.8.1 Growth of <i>Acinetobacter</i> spp. in LAM liquid with and without background contamination found in waste water	108
3.4.8.2 Growth of waste water samples on TSA and LAM agar	109
3.4.8.3 Membrane filtration	109
3.5 Results	110
3.5.1 Characterisation of Sussex isolates using microscopy	110
3.5.2 Gram staining of Sussex isolates	110
3.5.3 Oxidase test	110
3.5.4 Catalase test	111
3.5.5 Identification of nine clinical Sussex strains using the API20NE system	111
3.5.6 Growth of 9 clinical Sussex isolates at 42 and 44°C	112
3.5.7 Growth curves at 37°C, using Bioscreen-C	114
3.5.8 Antibiotic susceptibility of Sussex isolates using the BSAC method	118
3.5.9 LAM agar and liquid medium validation experiments	122
3.5.10 Growth characteristics of Sussex isolates on LAM with varying antibiotic compositions	126
3.5.11 Growth of clinical <i>Acinetobacter</i> isolates in LAM liquid	127
3.5.12 Growth of <i>Acinetobacter</i> spp. in LAM liquid with and without background contamination found in waste water	128
3.6 Discussion	131
3.6.1 Identification of 9 clinical Sussex strains using the API20NE system and growth at various temperatures of 9 clinical Sussex isolates	131
3.6.2 Antibiotic susceptibility of 9 Sussex isolates using the BSAC disc diffusion method	132
3.6.3 Growth characteristics of Sussex isolates on LAM with varying antibiotic composition and MLAM	133
3.6.4. Growth of Sussex isolates in LAM liquid without background contamination	134
3.6.5 Growth of Sussex isolates in LAM liquid with background contamination	135

3.7 Conclusion	136
Chapter FOUR	138
Attempted isolation of <i>Acinetobacter</i> spp. and phage from sewage and waste water	138
4.1 Introduction	138
4.1.1 Specific isolation protocols for <i>Acinetobacter</i> phage	140
4.1.2 Aim	142
4.2 Material and Methods	143
4.2.1 Bacterial strains	143
4.2.2 QVH Waste water samples	143
4.2.3 Sewage samples from Poynings, Scaynes Hill and other collection sites	144
4.2.4 Isolation of <i>Acinetobacter</i> spp and phage from Queen Victoria Hospital waste water samples	146
4.2.4.1 Surface spotting of filtered supernatant liquid 'Direct plate selection'	146
4.2.4.2 Overlay method using filtered supernatant liquid 'Direct plate selection'	146
4.2.4.3 Propagation of bacteria found in unfiltered supernatant liquid and pellet	147
4.2.4.4 Checking for growth of wastewater samples on TSA and subculture onto LAM	148
4.2.5 Isolation of <i>Acinetobacter</i> spp and corresponding phage from sewage samples	148
4.2.6 Attempts at obtaining bacteriophage from other sources	149
4.2.6.1 Culture collections	149
4.2.6.2 Contact with individuals in the phage community	150
4.2.7 Material obtained from the Pasteur Institute, Paris.	154
4.2.7.1 Investigating viability of Pasteur isolates	155
4.2.7.2 Method optimisation for bacteriophage host screening using multiple application devices	155
4.2.7.3 Production of bacterial lawns for phage screening	155
4.2.7.4 Method optimisation using a multipoint inoculator for phage screening.	156
4.2.7.5 Method optimisation using a multipoint pipettor for phage screening	156
4.3 Results	158

Contents

4.3.1 Surface spotting of filtered supernatant liquid from burns unit (QVH) samples	158
4.3.2 Overlay method using filtered supernatant liquid-QVH	158
4.3.3 Bacteria found in unfiltered supernatant liquids and pellets-QVH	158
4.3.4 Checking for growth of wastewater samples on TSA and subculture onto LAM -QVH samples	159
4.3.5 Sewage samples on LAM agar	160
4.3.5.2 Controls	162
4.3.5.3 Testing of sewage isolates subcultured on TSA	162
4.3.5.4 Phage isolation from sewage samples	163
4.3.6 Viability of Pasteur isolates	163
4.3.7 Results of multipoint inoculator for phage screening	165
4.3.8 Results of multipoint pipettor used for phage screening	165
4.4 Discussion	165
4.4.1 Isolation of <i>Acinetobacter</i> spp. and phage for waste water and sewage samples	165
4.4.2 Phage isolation methods	167
4.4.3 Difficulties encountered obtaining <i>Acinetobacter</i> phage with matching host	171
4.4.4 Pasteur material phage screening	172
4.4.5 Lack of suitability of multipoint inoculator for phage screening	174
4.5 Conclusion	174
Chapter FIVE	175
Investigation of temperature dependent lytic relationship of host and phage HER1424 and HER424	175
5.1 Introduction	175
5.1.1 Genetic profile of AP205 (HER424)	176
5.2.1 Propagation on arrival and long-term storage	177
5.2.2 Routine subculture of <i>Acinetobacter</i> HER1424	178
5.2.3 Phenotypic properties, identification of HER1424 with API20NE test system, microscopic examination and digital imaging using a bright-field light microscope	178

Contents

5.2.4 Antibiotic susceptibility of HER1424 using BSAC disc diffusion method	179
5.2.5 Growth characteristics of HER1424 on Leeds Acinetobacter Medium (LAM)	179
5.2.6 Growth characteristics of HER1424 at 20, 25 and 30°C	180
5.2.7 Growth curves of HER1424 at 25, 30 and 37°C	180
5.2.8 Log phase experiment at 37°C, using a Bioscreen-C	181
5.2.9 Growth characteristics of HER1424 at 4°C ± 1°C	181
5.2.10 Propagation of phage HER424 using host HER1424	182
5.2.10.1 Production of CaCl ₂ solutions- Solution 1	182
5.2.10.2 Production of CaCl ₂ solutions- Solution 2	183
5.2.10.3 Production of CaCl ₂ solutions- Solution 3	183
5.2.10.4 Phage propagation attempt 1	183
5.2.10.5 Phage propagation attempt 2	184
5.2.11 Temperature shifts affecting plaque formation of HER1424 and HER424	185
5.2.12 Production of a primary lysate using HER1424 and HER424	185
5.2.13 Apparent relationship between temperature and ability to form plaques of HER1424 and HER424 using crude phage preparation AEH019(4/6/3)3	186
5.2.14 Production of purified phage suspension of HER1424 (secondary lysates)	187
5.2.15 Diagram to show propagation of phage HER1424 after receipt	189
5.2.16 Investigating viability, sensitivity to chloroform and quantitative titration of purified phage suspension	189
5.2.17 Validation of quantitative phage estimation using the soft agar overlay method	190
5.2.18 Determination of loss of phage after filtration with 0.45 m syringe filter	190
5.2.19 Method optimisation for optimum optical density of inocula to yield best bacterial lawns for plaque counts	191
5.2.20 Impact of heat on phage activity	191
5.2.21 Temperature dependent relationship between <i>Acinetobacter haemolyticus</i> HER1424 and phage AP205 (HER 424)	192
5.2.22 Temperature switches affecting ability to form plaques	192

Contents

5.2.23 Downshifting of temperature after infection	193
5.2.24 Attempts to artificially create temperate phage	194
5.2.25 Investigating the possibility of clonal differences between plaques	194
5.2.26 Adsorption experiments at 25°C and 37°C	196
5.2.27 Screening Sussex isolates as potential hosts for AP205 at various temperatures.	197
5.2.27.1 Screening Sussex isolates as potential hosts for AP205 using the surface spotting method	197
5.2.27.2 Screening Sussex isolates as potential hosts for AP205 using the soft agar overlay method	197
5.3 Results	199
5.3.1 Phenotypic properties, identification of HER1424 with API20NE test system, microscopic examination and digital imaging using a bright-field light microscope	199
5.3.2 Antibiotic susceptibility of HER1424 using BSAC disc diffusion method	200
5.3.3 Growth characteristics of HER1424 on Leeds Acinetobacter Medium (LAM)	202
5.3.4 Growth characteristics of HER1424 at 20, 25 and 30°C	203
5.3.5 Growth curves of HER1424 at 25, 30 and 37°C	203
5.3.6 Log phase experiment at 37°C, using a Bioscreen-C	205
5.3.7 Growth characteristics of HER1424 at 4°C +/- 1°C	206
5.3.8.1 Production of CaCl ₂ solutions- Solution 1	206
5.3.8.2 Production of CaCl ₂ solutions- Solution 2	206
5.3.8.3 Production of CaCl ₂ solutions- Solution 3	206
5.3.8.4 Phage propagation attempt 1	207
5.3.8.5 Phage propagation attempt 2	208
5.3.9 Temperature shifts affecting plaque formation of HER1424 and HER424	209
5.3.10 Apparent relationship between temperature and ability to form plaques of HER1424 and HER424 using crude phage preparation AEH019 (4/6/3)3	211
5.3.11 Investigating viability, sensitivity to chloroform and quantitative titration of purified phage suspension	215

Contents

5.3.12 Validation of quantitative phage estimation using the soft agar overlay method	217
5.3.13 Determination of loss of phage after filtration with 0.45 m syringe filter	221
5.3.14 Method optimisation for optimum optical density of inocula to yield best bacterial lawns for plaque counts	222
5.3.15 Impact of heat on phage activity	223
5.3.16 Temperature dependent relationship between <i>Acinetobacter haemolyticus</i> HER1424 and phage AP205 (HER 424)	223
5.3.17 Temperature switches affecting ability to form plaques	228
5.3.18 Downshifting of temperature after infection	229
5.3.19 Attempts to artificially create temperate phage	231
5.3.20 Investigating the possibility of clonal differences between plaques	233
5.3.21 Adsorption experiments at 25°C and 37°C	237
5.3.22 Screening Sussex isolates as potential hosts for AP205 using the surface spotting method	238
5.3.23 Screening Sussex isolates as potential hosts for AP205 using the soft agar overlay method	241
5.4 Discussion	242
5.5 Conclusion	253
Chapter SIX	258
Identification and characterization of Canadian bacteriophages and corresponding host bacteria	258
6.1 Introduction	258
6.2 Material and Methods	259
6.2.1 Propagation of host bacterial species	259
6.2.2 Long term storage of bacterial cultures	259
6.2.3 Routine culture and growth of bacterial cultures	259
6.2.4 Phenotypic properties of bacterial cultures	260
6.2.5 Identification of bacterial cultures using API20NE test system	260
6.2.6 Growth of bacterial cultures at 25, 30, 37, 42 and 44°C	260
6.2.7 Antibiotic susceptibility of bacterial cultures using the BSAC disc diffusion method	260

Contents

6.2.8 Growth characteristics of bacterial cultures on Leeds Acinetobacter Medium (LAM) with varying antibiotic compositions	261
6.2.9 Determination of host for each bacteriophage	262
6.2.9.1 Optimization of surface spotting method	262
6.2.9.2 Soft agar spotting method	263
6.2.9.3 Production of stock bacteriophages lysates	263
6.2.10 Selection criteria of suitable phage and host for further experiments	264
6.2.10.1 Reliable plaque formation	264
6.2.10.2 Crisp plaques seen at 37°C	264
6.2.10.3 Production of circular clearance zones	265
6.2.10.4 Formation of sufficient amounts of recoverable precipitate	265
6.2.10.5 Phage titre > 10 ¹⁰ pfu/ml	265
6.2.11 Analysis of infection cycle of phage HER1151 in host bacteria	266
6.3 Results	267
6.3.1 Phenotypic properties of bacterial cultures	267
6.3.2 Identification of bacterial cultures using API20NE test system	267
6.3.3 Growth of bacterial cultures at 25, 30, 37, 42 and 44°C	269
6.3.4 Antibiotic susceptibility of Canadian isolates using the BSAC disc diffusion method	271
6.3.5 Growth characteristics of Canadian isolates on Leeds Acinetobacter Medium (LAM) with varying antibiotic compositions	274
6.3.6 Determination of host for each bacteriophage	279
6.3.7 Selection criteria of suitable phage and host for further experiments	282
6.3.8 Analysis of infection cycle of phage HER1151 in host bacteria	284
6.4 Discussion	286
6.4.1 Phenotypic tests and identification	286
6.4.2 Antibiotic susceptibility of bacterial cultures	287
6.4.3 Determination of host for each bacteriophage	291
6.4.4 Comparison of host screening methods	293
6.4.5 Selection criteria of suitable phage and host for further experiments	294
6.5 Conclusion	298

Chapter SEVEN	301
Effects of phage on mammalian cells in vitro	301
7.1 Introduction	301
7.1.2 In vitro cultivation of cells and tissues (definitions)	302
7.1.3 Advantages of using cell culture compared to whole tissue or animals	303
7.1.4 Disadvantages of using cell culture compared to whole tissue or animals	304
7.1.5 Senescence, 'Normal cells' and transformed cell lines	305
7.1.6 Continuous cell line versus primary cell line	306
7.1.7 Embryonic Swiss Mouse Fibroblast cells (3T3 line)	306
7.1.8 Rationale for using cell culture	307
7.1.9 Cytotoxicity and viable cell quantification	307
7.1.10. Miniaturized colourimetric methods to determine cytotoxicity	307
7.1.10.1. Lactate dehydrogenase activity (LDH)	309
7.1.10.2 Assays using MTS tetrazolium compounds	309
7.1.11 Colony formation assay (clonal assay)	310
7.1.12 Cell mediators	310
7.2 Aims & Objectives	312
7.3 Material and Methods	312
7.3.1 3T3 Swiss mouse fibroblast cell line (3T3)	312
7.3.1.1 Standard growth vessels for 3T3 Swiss mouse fibroblast cells	312
7.3.1.2 Standard growth-medium for 3T3 Swiss mouse fibroblast cells	312
7.3.1.3 Standard incubation of 3T3 Swiss mouse fibroblast cells	313
7.3.1.4 Trypsinisation of 3T3 mouse cells grown in T25 flasks	313
7.3.1.5 Trypsinisation of 3T3 mouse cells grown in 24-well plates	313
7.3.1.6 Standard seeding and passaging of growth vessels	314
7.3.1.7 Counting of mammalian cells with Trypan Blue stain	314
7.3.1.8 Hoechst propidium iodide staining (HPI)	315
7.3.1.9 Cumulative population doublings of 3T3 Swiss fibroblast cell line in 24-well plates over 72 hours	315
7.3.1.10 Cumulative population doublings of 3T3 Swiss fibroblast cell line in T25 flasks over 96 hours	316

Contents

7.3.1.11 Validation of phage activity in tissue culture conditions without mammalian cells present	316
7.3.1.12 Validation of phage activity in tissue culture conditions with mammalian cells present	316
7.3.1.13 Cytotoxicity and cell proliferation assays for phage HER401 BS46 using a 3T3 mouse fibroblast cell line	317
7.3.1.13.1 Cytotoxicity assay using Trypan Blue and 3T3 cells	318
7.3.1.13.2 Cytotoxicity assay using HPI staining and 3T3 cells	319
7.3.1.13.3 Cytotoxicity assay measuring LDH release of 3T3 mouse fibroblast cells	319
7.3.1.13.4 Cell proliferation assay measuring MTS formation of 3T3 mouse fibroblast cells (I)	320
7.3.1.13.5 Cell proliferation assay measuring MTS formation of 3T3 mouse fibroblast cells (II)	321
7.3.1.13.6 Microscopy of 3T3 mouse fibroblast cells	322
7.3.2 Propagation of 3T3 Swiss mouse fibroblast cells for feeder layers	322
7.3.2.1 Standard growth-medium for 3T3 cells for feeder layers	323
7.3.2.2 Gamma irradiation of 3T3 Swiss mouse fibroblast cells for feeder layers	323
7.3.2.3 Standard growth medium for isolated keratinocytes	323
7.3.3 Isolation of epithelial cells from human biopsy-keratinocytes	324
7.3.4 Isolation of epithelial cells from human biopsy-fibroblasts	326
7.3.4.1 Human primary fibroblasts - transfer from Blond McIndoe Centre to the University of Brighton	327
7.3.4.2 Harvesting and freezing of human fibroblasts SKF276	328
7.3.4.3 Standard incubation of human fibroblasts SKF276	328
7.3.4.4 Standard growth vessels for human fibroblasts SKF276	329
7.3.4.5 Standard growth-medium for Fibroblasts SKF276	329
7.3.4.6 Seeding SKF276 cells from frozen stock	329
7.3.4.7 Trypsinizing of human fibroblasts SKF276 in T25 flasks	329
7.3.4.8 Trypsinizing of human fibroblasts SKF276 in T75 flasks	330
7.3.4.9 Trypsinizing of human fibroblasts SKF276 in 24-well plates	330

Contents

7.3.4.10 Mycoplasma screening of human fibroblasts SKF276	330
7.3.4.11 Doubling times of human primary fibroblasts SKF276 in 24-well plates	331
7.3.4.12 Cell proliferation assay of SKF276 cells (I)	331
7.3.4.12.1 MTS assay	332
7.3.4.13 Cytotoxicity assay using human fibroblasts SKF276 (I)	332
7.3.4.13.1 LDH method using SKF276 cells	333
7.3.5 Preparation of samples containing endotoxin	333
7.3.5.1 Bacterial cells disrupted using a sonicator	334
7.3.5.2 Bacterial cells disrupted by phage	334
7.3.5.3 Bacterial cells lysed by antibiotic	334
7.3.5.4 Bacterial cells disrupted using a Bead-Beater	335
7.3.5.5 Bacterial cells disrupted using an autoclave	336
7.3.5.6 Endotoxin test	336
7.3.5.7 Cytotoxicity assay using human fibroblasts SKF276 (II)	338
7.3.5.8 Correlation of cell numbers to MTS absorbance readings	339
7.3.5.9 Cell proliferation assay using SKF276 cells (II)	339
7.3.5.10 Cytokine release of human primary fibroblasts SKN276 challenged with phage	340
7.3.5.10.1 Wash buffer for cytokine experiments	340
7.3.5.10.2 Multi-well plates	340
7.3.5.10.3 Preparation of IL-1, IL-6, IL-8 and TNF- α standards	340
7.3.5.10.4 Sample treatment and dilution of SKN276 supernatant liquids for IL-1 assay- pilot experiment (I)	341
7.3.5.10.5 Cytokine assays for IL-1 for HDF (II)	341
7.3.5.10.6 Cytokine assays for IL-6, IL-8 and TNF- α for HDF	343
7.3.5.10.7 Sample treatment and dilution of SKN276 supernatant liquids for IL-8 assay- pilot experiment (I)	343
7.3.5.10.8 Sample treatment and dilution of SKN276 supernatant liquids for IL-8 assay (II)	344
7.3.5.10.9 Microscopy of SKN276 human fibroblast cells	344

Contents

7.3.6 Cytotoxicity assay for human keratinocytes SKN0106 with 3T3 mouse fibroblast feeder layers	344
7.3.6.1 LDH assay for human keratinocytes SKN0106 with 3T3 mouse fibroblast feeder layers	346
7.3.6.2 MTS assay for human keratinocytes SKN0106 with 3T3 mouse fibroblast feeder layers	346
7.3.7 Colony formation assay using V79 hamster cells	347
7.3.7.1 V79 hamster cells	348
7.3.7.2 Standard growth vessels for V79 hamster cells	348
7.3.7.3 Standard growth medium for V79 hamster cells	348
7.3.7.4 Standard seeding of V79 hamster cells	348
7.3.7.5 Standard incubation of V79 hamster cells	349
7.3.7.6 Trypsinisation of V79 hamster cells grown in T25 flasks	349
7.3.7.7 Colony formation assay to assess the cytotoxicity of phage and lambda buffer on V79 cells	349
7.3.7.8 Colony formation assay to assess the cytotoxicity of phage, diluents and cell extracts with varying endotoxin concentrations on V79 cells	351
7.4 Results	352
7.4.1 Cumulative population doublings of 3T3 Swiss fibroblast cell line	352
7.4.2 Determination of cumulative population doublings over 96 hours in T25 flasks	353
7.4.3 Validation of phage activity in tissue culture conditions without mammalian cells present	354
7.4.4 Validation of phage activity in tissue culture conditions with mammalian cells present	356
7.4.5 Cytotoxicity assay using Trypan Blue and 3T3 cells	360
7.4.6 Cytotoxicity assay using HPI staining and 3T3 cells	366
7.4.7 Cytotoxicity assay measuring LDH release of 3T3 mouse fibroblast cells	369
7.4.8 Cell proliferation assay measuring MTS formation of 3T3 mouse fibroblast cells (I)	371
7.4.9 Cell proliferation assay measuring MTS formation of 3T3 mouse fibroblast cells (II)	372

Contents

7.4.10 Microscopy of 3T3 mouse fibroblast cells	376
7.4.11 Mycoplasma screening of human fibroblasts SKF276	377
7.4.12 Population history of SKF276, after receipt from the Blond McIndoe centre	377
7.4.13 Doubling times of human primary fibroblasts SKF276 in 24-well plates	379
7.4.14 Cell proliferation assay of SKF276 cells (I)	379
7.4.15 Cytotoxicity assay using human fibroblasts SKF276 (I)	382
7.4.16 Viable counts for samples containing endotoxins	384
7.4.17 Multodisc clearance areas	384
7.4.18 Endotoxin levels of various cell extracts	385
7.4.19 Cytotoxicity assay using human fibroblasts SKF276 (II)	387
7.4.20 Correlation of cell numbers to MTS absorbance readings	390
7.4.21 Cell proliferation assay using SKF276 cells (II)	393
7.4.22 Cytokine assays for IL-1 fibroblasts SKF276	396
7.4.23 IL-6 release by fibroblasts SKF276-pilot experiment	397
7.4.24 IL-6 release by fibroblasts SKF276	397
7.4.25 IL-8 release by fibroblasts SKF276-pilot experiment (I)	399
7.4.26 IL-8 release by fibroblasts SKF276	400
7.4.27 TNF- α release by fibroblasts SKF276	402
7.4.28 Microscopy of SKN276 human fibroblast cells	402
7.4.29 LDH assay for human keratinocytes SKN0106 with 3T3 mouse fibroblast feeder layers	404
7.4.30 MTS assay for human keratinocytes SKN0106 with 3T3 mouse fibroblast feeder layers	406
7.4.31 Colony formation assay to assess the cytotoxicity of phage and lambda buffer on V79 cells	409
7.4.32 Colony formation assay to assess the cytotoxicity of phage, diluents and cell extracts with varying endotoxin concentrations on V79 cells	411
7.5 Discussion	419
7.5.1 Cytotoxicity tests	419
7.5.2 Cytotoxicity testing using 3T3 cells	420
7.5.2.1 Validation of phage activity in tissue culture conditions	421

7.5.2.2 Cytotoxicity assays using dyes	422
7.5.2.3 Cytotoxicity and cell proliferation assays (LDH and MTS assays)	423
7.5.3 Bacterial cell extracts with varying endotoxin concentration	424
7.5.4 Cytotoxicity testing using SKF276 human dermal fibroblasts	426
7.5.4.1 Cytotoxicity and cell proliferation assays (LDH and MTS assays)	427
7.5.6 Cytokine assays for IL-1 released from human dermal fibroblasts SKN276	428
7.5.7 Cytokine assays for IL-6 released from human dermal fibroblasts SKN276	429
7.5.8 Cytokine assays for IL-8 release from human dermal fibroblasts SKN276	430
7.5.9 Cytokine assays for TNF- α release from human dermal fibroblasts SKN276	430
7.5.10 Cytotoxicity testing using SKF276 human dermal keratinocytes	432
7.5.11 Colony formation assay using V79 hamster cells	433
7.6 Conclusion	436
Chapter EIGHT	439
General Discussion	439
8.1 Antibiotic resistance of Acinetobacter spp. strains	439
8.2 Identification, taxonomic status of Acinetobacter spp.	440
8.3 Selective media for the isolation of Acinetobacter spp.	441
8.4 Availability of Acinetobacter phage	442
8.5 Narrow host range	444
8.6 Resistance to bacteriophage	447
8.7 Phage cocktails	448
8.8 Restriction and modification	449
8.9 Phage behaviour in-vitro compared to in-vivo	450
8.10 Lytic nature of phages and transduction	450
8.11 Lack of interest of the pharmaceutical industry in phage	451
8.12 Regulatory agency approval	454
8.13 Intellectual property	455
8.14 Safety of phage- safety of phage preparations	455
8.15 Cytotoxicity- Nanotoxicity	457

Contents

8.16 Cytokine release	458
8.17 Immunogenicity	458
8.18 Ethics	459
8.19 Paradigm shifts	460
8.20 Absence of rigorous proof of efficacy	461
8.21 Conclusion	461
8.22 Suggestions for future work	462
Appendices	
Appendix 1	465
Appendix 2	467
References	472
Abstract & conference proceedings	523

The potential of bacteriophage therapy in *Acinetobacter* spp. infections

Bacteria resistant to multiple antibiotics pose a number of therapeutic problems. This situation is likely to worsen in the future, as the development of new classes of antibiotics has declined sharply in recent years. *Acinetobacter* species are an example of antibiotic-resistant bacterial pathogens with increasing clinical significance, particularly with respect to infections in high-risk patients such as those with severe burns. The treatment options for these patients are severely limited, and they often can only be treated with highly toxic antibiotics such as colistin. It is therefore evident that there is a need to investigate alternative approaches to therapy in these cases. This thesis represents some preliminary investigations into the use of bacteriophages for the treatment of *Acinetobacter* infections of severe burns patients.

Bacteriophages are viruses that have bacteria as their host. They cannot attack human cells and indeed they are highly specific for given species of bacteria. The aim of this thesis was to investigate the potential use of bacteriophages in these infections and their possible toxic effects on mammalian (including human skin) cells. Since bacteriophages lytic against *Acinetobacter* species were not available through conventional culture collections it was necessary to identify other sources. Clinical isolates of *Acinetobacter* species were obtained from Sussex hospitals and characterised. Several unsuccessful attempts were made to isolate corresponding bacteriophages from the environment and ultimately 10 isolates of *Acinetobacter* spp. and their corresponding phage were obtained from Laval University, Canada. These bacterial strains were identified and host matched to each phage using a refined screening technique and purified phage preparations were produced for those potentially therapeutic phages. The results of this study formed the basis for the selection of a small number of phage to take forward for cytotoxicity studies.

Very little scientific evidence has been published on the cytotoxic potential of bacteriophage or their bacterial lysis products, although phages have been routinely used for therapeutic purposes over many years. The establishment of phages as a mainstream treatment choice for bacterial infections in the West will require stringent testing and proof that phage preparations are not harmful.

Human dermal fibroblast (HDF) and keratinocytes (HDK) were isolated from human biopsies. A 3T3 mouse fibroblast cell line, HDF and HDK with 3T3 feeder layers were exposed to dilutions of purified phage. The cytotoxic impact and effect on cell proliferation was measured using a range of assays. No statistically significant difference could be detected between controls and phage with the Trypan blue method (3T3 cells). Hoechst propidium iodide stain experiments remained inconclusive (3T3 cells). Lactate dehydrogenase (LDH) and MTS tetrazolium compound reduction (MTS) results showed no evidence of statistically significant cytotoxic effect of phage on 3T3 cells, HDF or HDK. Some data suggested phage may have some beneficial effect on cell survival of HDK and proliferation of HDF and 3T3 cells compared to controls.

Endotoxin levels present in phage lysates and purified phage preparations were compared to those found in bacterial suspensions subjected to lysis by other methods including autoclaving, bead beating, sonication and high concentrations of antibiotics. There was no evidence to suggest that bacteria lysed by phage liberated significantly more endotoxins than the other methods of cell disruption.

A variety of endotoxin preparations and phage lysates were incubated with HDF and cytotoxicity together with cell proliferation were measured using LDH and MTS assays. Changes in interleukin levels of the same samples were monitored, measuring IL-1 β , IL-6, IL-8 and TNF- α levels. IL-1 β or TNF- α could not be detected in any samples. Highly concentrated phage gave rise to significantly higher IL-6 outputs than any other samples. Ten-fold dilutions of the same phage preparation were not statistically different to any other samples, including controls. This suggested components introduced during the phage purification process, rather than the phage itself may have contributed to higher IL-6 readings. Purified and crude phage lysate gave rise to significantly higher IL-8 outputs than all other samples.

Colony formation assays utilising a V79 hamster cell line were used to indicate the cytotoxicity of purified phage in different diluents and provided comparison with endotoxin containing preparations used in other experiments. There was no statistically significant difference in terms of colony numbers or colony size (where measured) between phage samples and controls.

This thesis has therefore demonstrated that because of the lack of cytotoxic effect of phage on mammalian cells in culture, there is potential for therapeutic applications of bacteriophage.

List of Tables

Chapter 1		Page
Table 1.1	Some commercially available skin substitutes	21
Table 1.2	Risk factors in burn wound infection	24
Table 1.3	Phenotypic properties used for the identification of the genus <i>Acinetobacter</i>	34
Table 1.4:	Different species of <i>Acinetobacter</i> suggested by various sources	37
Table 1.5	Resistance rates (%) for <i>Acinetobacter</i> from ICUs 1995 and 2000 after Friedland et al. (2003).	41
Table 1.6	Susceptibility data (%) for <i>Acinetobacter baumannii</i> after Quale et al. (2003)	42
Chapter 2		
Table 2.1	List of 10 strains donated by Surrey and Sussex NHS Trust	73
Table 2.2	<i>Acinetobacter haemolyticus</i> strain and phage donated by Leiden University	74
Table 2.3	Bacterial isolates obtained from the Pasteur Institute, Paris	75
Table 2.4	Bacteriophage samples obtained from the Pasteur Institute, Paris	76
Table 2.5	Bacteriophage obtained from the Pasteur Institute, Paris	77
Table 2.6	Microbiological samples obtained from Laval University, Canada	77
Chapter 3		
Table 3.1	Isolation history of Sussex strains, information as provided with samples	94
Table 3.2	Colour of phenol red over a range of pH values	107
Table 3.3	Phenotypic properties of Sussex isolates	111
Table 3.4	Identification of Sussex strains using the API20NE system	112

Table 3.5	Growth of Sussex isolates at 42 and 44°C	113
Table 3.6	Antibiotic susceptibility of nine Sussex isolates using BSAC disc diffusion method	120
Table 3.7	Growth of Sussex isolates on LAM with varying antibiotic composition	122
Table 3.8	Growth of control strains on LAM with varying antibiotic composition	123
Table 3.9	Growth of Sussex strains on MLAM	126
Table 3.10	Optical density reading OD ₆₀₀ of Sussex isolates with varying inoculums concentration in LAM	127
Table 3.11	Optical density reading OD ₆₀₀ of samples A and B with varying concentrations of <i>Acinetobacter</i> spp. inocula	129
Table 3.12	Appearance of colonies after membrane filtration of dilutions of samples A and B	130
Chapter 4		
Table 4.1	Sample origins and corresponding short codes	143
Table 4.2	Origin, short codes and sampling dates of sewage samples	145
Table 4.3	Surface spread unfiltered supernatant liquid, appearance on LAM and further tests	158
Table 4.4	Bacteria found in pellet, cultured on LAM	159
Table 4.5	Appearance of 20µl surface spots when subcultured on LAM, including further tests	160
Table 4.6	Growth of subcultured sewage isolates on LAM	161
Table 4.7	Results of Catalase and Oxidase test and Gram- stain	162
Table 4.8	Outcome of API20NE test for selected isolates	163
Table 4.9	Viability of Pasteur isolates	164
Table 4.10	Growth of viable strains at 30°C and 37°C, colony size (mm) when grown at 37°C	164

Chapter 5

Table 5.1	Phenotypic properties of HER1424	199
Table 5.2	Antibiotic susceptibility of HER1424 using BSAC disc diffusion method	201
Table 5.3	Growth characteristics of HER1424 at 20, 25 and 30 °C	203
Table 5.4	Doubling times of HER1424 grown in TSB at various temperatures	204
Table 5.5	Incubation temperature affecting plaque formation	209
Table 5.6	Results of soft agar overlay method	212
Table 5.7	Optical density of overnight cell suspensions grown at respective temperatures	212
Table 5.8	Results for surface spotting method at a range of incubation temperatures	213
Table 5.9	Average phage titres of AEH023 (23/7/3)	216
Table 5.10	Plaque counts dilution 1	217
Table 5.11	Plaque counts dilution 2	217
Table 5.12	Mean and standard deviation for dilution 1	218
Table 5.13	Mean and standard deviation for dilution 2	218
Table 5.14	Plaque counts before and after syringe filtration of phage lysates	221
Table 5.15	Correlation of optical density of cell suspension to average plaque size	222
Table 5.16	Plaque appearance and average phage titre at different temperatures	224
Table 5.17	Effect of temperature switches on plaque formation	229
Table 5.18	Optical densities of subcultures after overnight incubation	229
Table 5.19	Incubation temperature and plaque visibility of samples	230
Table 5.20	Parameters of adsorption experiments	238
Table 5.21	Screening of Sussex isolates with AP205 at 22°C	240
Table 5.22	Screening of Sussex isolates with AP205 at 25 °C	240
Table 5.23	Screening of Sussex isolates with AP205 at 30 °C	241

Table 5.24	Screening of Sussex isolates with AP205 at 37 °C	241
Table 5.25	Plates incubated at 25 °C	242
Table 5.26	Plates incubated at 37 °C	243
Chapter 6		
Table 6.1	Phenotypic properties of Canadian isolates	267
Table 6.2	24 and 48 hours API20NE profile of <i>Acinetobacter</i> spp. strains	268
Table 6.3	Table 6.3 Identity of <i>Acinetobacter</i> strains	269
Table 6.4	Growth of <i>Acinetobacter</i> strains at various incubation temperatures on TSA	270
Table 6.5	Growth of bacterial cultures at higher temperatures in TSB	271
Table 6.6	Antibiotic sensitivities of bacterial cultures	272
Table 6.7	Antibiotic sensitivities of Canadian isolates	273
Table 6.8	Growth of Canadian strains on LAM with varying antibiotic components	274
Table 6.9	Control strains on LAM agar with varying antibiotic components	275
Table 6.10	Correlating growth on LAM agar with <i>Acinetobacter</i> species	277
Table 6.11	Results of two phage screening methods of Canadian host and phage pairs	279
Table 6.12	Results of two screening methods of previously unmatched host and phage	280
Table 6.13	Meeting of selection criteria of five bacteriophages for future work	282
Chapter 7		
Table 7.1	Population doublings for 3T3 cells, 24-well plates	352
Table 7.2	Population doublings for 3T3 cells, T25 flaks	354
Table 7.3	Cell count 24hr-Baseline for Plate 1 (seeding density 5×10^3 cells per well)	361
Table 7.4	Cell counts for Control (seeding density (sd) 5×10^3 cells/well)	361
Table 7.5	Cell counts for phage HER401 BS46 dilution 1 (sd 5×10^3 cells/well)	362

Table 7.6	Cell counts for phage HER401 BS46 dilution 2 (sd 5×10^3 cells/well)	362
Table 7.7	Cell counts for phage HER401 BS46 dilution 3 (sd 5×10^3 cells/well)	363
Table 7.8	Cell count 24hr-Baseline for Plate 2 (sd 2×10^5 cells per well)	363
Table 7.9	Cell counts for Control (sd 2×10^5 cells/well)	364
Table 7.10	Cell counts for phage HER401 BS46 dilution 1 (sd 2×10^5 cells/well)	364
Table 7.11	Cell counts for phage HER401 BS46 dilution 2 (sd 2×10^5 cells/well)	365
Table 7.12	Cell counts for phage HER401 BS46 dilution 3 (sd 2×10^5 cells/well)	365
Table 7.13	Mean percentage viable cells (n=6x6) 24 hours after phage addition	366
Table 7.14	Mean percentage viable cells (n=6x6) 48 hours after phage addition	367
Table 7.15	Mean percentage viable cells (n=6x6) 72 hours after phage addition	367
Table 7.16	Average percentages cell death of cells exposed to phage compared with controls (N=6)	369
Table 7.17	Percentages of live cells after 24 hour incubation	375
Table 7.18	Percentages of live cells after 48 hour incubation	375
Table 7.19	Percentages of live cells after 72 hour incubation	376
Table 7.20	Population history of SKF276	377
Table 7.21	Cell count before freezing and calculation of doubling times in 12x T25 flasks	378
Table 7.22	SKF276 cells harvested from 24-well plates	379
Table 7.23	Baseline count for SKF276, Cell proliferation assay	379
Table 7.24	Average percentages cell death of cells exposed to phage, sonicated cells or lambda buffer compared with controls (N=6)	383

Table 7.25	Diameters of zones of inhibitions (cm) of HER1151 using Multodiscs	384
Table 7.26	Diameters of zones of inhibitions (cm) of HER1401 using Multodiscs	385
Table 7.27	Calculated endotoxin concentrations of various samples	386
Table 7.28	Average percentages cell death of cells exposed to phage, or cell extracts	389
Table 7.29	IL-6 readings of SKF276 pilot experiment (pg/ml)	397
Table 7.30	IL-6 results of HDF (pg/ml)	398
Table 7.31	IL-6 results of HDF (pg/ml)	399
Table 7.32	IL-8 release of HDF (pilot experiment; pg/ml)	400
Table 7.33	IL-8 readings experiment I (pg/ml)	401
Table 7.34	IL-8 readings experiment II	401
Table 7.35	Percentage cell death of Keratinocytes with 3T3 feeder layers	406
Table 7.36	Statistical differences of mean colony numbers in colony formation assays	413
Table 7.37	Statistical differences of mean colony numbers in colony formation assays	414
Table 7.38	Statistical differences of mean colony numbers in colony formation assays	415
Table 7.39	Statistical differences of mean colony numbers in colony formation assays	416
Table 7.40	Statistical differences of mean colony numbers in colony formation assays	417
Chapter 8		
Table 8.1	List of companies commercially dealing with phage products	452
Table 8.2	List of companies commercially dealing with phage products	453

List of Figures

Chapter 1		Page
Figure 1.1	Diagram of human skin	2
Chapter 2		
Figure 2.1	Absorbance at 600nm correlated with colony forming units/ml in PBS	90
Figure 2.2	Absorbance correlated with cfu/ml in an overnight suspension of <i>Acinetobacter</i> HER1423	91
Figure 2.3	Absorbance correlated with cfu/ml in an overnight suspension of <i>Acinetobacter</i> HER1401	92
Chapter 3		
Figure 3.1	Acceptable ranges for inoculum densities	100
Figure 3.2	An <i>Acinetobacter</i> strain (AP205) with ideal inoculum density	101
Figure 3.3	Picture of R2751 using a Leitz Wetzler microscope	110
Figure 3.4	Growth curves of R1973 at 37°C	114
Figure 3.5	Growth curves of W6108 at 37°C	114
Figure 3.6	Growth curves of R4474 at 37°C	115
Figure 3.7	Growth curves of R46383 at 37°C	115
Figure 3.8	Growth curves of W6492 at 37°C	116
Figure 3.9	Growth curves of R3417 at 37°C	116
Figure 3.10	Growth curves of R45502 at 37°C	117
Figure 3.11	Growth curves of W5211 at 37°C	117
Figure 3.12	Growth curves of R2751 at 37°C	118
Figure 3.13	<i>Citrobacter freundii</i> ATCC 8090 on LAM agar, LAM agar, TSA plate	124
Figure 3.14	Typical appearance of LAM plate	124

Chapter 5

Figure 5.1	HER1424 photographed through a bright-field light microscope	200
Figure 5.2	HER1424 photographed through a bright-field light microscope	200
Figure 5.3	Growth curves of HER1424 at different temperatures	204
Figure 5.4	Growth curve of HER1424 at 37°C	205
Figure 5.5	Sample A with areas of confluent plaques	210
Figure 5.6	Temperature dependent plaque formation of HER1424 and HER425	224
Figure 5.7	Typical appearance of heterogeneous plaques of AP205	234
Figure 5.8	Plaque size distributions of viruses from the largest plaque	234
Figure 5.9	Plaque size distributions of progeny of the smallest phage	235
Figure 5.10	Q-Q plot of plaque size of viruses from the largest plaque (ProgenyL), demonstrating data deviation from normality	235
Figure 5.11	Q-Q plot plaque sizes of viruses from the smallest plaque (ProgenyS), demonstrating data deviation from normality	230
Figure 5.12	Adsorption of phage HER424 to HER1424 at 25°C and 37°C	238

Chapter 6

Figure 6.1	Growth behaviour of selected Canadian <i>Acinetobacter</i> species on LAM	276
Figure 6.2	Three Canadian bacterial species alongside three control species on LAM	276
Figure 6.3	Single step growth of HER1156, 4 samples	285

Chapter 7

Figure 7.1	Phage particles recovered after incubation in DMEM or lambda in tissue culture conditions	355
Figure 7.2	Phage count after 24hrs of incubation in tissue culture conditions (Dilution 1)	357
Figure 7.3	Phage count after 48hrs of incubation in tissue culture conditions (Dilution 1)	357

Figure 7.4	Phage count after 72hrs of incubation in tissue culture conditions (Dilution 1)	358
Figure 7.5	Phage count after 24hrs of incubation in tissue culture conditions (Dilution 2)	359
Figure 7.6	Phage count after 48hrs of incubation in tissue culture conditions (Dilution 2)	359
Figure 7.7	Phage count after 72hrs of incubation in tissue culture conditions (Dilution 2)	360
Figure 7.8	Overall mean percentages cell survivors 24, 48 and 72 hours after phage addition	368
Figure 7.9	LDH readings of 3T3 cells treated with phage	370
Figure 7.10	MTS formation of 3T3 cells exposed to three phage dilutions	372
Figure 7.11	Comparison of MTS formation of 3T3 cells after 24h incubation	373
Figure 7.12	Comparison of MTS formation of 3T3 cells after 48h incubation	374
Figure 7.13	Comparison of MTS formation of 3T3 cells after 72h incubation	374
Figure 7.14	3T3 mouse fibroblast cells, estimated 45% confluence	376
Figure 7.15	MTS readings of cells incubated with phage for 24 hours	380
Figure 7.16	MTS readings of cells incubated with phage for 48 hours	381
Figure 7.17	MTS readings of cells incubated with phage for 72 hours	381
Figure 7.18	MTS readings of SKF276 cells incubated with phage	382
Figure 7.19	LDH reading of SKF276 cells treated with phage	383
Figure 7.21	Calibration curve correlating endotoxin concentrations to absorbance	385
Figure 7.22	SKF276 LDH reading after 24 hours	388
Figure 7.23	SKF276 LDH reading after 48 hours	388
Figure 7.24	SKF276 LDH reading after 72 hours	389
Figure 7.25	MTS Calibration plot for SKF276 cells	390
Figure 7.26	Viable cell numbers of SKF276 incubated with reagents for 24 hours	391

Figure 7.27	Viable cell numbers of SKF276 incubated with reagents for 48 hours	391
Figure 7.28	Viable cell numbers of SKF276 incubated with reagents for 72 hours	392
Figure 7.29	MTS assay (II) 24 hours	395
Figure 7.30	MTS assay (II) 48hours	395
Figure7.31	MTS assay (II) 72hours	396
Figure 7.32	SKN276- seeding density 10,000 cells/well medium only	402
Figure 7.33	SKN276- seeding density 10,000 cells/well exposed to phage	403
Figure 7.34	SKN276- seeding density 20,000 cells/well exposed to phage	403
Figure 7.35	SKN276- seeding density 20,000 cells/well medium only	404
Figure 7.36	LDH assay Keratinocytes with 3T3 feeder layers 24,48&72 hours	405
Figure 7.37	MTS assay Keratinocytes with 3T3 feeder layers 24 hours	407
Figure 7.38	MTS assay Keratinocytes with 3T3 feeder layers 48 hours	408
Figure 7.39	MTS assay Keratinocytes with 3T3 feeder layers 72 hours	408
Figure 7.40	Percentage reduction of colony numbers grown with lambda or phage compared to colony numbers in DMEM	410
Figure 7.41	Percentage reduction in colony numbers dependent on supplementation	411
Figure 7.42	Percentage reduction in colony numbers dependent on supplementation	412

Acknowledgements

A heartfelt thank you goes to Prof. Geoff Hanlon, who has continuously and patiently provided supervisory support throughout the entire project and was always there for me when needed.

I would like to thank Prof. Stephen Denyer and Dr Jean-Yves Maillard for their additional supervisory support.

Mr Christopher Morris and Mrs Chinzia Dedi for technical help. Mr Duncan Livingstone, Mrs Jane Allan and Mr Matthew Ilsey for providing clinical and technical input. Dr Elizabeth James for allowing me to undertake part of my cell culture work at the Blond McIndoe centre, Queen Victoria Hospital.

Cefsulodin was provided by Gruenthal GmbH (Aachen, Germany) as a free sample.

Particularly cordial thanks are extended to Dr Katrin Jennert-Burston for her life-changing friendship, unfaltering faith in my progress and intellectual support.

Ms Nabila Al-Lawati for the fits of laughter and introducing me to unknown parts of my cultural heritage. Dr Neil McNair for his unwavering support in the last few months of thesis preparation.

My family and friends scattered over the globe in Austria, Egypt, Germany, Sweden, USA and the UK, who maintained friendships, provided support, courage and helped me grow, they are too many to list.

Thanks to my parents, Dr Fawzi and Erika Henein who managed to raise me better than a pack of wolves, emphasised the importance of academic achievements from childhood, paid for my undergraduate training, continuously ensure my life does not get too bland despite the geographical distance and who are very dearly loved.

Abbreviations

Dulbecco's Modified Eagle's Medium	DMEM
Foetal calf serum	FCS
Hanks' Balanced Salt Solution	HBSS
Hoechst Propidium Iodide stain	HPI
Interleukin-1 α	IL-1 α
Interleukin-6	IL-6
Interleukin-8	IL-8
Isosensitest Agar	ISA
Isosensitest Broth	ISB
Leeds Acinetobacter Medium	LAM
Modified Leeds Acinetobacter Medium	MLAM
Penicillin and Streptomycin	Pen&Strep
Phosphate buffered saline	PBS
Recombinant Human Epidermal growth factor	EGF
Rheinwald Green medium	R&G
Tryptone Soya Agar	TSA
Tryptone Soya Broth	TSB
Tumor necrosis factor- alpha	TNF- α
Leeds Acinetobacter medium	LAM
American Type Culture Collection	ATCC
Dimethyl sulfoxide	DMSO
Ethylenediaminetetraacetic acid	EDTA
Cumulative population doublings	CPD
Human β -defensins	HBD
Nuclear factor- κ B	NF- κ B
Tumor necrosis factor- α	TNF- α
Interleukin	IL
Toll-like receptors	TLR

Skin associated lymphoid tissue	SALT
Immunoglobulin G	IgG
Total body surface area burn	TBSA
Polymorphonuclear leukocytes	PML
Transforming growth factor	TGF
Fibroblast growth factors	FGF
Tumor necrosis factor	TNF
Enteral nutrition	EN
Systemic inflammatory response syndrome	SIRS
Thyronine	T4
3,5,3'-tridothyramine	T3
Cultured epithelial autografts	CEA
Collagen-glycosaminoglycan matrix	C-GAG
Colony-forming unit	CFU
Polymerase chain reaction	PCR
Meticillin resistant Staphylococcus aureus	MRSA
Vancomycin resistant enterococci	VRE
Interferon	IFN
Glomerular filtration rate	GFR
Health Protection Agency	HPA
Multi-resistant Acinetobacter baumannii	MRAB
Deoxyribonucleic acid	DNA
Intensive care unit	ICU
National Committee for Clinical Laboratory Standards	NCCLS
Minimum inhibitory concentration	MIC
Human immunodeficiency virus	HIV
U.S. Food and Drug Administration	FDA
National Collection of Type Cultures	NCTC
Bacterial cell wall hydrolases	BCWH
Antimicrobial peptides	AMP
World Health Organization	WHO
Plaque forming units	PFU

Fotodynamic therapy	PDT
Generally Recognized as Safe	GRAS
Modified Leeds Acinetobacter medium	MLAM
Tryptone soya agar	TSA
Tryptone soya broth	TSB
Isosensitest agar	ISA
Isosensitest broth	ISB
Phosphate buffered saline	PBS
Hoechst propidium iodide stain	HPI
Dulbecco's Modified Eagle's Medium	DMEM
Foetal calf serum	FCS
Hanks' Balanced Salt Solution	HBSS
Lactate dehydrogenase	LDH
MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3 carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolium	MTS
Optical density	OD
Polyethylene glycol	PEG
tris[hydroxymethyl]aminomethane	Trizma
Queen Victoria Hospital	QVH
British Society for Antimicrobial Chemotherapy	BSAC
The Swedish Reference Group for Antibiotics	SRGA
Clinical and Laboratory Standards Institute	CLSI
Too Numerous To Count	TNTC:
Queen Victoria Hospital	QVH
Nutrient Broth	NB
Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH	DSMZ
American Type Culture Collection	ATCC
National Collection of Type Cultures	NCTC
Central Public Health Laboratory	PHLS

The National Collection of Industrial, Marine and Food
Bacteria

NCIMB

The United Kingdom National Culture Collection

UKNCC

Chapter ONE

Introduction

1.1 Functions and structure of human skin

Human skin is considered the largest organ in the body and forms approximately 8% of total body mass. Its thickness varies from 1.5-4.0mm, depending on location and specific function (Gray, 1995). Skin provides a barrier against microbial assault and the environment (Fakhruddin et al, 2004) and is involved in homeostasis and biochemical synthesis. Body temperature regulation by control of heat loss from the circulation by increase or decrease of blood flow and sweating is an essential property of skin (Gray, 1995). When the integrity of the skin is compromised such as in burns this function can be lost, due to damage to blood vessels and nerve endings leading to hypothermia. Skin also plays an important part in non-verbal communication and is part of human self awareness and identity (Gray, 1995). Even minor scarring due to injury, particularly in the facial area can have significant psychological effects on an individual (Tebble et al., 2005).

1.1.1 Anatomy of normal skin

Human skin consists of epidermis, dermis and a layer of subcutaneous tissue, which can be termed the hypodermis (**Figure 1.1**).

The outer surface of the skin, the epidermis is made up of several layers and a variety of cell types. The predominant type of cells found in the epidermis is keratinocytes, which are essential to wound healing and act as a barrier against environmental factors (Fakhruddin et al, 2004). Other cell types found in the epidermis are melanocytes, involved in pigment formation, sensory nerve endings and Langerhans cells, which are responsible for some immune functions. Langerhans cells are partly responsible for skin

graft rejection. Merkel cells are considered to be differentiated keratinocytes. It is conventional to divide the epidermis into strata, which are not strictly speaking partitioned layers, but should be thought of as areas through which cells migrate as they differentiate (Gray, 1995).

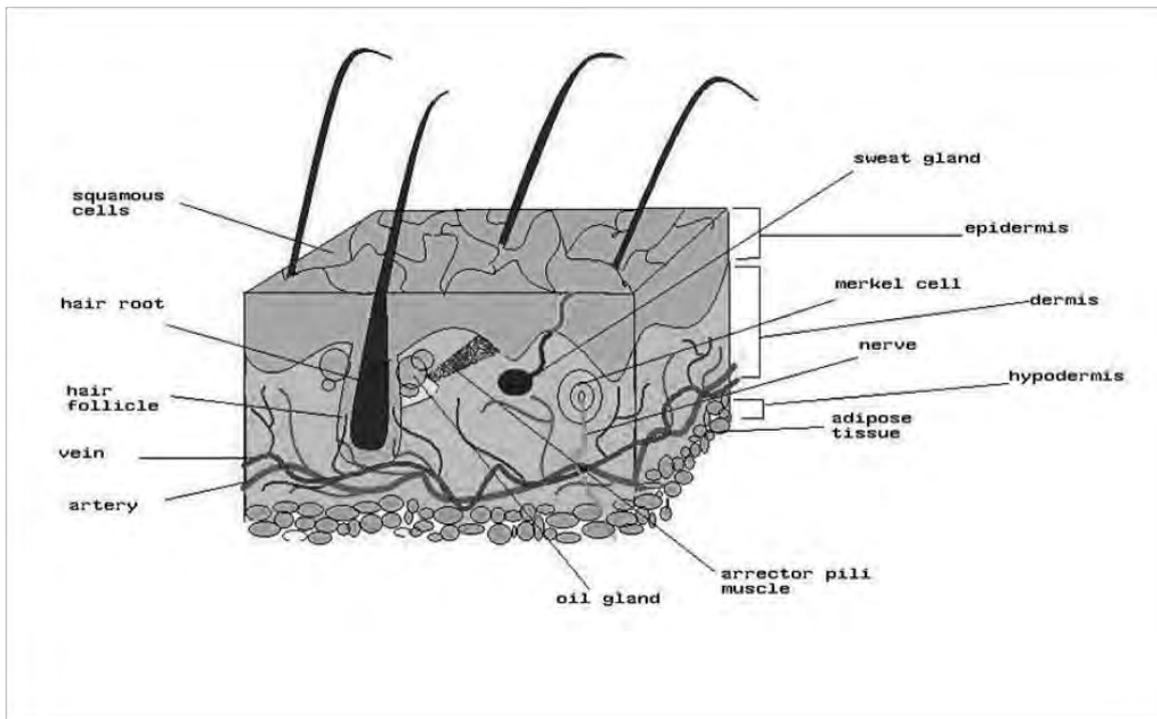


Figure 1.1 Diagram of human skin -Created by Crystal Mason and released under the GNU Free Documentation License

The healthy epidermis is a dynamic system undergoing continuous renewal to compensate for the amount of cells lost due to skin shedding. In the deepest layer, the stratum basale (basal cell layer), millions of skin cells are produced daily, which are pushed upwards by the dividing cells beneath them. As the cells pass through various strata to the surface, features such as shape and expression of signalling molecules change. The stratum corneum, as the top layer of the epidermis contains dead keratinized, squamous cells, their overlapping properties provide the water-proof character of human skin (Gray, 1995).

The dermis is an irregular soft connective tissue, which contains blood vessels, nerves, lymphatics, epidermal appendages and a variety of cells. It can be divided into two

zones: the thin superficial papillary layer and the deeper reticular layer, however there is no barrier between the two layers and one transitions gradually into the other.

The papillary layer's specialized functions include metabolic support, accommodating blood vessels, nerve endings and mechanical anchorage (Gray, 1995). This is important in the context of burns, as the extent of burns is still clinically assessed in part by skin sensitivity and bleeding which can be misleading (Atiyeh et al. 2005).

As the dermis accommodates sweat ducts and blood vessels, which are both involved in temperature control, damage to this structure leads to loss of control of this homeostatic feature. This problem is partly responsible for hypothermia encountered in severely burnt patients.

The dermis is essential to the maintenance of the epidermis, the variable amounts of adipose tissue, collagen and elastin fibres provides flexibility and tensile strength to the skin. By interweaving with collagen bundles elastic fibres form a mesh throughout the dermis (Gray, 1995).

Cells of the adult dermis can be divided into two categories: cells forming part of a structure such as blood vessels (smooth muscle cells, endothelial cells, pericytes) nerves (Schwann cells) and cells forming the erector pili muscles- and an ever changing population of cells of different origins and functions (Gray, 1995). Fibroblasts are the major cellular component of the dermis. Other cell types populating this tissue type are mast cells, macrophages, eosinophils, neutrophils and T- and B-lymphocytes (Gray, 1995).

1.1.2 Host defence of intact human skin

Historically the skin was mainly seen as a physical barrier to the environment (Allgower, 1995), however, currently it is seen as an endocrine organ and is recognized for its involvement in immune surveillance and immune response (Gray, 1995).

Undamaged skin is capable of producing a complex array of peptides and proteins with antimicrobial action which are part of the innate defence of skin. These agents include human β -defensins (HBD), human cathelicidin LL-37, lysozyme (muramidase),

RNase7, elafin, psoriasin, dermicin, adrenomedullin, secretory leukocyte protease inhibitor and neutrophil elastinase-associated lipocalin (Niyonsaba & Ogawa, 2005). Some of these natural antimicrobials have an innate affinity for lipopolysaccharides and displace divalent cations, which consequently disrupts the outer membrane of Gram-negative bacteria (Hancock, 1997). Other agents cause pore formation and change the permeability of bacterial cells. These agents often show synergy with one another and are capable of inducing several mechanisms of the inflammatory pathway and as a consequence an immune response to invading microorganisms (Niyonsaba & Ogawa, 2005).

Disruption of human skin host defence by burns may not only destroy the physical barrier the skin provides, but also destroy this natural antimicrobial shield, predisposing the patient to infection. It has been shown that Human β -defensin-2, a naturally occurring human antimicrobial agent commonly found in keratinocytes and epithelial cells (Niyonsaba & Ogawa, 2005), is undetectable in human burn blister fluid and lost in full thickness burns (Ortega et al., 2000), demonstrating how burns adversely affect innate immune mechanisms.

Other inherent defence mechanisms include the secretion of cytokines, particularly the nuclear factor- κ B (NF- κ B) in keratinocytes, which can in turn activate several cellular signalling pathways, responsible for regulating genetic expression for chemokines, cytokines, defensins, E-selectin and others (Kis et al., 2006). NF- κ B responses can be elicited by a variety of stimuli such as Tumor necrosis factor-alpha (TNF- α) and IL-1, T-cell activation signals, growth factors and stress inducers. In turn NF- κ B, which acts a transcription factor, is responsible for the regulation of TNF- α expression (Baldwin, 2001). Detection of pathogen-associated molecular patterns by keratinocytes through Toll-like receptors (TLRs), which are expressed on the cell surface, can activate the NF- κ B pathway. TLR2 and TLR4 are found in intact human epidermis *in vivo* and keratinocytes have been found to express both Toll-like receptors at the mRNA and protein levels *in vitro*. It was also found that expression of TLRs is dependent on detection of microbial components, such as lipopolysaccharides, peptidoglycans and mannans (Kis, 2006).

At a cellular level skin associated lymphoid tissue (SALT) consists of Langerhans cells, T-lymphocytes, keratinocytes, peripheral lymphatics and associated lymph nodes. Langerhans cells found throughout the epidermis are antigen-presenting cells, with a characteristic dendritic shape. They are non-keratinocytes and do not become keratinized. They carry receptors for the Fc portion of IgG, express a number of antigens and are involved in skin graft rejection from histo-incompatible animals. Interaction of keratinocytes within SALT has been documented (Gray, 1995) where it has been shown that via the production of cytokines keratinocytes can up- and down-regulate T-cells. Fibroblasts respond to cytokines and direct cell contact by immunocytes, hence this may in turn have an effect on wound healing and inflammation (Spörri et al., 1996).

1.1.3 Microflora

Exposed sites such as skin are colonised by a large number and variety of microorganisms, which are highly adapted to their particular environment, constituting the normal flora.

The majority of microorganisms found in the skin survive in the most superficial layers of the epidermis and the upper parts of the hair follicles. They consist largely of *Staphylococcus epidermidis*, *S. hominis*, *Micrococcus* sp. and corynebacteria (Chiller et al., 2001). Gram-negative bacteria like *Acinetobacter* spp. are also considered part of the normal skin flora (Taplin et al., 1963; Chiller et al., 2001; Hanlon, 2005), as well as the upper respiratory (Rosenthal & Tager, 1975) and gastrointestinal tract (Corbella et al., 1996).

Microbes may be components of the normal flora at certain anatomical sites, but are considered pathogens when isolated from other sites (Chiller et al., 2001). Some bacteria like *Acinetobacter* spp. can become problematic when the host becomes immuno-compromised, causing opportunistic nosocomial infections (Hanlon, 2005), which may be aggravated by strains being intrinsically or becoming antibiotic resistant (Wong et al., 2002; Hanlon, 2005).

1.2 Burns

In its worst form burn injury is perceived to be the most severe form of injury which can be survived. The term burn injury covers a large variation in cause and variability of injury and affects all age groups. A burn can be caused by thermal injuries, brought about by scalds, flames or flash thermal injuries. Chemical and electrical injuries may often have a small entry port, with only small areas of skin damaged, but with severe systemic effects and damage in deeper tissues. It includes large and severe sunburns, radiation injury and frostbite (National Burn Care Review Committee, 2002).

1.2.1 UK Burns statistics

Currently there is no national database operational in the UK which provides absolute numbers on the incidence rate and severity of burns. According to the National Burn Care Group, which is part of the NHS, a database is planned which integrates Hospital Episode statistics, the British Isles Burn Injury database and the National Burn Bed Bureau information to form the National Burn Injury Database (National Burn Care Review Committee, 2006).

Approximately 250,000 people suffer burn injuries in the UK each year. 175,000 seek medical help in Accident and Emergency Departments and 13,000 are admitted as inpatients to hospital, however not always automatically to a specialist burns unit. An estimated 1,000 burn patients require fluid resuscitation and 500 from this group are children less than 16 years. Despite the fact that the chances of survival have increased markedly over the last 25 years, an estimated 300 deaths per year are attributed to burns in the UK (National Burn Care Review Committee, 2001).

Since the National Burn Care Review (2001) recommended stratification of services (Judkins, 2000), major burn cases are managed nationally by a few specialist Burns Centres, one of which is the Blond McIndoe Burns Centre at the Queen Victoria Hospital, East Grinstead. It accepts patients from a 3.5 million person catchment area, with its own admission rate of patients requiring fluid resuscitation being reflected in national statistics (Ashworth et al., 2001). In a single year adults with 15% burns and

children with 10% were included, of which 12 adults and two children suffered flame burns; two adults endured flash injury.

One adult suffered contact burns while 5 adults and 8 children suffered scalding. 13 of the 31 patients included in the study died, of which three were elderly with scalds of 25% body surface area or less (Ashworth et al., 2001). The complexity of a serious burn requires multidisciplinary specialist input preferably in a specialist burn unit (Judkins, 2000).

90% of burn injuries are considered preventable (Hettiaratchy & Dziewulski, 2004 II).

1.2.2 Blond McIndoe Research Foundation

This medical research charitable trust is associated with the burns unit at the Queen Victoria Hospital, East Grinstead allowing clinical practice and research to be integrated. A wound healing group focuses on both pre-clinical and clinical studies-with patients receiving cultured keratinocytes.

Plastic Surgery Consultant Sir Archibald McIndoe conducted groundbreaking burns reconstruction on burnt airmen during WWII, who dubbed themselves „The Guinea Pig Club“, the results of his work were the foundations of the Blond McIndoe Research centre (Meikle, 2006).

1.2.3 Quantification of Burn Injury

Accurate and early assessment of burn wounds is essential for providing adequate specialist care. Two main parameters affect the choice of therapy:

- Total body surface area burn (TBSA), which is important in the initial assessment as the percentage TBSA (%TBSA) is needed to calculate fluid and nutritional requirements for the individual patient.
- Burn depth (superficial dermal, deep dermal or full thickness) determines whether drastic surgical intervention becomes necessary (Atiyeh et al., 2005).

1.2.3.1 Total body surface area burn

Different methods exist to calculate TBSA, which are not equally applicable to adults and children (Atiyeh et al., 2005). It is generally agreed that assessments of burn areas are often done badly, even by specialist staff (Ashworth et al., 2001; Hettiaratchy & Papini, 2004)

Simple erythema should not be included in the calculation, however it is often impossible to establish where the burn ends and reddening starts, hence an initial overestimation of the burned surface before subsidence of erythema is common (Hettiaratchy & Papini, 2004). The Lund and Bowder method allows the most accurate calculation of % TBSA burn, if used correctly (Hettiaratchy & Papini, 2004).

This method assigns a number, reflecting a percentage of total surface area, to each body part. It is used at the Blond McIndoe Burns Centre, Queen Victoria Hospital.

1.2.3.2 Classification of burn depth

Superficial burns require less attention and generally heal spontaneously, however deep burns require extensive surgical excision and grafting (Atiyeh et al., 2005).

Various methods can be used to assess burn wound depth, including digital imaging, biopsies, measurement of tissue perfusion, photo-optical measurements, however clinical evaluation on its own remains unreliable (Ashworth et al., 2001; La Hei, 2006). Classifications of first, second and third degree burns have been replaced with superficial, superficial partial thickness, deep partial thickness burns and full thickness burns (Morgan et al., 2000).

In **superficial (epidermal) burns** only the epidermis is affected, as typified by sunburn and these generally only require supportive therapy including analgesia and dressing. Only if a large TBSA is affected will intravenous fluids be needed in addition. Healing is generally spontaneous and rapid, within a week undamaged keratinocytes regenerate the wound, without intervention (Papini, 2004).

Superficial partial thickness burns arise when the upper layers of the dermis and epidermis are affected. Due to superficial nerves being exposed within the tissues but the neural network remaining functional, these types of burns can be painful. Healing of this type of burn is expected to take approximately two weeks, with the cells responsible for wound closure being mainly epidermal cells, keratinocytes within hair follicles and sweat glands. Hairy (scalp) or thick skin generally heals faster than hairless skin such as eyelids (Papini, 2004).

Deep partial thickness burns occur when the epidermis and the majority of dermis are destroyed. This type of burn can present a challenge in terms of treatment and diagnosis as this type of injury often appears to be superficial. Some partial thickness burns will heal without surgical intervention provided the wound is kept moist, warm and free from infection. If wounds of this type are left to heal by themselves the healing process is slow and regularly leads to wound contraction which can impair function or be aesthetically unpleasant. If burns areas are large or in functionally or cosmetically critical areas the best practice is to surgically excise the eschar and apply a skin graft (Papini, 2004).

In **full thickness burns** all cell layers with regeneration potential are obliterated. Endogenous healing can only occur from the wound edges and considerable contraction to the point of loss of function can be expected. All injuries larger than 1cm in diameter need to be excised and grafted (Papini, 2004).

1.2.4 Prognosis

Chances of survival for burns victims in the western world have increased dramatically since WWII. In 1940 only half of patients with a 30% or above TBSA survived (Rivara, 2006).

In a US retrospective study investigating paediatric burn survival no child died with 40%-50% TBSA between 1991 and 1997. Deaths occurred only once TBSA reached 60% or above and affected 14.3% of the children included in the study (Sheridan et al., 2000). A similar study conducted on children and adults admitted from 1990 to 1996

with a mean of 14-20 % TBSA showed that 96% lived to be discharged (Ryan et al., 1998). 50% of adult patients with 80% TBSA burns are likely to survive (Saffle, 1998).

Three risk factors for death caused by burns injury are: inhalation injury, age above 60 years and more than 40% TBSA. Depending on whether zero, one, two or three risk factors are present the chances of survival can be estimated to be 90, 33, 3 and approximately 0.3% respectively (Ryan et al., 1998).

Elderly patients as a particularly vulnerable group have decreased chances of survival after suffering a burn injury but exactly how all the risk factors interrelate is unclear (Hettiaratchy & Dziewulski, 2006). Aggravating factors such as psychiatric or underlying medical illness, alcoholism and drug abuse can compromise the victim's health and chances of surviving a burn (Ali et al., 2006; Hettiaratchy & Dziewulski, 2006). Once the patient has been stabilised the acquisition of multiply resistant strains of pathogenic bacteria in burns wounds has been statistically correlated with mortality (Wong et al., 2002).

1.2.5 Pathophysiology of burn injuries

Severe burn injury is a potentially fatal insult on the human body, as most homeostatic responses are unable to cope, leading to ultimate system shutdown without medical intervention. Thermal injury causes obvious cell death, at temperatures above 45°C it is progressive, once it reaches 60°C it is instantaneous. As the heat is conducted into the surrounding tissues sub-lethal inflammatory injury occurs on a cellular level (Ayers & Kay, 2005). As a response to a burn the body can enter a hypermetabolic state, operating at up to three times its original metabolic rate resulting in a multitude of complications including poor wound healing (Hettiaratchy & Dziewulski, 2004; Pereira et al., 2005).

The very same inflammatory mediators which are essential to wound healing and cell migration to support wound healing can cause additional tissue damage, catabolism and immune suppression (Drost & Burlison, 1993). Increased capillary permeability and loss of intravascular fluid are the consequence of this inflammatory process taking place (Ayers & Kay, 2005). Inflammatory mediators cause bronchoconstriction, which can

lead to acute respiratory distress syndrome (Hettiaratchy & Dziewulski, 2004). Myocardial contractility is decreased, possibly due to release of TNF- α . As the permeability of the capillaries is increased intravascular proteins are lost, which changes osmolarity and in turn causes intravascular fluid loss. As a homeostatic mechanism peripheral and splanchnic vasoconstriction occurs.

The combination of these changes can lead to systemic hypotension and underperfusion of end organs (Hettiaratchy & Dziewulski, 2004).

In addition a non-specific down regulation of humoral and cell mediated immune function occurs (Hettiaratchy & Dziewulski, 2004). Once a TBSA of 15% in adults and 10% in children is burnt any compensatory mechanism can no longer cope and fluid resuscitation needs to be started to avoid the patient going into hypovolaemic shock (Ayers & Kay, 2005). Once the injuries affect 25-30% TBSA, the amount of inflammatory mediators released is so great that progressively a systemic inflammatory response syndrome (SIRS) can occur, which can be further aggravated by toxins released from the burn wound (Ayers & Kay, 2005).

1.2.6 Histological and histochemical aspects of wound healing

A healing burn wound is a complex physiological process, including re-growth of different tissue types and revascularisation. The healing process in human burns wounds in vivo is dependent on many variables including depth and area of burn, cause of burn (chemical, electrical, heat source) which in turn affects the nature of the burn, nutritional status of the patient, circulation and age, surgical procedures undertaken, wound management procedures and infections

One of the first responses to wounding is an increase in blood flow, caused by regional vasodilatation (Frantz et al., 2005). As a temporary means of repair a temporary clot forms which has a protective function as an intermediate barrier and provides a scaffold for cell migration (Clark, 1996). The clot is a mixture of platelets embedded in a matrix of fibrin fibres with small quantities of plasma fibronectin, thrombospondin and vitronectin. It also contains growth factors and cytokines, which are slowly released as the activated platelets degranulate and play an essential part in the chemotactic process

of wound healing (Clark, 1996). Other cell types are recruited to the wound site by various signals (Clark, 1996). Monocytes and neutrophils migrate to the wound in response to growth factors released by the degranulating platelets and formyl methionyl peptides cleaved from bacterial proteins and other chemicals released by the degradation of matrix components (Clark, 2006). Macrophages and polymorphonuclear leukocytes (PML) are the most important sources of pro-inflammatory cytokines in the wound healing process. PML are primarily associated with host defence against bacteria and parasites. They are heavily involved in signalling for the proliferative process of healing by signalling to nearby fibroblast and keratinocytes. They may play an essential role in the transition between inflammation and wound repair (Hübner et al., 1996). In addition to their signalling function macrophages remove obsolete neutrophils, bacterial, human cell and matrix debris by phagocytosis. Absence of macrophages at the wound site has been proven to cause severe impairment in wound healing in guinea pig models (Leibovich & Ross, 1975). Activated macrophages similarly to neutrophils release a variety of signalling molecules including transforming growth factor (TGF)- α & β 1-3, Fibroblast growth factors (FGFs) 1, 2 and 4.

1.2.6.1 Role of cytokines in wound healing

Cytokines are often referred to as growth factors and are essential to inter- and intracellular communication in wound healing. Investigating actions and control of their action is an essential part of wound research and understanding their functions is far from complete, including difficulties in determining which cytokines are secreted during wound healing (Ono et al., 1995).

Human burn blister fluid contains cytokines with measurable levels of IL-1 α , IL-1 β , IL-6 and IL-8, with evidence that when 1% blister fluid added to keratinocytes *in vitro*, growth was significantly stimulated (Ono et al., 1995).

The pro-inflammatory cytokines IL-1 α , IL-1 β and IL-6 and Tumor necrosis factor (TNF)- α are regarded as playing an important role in wound repair, with particular functions of keratinocyte stimulation, fibroblast proliferation and chemotaxis and immune response (Werner & Grose, 2003). The coordinated expression *in vivo* of these

cytokines is believed to be essential for wound repair (Ono et al., 1995). Expression of these cytokines was largely impaired in healing-impaired glucocorticoid treated mice (Hübner et al., 1996), and expression of IL-1 β and TNF- α was unusually prolonged in genetically diabetic *db/db* mice with severe healing deficits, indicating a prolonged inflammatory phase (Wetzler et al., 2000).

IL-6 is believed to have a very important role in acceleration of keratinocyte growth, showing mitogenic effects on wound edge keratinocytes and chemotactic effects on neutrophils in mouse models.

IL-6 knock-out mice showed severe inhibition in wound healing which could only be remedied with administration of recombinant murine IL-6 protein (Lin et al., 2003).

The role of TNF-Rp55 for TNF- α expression on non- haematopoietic cells types was elucidated with a knock-out mouse model. Mice with excisional wounds lacking TNF-Rp55 expression showed enhanced angiogenesis, re-epithelialisation and collagen content with reduction of mRNA expression of cytokines. This implies that non-expression of TNF- α resulted in accelerated healing of wounds and promotion of reepithelialisation.

IL-8 elicited responses in keratinocytes and endothelial cells, by stimulating human keratinocyte migration and proliferation. There is evidence that interaction of keratinocytes CXCR2 with their ligands, IL-8 in humans has an important role in wound healing. Topical application of IL-8 to mouse wounds has been shown to have favourable effects (Rennekampf et al., 1997, 2000). *In vitro* IL-8 release in human fibroblasts was shown to be stimulated by IL-1 α and TNF- α (Sticherling et al., 1993).

Some commercially available wound cover products contain cytokines, which supposedly aid wound healing (Pruitt & Levine, 1984; Papini, 2004; Clark et al., 2007), such as Orcel (Ortec, Ortec International, Inc., New York, NY) or Transcyte, (Smith & Nephew UK Limited, London).

1.2.6.2 Modulation of wound healing

The insult of a severe burns injury leads to hormonal and metabolic derangements, leading to hypermetabolism, progressive weight loss, muscle wasting (Pereira et al., 2005), poor wound healing, and an impaired immune system which increases the susceptibility to infections. These changes can directly affect wound healing and can be partly influenced by nutritional, pharmacological and environmental means.

When the burn size is greater than 20-30% TBSA the metabolic rate can be increased to up to 150% of normal levels (Muller et al., 2001) and this can further increase when wound sepsis occurs. Hypermetabolism can be affected by patient-controlled temperature of air supply or environment and adequate nutritional support. Weight loss of 30% has been reported in patients with moderate sized burns.

Once more than 20% of pre-burn weight is lost mortality rates are higher and wound healing is compromised. Historically, parenteral nutrition was the favoured choice of meeting patients' increased calorific needs (Muller et al., 2001). However trauma patients are at higher risk of developing sepsis when receiving total parenteral nutrition, partly due to higher plasma glucose levels than patients fed with enteral nutrition (EN) (Jeejeebhoy, 2001).

EN is currently favoured due to safety considerations. Theories based on animal models that EN is less likely to cause mucosal atrophy and thereby limits bacterial translocation are not proven in clinical human studies (Jeejeebhoy, 2001). Enteral feeds containing approximately 20% protein, 50% carbohydrate and low levels of linoleic fat will help prevent weight loss and in turn support wound healing. Linoleic fat in enteral feeds has been associated with wound infection and increased hospital stay. After burn injury, as is found with other critical illnesses sick, euthyroid syndrome can be seen. Clinically, free thyronine (T4) and free 3,5,3'-tridothyramine (T3) serum levels are abnormally low, whilst reverse T3, an inactive metabolite increases. Clinically thyroxine therapy does not affect hypermetabolism or affect mortality, however it may affect wound healing of a burn by limiting unsightly scars. In animal studies on guinea-pigs with thyroidectomy, intermediate doses of thyroxine improved burn wound healing.

1.2.7 Management of burns wound of various depths

Traditionally burn wound management consisted of covering the wound with antimicrobial agents and applying dressings, until the eschar separated from the wound bed 3-5 weeks after the burns injury (Ong et al., 2006). Eschar is an expression for the necrotic burnt tissue left after a burns injury, which is unperfused hence prevents the migration of immune cells and the distribution of systemically administered antibiotics. It is a moist, protein rich environment and consequently is the perfect breeding ground for microorganisms. The eschar separation from the wound bed is mainly caused by the digestion of burnt tissue by proteolytic enzymes released by pathogens, particularly pseudomonads and β -haemolytic streptococci (Nguyen et al., 1996). Unsurprisingly, those patients suffering major burns treated in the traditional way were more likely to die from sepsis, due to an overwhelming release of inflammatory mediators leading to systemic inflammatory response syndrome (SIRS) (Ong et al., 2006). In 1970 Janzekovic demonstrated that an excision technique removing the superficial necrotic burnt tissue while preserving the underlying viable tissue was a major improvement compared to traditional burn management (Janzekovic, 1970). A recent meta-analysis demonstrated that early excision of burn eschar reduces mortality in patients provided they have not suffered inhalation injury (Ong et al., 2006). Ideally burn grafting should be done within five days of burn, to reduce blood loss during eschar excision (Papini, 2004).

The rationale supporting early excision is that it decreases the chances of bacterial colonisation in the wound hence reduces the chances of inflammatory mediators being released and the occurrence of SIRS, which in turn reduces the likelihood of sepsis, multi-system organ failure and metabolic abnormalities (Nguyen et al., 1996). Consequently early excision has become the standard method of major burn wound management (Ong et al., 2006).

As large areas of eschar are removed, the surgically prepared wound requires prompt (temporary) wound closure and several approaches have been made to achieve optimum wound coverage to facilitate a closure of wound by healing.

1.2.8 Burn wound cover and closure

Some methods providing wound coverage are still controversial and various burns centres prefer different methods worldwide. In the ideal case a burns wound would be covered with the patient's own skin (Shakespeare, 2005). In reality this is rarely possible, particularly when extensive areas have been burnt. Currently there is no artificial replacement for patient's own skin, which has equal properties to this autogenous biological material (Shakespeare, 2005). Interestingly some authors refer to cultured keratinocytes as skin, which is anatomically incorrect (Breidahl et al., 1989; Shakespeare, 2005). The ideal wound cover universally suitable for all types of burns simply does not exist. Depending on the layers of skin and area damaged an individual decision for each patient needs to be made. Limitations in usefulness are availability of material, cost, size of burn wound to be covered, shelf-life of material and degree of burn.

Several different approaches are used to aid cutaneous wound repair and in some cases different methods are combined in the hope of providing a better outcome for the patient. Expectations as to the main properties of a skin substitute vary, as different graft sites require materials with different properties, depending on thickness of burns or required aesthetic outcome (Jones et al., 2002; Atiyeh et al., 2005). Some authors differentiate between wound cover and wound closure (Herndon, 1996). Wound closure includes material into the healing wound and restores the barrier function of the epidermis (Jones et al., 2002). Wound cover requires the ingrowth of granulation tissue to achieve adhesion and can be only temporary, in order to create a suitable environment for wound healing (Jones et al., 2002).

The benefits of at the very least covering the wound as opposed to leaving it exposed are obvious, it aids microbial control, helps regulate evaporation and temperature to keep the wound moist, which in turn aids healing, it physically protects the newly developing granulation tissue from airborne contamination, reduces wound pain and allows the patient to undergo physiotherapy earlier (Pruitt & Levine, 1984).

1.2.8.1 Amniotic membrane

The innermost layer of the foetal tissue, despite its fragility has been used as burns wound coverage since 1910 (Rejzek et al., 2001). It is reputed to be an excellent wound dressing due to decreasing the drain of fluid, proteins and electrolyte whilst permitting gas permeability (Rejzek et al., 2001). Various treatments including gamma-irradiation and glycerol preservations generally rid the amnion of bacterial contamination originating during delivery (Rejzek et al., 2001; Gajiwala & Gajiwala 2004). Risk of disease transmission, despite being very low in practice, needs to be considered when using amniotic membrane (Atiyeh et al., 2005). It can be used for second degree burns as temporary cover until complete healing is achieved (Gajiwala & Gajiwala, 2004).

1.2.8.2 Autologous skin grafts

Autologous skin graft or autograft is where the donor and recipient is the same person; healthy skin is harvested from an unburnt site and used as a skin graft over the burnt area to promote healing.

Autografts have been described as the gold standard for extensive burns (Lee et al., 2005) and have been praised as the ideal burn wound cover which also allows wound closure (Papini, 2004). The thickness of the skin can be tailored for best cosmetic effects, however thin grafts are considered more prone to contraction (Papini, 2004). Problems arise in cases where there is not enough unburnt tissue to harvest sufficient autologous skin. Various expansion methods can increase the surface area (Lee et al., 2005). The graft can be stretched by perforation with a mesher (Papini, 2004) or using a „Flypaper technique“ where pre-cut skin pieces the size of postage stamps are used to patch over the wound (Lee et al., 2005). Once the wound has healed the resulting pattern is permanent and cosmetically displeasing (Papini, 2004).

The amount of times one donor site can be harvested to provide an autograft is limited. When donor sites are in short supply a rotation of donor sites can be practiced, where the unexcised burn is covered with antimicrobial cream. A temporary cover of the debrided wound is achieved in the form of a human cadaver allograft, xenograft, skin substitute or cultured epithelial cells (Papini, 2004).

1.2.8.3 Human cadaver allografts and xenografts

Cadaveric allografts, skin harvested from deceased humans, can be useful as temporary wound closure. They are generally provided by skin banks, in a cryopreserved state (Bravo et al., 2000) and only used after vigorous screening (Jones et al., 2002; Atiyeh et al., 2005). Using human cadaver allografts is a second rate option explored when the patient does not have enough donor sites to allow an autograft or when the patient's condition does not allow coverage with autologous skin (Atiyeh et al., 2005). During the early stages of severe burn injury cadaveric allografts are not rejected, as the immune system is severely compromised.

Xenografts where donor species include reptiles, rabbit, dog and pig have been used for centuries (Atiyeh et al., 2005). Unless therapeutic doses of immunosuppressants are given allografts and xenografts are ultimately rejected (Atiyeh et al., 2005) and are only useful to bridge a critical time gap in the early phases of burns treatment.

1.2.8.4 Cultured human dermal keratinocyte sheets

The shortcomings of autologous skin grafts, particularly when skin donor sites were unavailable led to a search for alternative options. Rheinwald and Green had successfully cultured and subcultured keratinocytes in clonal cell densities on a feeder-layer of lethally irradiated mouse fibroblasts (Rheinwald & Green, 1975). Green suggested that these cells were suitable for therapeutic use (Green, 1979; Green, 1991). The first report of therapeutic use of cultured epithelial keratinocytes (which are referred to as „cultured epithelial autograft“ in the literature) in major burns was by O'Connor et al., (1981).

O'Connor's method involves growing human dermal keratinocytes to confluent sheets, also known as „sheet grafts“ which can be applied to the prepared burns wound directly or on a cell carrier. When cells have covered the entire surface available for growth, they are considered as having reached confluence. A major drawback is the time required to grow the cell sheets to confluence (Wood et al., 2006). It takes approximately 3-5 weeks to grow confluent cell sheets suitable for grafting which is a significant limitation to the usefulness of this method (Rab et al., 2005).

The advantage of this method is that a replacement epidermis can be grown to sufficient quantities to cover an entire body from a single small skin biopsy a few millimetres in diameter (Horch et al., 2005). Cultured epithelial autografts (CEA) are reported to be more susceptible to clinically significant bacterial contamination than meshed skin grafts (Horch et al., 2005). Increased vulnerability to infection is a serious limitation, as it is a major cause of graft failure (Wood et al., 2006).

The main issues associated with this technique besides the long culture time are the necessary preparation of a wound bed, a difficulty in assessing graft take, high susceptibility to infection and long term fragility (Wood et al., 2006).

Other problems with whole keratinocyte sheets are that they are fragile. To remove them from the substrate on which they have been cultured requires a proteolytic enzyme, which can cause graft contraction and restricted proliferation (Jones et al., 2002).

CEAs are considered a reliable method for partial thickness burns in childhood scalds (Rab et al., 2005) after promising pioneering results in children with 53-98% TBSA burns (Compton et al., 1989; Robson et al., 1992) and are considered the gold standard by many burns centres.

1.2.8.5 Keratinocyte cell sheets on supports

To combat the issue of graft fragility research groups have taken to using sub-confluent keratinocyte cell sheets attached to polyhydroxyethylmethacrylate supports, which reduces culture time to 5-8 days (Dvoránková et al., 1998). Alternatively keratinocytes were seeded *in vitro* on top of a hyaluronic acid membrane, with laser drilled perforations. The skin substitute was placed membrane side down onto the wound to allow cell migration into the wound bed. It is suggested the hyaluronic acid may promote cell migration and the membrane not requiring inversion unlike CEA sheets did not require the cells to re-orientate in the wound (Jones et al., 2002).

1.2.8.6 Fibrin-glue and keratinocytes

Various combinations involving fibrin and keratinocytes have been assessed for clinical efficacy. In some cases keratinocytes were grown embedded in a fibrin matrix, rather than producing CEA sheets, which allows grafting earlier, when the cells are in an actively proliferative state. Stable skin with good mechanical qualities was formed, with complete healing achieved within 14-21 days (Jones et al., 2002).

1.2.8.7 Sprayed keratinocyte cell suspension

Pre-confluent keratinocytes can also be delivered by aerosol (Duncan et al., 2005). A team working at the Blond McIndoe Centre tested a combination of autologous keratinocytes and autologous fibrin sealant in a porcine wound model (Grant et al., 2002). The keratinocytes were delivered as a spray intermixed with fibrin sealant on full-thickness wounds, in an entirely autologous system, where fibrin was isolated from whole blood. The wound had been prepared with Integra® to provide the dermal element, 14 days after application a proliferated, but non-differentiated epithelium was created.

1.2.8.8 Skin substitutes

The term skin substitutes is widely used in literature, often describing commercial products with distinctly different properties, some considered suitable for wound closure, others only for wound cover (Jones et al., 2002). Combinations of skin substitutes with CEA have been reported.

Skin substitutes cannot replace autologous human skin or CEA, but do have a therapeutic function in terms of protection, procrastination (until permanent graft material becomes available), promotion or provision (Shakespeare, 2005). Skin substitutes mimic parts of the skin's structural matrix and may contain fibroblasts and keratinocytes, however melanocytes, Langerhans' cells, macrophages, and lymphocytes, or other structures such as blood vessels, hair follicles or sweat glands are obviously absent. Pruitt & Levine (1984) compiled a list of desirable properties of skin substitutes.

SUPPORT MATERIAL	CELLS	BRAND NAME	MANUFACTURER
Cadaveric dermal matrix	No viable cells	Alloderm®	Lifecell Corporation, Branchberg, NJ
Outer silicone film and inner layer of nylon and collagen	No viable cells	Biobrane™	Dow Hickham/Bertek Pharmac. Sugar Land, TX, USA
C-GAG on silicone foil support	No viable cells	Integra®	Integra Life Science, Plainsboro, NJ, USA
Polymer membrane with nylon mesh, extracellular matrix protein from Fib	No viable cells	Transcyte®	Smith & Nephew UK Limited, London).
Bovine collagen analogue	No viable cells	Terumo™	Terumo, Tokyo, Japan
Polyglycolic acid/Polyglactin-910	Fib	Dermagraft®	Smith & Nephew, La Jolla, CA, USA
Hyaluronic acid membrane	Fib	Hyalograft™	Fidia Advanced Biopolymers, Padua, Italy
Fibrin sealant	Autologous Ker	Bioseed™	BioTissue Technologies, Freiburg, Germany
Petroleum jelly	Autologous Ker	Epicel™	Genzyme Biosurgery, Cambridge, MA, USA
Benzyl esterified hyaluronic acid	Autologous Ker	Laserskin™/ Vivoderm™	FIDIS Advanced Polymer, Aban Terme, Italy/ ER Squibb& sons Inc., Princeton, NJ, USA
Bovine collagen	Ker + Fib	Apligraf®	Organogenesis, Canton, MA, USA
Bovine collagen sponge	Ker + Fib	Orcel®	Ortec International, Inc., New York, NY, USA

Table 1.1 Some commercially available skin substitutes

Legend Table 1.1: *Cells used: Ker – human Keratinocytes, Fib – human fibroblasts*

The ideal wound covering should be non-antigenic, show little toxicity, be tissue compatible, flexible and elastic but at the same time resistant enough to sustain sheer stress and lead to a cosmetically satisfactory outcome. It needs to exclude microorganisms yet allow vapour transmission, be biodegradable to avoid further surgical interventions and permit ingrowth of fibroblasts and vascular tissue (Pruitt & Levine, 1984). **Table 1.1** lists some commercially available skin substitutes.

Using skin substitutes as a source and construct for cytokine delivery is claimed to improve graft take and wound healing (Kearney, 2001). Such agents include cytokines and growth factors for example IL-1 α , IL-1 β , IL-6, TGF- α in Orcel (Ortec, Ortec International, Inc., New York, NY). Reports of improved graft performance and healing rates due to supplementation with antibiotics and bacteriophages have been reported (Häusler, 2003; Kutter & Sulakvelidze, 2005).

A variety of artificial skin substitutes are available, some ready seeded with autologous or allogeneic human fibroblast and/or human keratinocytes, some manufacturers have taken to using neonatal foreskin fibroblasts (Dermagraft, Apligraf) rather than dermal fibroblasts obtained from punch biopsies (Atiyeh et al., 2005).

Skin replacements often contain allogeneic keratinocytes (as opposed to autologous in CEA), which ultimately cannot achieve wound closure, however they release growth factors and cytokines which enhance wound closure. Purists may not consider these products skin replacements as they are mere autologous keratinocyte grafts attached to a support, which is later removed.

Limiting factors for skin substitutes are cost, suitability for limited circumstances, licensing only for uses other than burns and reliance on empirical data.

1.2.8.9 Combinations of fibroblasts and keratinocytes

Keratinocytes on their own regardless of the delivery method are unsuitable for third degree burns, as the dermal element is missing (Wood et al., 2006). Various avenues of introducing a dermal element with keratinocytes in full thickness burns have been explored. Efforts have been made to circumvent this dilemma by combining

keratinocytes with fibroblasts in a collagen-glycosaminoglycan matrix (C-GAG) (Horch et al., 2005).

Researchers have explored the possibility of combining fibroblasts and keratinocytes in one product by a series of *in vitro* experiments (Kamolz et al., 2005). A layer of fibrin glue containing fibroblasts was sandwiched between layers of human dermal fibroblasts, adding a layer of primary keratinocytes obtained from skin biopsies on top, with the advantage of reduced culture time.

1.3 Burn wound infection

Due to improved burns aftercare and healthcare provisions the survival rate of burns victims has dramatically improved since the beginning of the 20th century. Increasing numbers of patients with over 60% burns of total body surface area now have realistic chances of survival (Edwards-Jones & Greenwood, 2003).

Innovations in plastic surgery, scar management and rehabilitation have improved patients' quality of life, if the patient survives the critical period (Edwards-Jones & Greenwood, 2003).

A major problem arises when burns become infected, which causes over 50% of burns deaths (Edwards-Jones & Greenwood, 2003). An older, still often cited source estimates that 75 per cent of all deaths following burn injuries are related to infection (Polk, 1979). Wounds provide a favourable environment for microbial proliferation, if devitalised tissue is present and the immune system compromised it provides optimal conditions for microbial growth. Improved wound and clinical management has reduced the prevalence of certain pathogens causing wound infection.

1.3.1 Sources of burn wound contamination

Directly after the burn incident the wound is generally considered free from contamination (Vindenes & Bjerknes, 1995). Wound contaminants originate from three main sources: the environment, the normal microflora of the skin and endogenous sources such as mucous membranes, particularly the oral and gastrointestinal mucosae (Bowler et al., 2001). Gram positive bacteria may survive the burn in sweat glands and

hair follicles, which can colonize the wound within 48 hours (Vindenes & Bjerknes, 1995).

1.3.2 Factors predisposing to microbial proliferation in burns

PATIENT FACTORS	MICROBIAL FACTORS
Extent of burn >30 % of body surface	Presence of over 10^5 organisms per gram of tissue
Depth of burn	Motility
Patient age	Metabolic products
Pre-existing conditions	Endotoxins
Wound dryness	Exotoxins
Secondary impairment of blood flow	Permeability factors
Acidosis	Other factors
Number of vascular catheters	Antimicrobial resistance

Table 1.2 Risk factors in burn wound infection (Pruitt & Levine 1984; Wong et al., 2002)

Burns patients are often immune suppressed and in addition the blood supply to facilitate transportation of antibodies to the burns wound is frequently non-existent as blood vessels have usually been damaged. The eschar, (that part of the necrotised skin destroyed through the burn) is no longer vascularised and due to its protein-rich properties is an ideal breeding ground for microorganisms. This forms part of the rationale of early surgical removal of eschar as part of after-burns therapy (Edwards-Jones & Greenwood, 2003).

The patient's age is correlated with the risk of infection; young adults are at a lower risk of burn infection than groups at either end of the demographic scale such as children or elderly patients (**Table 1.2**). Pre-existing diseases can dramatically affect chances of recovery from burns, as seen in patients with diabetes, who are more prone to fungal infection. Some risk factors are interlinked. Patients with underlying cardiopulmonary diseases and those with inhalation injuries are more prone to bronchopneumonia. Immunological and metabolic abnormalities, as seen in obesity or malnutrition can also increase the risk of burns infection.

Functioning blood supply to the burn wound can depend on the thickness of burns. Avascularity can lead to a diminished immune response, affect wound pH and impede transport of antimicrobials to the wound.

Un-excised full-thickness burn wound provides the perfect protein-rich substrate for microbial growth due to a lack of blood supply. Moisture levels of the wound (Bowler et al., 2001) and its temperature can both affect microbial growth (Sessler, 2006).

The nature and consequences of a burn can mimic signs and symptoms of infection.

Hyperthermia, tachycardia and hyperventilation as well as central nervous dysfunctions could be a sign of infection or the consequence of the burns injury (Edwards-Jones & Greenwood, 2003).

Intensity of care required by the patient was associated with the acquisition of multiple antibiotic resistant bacteria, particularly *Acinetobacter baumannii* (Fierobe et al., 2001; Wong et al., 2002). The number of indwelling devices, particularly vascular catheters were identified as risk factors for colonisation and infections with *A. baumannii* in burns patients (Wong et al., 2002).

1.3.3 Significance of microorganisms present in the wound

„Infection occurs when virulence factors expressed by one or more micro-organisms in a wound outcompete the host natural immune system“ (Bowler et al., 2001). One school of thought believes that once the microbial load exceeds 10^5 cfu of bacteria per gram of tissue an acute or chronic infection exists (Bowler et al., 2001). Other investigators

report that even quantitative cultures are not useful in differentiating between burn wound colonisation and infection but recommend histological analysis to diagnose invasive burn wound infection (Pruitt, 1998). In general quantitative microbiology can reliably predict the risk of infection and wound healing, however controversy exists as to whether invasive techniques or non-invasive procedures such as the velvet pad technique and the quantitative swab are preferable (Bowler, 2001). In practice the term „burn wound infection“ is only used when a pathologist identifies the presence of microorganisms in unburned tissue (Pruitt, 1980).

A variety of methods exist which allow early detection of bacterial presence in wound such as polymerase chain reaction, aromascan technology and intact cell mass spectrometry, which would allow early detection and early intervention, before serious illness and further wound damage occurs, however in practice these methods are not used routinely in a clinical setting (Edwards-Jones & Greenwood, 2003). The presence of bacteria in the wound (colonisation) has been demonstrated to interfere with wound healing (Bowler, 2001) and may affect graft performance. Burn wound avascularity and the fact that microbes are introduced through the portal of the wound can, at a later stage, lead to systemic infections and associated complications (Pruitt, 1980).

20-fold (Lortholary et al., 1998) and two-fold increases (Wisplinghoff et al., 1999) in mortality rates in burns patients have been correlated with colonisation and subsequent infection of *Acinetobacter* spp.

1.3.4 Graft failure due to microbial contamination of the wound

CEA or other cultured graft options do not contain immune cells and are not vascularised. It has been shown that cultured skin grafts are more sensitive to topically applied antimicrobial agents than split thickness grafts. Cultured skin grafts are grown under aseptic conditions, after transplantation into the wound bed an abrupt transition into a non-sterile environment occurs (Erdag & Morgan, 2002).

CEAs have shown increased vulnerability to infection, compared to other wound closure systems (Wood et al., 2006). Colonisation and infection of the wound bed have been identified as major causes of graft failure (Wood et al., 2006). It is well

documented that skin grafts do not „take“ on experimental or clinical wound beds containing 10^5 cfu/g tissue or above (Pruitt, 1984; Edwards-Jones & Greenwood, 2003).

Some evidence suggests that the occurrence of bacterial products alone, without the presence of viable bacteria may contribute to graft failure (Taylor et al., 1990). Fibroblast and keratinocyte tissue cultures, exposed to bacterial filtrates of clinical isolates including three isolates of *Acinetobacter* spp., were effective in causing detachment and death of both cell types.

With increased chances of survival on successful grafting and costs of up to 425,000 Euros for the successful treatment of one patient (Horch et al., 2005), microbial contamination or infection of the wound are highly undesirable.

There is evidence to suggest that cultured keratinocytes show enhanced inhibition of bacterial growth when pre-treated with certain cytokines (Erdag & Morgan, 2002).

1.3.5 Bacteria associated with wound infection

Burn wound flora changes occur over time; initially Gram-positive organisms predominate which are replaced with Gram-negative bacteria generally during the first post-burns week. Colonising microbes are generally a mixture of surviving resident bacteria and microbes picked up from the environment.

Initially the density of the microbes is low, however if uninhibited proliferation occurs a density of 10^5 organisms per gram of viable tissue can be reached. This may in turn lead to systemic infection and rejection of skin grafts (Pruitt, 1980).

Bacterial armoury such as enzyme production, presence of endotoxins, exotoxins, motility, capsule formation or slime production all aid bacterial survival or proliferation. Gram-positive bacteria appear to proliferate less rapidly in necrotic tissue than their Gram-negative counterparts and infrequently infiltrate fascial layers.

Common pathogens isolated from a burn wound are *Staphylococcus aureus* (75%), *Pseudomonas aeruginosa* (25%), *Streptococcus pyogenes* (20%) and a variety of coliform bacteria (5%). Anaerobic organisms, other streptococci and fungi, such as

Candida albicans and *Aspergillus fumigatis* can also cause infections (Edwards-Jones & Greenwood, 2003). Bowler et al. disagrees with the widespread opinion that aerobic or facultative pathogens mentioned above are the primary causes of delayed healing and infection and shifts the emphasis towards anaerobic bacteria (Bowler et al., 2001). Another group reported different ratios of commonly found isolates including *Pseudomonas* spp. (36%), *Staphylococcus aureus* (19%), *Klebsiella* spp. (16%), *Proteus* spp. (11%), *Enterococcus faecalis* (9%), *Escherichia coli* (5%), *Salmonella senftenberg* (0.8%) *Acinetobacter* spp. (1%) and others (Revathi et al., 1998). Different predominant species being reported in various burns centres is common; shifts in occurring patterns of microbial species found and their antibiotic susceptibility may be correlated to changing clinical management (Revathi et al., 1998).

Staphylococcus aureus produces proteinases, collagenases and hyaluronidase which can virtually digest the extra-cellular matrix, which is an essential structure for wound healing (Edwards-Jones & Greenwood, 2003). In addition excretion of exotoxins, such as enterotoxins A, B and C and toxic shock syndrome toxin-1 can lead to severe clinical complications. It is also renowned for causing graft failure and local necrosis (Edwards-Jones & Greenwood, 2003).

Most *P. aeruginosa* strains produce a toxic pigment (pyocyanin) and some strains produce exotoxin A in addition, which can lead to inhibition of protein synthesis and cell death.

The consequences can be local necrosis, septicaemia (Edwards-Jones & Greenwood, 2003) and graft destruction or failure (Soothill, 1994). *Acinetobacter calcoaceticus* has been isolated in acute and chronic wounds (Bowler et al., 2001).

Acinetobacter baumannii, besides being implicated as a causative organism in a variety of nosocomial infections, can be a regular cause of wound infections (Martín-Lozano et al, 2002). Multiply resistant *Acinetobacter* spp. and pseudomonads, meticillin resistant *Staphylococcus aureus* (MRSA), and vancomycin resistant enterococci (VRE) are increasingly isolated in burns units and are responsible for a range of clinical problems (Edwards-Jones & Greenwood, 2003).

1.3.6 Burn wound infection management

A multitude of approaches to combat burn wound infection and colonisation have been suggested, with varying success (Edwards-Jones & Greenwood, 2003) and there is a need to weigh the benefit achieved against the toxicity risks.

1.3.6.1 Early closure of wounds

An obvious major factor in the prevention of infection is early wound closure by grafting, use of artificial skin or skin substitutes. The risk of infection can be reduced by generating a physical barrier to the influx of pathogens (Edwards-Jones & Greenwood, 2003). In many cases early wound closure cannot be achieved, as colonisation or even infection may have occurred beforehand, which in turn would most likely lead to graft rejection.

1.3.6.2 Topical antimicrobial agents

In the 1960s topical silver containing preparations revolutionised burn care by reducing morbidity and mortality, however silver deposits in tissue can be problematic (Edwards-Jones & Greenwood, 2003).

Other antiseptics such as chlorhexidine, cerium nitrate and povidone iodine are still widely used in burns wounds (Edwards-Jones & Greenwood, 2003). Some antimicrobial agents have been shown to have cytotoxic potential against primary cultures of human keratinocytes (Kautzky et al., 1996).

1.3.6.3 Regulatory molecules

Novel therapeutic treatments including regulatory molecules have been explored in the laboratory. These molecules may be used to prevent the production of virulence factors, which are affected by inducer molecules. A density dependent phenomenon (quorum sensing) signals the expression of toxins and repression of other factors (Williams, 2000). A sensor or transcriptional activator recognizes these molecules and activates or deactivates the expression of certain virulence factors. It may be feasible to design

molecules which would block this cell-to-cell communication and thereby attenuate virulence (Williams, 2004).

1.3.6.4 Bacterial interference

This remains an ethically debatable approach to preventing infection. „Harmless“ strains were seeded into wounds in a mouse model to prevent colonisation with more virulent strains. Some antagonistic effect was achieved, however in some mixtures of pathogens the infection was more pronounced (Edwards-Jones & Greenwood, 2003).

Consideration of whether the acquisition of *Acinetobacter baumannii* had a protective role in preventing colonisation by other species in burns patients (Sherertz & Sullivan, 1985) was disproven when mortality rates were found to be significantly higher than in controls (Lortholary et al., 1995; Wisplighoff et al., 1999).

1.3.6.5 Natural agents

Sugar and honey have been rediscovered as effective antibacterial agents for wound dressing. The effect has been attributed to high osmolarity, release of hydrogen peroxide and other substances. Manuka honey, which is derived from *Leptospermum scoparium* has been reported to be effective against *S. aureus* and *P. aeruginosa* (Edwards-Jones & Greenwood, 2003).

1.3.6.6 Cytokines

Cytokine treatment of cultured composite keratinocyte grafts augmented their antibacterial properties in an experimental setting (Erdag & Morgan, 2002). In particular IL-1 α , IL-6, TNF- α and lipopolysaccharides decreased bacterial numbers.

More specifically composite grafts artificially contaminated with *S. aureus* and *P. aeruginosa* which had been pre-treated with IL-1 α and IL-6 significantly inhibited growth of these two bacteria (Erdag & Morgan, 2002).

1.3.6.7 Antibiotics

Small numbers of burn units routinely use erythromycin and flucloxacillin as prophylactic antibiotics, however the general consensus is that they should not be routinely used as a preventative measure due to resistance, high cost and adverse drug effects (Edwards-Jones & Greenwood, 2003). The Blond McIndoe Centre does not use routine antibiotics prophylactically (Personal communication Jane Allan, Chief Pharmacist, 22 January 2003).

1.3.7 Pharmacokinetics of antibiotics in burn patients

Insufficient data are available to describe the pharmacokinetics of many antibiotics in burns patients (Weinbren, 2001). In burns patients pharmacokinetics may be significantly altered, the exact effect of the thermal insult on drug pharmacokinetics is not completely understood for some drugs. There are two phases of pharmacological changes, in the acute phase, within 48 hours of injury capillary changes occur, protein-rich fluid is lost from the vascular system, hypovolemia, tissue hypoperfusion and decreased cardiac output occur, resulting in a decreased glomerular filtration rate (GFR). After 48 hours the second hypermetabolic phase occurs, provided adequate fluid resuscitation was undertaken the cardiac output increases with resulting increased perfusion of liver and kidneys. Consequently an increased GFR and creatinine clearance can be observed. Drug metabolism is affected by an acute depression of microsomal activity, however conjugative metabolic action is unaffected or can be increased. Due to the changes between the two phases high inpatient physiological variability occurs over short periods of time (Weinbren, 1999).

Underlying conditions, inadequate renal perfusion during resuscitation, administration of potentially nephrotoxic drugs and sepsis can influence renal clearance. Changes in plasma protein levels caused by the burns injury can affect drug pharmacokinetics and may warrant loading doses which may otherwise not be required in an unburnt patient.

Pharmacokinetics studies in burns patients are lacking in standardisation as adult and paediatric patient populations are not comparable. Additionally studies including

patients in the acute phase of injury should not be compared to patients in the hypermetabolic phase (Weinbren, 1999).

1.4 Antibiotic resistance and resulting clinical problems

Increasingly bacteria are becoming multiply antibiotic resistant thereby reducing clinical options dramatically. Methicillin resistant *Staphylococcus aureus* (MRSA) is the major cause of nosocomial infections in Europe and its prevalence in burns units is rising (Edwards-Jones & Greenwood, 2003). In addition, multiply resistant *Acinetobacter* spp., pseudomonads and vancomycin resistant enterococci (VRE) are increasingly isolated in burns units. These organisms cause a major problem because eradication of the infection or even the colonisation is extremely hard to achieve.

Handling of the patient during nursing care becomes an infection control issue as this can easily disseminate organisms to other patients (Edwards-Jones & Greenwood, 2003).

1.5 *Acinetobacter* species

Acinetobacter is an opportunistic pathogen with increasing clinical importance particularly in intensive therapy units, causing infections as diverse as septicaemia, meningitis, eye and urinary tract infections, endocarditis and pneumonia predominantly in immunocompromised patients. In 39 French intensive therapy units *Acinetobacter* spp. have become the third most common Gram-negative bacteria causing infections, with only *E. coli* and *Klebsiella* spp. infections being reported more frequently (Garau, 1998). A national survey system for Spain named *A. baumannii* second in the league table for causing ventilator-associated pneumonia (Rello, 1999).

Health Protection Agency (HPA) figures demonstrate the increasing incidence of reported bacteraemia caused by *Acinetobacter* spp. in the UK. (Voluntary surveillance data, 2003-2007, HPA)

An increasing antibiotic resistance of clinical strains has been reported in several countries, which is in agreement with HPA findings in the UK. (Bergogne-Berezin & Joly-Guillou, 1985; Guardabassi et al., 1998; Bou et al., 2000; Simhon et al., 2001;

Hsueh et al., 2002). *Acinetobacter* spp. are recognised to cause failure in skin grafting of deep burns patients (Bergogne-Berezin & Towner, 1996). Subsequent colonisation leads to septicaemia and pulmonary infections with very poor prognosis; Such patients need barrier nursing and cannot be transferred to other units (Khanchanpoom & Khardori, 2002).

The Blond McIndoe specialist burns centre at East Grinstead, Queen Victoria hospital receives some of the most severe burns injury patients in the UK and is recognising intrinsic and acquired antibiotic-resistance in these pathogens (personal correspondence Jane Allen, Clinical Pharmacist, 3 June 2004). Reports of this phenomenon and occurrences of carbapenem-resistant strains of *Acinetobacter* spp. have been described in many countries (Corbella et al., 2000). Treatment of *Acinetobacter* infections in burns patients is often limited to colistin, sulbactam combinations and doxy-or minocycline (Coelho et al., 2004), particularly with colistin having poor toxicological profiles and little published evidence (Coelho et al., 2004).

Acquisition of multi-resistant *Acinetobacter baumannii* (MRAB) is an independent risk factor for mortality in burns patients (Wong et al., 2002). A US study estimated total costs of treatment in a burn unit of a public university-affiliated teaching hospital of patients who acquired multi-drug resistant *A. baumannii* to be \$98,575. The average length of stay was 11 days longer for cases affected by MRAB than control cases (Wilson et al., 2004).

None of the recently licensed antibiotics in the UK are effective against this Gram-negative bacterium (Medicines Compendium, Anon, 2006) and none are likely to enter the market in the future (Spellberg, 2004)- there is an urgent need to identify alternative antibacterial therapies to address this problem.

1.5.1 Morphological, metabolic and cultural characteristics of *Acinetobacter* spp.

The genus *Acinetobacter* contains a number of species that are opportunistic pathogens with increasing clinical importance particularly in intensive therapy units. *Acinetobacter* species are non-spore forming, non-fermenting bacteria. They are strictly aerobic, which is due to a strictly respiratory type of metabolism with oxygen functioning as the terminal electron acceptor (Table 1.3).

Gram stain	Negative
Microscopic appearance	Rods (coccobacilli in stationary phase)
Oxidase	Negative
Catalase	Positive
Motility (hanging drop preparation)	Non-motile, possible twitching
Growth requirement	Strictly aerobic
Glucose O-F	Oxidative or no acidification, no fermentation
Pilus formation	Yes
Spore formation	No

Table 1.3 Phenotypic properties used for the identification of the genus *Acinetobacter*

This genus is known to be non-motile as swimming motility does not occur, however a „twitching motility“ of cells may be seen, which may be attributed to the presence of polar fimbriae (Holt et al., 1994).

They are Gram-negative, though sometimes difficult to destain, catalase positive and oxidase negative (**Table 1.3**) which easily differentiates them from *Pseudomonas aeruginosa* (Collins et al., 1995). In most papers *Acinetobacter* are referred to as coccobacilli, however strictly speaking this genus presents as rod with a diameter of 0.9-1.6 μm and a length of 1.5-2.5 μm , but becomes spherical in the stationary phase of growth (Holt et al., 1994)

1.5.2 Growth temperatures

Members of the genus *Acinetobacter* generally grow well on complex bacteriological media with a temperature optimum of 20-30°C without the need for extra growth factors. Holt et al. (1994) report growth temperature optima in the region of 33-35°C for most strains. However clinical isolates particularly *A. baumannii* grow at 37°C or higher (Bergogne-Berezin & Towner, 1996). Other genomic species prefer lower temperatures or only exclusively grow at such temperatures. A general growth temperature of 30°C has been recommended (Hugh, 1978) however in practice a combination of temperatures may be used depending on the origin and type of the strain.

1.5.3 Growth media

Acinetobacter species are capable of utilising a variety of carbon sources for growth which enables bacteria of this genus to thrive in rather hostile conditions and contributes to the wide range of isolation sources. The bacteria can be easily grown on standard media such as nutrient agar and tryptic soy agar. *A.baumannii* is saccharolytic, many strains acidify most oxidative and fermentative carbohydrates; it can also rapidly produce acid from lactose (Koneman et al., 1994).

D-Glucose is the only hexose sugar some strains are capable of utilising, however most strains are able to grow in a simple mineral medium containing ammonium or nitrate salts and a single carbon source such as acetate, lactate or pyruvate.

Some strains can also utilise the pentoses D-ribose, D-xylose and L-arabinose as a carbon source (Holt et al., 1994). These varying metabolic requirements are helpful during biotyping which aids differentiation of the species of the genus *Acinetobacter*.

Apart from clinical sources *Acinetobacter* has been found in pig manure, sewage, waste water, surface water, vegetables (Berlau et al., 1999), soil, surfaces and other locations.

1.5.4 Isolation from clinical samples/ Isolation of *Acinetobacter* spp. from various sources

For isolation from any source which may contain background contamination from other bacteria a selective medium, which suppresses the growth of other microorganisms is useful.

Commercially available Herellea agar (Difco) has been reported as a useful isolation tool, however more recently Leeds Acinetobacter Medium has been used (Bergogne-Berezin & Towner, 1996)

1.5.5 *Acinetobacter* taxonomy

Bacteria belonging to what is currently recognised to be the genus *Acinetobacter* have undergone a long history of taxonomic changes. Bacteria within this genus have been known previously under a confusing variety of different names: *Bacterium anitratum*, *Herellea vaginicola*, *Mima polymorpha*, *Achromobacter*, *Alcaligenes*, *Micrococcus calcoaceticus*, *B5W*, *Moraxella glucidolytica* and *Moraxella lwoffii* (Bergogne-Berezin & Towner, 1996); *Diplococcus mucosus* (Lingelsheim 1908 cited in Gerner-Smidt, 1994), *B5W* (Stuart et al., 1949 cited in Gerner-Smidt, 1994), *Neisseria winogradskyi* (Lemoigne et al., 1952 cited in Gerner-Smidt, 1994), *Cytophaga anitrata* (Lautrop, 1961 cited in Gerner-Smidt, 1994) and *Lingelsheimia anitrata* (Seeliger et al., 1966 cited in Gerner-Smidt, 1994).

Originally the genus *Acinetobacter* was a heterogeneous group of Gram-negative non-motile, oxidase-positive and negative bacteria, which did not produce pigmentation. In the 1960s extensive nutritional studies made it apparent that oxidase-negative strains were significantly different from the oxidase-positive strains (Baumann et al., 1968 I&II). In 1971 the Subcommittee on the Taxonomy of *Moraxella* and Allied Bacteria recommended only oxidase-negative strains should be included in the genus *Acinetobacter* (Bergogne-Berezin & Towner, 1996).

Species name	Reference
<i>A. baumannii</i>	Bouvet & Grimont 1986 (amended)
<i>A. bouvettii</i>	Carr et al., 2003
<i>A. baylyi</i>	Carr et al., 2003
<i>A. calcoaceticus</i>	1)Approved List of Bacterial Names
	2)Bergey's Manual
	3)Bouvet & Grimont 1986 (amended)
<i>A. gernerii</i>	Carr et al., 2003
<i>A. grimontii</i>	Carr et al., 2003
<i>A. haemolyticus</i>	Bouvet & Grimont 1986 (amended)
<i>A. lwoffii</i>	1)Approved List of Bacterial Names
	2)Bouvet & Grimont 1986 (amended)
<i>A. parvus</i>	Nemec et al., 2003
<i>A. radioresistens</i>	Nishimura et al. 1988
<i>A. schindleri</i>	Nemec et al., 2001
<i>A. tandoii</i>	Carr et al., 2003
<i>A. tjernbergiae</i>	Carr et al., 2003
<i>A. townneri</i>	Carr et al., 2003
<i>A. ursingii</i>	Nemec et al., 2001

Table 1.4 Different species of *Acinetobacter* suggested by various sources

Legend to Table 1.4: (amended) denotes the same name being used by two different authors, with different criteria for the genospecies described by that name.

At present the generic description of *Acinetobacter* is satisfactory to allow unambiguous identification to genus level, the present species delineation however is lacking.

The genus *Acinetobacter* has been documented to be biochemically and genetically diverse since the 1960s, it was demonstrated that glucose oxidation was not a criterion sufficient enough to separate acinetobacters taxonomically (Baumann et al., 1968 II ; Gerner-Smidt, 1994). Despite this the Approved List of Bacterial Names (Skerman et al., 1989) only mentions *Acinetobacter calcoaceticus* and *Acinetobacter lwoffii* (**Table 1.4**). Worse still, Bergey's Manual of Systemic Bacteriology recognises only *Acinetobacter calcoaceticus* (Bouvet & Grimont, 1986).

Several attempts have been made to define several species of *Acinetobacter* based on nutritional characteristics (Baumann et al., 1968 I,II) deoxyribonucleic acid (DNA)-DNA hybridisation (Johnson et al., 1970) a combination of biochemical properties and DNA relatedness (Bouvet & Grimont, 1986), genomic fingerprinting and 16S rDNA sequence data (Carr et al., 2003). Throughout literature *A. anitratus* describes glucose-oxidising acinetobacters and the name *A. lwoffii* used for asaccharolytic strains (Gerner-Smidt, 1994). Some research groups also use the term *A. calcoaceticus*- *A. baumannii* complex which illustrates how extremely close the relationship of these two species is.

Gerner-Smidt (1994) identified that the phenotypic identification is problematic and despite claims of commercial identification system manufacturers that their product is able to discriminate between DNA groups this has never been supported by published genotypic data. Only identification kits including assimilation reactions are of theoretical use in *Acinetobacter* identification.

As the differences between species are so small, reliably assigning a strain to one of the many officially unrecognized species becomes very difficult if not impossible; No available phenotypic identification system can identify all acinetobacters to DNA group level (Gerner-Smidt, 1994.), some genospecies are unnamed (Bouvet & Grimont, 1986).

1.5.6 Mechanisms of resistance

Non-fermenting Gram-negative bacteria often utilise different mechanisms of resistance in combination, making multidrug resistance commonplace.

This group of bacteria display intrinsic resistance, partly due to the relative impermeability of their outer membrane to macrolides and β -lactams, in comparison with other Gram-negative organisms such as *E. coli*. Efflux pumps are a different mechanism contributing to the intrinsic resistance mechanisms (McGowan, 2006).

1.5.6.1 β -lactam resistance

The high incidence of β -lactam resistance found in *Acinetobacter* spp. is partially due to effective efflux pumps and an impermeability of their outer membrane to these agents. This genus is considered to have developed resistance to macrolides using similar mechanisms (McGowan, 2006). Small outer membrane pore size and limited porin production are considered contributing factors to resistance to β -lactam antibiotics in *Acinetobacter* spp. (Bergogne-Berezin et al., 1996). β -lactamases, both plasmid-associated and particularly chromosomal, in combination with altered penicillin binding proteins have been important in the β -lactam resistance of *Acinetobacter* spp. (Bergogne-Berezin et al., 1996).

1.5.6.2 Imipenem (Carbapenem) resistance

Several mechanisms of carbapenem resistance of *A. baumannii* have been described such as loss of outer membrane proteins and altered penicillin-binding proteins. *A. baumannii* is also capable of acquiring carbapenemases, including metallo- β -lactamases and class A and D enzymes (Da Silva et al., 1999; Quale et al. 2003). Plasmid-encoded imipenem resistance has been reported in *Acinetobacter* spp., which in turn was the first European plasmid-encoded imipenem resistance reported in any bacterial species (Bergogne-Berezin et al., 1996).

1.5.6.3 Aminoglycoside resistance

Most commonly aminoglycoside resistance arises from the activity of plasmid-or transposon-encoded aminoglycoside-modifying enzymes, some evidence points towards plasmid-borne aminoglycoside resistance genes (Bergogne-Berezin et al., 1996). Transposon-encoded aminoglycoside resistance in *Acinetobacter calcoaceticus* was first reported by Devaud et al. (1982).

1.5.6.4 Fluoroquinolone resistance

Resistance to fluoroquinolones has been attributed to mutations in genes of DNA gyrase (Vila et al., 1995). 4-quinolones act upon bacterial replication by inhibiting DNA gyrase, which promotes the negative supercoiling of DNA (Oram & Fisher, 1991).

It was found that *gyrA* mutations at a codon equivalent to Ser-83 in *E.coli* were responsible or at least contributed to resistance of *A. baumannii* to ciprofloxacin and nalidixic acid (Oram & Fisher, 1991). Another mechanism of resistance to fluoroquinolones is genetic mutation leading to changes in the influx and efflux system (Pidcock, 1995). These changes can be due to reduced formation of outer membrane proteins, leading to reduced quinolone influx or a stimulation of the cellular efflux system, leading to increased removal of quinolones (Heinemann et al., 2000).

1.5.6.5 Phenotypic antimicrobial resistance patterns of *Acinetobacter* spp. reported by various centres

In a multicentre, multistate intensive care unit (ICU) surveillance study from the USA susceptibility testing was performed using microdilution panels according to the National Committee for Clinical Laboratory Standards (NCCLS).

As expected resistance patterns changed over a period of 5 years and resistance was higher in 2000 than in 1995 (**Table 1.5**). The two most active drugs overall were found to be imipenem and amikacin (Friedland et al., 2003). In another US study, by Quale et al. (2003) unique patient isolates were collected from 15 hospitals in Brooklyn, New York City from December 2000 to February 2001. The agar dilution technique was

chosen to determine antibiotic susceptibilities according to NCCLS. Only half of all 433 isolates were susceptible to ampicillin-sulbactam and amikacin (**Table 1.6**). Only one third was susceptible to meropenem, with most minimum inhibitory concentrations (MIC) close to the breakpoint. Considering the small time frame and relatively small area in comparison with a national or international study, the results are worrying.

Antimicrobial	1995 (N=210)	2000 (N=375)
Imipenem	1.9	5.1
Amikacin	4.3	13.3
Tobramycin	12.4	25.1
Ticarcillin-clavulanate	13.3	30.9
Ampicillin-sulbactam	13.8	21.6
Piperacillin-tazobactam	-	21.1
Ceftazidime	23.3	45.1
Cefepime	-	34.1
Ciprofloxacin	31.0	57.1
Aztreonam	48.1	65.3

Table 1.5 Resistance rates (%) for *Acinetobacter* from ICUs 1995 and 2000 after Friedland et al. (2003).

In 2000 a range of then recently developed fluoroquinolones was seen as promising in the fight against *A. baumannii* infections. While cinafloxacin, gatifloxacin, gemifloxacin, levofloxacin, moxifloxacin and trovafloxacin were active at lower MICs against *A. baumannii* strains when compared to ciprofloxacin, there were only minor differences regarding the percentage of strains fully susceptible.

Antimicrobial	Susceptible	Intermediate	Resistant
Ampicillin-sulbactam	54	9	37
Piperacillin-tazobactam	19	20	61
Ceftriaxone	1	16	83
Ceftazidime	18	3	79
Cefepime	14	26	60
Imipenem	63	23	14
Meropenem	32	39	29
Ciprofloxacin	12.5	0.5	87
Levofloxacin	14	2	84
Gatifloxacin	14	12	74
Amikacin	56	30	14
Polymyxin	94.5	-	5.5

Table 1.6 Susceptibility data (%) for *Acinetobacter baumannii* after Quale et al. (2003)

It was also observed that outbreak related *A. baumannii* strains from a variety of centres were significantly more resistant to any fluoroquinolones than sporadic strains (Heinemann et al., 2000). This correlates with the findings that previous administration of a fluoroquinolone was an independent risk factor for endemic *A. baumannii* infection in a retrospective cohort study combined with three case-control studies. It was found that selection pressure caused by the indiscriminate use, including empirical therapy of fluoroquinolones was responsible for the persistence of multi-drug resistant clones for at

least 5 years. This selection pressure was greater when fluoroquinolones were administered intravenously rather than orally (Villers et al., 1998).

1.5.7 The role of *Acinetobacter* spp. in human disease

Acinetobacter spp. can form part of the human skin flora, particularly in moist regions such as groin, axillae and toe webs of healthy individuals (Bergogne-Berezin et al., 1996). Skin carriage has been reported at an incidence rate of 20-25% (Taplin et al., 1963; Somerville & Noble, 1970) This genus has been isolated from the oral cavity, respiratory tract and rectum (Bergogne-Berezin et al., 1996; Forster & Daschner, 1998) and is considered to be of low virulence with a low incidence of causing infection in otherwise healthy individuals (Bergogne-Berezin et al., 1996).

Acinetobacter spp. have received ever increasing recognition as nosocomial pathogens, accounting for up to 10% of nosocomial bacteraemias reported in ICUs (Bergogne-Berezin et al., 1996; Garau, 1998).

Most infected or colonized patients are reported high-risk surgical patients, burns patients and ICU patients (Bergogne-Berezin et al., 1996).

The escalation of the clinical importance of this genus has been attributed to a combination of factors: the widespread resistance to antimicrobials, which fosters their selection in hospital settings or a patient's own microflora under the selective pressure of multiple antibiotic regimes, the inherent ability to colonize skin and mucous membranes, the ability to survive for long periods of time under unfavourable conditions (Bergogne-Berezin et al., 1996).

The clinically most important species is *Acinetobacter baumannii* (Forster & Daschner, 1998; Garau, 1998). Published identification rates of *Acinetobacter baumannii* require cautious interpretation, due to unreliable identification methods, as species differentiation can be difficult (Forster & Daschner, 1998). Most frequently isolated species are considered *A. lwoffii*, *A. johnsonii*, *A. radioresistens* and DNA group 3 (Seifert et al., 1997). Seasonal increases in nosocomial infections caused by

Acinetobacter spp. in the summer months have been reported, with increases in temperature and humidity being proposed factors (Bergogne-Berezin et al., 1996).

The most common sites of infections reported are respiratory tract, especially in ventilated patients (like burns victims), bloodstream, urinary tract, surgical wounds, central nervous system, peritoneum, skin and eye (Forster & Daschner, 1998).

1.5.7.1 *Acinetobacter* spp. in wounds and burns

Wound colonisation with *Acinetobacter* may lead to skin graft rejection while worse wound infection may lead to bacteraemia (Bergogne-Berezin et al., 1996). In cases of multiply resistant strains or pan-resistant strains this clinical problem may become untreatable (Wong et al., 2002). Burns patients are particularly prone to infections due to loss of protective barrier, the eschar providing a substrate for microbial proliferation and decreased immunity and impaired physiological responses.

Historically there has been controversy over whether colonization and infection with *Acinetobacter baumannii* was a cause for morbidity and mortality, irrespective of other factors (Wong et al., 2002). Sheretz & Sullivan (1985) reported lower incidences of mortality in burns patients with *Acinetobacter* bacteraemia compared to bacteraemias caused by other organisms, leading to speculations whether *Acinetobacter* had a protective role in prevention of colonization by other species. These conflicting data may be due to differences in statistical methodology (Wong et al., 2002). Other studies found mortality rates in patients with *A. baumannii* to be twice as high as controls (Wisplinghoff et al., 1999). A systematic review including US and European data of burns patients suggested an association between *Acinetobacter baumannii* colonization and infection and considerably increased mortality (Falagas et al., 2006). Other authors correlated the outcome of *Acinetobacter* bacteraemia more closely with the underlying illness, investigating a variety of clinical settings; however the study only included four burns patients with half having died (Tilley & Roberts, 1994). A twenty-fold increase in mortality was attributed to the acquisition of and infection by multiply resistant *Acinetobacter baumannii* (Lortholary et al., 1995).

1.6 Alternatives to antibiotics

Antimicrobial resistance is a major international public health threat. It can severely limit treatment choice in clinical settings and consequently makes infections more difficult to treat. There are even suggestions that a return to pre-penicillin days is a possibility if adequate steps are not taken (Langleben, 2001; Sulakvelidze, 2001).

The success of antimicrobial medicines created the illusion in the late 1960s and early 1970s that infectious diseases had been overcome (Spellberg et al., 2007). Infectious diseases have made a worrying comeback since that time and as a consequence alternatives to antibiotics are now being explored or re-evaluated resulting in the House of Lords Select Committee on Science and Technology publishing its report „Resistance to Antibiotics and other Antimicrobial Agents“ in April 1998, recognizing the severity of the situation.

Following that the Department of Health published „UK Antimicrobial Resistance Strategy and Action Plan“ (2000), which defined eight action areas, one of which was „to encourage the development of new and novel agents/technologies to detect, prevent and treat infection to overcome resistance“.

Department of Health documents (UK Antimicrobial Resistance Strategy and Action Plan, 2000) state very clearly that alternatives to antibiotic treatment must be sought in the fight against antimicrobial resistance. The development of new classes of antibiotics is not progressing at the same pace as that at which bacteria are developing resistance. Already there have been reports of bacteria isolated in hospitals that are virtually resistant to every known antibiotic (Souli et al., 2008).

1.6.1 Reasons for a lack of new antibiotics

Research and development for one approved agent can amount to an estimated \$400-\$800 million, which can constitute a hindrance to any new drug development. (DiMasi et al., 2003) Additionally antimicrobial agents are less lucrative than other drug classes as they are mostly only required for short courses, rather than a continuous therapy as required for chronic conditions (Projan, 2003) Due to ageing populations drug

discovery efforts are focussed on medicines which treat chronic medical conditions, which commonly occur in the elderly. In addition it is in the interest of public health to limit the use of broad-spectrum antibiotics, as can be done by the introduction of antibiotic formularies and enforcement of prescribing protocols.

As an unwanted effect these measures can discourage the more widespread use of newly developed, more expensive antimicrobials, which in turn can negatively impact on sales (Spellberg, 2004). For these reasons some large pharmaceutical companies have indicated that they are limiting or entirely abandoning anti-infective research. Spellberg (2004) reported that the number of FDA approved new antimicrobial agents from 1980 to 2002 decreased by 56%, compared with the period from 1983-1987. Of the 9 newly approved antibacterial agents since 01/1998 only two, namely linezolid and daptomycin had entirely novel modes of action. From 1998 to 2002 nine new antivirals were approved, 5 were Human immunodeficiency virus (HIV) specific. From 1998-2003 the same number of antibacterial agents was approved by U.S. Food and Drug Administration (FDA) as the number of HIV specific treatments.

In 2004 twice as many anti-HIV agents as antibacterial medicines were approved, half of the HIV drugs had novel modes of action, compared with zero identified for antibacterials. The decrease in antibacterial drug development is not reflected in an overall reduction of research and development activity or expenditure overall. There is no reversal of this trend in the foreseeable future, which may potentially have a serious impact on public health.

1.6.2 Photodynamic therapy (PDT)

Photodynamic therapy has been suggested as an alternative treatment option in microbial infection, whereby the combination of photosensitising dye with light at the appropriate wavelength causes the production of reactive oxygen species (Wainwright, 1998). Proposed fields of interest include treatment of chronic ulcers and infected burns (Jori et al., 2006). Gram-negative bacteria have been proven to be poorly responsive to PDT with traditional photosensitising agents, due the impermeability of the outer cell walls (Jori et al., 2006). Studies involving pretreatment, which constituted removal of

bivalent cations, enhanced bacterial cell photoinactivation (Jori et al., 2006). *In vitro* studies have been limited to wound infections in mice; combined use of photosensitisers with antibodies specific for the target microorganism gave superior results to photosensitisers alone in *P. aeruginosa* infected dorsal skin models in mice (Jori et al., 2006). PDT has been suggested in the treatment of wild-type and multiply resistant strains. A potential advantage in wound treatment could be that PDT has been shown to up-regulate growth factor expression, which in turn stimulates wound healing at low light doses (Jori et al., 2006).

PDT appears to have a range of shortcomings: cells in the stationary phase were less susceptible than cells in logarithmic growth; the presence of serum reduced the binding of most photosensitisers to microbial cells (Lambrechts et al., 2005) and any phototoxic effects of PDT on host cells or skin grafts remain unexplored (Wainwright, 1998). Overall this method is considered to be in its experimental stages (Jori et al., 2006).

1.6.3 Phytomedicines

Essential oils and plant extracts have been proposed as topical antiseptics (Hammer et al., 1999) and some oils have shown antimicrobial activity against a range of Gram-negative bacteria including *Acinetobacter baumannii* NCTC 7844 at concentrations of less or equal to 2% v/v (Hammer et al., 1999). In particular tea tree oil is known to have antimicrobial effects, even against multiple antibiotic resistant strains, however use on open wounds is not recommended (Edwards-Jones & Greenwood, 2003) due to toxicity.

The lack of standard, reproducible methods for assessing oils has been criticised (Carson et al., 1995; Mann & Markham, 1998), and the composition of plant oil extracts are known to vary depending on climactic and environmental conditions (Janssen et al., 1987; Sivropoulou et al., 1995).

The toxicity and irritant properties of essential oils (Jacobs & Hornfeldt, 1994) including dermal sensitivity and in particular contact dermatitis (Selvaag, 1995) have been highlighted. Concerns have been raised about sterility and purity of essential oils, which are not regulated as pharmaceutical products (Halcon & Milkus, 2004).

1.6.4 Honey

Particularly in wound care honey has been used for its antibacterial properties, with its activity attributed to high osmolarity, low pH and presence of hydrogen peroxide. The identity of the main antimicrobial constituents of some honeys and mechanism of action remain unclear (Carson & Riley, 2003). Case studies by Visavadia et al. (2008) demonstrated the effectiveness of manuka honey in chronic wound infections against MRSA in split and full thickness skin grafts.

1.6.5 Antibodies

Serum therapy whereby immune serum from immunized animals or recovering humans is administered for the prevention or treatment of infectious diseases is not a new concept (Buchwald & Pirofski, 2003).

Major obstacles constitute the existence of bacteria with multiple serotypes (Berghman et al., 2005), the potential of serum sickness and anaphylaxis, risks of disease transmission and variations in antibody content (Casadevall, 1996). Despite narrow specificity being an advantage in terms of maintaining normal microflora, it decreases the marketing potential for such a pharmaceutical product (Berghman et al., 2005). Additionally systemic administration would be required, unless for treatment of enteric infections, which may cause a multitude of issues including anti-isotope immune reactions against the therapeutic agent (Casadevall & Scharff, 1994).

1.6.6 Bacterial cell wall hydrolases (BCWH)

BCWH are peptidoglycan-degrading enzymes which cause bacteriolysis and potential sources can be eukaryotic cells, prokaryotic cells or bacteriophages. BCWH of eukaryotic origin are usually referred to as lysozymes and form part of the host defence mechanism. In humans lysozymes are present in a variety of tissues and secretions including skin, tears and milk. Autolysins are bacterium-encoded BCWH, with each bacterial species containing one or more autolysins, which facilitate physiological cell functions like cell adhesion or cell wall biosynthesis and virulence. Autolysins can cause bacterial cell death by a variety of mechanisms (Parisien et al., 2008).

Virolysins are BCWH encoded by lytic dsDNA phages, produced during the infection of bacterial cells and facilitate the release of virions by degradation of peptidoglycan at the end of the phage infection cycle (Parisien et al., 2008). Virolysins are referred to by a confusing array of terms such as endolysins, lysins, (Hermoso et al., 2007) lysozymes and lytic enzymes (Parisien et al., 2008). Despite generally having a narrow antibacterial spectrum, with few exceptions functional only against the host bacterial species infected by the phage (Hermoso et al., 2007), virolysins have been proposed as future antimicrobials against multiply antibiotic resistant strains. Lysozymes have a broader spectrum of activity, yet are not functional against Gram-negative bacteria owing to their outer membrane (Parisien et al., 2008). Resistance of some Gram-positive bacteria has been reported against lysozymes, whilst no resistance has been reported to virolysins (Parisien et al., 2008). Using (synergistic) combinations of purified BCWH has been proposed to eliminate colonisation and in the prevention or treatment of infections (Hermoso et al., 2007).

1.6.7 Antimicrobial peptides (AMP)

AMPs comprise a diverse range of antimicrobial, naturally occurring peptides of eukaryotic, bacterial or phage origin. Eukaryotic AMPs are naturally occurring cationic peptides possessing a net positive charge at physiological pH, which form pores in the cytoplasmic membrane, leading to loss of cell content (Parisien et al., 2008).

Bacteriocins are AMPs of bacterial origin with narrow killing spectra. Klaenhammer (1988) has proposed that if enough research was devoted to this area at least one bacteriocin could be discovered for each different bacterium.

Phage-tail like bacteriocins are bacterial produced peptides, with striking genetic and morphological similarities and common ancestry to phage tails (Damasko et al., 2005; Sicard et al., 2005). Two types of phage encoded AMPs are known: phage tail complexes and phage-encoded lytic factors. The latter have a similar function to the virolysin-holin system of phage enzymes namely the induction of bacteriolysis, however they operate via entirely different, non-enzymatic mechanisms. Phage tail complexes are large peptide subunits, capable of adsorption to sensitive bacteria,

diffusion through the outer membrane in Gram-negative cells, local lysis of peptidoglycan and injection of the phage genome into the host cell. Parisien et al. (2008) proposed that phage tail complexes may have a realistic potential as antibacterials.

1.6.8 Bacteriophages

Bacteriophages or phages are viruses, which only infect bacteria. Phages are absolute parasites; they carry all the genetic information necessary for reproduction yet are not capable of producing proteins or energy creation. They are the most abundant living entities on earth and are found in large numbers where their hosts reside, usually sewage, faeces, soil or water. Phages can be divided into virulent and temperate. Virulent phages can only multiply through a lytic cycle: the virion adsorbs to the bacterial surface through interaction of the phage tail fibres and a specific attachment molecule on the host cell surface, which makes phage attachment and infection of bacteria specific to its hosts. After attachment the phage injects the phage genome which takes over the bacterial host metabolism to produce more phage, leading ultimately to lysis and killing of the bacterial cells.

Temperate phages can switch between reproductive modes either lytic or lysogenic, where instead of replication the phage genome assumes a quiescent state (prophage) which is often integrated into the bacterial chromosome or sometimes remains in the bacterial cell as a plasmid. Temperate phages are considered unsuitable for phage therapy. Phages for clinical use must be virulent and non-lysogenic (Chanishvilli et al., 2001).

By their very nature bacteriophages appear to be ideal alternatives to antibiotics: they are usually specific to one bacterial species or strain, leaving the natural host flora undisturbed (Chanishvilli et al., 2001); they increase in titre during infection (Payne & Jansen, 2001) and are reputedly non-toxic to animals and plants (Chanishvilli et al., 2001).

The therapeutic use of these biological agents is a field of research that has acquired significantly increasing importance in recent years due to the advent of enhanced

bacterial resistance to antibiotics (Chanishvili et al., 2001). The question as to why phages are not routinely used as a therapeutic tool in bacterial infections has been raised (Summers, 2001).

1.6.8.1 Phage display

Heterologous peptide and proteins can be displayed on the surface of phages, through transcriptional fusion with a coat-protein gene (Smith, 1985). Multiple applications of phage display have been demonstrated including production and purification of proteins, antibodies, enzyme substrates and inhibitors (Smith, 1985; Benhar, 2001) for the detection of pathogens (Petrenko & Vodyanoy, 2003) and directed evolution of enzymes (Fernandez-Gacio et al., 2003).

1.6.8.2 Bacterial typing and detection

Bacteriophages have proven useful in the detection and susceptibility testing of *M. tuberculosis* (Carriere, 1997; Hazbon et al., 2003; Rybniker et al., 2006) *M. ulcerans* (Kramme & Small, 2006), *Listeria monocytogenes* (Loessner et al., 1996) and *Escherichia coli* O157:H7 (Goodridge et al., 1999).

1.6.8.3 Phage-delivered medicinal substances

1.6.8.3.1 Vaccines

Two potential uses of phage as vaccine delivery vehicles have been suggested; directly vaccinating with phages carrying antigens on their surface or by the use of phages delivering a DNA vaccine expression cassette in their viral genome (March et al., 2004; Clark & March, 2006). DNA vaccines have been shown to be more efficient delivery vehicles than standard plasmid DNA vaccination, as the DNA is protected from degradation by the phage coats (Jepson & March, 2004; Clark & March, 2006).

1.6.8.3.2 Antimicrobials

Filamentous phages, which are considered unsuitable for phage therapy, have been utilised for targeted antimicrobial drug delivery of toxic antibiotics such as chloramphenicol (Yacoby et al., 2007).

1.6.8.3.3 Genes

Filamentous bacteriophages, which have no tropism for mammalian cells, have been genetically engineered to deliver fibroblast growth factor specifically to monkey kidney cells (Larocca et al., 1999).

1.7 Bacteriophage therapy

Prior to the discovery of antibiotics and their widespread use bacteriophage therapy was a popular method to treat a variety of life-threatening bacterial infections. Felix D'Hérelle, a French-Canadian microbiologist treated a number of severe cases of dysentery with phage preparations, with clinical success as long ago as 1919. He continued using phage preparations in patients suffering from bubonic plague, cholera and other illnesses, regularly with apparently very good clinical results (D'Hérelle, 1926).

Following the discovery of antibiotics, the therapeutic use of bacteriophage was abandoned in the Western world, amid controversies over uncontrolled trials and wildly exaggerated claims of commercial bacteriophage preparations. Phages continued to be used solely or concurrently with antibiotics in the Soviet Union and Eastern Europe to treat infections in humans (Chanishvili, 2001; Sulakvelidze, 2001).

Media coverage and articles in the scientific and lay press from the 1980s onwards, combined with emerging pan-drug resistant bacteria have sparked renewed interest in phage research in the West. Bacteriophages have been identified as an option in the treatment of antibiotic-resistant infections (Barrow & Soothill, 1997; Alisky et al., 1998; Barrow, 2001; Chanishvili et al., 2001; Sulakvelidze et al., 2001; Summers, 2001; Taylor et al., 2002; Thiel, 2004; Kropinski, 2006). The prospect of phage therapy has

stimulated a large amount interest within the scientific community. Some scientists remain pessimistic, such as Projan (2004) who accuses scientists of creating „a cult of phage therapy followers“, claims the little animal efficacy data there is in the literature can charitably be described as meagre“, „.....this silence (on animal efficiency data) speaks volumes“ and „...anecdotal testimonials of former patients“.

Bacteriophage may offer a selective method for the decolonisation of the skin of infected burns patients and potentially a novel approach to treating topical and systemic infections. Preliminary attempts have been made to control *Pseudomonas* and other topical infections in burns patients (Soothill, 1994).

1.7.1 History of phage therapy

In 1896 Ernest Hankin reported the presence of antibacterial activity of filtered water collected from the river Ganges against *Vibrio cholerae* (Hankin 1896 cited by Sulakvelidze, 2001), followed two years later by Nikolay Fyodorovich Gamaleya, who reported similar observations in *Bacillus subtilis* (Hermoso et al., 2007). In 1915, Frederic Twort, who is credited with the discovery of bacteriophages, hypothesised that such antibacterial activity could be due to the presence of a virus (Twort, 1915; Twort, 1920; Duckworth, 1976; Dixon 2001).

Twort however, did not support his hypothesis by experimental work (Dixon, 2001) unlike Felix d'Hérelle, who (reputedly) independently from Twort, discovered phage in 1917 (Duckworth, 1976) and realised the potential of phage therapy. D'Hérelle recognized that the bacteriotoxic phenomenon was caused by a virus capable of parasitizing bacteria and termed these viruses „bacteriophages“ which literally means „bacteria eaters“.

1.7.2 Early Clinical application of phage therapy in scientific literature

It was D'Hérelle who recognised the clinical importance of his discoveries. He initially conducted a range of animal experiments and „safety“ studies involving administration of purified phage preparations on himself and members of his family and colleagues.

He successfully treated paediatric dysentery patients with phage preparations made by himself as early as 1919 (D'Hérelle, 1926). His approach was to produce bacterium-free filtrates of the patient's faecal samples, which were mixed with the bacterial strains causing the infections for enrichment and again purified, mainly through multiple filtrations, before administration (D'Hérelle, 1926).

D'Hérelle soon recognised that each bacterial infection may have a specific virus (Sulakvelidze, 2001; D'Hérelle, 2007) which may be used to treat such infections, a theory disputed for decades by his opponents, which may have contributed to the demise of phage therapy. The rapid translation of laboratory findings into the clinical field was typical of the Pasteurian approach (Summers, 2001) and was apparently supported by the director of the Pasteur Institute at the time, Emile Roux (D'Hérelle, 1926). Despite D'Hérelle prolific experimental work he failed to immediately publish his findings, which made Richard Bruynoghe and Joseph Maisin, who used phage to treat staphylococcal skin disease, the first authors of bacteriophage therapy in 1921 (Bruynoghe & Maisin, 1921).

The clinical success which led to D'Hérelle work being popularised were four cases of laboratory-diagnosed bubonic plague, which he treated in Egypt. Injection of phage preparations into the lymph nodes led to a remarkable recovery of the four men, which was published in a widely read journal „La Presse Médicale“ (Summers, 2001).

Due to this work the British government invited D'Hérelle to conduct further research on phage therapy on initially plague in India. This led to „The Bacteriophage Inquiry“ in India, concerned with phage therapy of cholera, under the patronage of the Indian Research Fund Association (Summers, 2001). The particular focus of this project was on combating regularly occurring cholera epidemics in India, which led to World Health Organization (WHO) sponsored studies in Pakistan (Pollitzer, 1959; Monsur et al., 1970; Marcuk et al., 1971). Both studies conducted in the 1970s concluded that bacteriophage was not as effective as tetracycline, however may have uses in eliminating *Vibrio cholerae* without affecting the intestinal flora. D'Hérelle found an eager co-worker in the Georgian scientist Giorgi Eliava, which led to the establishment of what is now known as the Eliava Institute in Tbilisi, Georgia in 1923 (Sulakvelidze,

2001; Thiel, 2004). This scientific union led to phage therapy being taken to a different level, away from application in individual cases, to teams of scientists (up to 1,200) investigating the application of phage in a multitude of infections and large scale productions of several tons a day (Sulakvelidze, 2001). D'Hérelle laboratory in Paris and the Eli Lilly Company (Indianapolis, Ind.) produced several phage preparations for commercial use, however, the efficacy of commercially available phage preparations were controversial, due to inability to understand the spectrum of phage activity and biology of the phage cycle (Barrow & Soothill, 1997; Sulakvelidze, 2001).

Phages were used by many investigators for a variety of bacterial infections and much work was published which may have been considered of appropriate standard at the time, however would not meet modern standards of clinical trials or case studies due to lack of thorough documentation. In 1933 Stout claimed "The medical profession is not yet well enough informed to make intelligent use of this form of therapy", warning that indiscriminate use of phage preparations would lead to a detriment of success of this principle. Such a large number of reports on phage therapy were published by the 1930s that the Council on Pharmacy and Chemistry undertook an evaluation of phage therapy. The report published in 1934 by Bayne-Jones and Eaton concluded with an ambiguous assessment of the literature published on phage therapy.

It was recognised in the report that positive as well as negative reports on phage therapy were found in literature, the main concerns were lack of understanding of the biological nature of bacteriophage, absence of standardised phage preparations or criteria for purity and potency; these factors aggravated the task of comparing the published studies (Eaton & Bayne-Jones, 1934). Under different circumstances this may have spurred investigators to conduct more research and apply the recommendations voiced by the report, however the dawn of the Second World War and the discovery of antibiotics led to efforts being focussed elsewhere. The situation was aggravated by Eliava being executed due to political changes in Georgia (Thiel, 2004) and D'Hérelle, who had returned to Europe fearing for his own life, being held under virtual house arrest in France (Summers, 2001), which silenced the most fervent pioneers of phage therapy.

1.7.3 Clinical applications of phage therapy

Whilst the use of antibiotics increased rapidly in the USA and Western Europe, phage preparations continued to be the therapeutic choice in the Soviet Union, possibly due to economic or ideological reasons (Summers, 2001). It was speculated that the concept of phage therapy may have become politically tainted in the post war period because of political differences between the paradigms of the Soviet science and the capitalist West (Summers, 2001; Kutter & Sulakvelidze, 2005). Phage therapy trials ceased in the USA and most of Western Europe after WWII, but remained in clinical use in the Soviet Union and parts of the Eastern bloc.

The clinical application of bacteriophages from Eastern Europe and the former Soviet Union is generally poorly documented. The early records of therapy (1920s-1940s) are either not documented in sufficient detail or do not fit modern standards of clinical trials or treatment protocols. More recent clinical research has either not been published at all or not published in Western European journals. Clinical work with various patient groups and disease states has been done in Georgia (Eliava Institute) and Poland (Ludwik Hirszfeld Institute). It is almost impossible to source this information which is generally published in non-English literature. The exceptions are a series of trials published in English containing insufficient details to allow inclusion into meta-analysis (Mulczyk & Slopek, 1974; Cislo et al., 1987; Kucharewicz-Krukowska & Slopek, 1987; Slopek et al., 1983, 1984, 1985(I, II, III), 1987; Weber-Dabrowska et al. 1987, 2000).

Most reviews and trial articles include brief details on phage therapy, diagnosis, patient numbers, investigators and outcome, unfortunately it rarely goes beyond that. Reviews by Alisky et al. (1998); Chanishvili et al. (2001); Sulakvelidze et al.(2001) and a textbook by Kutter & Sulakvelidze (2005) give an overview of the versatility of phage therapy and the ingenuity of some of the investigators, who seem to have used phage for almost any conceivable infection using unusual methods of delivery. Great care has been taken in these publications to provide information, which would otherwise be inaccessible. However, it becomes apparent that some of the authors writing reviews have not had access to the original reference source in a few cases, partly due to clinical studies being conducted and owned by the Soviet military, which kept results secret.

Many papers on use of phage in humans are of limited usefulness, due to different investigative standards, absence of details or control groups. More recently a small number of papers were published in English, regarding the successful clinical application of phages in animal experimentation.

1.7.4 Animal experiments

The efficacy of treating experimental bacterial infections with phage in animal models has been demonstrated for *Acinetobacter baumannii* (Soothill, 1992), *Clostridium difficile* (Ramesh et al., 1999) *Enterococcus faecium* (Biswas et al., 2002), *Escherichia coli* (Smith & Huggins, 1982; Smith & Huggins, 1983; Smith et al., 1987a,b; Merrill et al., 1996; Barrow et al., 1998;), *Pseudomonas aeruginosa* (Soothill, 1992; Soothill, 1994; Hagens et al., 2004; McVay et al., 2007), *Salmonella enterica* serovar Typhimurium (Berchieri et al., 1991; Merrill et al., 1996), *Staphylococcus aureus* (Wille et al., 2005) *Vibrio vulnificus* (Cervený et al., 2002).

Reviews of bacteriophages and plant pathogens were published by Okabe & Goto (1963) and Gill & Abedon (2003). Successful experimental treatments of bacterial infections with phage in aquacultured fish were reported (Nakai et al., 1999; Park et al., 2000; Nakai & Park, 2002; Park and Nakai, 2003).

1.7.5 Phage therapy in wounds- animal data

In a rabbit wound model infection induced by *Staphylococcus aureus*, simultaneous injection of 2×10^9 pfu (plaque forming units) of staphylococcal phage prevented abscess formation (Wills et al., 2005). The authors compared titres used in previous investigations (Smith & Huggins, 1982; Soothill, 1992) and postulated that protection with phage against Gram-negative infections may be even more effective than seen with *Staph. aureus*.

In a mouse model 10^2 pfu of *Acinetobacter* phage protected the animals against 5 LD₅₀ (1×10^8 cfu) of a virulent strain of *A. baumannii*, with phage titres multiplying in the mice during the experiment. Similar results were observed with *Pseudomonas aeruginosa* and corresponding phage, but not *Staph. aureus* (Soothill, 1992).

Similarly promising results were seen in a mouse burns wound model (McVay et al., 2007). Thermally injured mice were injected with fatal doses of rifampicin-resistant *P. aeruginosa* and single doses of a phage cocktail containing three *P. aeruginosa* phages.

Phage administration affected survival significantly; the route of administration was critical in the efficacy of treatment. The intraperitoneal route proved to provide the most significant protection.

The animal data most relevant to this investigation were presented by Soothill (1994) in a guinea pig wound model, using *P. aeruginosa* strain 3719, a clinical isolate from a UK burns unit. Full thickness and partial thickness defects were created by excision; comparable with that of excised burns wounds. Wounds were inoculated with 6×10^5 cfu of *P. aeruginosa* and 1.2×10^7 cfu of phage BS24, covered with excised skin and dressed, appropriate controls were conducted. Grafts treated with bacteria were significantly protected from graft failure by application of the phage.

1.7.6 Phage therapy in wounds –human data

In 1921 Bruynoghe and Maisin (cited in Sulakvelidze et al. 2001) used bacteriophages to treat staphylococcal skin disease in humans, by injecting bacteriophage into and around surgically opened lesions. The authors reported a regression of infection within one to two days. Military bearing the German and Soviet Union flags utilised phage extensively to treat the war-wounded (Summers, 2001) during WWII. Summers (2001) claims that phage therapy was almost immediately applied to wound infections after the discovery of phage, particularly staphylococcal infections, including acute traumatic and surgical wounds, refractory skin ulcers and osteomyelitis with reports of favourable outcomes. Cislo, et al. (1987) described the treatment of suppurative skin infections in 31 patients; the bacterial isolates were mixed and some patients experienced poly-infections. The phage was applied topically as moist application on skin lesions three times daily and additionally administered orally three to four times daily. The patients were given substances to neutralise the gastric acid before oral phage application. Patients with trophic leg ulcers received vasodilators to facilitate topical phage therapy. At the time of bacteriophage treatment none of the patients used any antibiotics or local

disinfectants. The authors report outstanding therapeutic results in 16 cases, marked improvement in seven and transient improvement in two. Seven patients dropped out of the study due to nausea and vomiting or other complaints. These results appear impressive, however the authors do not disclose success criteria. Unfortunately this study does not appear to be designed as a clinical trial, with no mention of matching the patient population with another group who received gold-standard antibiotic treatment.

The paper does not mention who assessed the therapeutic progress of the patients, which success criteria were used, and it does not give sufficient detail about the past medical history of all patients included in the study. Additionally the patient population was too small and too heterogeneous to allow meaningful statistical analysis of the results.

Slopek, et al. (1983) report clinical results for 138 patients treated with bacteriophage for various infections ranging from immunological disorders to pericarditis, chronic bronchitis, pneumonia, gastrointestinal and urinary tract and other infections. The paper demonstrates the versatility of phage treatment, but due to the breadth of different types of infections in the report there was a lack of attention to fine detail. The assessment criteria were not clearly defined, which rendered the data difficult to interpret. Individual medical histories of patients were generally not discussed, except for cases which were described as difficult to evaluate. Unfortunately the authors include disease states of different severity in this study for example otitis media and pericarditis and did not define what constituted a satisfactory result in each case. The authors included chronic and acute infections, mono- and poly-infections and infections previously treated with antibiotics in the study. In some cases the authors claimed that antibiotic therapy was ineffective, without giving further detail on chosen antimicrobial therapy, administration route, doses or treatment duration. It was also unclear if an unsatisfactory response to antibiotics was based on clinical observations or microbiological results and if antibiotic administration was supervised. Unfortunately no control group was included in the study receiving only antibiotics or a concurrent combination of antibiotics and phage. The authors may have considered one option unethical or not cost effective, the motivation for treatment choices was not discussed. Details about the

patient population were not clarified, which only allowed assumptions that all patients concerned were adults and constituted a mixed age and gender population. It was also unclear how the patients were recruited into the studies, how many individuals were involved in making a diagnosis, what their qualifications were and who assessed disease progress based on which criteria.

Weber-Dabrowska et al. (2001) claim that bacteriophages have been used since 1987 in the L. Hirszfeld Institute of Immunology in Poland to treat more than 1400 patients. The team described 20 cases of cancer patients, 17 with solid tumours, three with haematological malignancies who experienced concurrent bacterial infections.

All patients had already been treated with antibiotics without apparent response. Bacteriophages were administered orally three times daily and some patients received topical phage treatment additionally. The reported outcome was good in 17 patients and very good in three patients. Assessment criteria for positive outcome were given as cessation of suppuration, closure of wounds, and eradication of pneumonia. It was claimed that bacteriophage can not only cure bacterial infections, but may also upregulate immune response in patients (Weber-Dabrowska et al., 2001).

Thirty cases of documented *Pseudomonas aeruginosa* burn wound sepsis treated with phage preparations were described in another study (Abul-Hassan et al., 1990). The authors provided details of age, burn surface area and gender.

Unfortunately, ongoing therapy and depths of burns were not reported. It would have been helpful if detail about the cause of burn, surgical interventions taken, type of dressings and topical antimicrobials used had been included. According to the authors all *Pseudomonas* infections treated with phage showed resistance to all available antibiotics and chemotherapeutic agents. Unfortunately there was no further information to support this statement, as it was not clear which antibiotics were available and if therapeutic doses had been used previously. It was also not clarified if antibacterial resistance to antibiotics was established by clinical observation or culturing the microorganism, although it was reported that swabs were taken. Gauze soaked in phage preparation was applied three times daily, phage was not administered orally.

Assessment of a positive outcome was based on post-phage cultures, temperature, and general appearance of the patient, local condition of the raw area, discharge and graft take. As a point of criticism temperature is not always a reliable indicator of infection in burns patients and „general appearance“ could be considered a subjective indicator of improvement. In 18 cases graft take was described as „with good result within a reasonable period of time“. The authors claimed that phage stock takes a long time to prepare and was expensive, however the authors did not elaborate on the origins of the phage preparation used or how it was prepared.

Clinical reports of the benefit of phage therapy in wound infections exist, which are either written in a fashion which does not allow scientific evaluation of the data (Häusler, 2003) or are not accessible in Western Europe like Kutter & Sulakvelidze (2005) citing Kochetkova et al., (1989) with 131 cancer patients with post-surgical wound infections (including staphylococci and pseudomonads).

65 patients received phages, as monotherapy or in conjunction with antibiotics, the rest received antibiotics. Phage treatment was successful in 82%, antibiotic therapy in 61%.

Ahmad (2002) hypothesised that bacteriophages are highly suitable and can be effectively used to treat post-burn infections.

1.7.7 Safety of bacteriophage

To date there is no published study formally investigating the cytotoxic properties of bacteriophage. Much has been speculated as to the safety of phage with some authors asserting that bacteriophages are likely to be safe agents, as they are constantly present in the environment in high quantities (Kutter & Sulakvelidze, 2005). Bacteriophages are present in clean water at approximately 2×10^8 pfu/ml (Bergh et al., 1989), with the total number of phages present on earth to be estimated at 10^{30} - 10^{32} (Brüssow & Hendrix, 2002).

Phages are considered part of the normal human commensal flora, and are found in samples taken from skin, urine, saliva, dental plaque and the gastrointestinal tract (Merril et al., 1972; Geier et al., 1975; Milch & Fornosi, 1975; Moody et al., 1975), yet

therapeutic application of concentrated phage preparations, with systemic exposure may have different effects to environmental exposure.

In the US one phage preparation used as veterinary vaccine, which was licensed for human use from the 1950s-1990s, contained confirmed active phage at 10^8 - 10^9 pfu/ml (Kutter & Sulakvelidze, 2005). During safety trials and clinical use no anaphylactic reactions were reported (Kutter & Sulakvelidze, 2005).

An assessment of the safety of phage T4 administered orally to 15 healthy volunteers led to the conclusion that no significant adverse effects were reported, and serum transaminase levels remained normal (Bruttin and Brüssow, 2005).

There is some evidence suggesting that phage therapy is associated with modulated cytokine production and immune stimulation (Weber-Dabrowska et al., 2000; Weber-Dabrowska et al., 2002).

The report of one patient experiencing liver area pain whilst being treated with a phage preparation has led to the speculation that side effects of phage therapy are attributed to the *in vivo* lytic activity of phages, leading to the extensive release of endotoxins from the lysed bacteria causing the infection (Slopek et al., 1987; Kutter & Sulakvelidze, 2005). No *in vitro* evidence supporting the notion that phage leads to significantly higher quantities of endotoxins than antibiotics has been published.

The safety of bacteriophage needs to be considered separately to the safety of individual phage preparations, which may contain chemical impurities or high levels of endotoxin (Naidu, 1932), due to insufficient purification methods.

L. monocytogenes phage has been approved for use in food and is considered GRAS (Generally Recognized as Safe) by the FDA (GRAS Notice No. GRN 000218, Agency Response Letter, FDA, June 22, 2007), however this only implies a reasonable certainty that a substance is not harmful under the intended conditions of use. The same principle may not be automatically applied to phage therapy which employs concentrated phage preparations used by additional routes to oral administration.

1.7.8 Cytotoxicity testing

Cultured skin substitutes like CEAs, which are used to cover and close burns wounds have been used as alternatives to animal testing for toxicological and pharmacological testing (Boyce, 2004). *In vitro* cultures of fibroblasts or keratinocytes have been used as models to investigate the proliferative and cytotoxic potential of therapeutic agents, including measurements of cytokines involved in wound healing (Burch & McMillan, 1991; Zhang et al., 1994).

1.8 Bacteriophage isolation

Different methods have been used to isolate various bacteriophages from a variety of samples, for brevity only a few methods and sampling sources will be mentioned here. There is no single repeatable specific method for the isolation of bacteriophage which will lyse clinical isolates. Protocols for general phage isolation are available, however other factors like operator know-how, seasonal changes and quality of samples can affect the outcome of any isolation attempt.

Bacteriophages can be isolated wherever phage-specific bacteria are present: from bodily excretions, environmental sources such as soil, waste or sea-water and other sources. In some cases discovery of bacteriophages may be accidental and undesirable (Dallas, 1997; Randerson, 2003). Scientists intending to test a range of antibiotics on *E.coli* O157:H7, faced a problem when the bacteria disappeared rapidly from the infected sheep tissue due to the presence of bacteriophages (Randerson, 2003).

Dallas (1997) described the undesired isolation of plaques on primary isolation media of a urine culture growing *E. coli*. These observations demonstrate that phages can be isolated in a clinical setting together with the host and emphasises the importance of establishing a communication link with local microbiologists, who can detect the presence of phages in clinical samples and may be able to supply them for further research.

Lactobacillus plantarum bacteriophage with an isometric head (59 nm in diameter), a 182 nm long non-contractile tail and a complex base plate could be isolated using a spot

test method to detect lytic activity of phage, requiring only small volumes of sample (Lu et al., 2003). 3ml of soft agar were seeded with 0.1ml of host culture, mixed gently and poured onto an agar plate. After solidification of the top agar 10 µl of phage lysate were spotted onto the surface. After drying, the plate was incubated overnight, a clear zone indicated the presence of lytic phage. The authors employed the technique of successive single plaque isolation, whereby a single plaque was picked from the lawn, inoculated directly into a host culture and the lysate plated again. Lysate was centrifuged at 4000xg for 10 minutes at 4°C and filtered using a 0.45-µm pore size syringe filter. Phage stock was stored with chloroform (5% by volume) at 4°C, and aliquots were frozen at -84°C in broth containing 16% glycerol.

Rubires et al. (1998) centrifuged sewage and enriched the supernatant with a bacterial host culture using several strains independently. After overnight incubation, bacteria were removed by centrifugation and filtration; the authors do not provide details on centrifugation force, filter type and size. The filtered supernatant liquids were plated with the host strain using the soft agar overlay method. Plaques were stabbed and eluted with a small volume of phage buffer.

The same authors described an alternative method to isolate bacteriophage resistant mutants, despite using the spot test to determine the host range. A mixture containing 10^8 cfu of bacteria and 10^9 pfu of phage was spread on TSA, incubated at 30°C for 48 hours. Mutants were picked, purified by streaking and cross-streaked against the same bacteriophage to confirm resistance. Borrego et al. (1991) mentioned various methods to isolate bacteriophages from aquatic environments, such as the direct assay, enrichment techniques, polyethylene glycol precipitation, adsorption and elution and differential centrifugation. The latter method introduced by the authors was apparently suitable to detect low numbers of phages in environmental samples, by concentrating phage involving an adsorption and elution stage. Various electronegative and –positive filters were used; aliquots of one litre were passed through the various filters then eluted using 10ml of a beef extract supplemented with 1 M NaCl and by the drop-by-drop method, which involved passing the eluent through the filter at 0.5ml/min using positive pressure. The authors conclude that the best method for the adsorption and recovery of

the phage was a DE-plus-Nalco filter placed on epoxyfiber glass or Virosorb 1-MD filters as holders and elution by the drop-by-drop method. This particular method was considered optimal when environmental water, hospital or domestic waste water was used for virus isolation. This method is limited by presence of organic matter which is generally found in waste water.

Kutter & Sulakvelidze (2005) described various methods for phage isolation from natural sources, which include direct plate selection similar to Lu et al. (2003) whereby samples were spotted onto bacterial lawns, plaques are removed and eluted in buffer and used to infect the host bacteria. Kutter & Sulakvelidze (2005) listed classic enrichment protocols where samples are either incubated with the indigenous host or after addition of one or several bacterial hosts in 10-times concentrated broth. Incubation conditions are generally dictated by the growth requirements of the host. Following incubation the samples were centrifuged and the supernatant which was expected to contain phage spotted onto lawns of host bacteria.

1.9 Thesis Aim and Objectives

1.9.1 Aim

Exploration of the potential for bacteriophage to be used as a suitable therapeutic tool in the treatment of *Acinetobacter* spp. colonisation or infection, particularly in burns wounds.

1.9.2 Objectives

- Acquisition of clinically relevant *Acinetobacter* spp. isolates from local burns and ICUs
- Initiating an *Acinetobacter* spp. culture collection at the University of Brighton
- Isolation or acquisition of phage material specific for *Acinetobacter* spp.
- Matching of bacterial host and phage pairs
- Exploration of phage breeding protocols

- Exploration and refinement of phage purification protocols
- Production of purified, concentrated phage preparations of suitable stability, with a view to potential clinical use
- Testing the ability of available phages to kill
- Exploration of some phage-host specific parameters and interactions and selection of at least one phage and host pair as a model system, reflective of clinical use
- Isolation and culturing of primary human dermal fibroblast and keratinocytes
- Development of several cytotoxic models involving cell lines
- Development of several cytotoxicity models, involving human primary dermal cells used in burn wound grafting

Chapter TWO

Materials and Methods

2.1. Materials and Equipment

2.1.1 Chemicals and Microbiological Media

All chemicals used were of analytical grade or ultrapure and obtained from Sigma (Poole, England), and Fisher Scientific UK (Loughborough, England) with the following exceptions: NaCl was purchased from Acros Organics (Geel, Belgium) mannitol and phenol red from BDH Chemicals Ltd., (Poole, England).

Antibiotics for the production of Leeds Acinetobacter medium (LAM) and modified Leeds Acinetobacter medium (MLAM) were licensed products suitable for human intravenous injection: Vancomycin HCl for injection BP, Eli Lilly (Basingstoke, England), ceftazidime pentahydrate for injection, GlaxoSmithKline (Uxbridge, England), cefradine arginine blend for injection, E.R.Squibb & Sons Ltd., (Hounslow, England) and marketed by B.My.Squibb (Dublin, Ireland). Cefsulodin was kindly provided by Grünenthal GmbH (Aachen, Germany) as a free sample.

Dried media and media components, including technical grade agar (Agar Technical No.1 & 3), were purchased from Oxoid Ltd., (Basingstoke, England) and subsequently from Fisher Scientific UK (Loughborough, England). Tryptone soya agar (TSA), tryptone soya broth (TSB), Isosensitest agar (ISA) and Isosensitest broth (ISB), phosphate buffered saline (PBS), were prepared following the manufacturer's (Oxoid) instructions. Reconstituted powders were autoclaved at 121°C for 15minutes.

Whenever water or sterile water is mentioned in the text it refers to demineralised water.

Whenever autoclaving is mentioned in the text it refers to steam sterilisation where a pressure of 103 kPa (15 psig) and temperatures of 121°C are maintained for at least 15 minutes.

Lambda buffer was prepared using the following constituents:

6ml 1M Trizma Base, 2.5ml of 2%w/v gelatine dissolved in water
and 2.5g MgSO₄·7H₂O.

The volume was made up to 1l with water using a volumetric flask, the contents were dissolved, dispensed into 100 or 200 ml glass bottles with plastic caps and sterilised by autoclaving at 121°C for 15 minutes.

1M Trizma base (tris[hydroxymethyl]aminomethane) was prepared by adding 12.11g of Trizma base (Sigma) to 100ml of demineralised water. The pH of this preparation was adjusted to 7.2 with HCl and autoclaved at 121°C for 15minutes.

2.1.2 Equipment-Microbiology

Optical density measurements were routinely made using a Unicam, Helios ε, Unicam (Cambridge, UK).

Repeated optical density readings of samples in 100 well plates were taken by Bioscreen-C (Growth curves USA, NJ, USA), routinely set to 600nm.

Centrifugation of volumes up to 50ml was carried out in a Centaur 2 MSE centrifuge, Sanyo Electric Biomedical Co., (Sakata, Gunma, Japan) at 2011g. Fisherbrand 50ml polypropylene centrifuge containers were used with the Centaur 2 centrifuge, even when small amounts of chloroform were contained in the samples. Liquids containing more than 0.5ml of chloroform were centrifuged using 50ml IWAKI centrifuge tubes (Asahi technology, Chiba, Japan).

For lysate production and purification a Sorvall RC-5B Refrigerated Superspeed Centrifuge (Du Pont Instruments) in conjunction with a GSA rotor was used. In the final step of purified lysate production a refrigerated ALC 4237R refrigerated centrifuge, ALC International Srl., (Cologno Monzese, Italy) was used. The speed was always

1000g. All lysates were stored in clear glass containers or when ready diluted in Eppendorf tubes at $4^{\circ}\text{C} \pm 1^{\circ}\text{C}$.

Cell suspensions and liquids which required incubation and agitation at 25°C were routinely incubated in an Innova 4230 refrigerated incubator using an Innova 2000 Platform shaker, Brunswick Scientific (St. Albans, Hertfordshire) at 120rpm.

Samples requiring incubation and agitation at $30\text{-}37^{\circ}\text{C}$ were routinely incubated in a Jencons PLS shaking platform incubator (Jencons Scientific Ltd., Leighton Buzzard, Bedfordshire) at 120rpm.

For syringe filtrations Minisart Sartorius AG (Göttingen, Germany) single use sterile filter unit, non-pyrogenic, $0.45\mu\text{m}$ average pore size, were used in conjunction with Plastipak sterile syringes, Becton-Dickinson (Franklin Lakes, NJ, USA) (10ml Luer lock), and Terumo Neolus (Leuven, Belgium) sterile needles, (Nr.2, luer $21\text{G}\times 1.5''$, 0.8×40).

For pouring agars and drying agar plates a Microflow Horizontal Laminar Flow workstation was always used.

2.1.3 Chemicals and Materials-Cell culture

Trypan Blue solution for microscopy $0.4\%w/v$ containing $0.81\%w/v$ sodium chloride and $0.06\%w/v$ potassium phosphate (Fluka, Buchs, Switzerland, through Sigma-Aldrich, Gillingham, UK) was routinely used for the Trypan Blue counting technique.

Hoechst propidium iodide stain (HPI) was produced by mixing $900\mu\text{l}$ of cell medium with $50\mu\text{l}$ of a propidium iodide working solution (1mg/ml in 10ml demineralised water) and $50\mu\text{l}$ of bisbenzimidazole (Hoechst 33342), Sigma-Aldrich, Poole, Dorset.

Trypsin EDTA liquid $0.05\%w/v$ Cambrex, BioWhittaker, East Rutherford, NJ, USA was always used to trypsinise cells at the University of Brighton. Trypsin EDTA (10X) liquid $0.5\%w/v$ BD Difco, Becton, Dickinson and Company, Oxford, UK, was used once during the isolation of keratinocytes only. Trypsin EDTA (1X), liquid 0.05% Gibco, Invitrogen, Paisley, UK, was used for keratinocyte and fibroblast passaging at the Blond McIndoe Centre, Queen Victoria Hospital, East Grinstead.

DMEM (Dulbecco's Modified Eagle's Medium) with 1000mg glucose/L, L-glutamine and sodium bicarbonate (Sigma Aldrich, Gillingham, UK) supplemented with 10% foetal calf serum (FCS), Biosera, Ringmer, UK 1% (v/v) penicillin and streptomycin (Sigma Aldrich, Gillingham, UK) was routinely used for cell cultivation of 3T3 and V79 cells, unless otherwise stated. DMEM+1000mg/l of glucose, Glutamax, pyruvate (Gibco, Invitrogen, Paisley, UK) and 10% FCS, (Biosera, Ringmer, UK) without antibiotic supplementation was exclusively used for 3T3 cells grown at the Blond McIndoe Centre, Queen Victoria Hospital, East Grinstead.

Penicillin-streptomycin (Pen/Strep), containing 10,000 units of penicillin (base) and 10,000 µg of streptomycin (base)/ml utilizing penicillin G (sodium salt) and streptomycin sulphate in 0.85%w/v saline, Gibco, Invitrogen, Paisley, UK was used for initial keratinocyte isolation.

Hanks' Balanced Salt Solution (HBSS) (1X) liquid (Gibco, Invitrogen, Paisley, UK) was used in cell culture work with human dermal cells at the Blond McIndoe Centre.

HBSS (1X) liquid (Sigma Aldrich, Gillingham, UK) was used in cell culture work with human dermal cells at the University of Brighton.

Dispase stock solution was prepared from the non-sterile, lyophilized *Bacillus polymyxa* enzyme (Gibco, Invitrogen, Paisley, UK), which was dissolved in PBS (Ca²⁺/Mg²⁺-free) to produce a stock concentration of 100mg/ml or 0.95units/mg. This was filter sterilised using a 0.22 µm filter membrane.

Rheinwald Green medium (modified after Rheinwald & Green, 1975) was exclusively used for the cultivation of Keratinocytes and was prepared using the following protocol:

One bottle of DMEM 500ml Gibco, Invitrogen, Paisley, UK, was freshly opened and the following ingredients added:

- 100ml F-12 Nutrient Mixture (Ham) (1X), liquid - with L-glutamine, (Invitrogen, Paisley, UK)
- 200µg Hydrocortisone (Sigma, Poole, England)
- 100ml FCS (Biosera, Ringmer, UK)

- 5µg Recombinant Human Epidermal growth factor, (Invitrogen, Paisley, UK)
- 5ml of a 1×10^{-10} M working solution of recombinant *E. coli* cholera toxin, azide free (Gentaur Molecular Products, Brussels, Belgium).

2.1.3.1 Plasticware

Standard culture vessels for cell culture were always sterile T25, T75 culture flasks with filter cap or 24-well plates suitable for tissue culture by Nunclon, Nunc, Roskilde, Denmark, unless otherwise indicated.

Sterile Cellstar 25 and 75cm, standard cell culture flasks, Greiner Bio-One, Stonehouse, Gloucestershire, UK were used for keratinocyte culture and growth of 3T3 feeder layers at the Blond McIndoe Centre.

50ml BD Falcon conical sterile centrifuge tubes, Becton, Dickinson and Company, Oxford Science Park, Oxford, UK were routinely used.

2.1.4 Equipment- Cell culture

Routine cell culture work was carried out under sterile conditions in a class II cell culture laminar flow cabinet (Cyttox II, Envair Ltd., Lancaster, UK).

Incubation of cells routinely took place in a humidified Heraeus Heracell, Thermo Fisher Scientific, cell incubator in air with 5% CO₂ saturation; Primary material transferred from Blond McIndoe Centre, Queen Victoria Hospital, East Grinstead was incubated at 10% CO₂ saturation. Incubation of primary cells at the point of isolation at the Blond McIndoe Centre, Queen Victoria Hospital, East Grinstead routinely took place in a humidified Leec Research GA3000 cell incubator (Leec, Cardiff, UK) in air with 10% CO₂ saturation, at 37 °C.

Cell centrifugation of volumes up to 1.5ml was undertaken in a Biofuge Pico, Heraeus, Thermo Fisher Scientific at 400g for 5 minutes.

Cell centrifugation of volumes above 1.5ml was undertaken in a Multifuge 3s, Heraeus, Thermo Fisher Scientific at 500g for 5minutes. Centrifugation at the Blond McIndoe

Centre, Queen Victoria Hospital, East Grinstead was routinely undertaken using a Jouan B4i centrifuge, Thermo Fisher Scientific, Basingstoke, UK.

For γ -irradiation of 3T3 cells a Gammacell 1000 (Isomedix Inc., Parsippany, New Jersey, US) Cs137 source was used set to dose-factor 100, emitting 3,000 rads.

Nalgene Cryo 1°C 'Mr Frosty' (Nalgene, Rochester, NY, USA) freezing containers were used for freezing of cell samples

96-well plates were routinely read in an ICN Flow Titertek Multiskan plus MKII plate reader (ICN Flow, Herts, UK), at 492 nm.

For sonication of bacterial cell samples a Sonicolor Ultrasonic Processor XL (Misonix, USA) was used.

2.1.4.1 Microscopy

For routine tissue culture microscopy an inverted light microscope (Wilover, Will, Germany) at 100 x magnification was used. For Hoechst Propidium Iodide stained samples a Karl Zeiss, Axiovert 25 (Karl Zeiss Ltd., Welwyn Garden city, UK), inverted fluorescence microscope with 420nm filter was used. For photography of tissue culture samples a Nikon Eclipse, TE 200-U inverted phase contrast light microscope and Nikon D1x digital camera using 400 x magnification were used (Nikon UK Ltd., Surrey, UK).

2.1.5 Molecular biology kits and bacterial identification kits

For interleukin assays measuring levels of IL-1 β , IL-6, IL-8 and TNF- α BD OptEIA Elisa kits BD Biosciences (San Diego, CA) were used according to manufacturer's instructions in conjunction with IWAKI 96-well Elisa plates (Asahi technology, Chiba, Japan). To measure endotoxin levels in samples a Limulus Amoebocyte Lysate QCL-1000 kit was used according to manufacturer's instructions (Cambrex, BioWhittaker, East Rutherford, NJ, USA).

For the identification of bacteria API20NE and API20E kits bioMérieux (Lyon, France) were used according to manufacturer's instructions.

For MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, USA) was used according to manufacturer's instructions.

For LDH assay CytoTox 96 Non-Radioactive cytotoxicity assay, Promega, Madison, USA was used according to manufacturer's instructions

2.2 Bacterial strains and phage material

Microbiological cultures used are listed according to their place of origin and are shown in chronological order of receipt.

2.2.1 Clinical isolates of *Acinetobacter* species.

Strain short codes
R1973
R2751
R3417
R4474
R45502
R46383
W5211
W6108
W6492

Table 2.1 List of 10 *Acinetobacter* species donated by Surrey and Sussex NHS Trust

Ten clinical isolates of *Acinetobacter* species (**Table 2.1**) were received in frozen form from Dr. J. A. Child, Consultant Microbiologist at the Surrey and Sussex NHS Trust at time of receipt. Strain R3834 was not viable and could not be propagated. Strain short codes were assigned by the originator. Some isolation history and disc sensitivity

results, without specific instructions on methods used, were provided with the material (Appendix 2).

2.2.2 *Acinetobacter haemolyticus* (Leiden strain) and corresponding bacteriophage

A strain of *Acinetobacter haemolyticus* designated (HER1424) and the corresponding bacteriophage (Table 2.2) were received from the Department of Biochemistry, Gorlaeus Laboratories, Leiden University, PO Box 9502, 2300 RA Leiden, The Netherlands with corresponding reference: J. Klovins, G. P. Overbeek, S. H. E. van den Worm, H.-W. Ackermann and J. van Duin, Journal of General Virology (2002), 83, 1523-153.

Strain short codes	Matching phage material received
HER1424	AP205 (HER423)

Table 2.2 *Acinetobacter haemolyticus* strain and phage donated by Leiden University

The bacterium was received at ambient temperature in transport agar, and the bacteriophage as an unpurified, but filtered liquid lysate.

The authors have reported that this phage and host-pair are held in the Félix d'Hérelle Reference Centre for Bacterial Viruses under the accession numbers HER424 and HER1424 respectively, phage material is routinely referred to as AP205.

2.2.3 Microbiological samples obtained from the Pasteur Institute, Paris.

A range of microbiological samples (**Tables 2.3-2.5**) was donated by the Collection Nationale de Cultures de Microorganismes, Institut Pasteur, 25 rue du Docteur Roux, Paris F-75724 Cedex 15, France.

Strain short codes
Ac 76.426 E20 1989
Ac 74.97 E4 1989
Ac 75.53 E13 1989
3 B Ac 75.126 E14 1986
E2/Ac 74-94(2) 1995
Ac 75.37 E11 1989
E1/Ac 74-93(41) 1995
Ac. 74.107(phE7) 1995
Ac. 74.110 E8 1989
Ac 74.101(phE6) 1995

Table 2.3 Bacterial isolates obtained from the Pasteur Institute, Paris.

The bacteria (*Acinetobacter* species) of uncertain provenance had been maintained for a number of years at room temperature on agar slopes. Phage liquid lysates contained in sealed cryovials, with some numbering on lids in black ink from 1-8, 12-15, numbering in red ink on lids ranging from 3-4, 8, 11-12, 20. Short codes are presented as written on each sample. One preparation was contained in a sealed glass vial labelled ‘Hλ .φGP 5.9.72’. Bacterial strains and phage material were obtained, however due to lack of

documentation it was unclear which strains belonged to which phage, or if the strains and phage material had ever been matched. Bacterial strains Ac 74.98 E5 1989 and E15 Mx. 75.127 2.78 could not be propagated.

Number	Bacteriophage samples
-	Hλ .φGP 5.9.72
1	φn°1 <i>Ac. johnsonii</i> 88-26
2	φn°2 <i>Ac. johnsonii</i> 88-22
3	φn°3 <i>Ac. johnsonii</i>
4	φn°4 <i>Ac. johnsonii</i>
5	φn°5 <i>Ac. johnsonii</i>
6	φn°6 <i>Ac. johnsonii</i>
7	φn°7 <i>Ac. johnsonii</i>
8	φn°8 <i>Ac. johnsonii</i>
12	φn°12 <i>Ac. johnsonii</i>
13	φn°13 <i>Ac. johnsonii</i>
14	φn°14 <i>Ac. johnsonii</i>
15	φn°15 <i>Ac. johnsonii</i>

Table 2.4 Bacteriophage samples obtained from the Pasteur Institute, Paris

Number	Bacteriophage samples
3	φ <i>Ac. johnsonii</i> n°3 / <i>Ac. baumannii</i> 88-231
4	φ <i>Ac. johnsonii</i> n°3/ <i>Ac. baumannii</i> 88-167
8	φ <i>Ac. johnsonii</i> n°8 / <i>Ac. baumannii</i> 88-167
11	φ <i>Ac. johnsonii</i> n°11/ <i>Ac. baumannii</i> 88-126
12	φ <i>Ac. johnsonii</i> n°12/ <i>Ac. baumannii</i> 88-154
20	n°20/ Ac 86.104 <i>Ac. Johnsonii</i>

Table 2.5 Bacteriophage obtained from the Pasteur Institute, Paris

2.2.4 Microbiological samples obtained from Laval University, Canada.

Strain short codes	Phage
HER1032	HER32531
HER1033	HER33 A3/2
HER1050	HER50 A10/A45
HER1096	HER96 B9PP
HER1151	HER151 E13
HER1156	HER156 E14
HER1162	HER162 B9GP
HER1401	HER401 BS46
HER1423	HER423 133
HER1425	HER425 2213173

Table 2.6 Microbiological samples obtained from Laval University, Canada

Microbiological samples of various *Acinetobacter* strains and bacteriophages (**Table 2.6**) were received from the Félix d'Hérelle Reference Centre for Bacterial Viruses, Department of Microbiology, Medical Faculty, Laval University, Québec, Canada G1K 7P4, with corresponding reference: H.-W. Ackermann, G. Brochu and H. P. Konjin, Archives of Virology (1994), 135, 345-354.

2.2.5 Long-term storage of *Acinetobacter* spp

All bacterial samples were streaked onto TSA plates to obtain single colonies and were incubated for 16-18 hours, standard incubation temperatures were at 37°C or 25°C unless otherwise stated.

Three colonies were removed from freshly incubated TSA plates, subcultured into 100ml of TSB in 250ml conical flasks with loose fitting lids and incubated at optimum growth temperature (routinely 37°C) for 16-18 hours, whilst being aerated at 120rpm on a shaking platform. After incubation 10% sterile glycerol (v/v) was added aseptically and 1ml aliquots were dispensed into screw-top cryovials, which were frozen overnight at -10°C and transferred afterwards to a -80°C freezer for long-term storage.

2.2.6 Routine subculture of *Acinetobacter* spp

Specimens of each strain were kept frozen at -80°C (10% v/v glycerol in TSB) in cryovials. Stock cultures were made by defrosting one cryovial each month, using one loopful to streak onto four TSA plates for single colonies, incubating at 37°C (unless stated otherwise) for 16-18 hours. The resulting plates were sealed and kept refrigerated at 4°C for 4 weeks. The agar plates were subcultured weekly and then discarded.

2.2.7 Routine culture of overnight suspensions of *Acinetobacter* spp.

Bacterial inocula were prepared from one single isolated colony and were vigorously dispersed in 10ml TSB. Cells were grown for 16-18 hours in loosely capped glass universal bottles on a shaking platform rotating at 120rpm. Standard incubation temperatures were at 37°C, with exceptions of HER1424 and HER1423, which were routinely incubated at lower temperatures of 25°C in an Innova 4230 Refrigerated Incubator shaker, New Brunswick Scientific, NJ, USA.

2.3 Preparation of agar plates and overlay agar

2.3.1 Preparation of TSA plates for viable counts

TSA was prepared following company instructions, autoclaved at 121 psi for 15 minutes and allowed to cool to 50-60°C in an incubator. TSA was poured into plastic dishes and allowed to dry for 10 minutes in a laminar flow cabinet (ambient temperature 17-19°C). Drying times were adjusted accordingly, if ambient temperature was higher or lower.

Plates were then transferred to a drying cabinet and overdried for 25 minutes on 'full' setting, until the agar surface showed typical wrinkled appearance.

In cases where the drying cabinet was unavailable plates were placed in an incubator set to 37°C for 2-3 hours and used imminently.

2.3.2 Preparation of TSA plates for use with soft agar overlays (agar overlay method)

TSA was prepared following the manufacturer's instructions, autoclaved at 121 psi for 15 minutes and allowed to cool to 50-60°C in an incubator.

TSA was poured into plastic Petri dishes and allowed to set for 10 minutes in a laminar flow cabinet. Plates were stored in plastic bags at ambient temperature and used within 48 hours of preparation. In cases where ambient temperature was above 19-21°C, plates were stored overnight at 4°C and allowed to equilibrate to ambient temperature 2 hours before use.

2.3.3 Soft agar

Soft agar was prepared by mixing 0.8g technical agar No. 3 (Oxoid) with 100ml purified water which was heated until the agar was dissolved. 6g of TSB was dissolved in 100ml purified water, warmed up to 50°C and combined with the dissolved agar. The product was stirred, filled into 15ml bijoux glass bottles in 5ml increments and autoclaved at 121°C for 15 minutes. This method was used because mixing the two components before heating resulted in caramelising of the sugars in TSB before the agar

had dissolved. Mixing the two ingredients without heating the technical agar to boiling point resulted in inconsistent soft agar viscosities and variable plaque numbers. Using different size glassware and closely fitted metal caps routinely led to condensation and did affect agar quality and consequently result variability.

2.3.4 Soft agar overlay method

A routine culture of overnight suspension of *Acinetobacter* spp. was produced (**Section 2.1.4.3**) and the optical density OD₆₀₀ adjusted to 0.5 using sterile TSB (**Chapter 5**).

5ml of previously prepared cooled sterile soft agar (0.4% w/v technical agar No.3, Oxoid in TSB, Oxoid) in 15ml bijoux bottles were melted at 100°C in a boiling waterbath for at least 5 minutes, to avoid the formation of microcrystals. Condensation was removed from the bijoux bottle lids, any caps loosened during the boiling process were tightened and all bottles were placed in a preheated 50°C waterbath for at least one hour before use. Any bottles which did not have their necks above water at any time of the procedure or which had accumulated condensation on the inside were discarded.

After cooling to 50°C the bottles were removed, one at a time, 100µl of phage preparation was added, then vortexed for 3 seconds, 100µl of overnight bacterial culture (adjusted to approx. 1×10^8 cfu/ml using TSB) were added and vortexed again for 5 seconds. The flask was placed on the bench for 2-3 seconds and flamed between each manual handling step to avoid excess foam formation. The content of the bottle was then carefully poured onto a TSA plate for use with soft agar overlays (see **Section 2.1.5.2**) and allowed to set untouched for over 60 minutes, to avoid rippling of the overlay agar. Depending on ambient temperature, the agar would set after approximately 1 hour and the plate could then be moved. All plates were incubated overnight at optimum temperature in inverted position. After incubation plates with dilutions high enough to yield a countable number of plaques were selected (40-300 depending on plaque size) and plaque numbers enumerated using magnification and direct illumination.

2.3.5 Selective Media

2.3.5.1 Leeds *Acinetobacter* Medium (LAM) (Jawad et al. 1994)

Agar technical No. 1, 12g/l

acid casein hydrolysate 15g/l

neutralised soya peptone 5g/l

sodium chloride 5g/l

D-fructose 5g/l

sucrose 5g/l

mannitol 5g/l

L-phenylalanine 1g/l

ferric ammonium citrate 0.4g/l

phenol red 0.02g/l

All the components were mixed in a glass vessel and dissolved in 1 L water. The medium was adjusted to pH7 and steamed on boiling water to dissolve the ingredients. The LAM was then autoclaved for 15min at 121°C. After cooling to 50-55°C the following syringe-filtered antibiotics, dissolved in water were added under aseptic conditions: vancomycin HCl 10mg/l, cefsulodin 15mg/l and cefradine 50mg/l.

The container was gently but thoroughly agitated by rolling over a surface and LAM plates poured into 9cm Petri dishes and allowed to set for 10 minutes in a laminar flow cabinet. Plates were stored in plastic bags at 4°C. For the production of LAM liquid addition of technical agar was omitted.

2.3.5.2 Leeds *Acinetobacter* Medium with varying antibiotic compositions

LAM agar with varying antibiotic constituents was produced in the following way: After autoclaving and cooling to 50°C, 50ml aliquots were dispensed aseptically into sterile centrifuge tubes. Antibiotic powder was weighed and each dissolved in 10ml sterile water and syringe filtered. 0.5ml of filtered antibiotic solution was added to each relevant tube, gently mixed and poured into 9cm Petri dishes. The agar was allowed to set for 10 minutes in a laminar flow cabinet. Plates were stored in plastic bags at 4°C

2.3.5.3 Modified Leeds *Acinetobacter* Medium (MLAM)

Agar Technical No. 3, 12g/l

acid casein hydrolysate 15g/l

peptone Water 7.5g/l

NaCl 2.5g/l

D-fructose 5g/l

sucrose 5g/l

mannitol 5g/l

L-phenylalanine 1g/l

ferric ammonium citrate 0.4g/l

phenol red 0.02g/l

All dried components were mixed in a glass vessel, dissolved in 1 L water, the medium adjusted to pH7 and steamed on boiling water to dissolve the ingredients. The MLAM was then autoclaved for 15min at 121°C. After cooling to 50-55°C the following syringe-filtered antibiotics, dissolved in water were added under aseptic conditions: vancomycin HCl 10mg/l, ceftazidime 15mg/l and cefradine 50mg/l (final concentration). The container was gently but thoroughly agitated by rolling over a

surface and MLAM plates poured. MLAM plates were stored in plastic bags at 4°C for up to 14 days and equilibrated to room temperature for 1 hour before use.

2.4 Identification methods for bacterial strains

2.4.1 Characterisation using microscopy

Stock cultures of all isolates were freshly subcultured on TSA. A sample of bacterial growth was mixed with 10µl water and the appearance and shape of individual cells and motility observed using phase contrast microscopy. Photographs of the prepared specimens were taken using a Leitz Wetzler microscope (Biomed, Germany) with a 100x objective and an Olympus DP10 microscope digital camera system.

2.4.2 Motility

National standard method BSOP TP 21 (Hanging drop method) was used to test for motility.

2.4.2 Gram staining

Small samples of growth were removed from single colonies of each isolate cultivated on an overnight TSA plate (37°C). The samples were used to prepare smears on glass slides which were heat fixed and then stained using the Gram staining procedure (Lansing et al. 2002). The slides were viewed under bright field microscopy.

2.4.3 Oxidase test (including validation)

National standard method BSOP TP 26 (Filter paper method) was used. Freshly grown overnight culture of confirmed *Pseudomonas aeruginosa* Glaxo (on TSA, 37°C) and confirmed *Acinetobacter*, clinical isolate strain R46383 were used as controls.

2.4.4 Catalase test

National standard method BSOP TP 8 (Cover-slip method) was used.

2.5 Viable count methods

Viable counts were routinely performed using the method after Miles & Misra (1938).

Several ten-fold dilutions of a vortexed overnight bacterial suspension were prepared using PBS as the standard diluent. Each dilution was mixed after diluting and before pipetting onto agar. Agar plates were divided into numbered sectors. Six replicates of the respective dilutions were deposited in 20 μ l drops from a height of 2.5cm onto the agar surface. Droplets were allowed to soak into the agar and the plates incubated for 16-18 hours at standard incubation temperatures (25, 30 or 37°C).

2.6 Phage screening methods

Two different methods were routinely used referred to here as the revised surface spotting method and the soft agar spotting method.

The revised surface spotting method generally gave defined areas of clearance with lytic phage, which were easy to see, however the soft agar spotting method appeared to be useful when the tested phage was potentially lysogenic and no clearance areas could be detected with the other method.

Most protocols which utilise the surface spotting technique use simple undiluted overnight cell suspensions for phage screening (Kutter & Sulakvelidze, 2005). One published method termed the Modified Method 1601, (US Environmental Protection Agency, EPA 821-R-01-030 spot plates, 12.3, p.20, 2001) employed undiluted overnight bacterial suspensions, and described a protocol for phage spotting using far more detail than other references. This method was used as the basis for the optimisation of the phage screening protocol used in this thesis. The method stipulated that the optical density of the overnight suspension should be between 0.1 and 0.5 at a wavelength of 520nm. In some cases following this method resulted in lawns being too thin to allow interpretation of results, whilst in other cases the lawn was too thick to allow detection of lysogenic phage. This led to the development of the revised surface spotting method (**Section 2.5.2**), where a washed cell suspension of a standardised optical density was used to yield reproducible lawns of excellent quality.

2.6.1 Basic surface spotting method

10ml TSB were inoculated with one loopful of freshly thawed stocks of bacterial isolates incubated for 16-18 hours at 20, 22, 25, 30 or 37°C respectively, on a shaking platforms set to 120rpm. The optical densities of the overnight suspensions were adjusted with TSB to read between OD₆₀₀ 0.1 and 0.5. 100µl of the undiluted respective overnight bacterial suspensions were surface spread onto overdried TSA plates and 10 µl spots of phage applied in triplicate alongside 10 µl spots of broth or lambda buffer as blank.

The surface spread plates with the spots applied were left at ambient temperature for 1 hour and placed in an incubator at 20, 22, 25, 30 or 37°C respectively for 16-18 hours. On the following day the plates were checked for clearance zones, overall quality of lawn and other characteristics indicative of lysis.

2.6.2 Revised Surface spotting method

Universal bottles containing 10ml Tryptone Soya broth were inoculated with 1-3 colonies of *Acinetobacter spp.* and incubated at the appropriate temperature for 16-18 h on a shaking platform rotating at 120rpm. This overnight suspension was centrifuged at 2011g in a Centaur 2 centrifuge for 10 minutes. The supernatant liquid was decanted and the cell pellet re-suspended in 10ml PBS. The OD₆₀₀ was adjusted to read 0.3-0.4 with PBS corresponding to 1×10^8 - 1.3×10^8 cfu/ml of *Acinetobacter* R46383. This suspension was used without further dilution. Sterile cotton buds were dipped into the dilute cell suspension and streaked over the TSA plate in a 4 way fashion, to ensure the plate was evenly inoculated. The plates were incubated for 2 hours at the appropriate temperatures to yield an even lawn. The standard incubation temperature was 37°C, with exceptions of HER1424 and HER1423, which were routinely incubated at lower temperatures of 25°C. After two hours the position of phage droplets to be applied was marked on the back of the plates using a template and 10-fold dilutions of the phage suspensions were applied as 5 µl drops using a multipoint pipette. The droplets were allowed to soak into the agar and all plates were incubated in inverted position for 16-18 hours. Circular areas containing no bacterial growth or individual colonies (Clearance

zones) implied lytic activity of phage. Thinning of the bacterial lawn implied some lytic activity or was seen as a sign for potential lysogeny.

Irrespective of the type of method used for phage spotting, all phage typing plates were read with a magnifying lens through the bottom of the plates using both direct and oblique illumination.

2.6.3 Soft agar spotting method

Universal bottles containing 10ml Tryptone Soya broth were inoculated with 1-3 colonies of *Acinetobacter spp.* and incubated at the respective temperature for 16-18 h on a shaking platform rotating at 120rpm. This overnight suspension was centrifuged at 2011g in a Centaur 2 centrifuge for 10 minutes. The supernatant liquid was decanted and the cell pellet re-suspended in 10ml PBS. The OD₆₀₀ was adjusted to read 0.3-0.4 with TSB.

Sterile 5ml aliquots of 0.4% w/v technical agar No.3 in TSB (soft agar) in 15ml bijoux glass bottles, were melted in a boiling waterbath at 100°C for 5minutes and thereafter maintained at 50°C in a heated waterbath for at least 90 minutes to allow the agar to cool but stay liquid. 100µl of the cell suspension were used to inoculate 5ml aliquots of the soft agar, which were vortexed for 5 seconds and poured over a set plate of TSA. After letting the soft agar set for 60 minutes, 5 µl drops of phage preparation were applied using a multipoint pipette.

2.7 Production of crude filtered lysates

In order to produce sufficient stocks of phage to conduct experiments the liquid lysate method was employed to produce at least 50ml of crude filtered lysate for each phage.

2.7.1 Liquid Lysate Method

1-3 colonies of host bacteria were used to inoculate 10ml sterile TSB, which was incubated at the optimum temperature (routinely 37°C- except for HER1423 and HER1424, which were incubated and infected at 25°C) for 16-18 hours on a shaking platform at 120rpm. 1-4ml of the resulting bacterial suspension was added to 100ml of sterile TSB contained in a 250ml conical flask.

The inoculated broth was incubated at the appropriate temperature on a shaking platform at 120rpm until the OD₆₀₀ was 0.5, which was generally after 3-5 hours. To infect the cells with phage 100µl of the original phage lysate were diluted tenfold with lambda buffer. The resulting 1ml of phage preparation of unknown titre was added to the bacterial suspension. The flask containing bacteria and phage was placed in a static incubator for 15 minutes at the appropriate temperature to facilitate infection of the bacteria.

Thereafter the flask was transferred to a shaking platform and incubated for 16-18 hours at 120 rpm at the optimum temperature. The following day the macroscopic appearance of the culture was noted and this varied from a fine, splintery appearance or large, stringy clumps of sediment at the bottom of the flask to the content of the flask appearing to be clear, quite in contrast to the appearance of an uninfected overnight bacterial suspension.

10ml of chloroform were added to the conical flask in a fume hood and incubated for 10 minutes at 120rpm at the optimum temperature. In order to obtain a crude lysate the bacterial debris was removed by centrifugation at 2500g for 10minutes and the supernatant filtered using a Millipore 0.45 µm syringe filter. Filtered lysates were stored in sealed glass bottles, protected from light at 2-8°C.

2.7.2 Production of purified lysates

When lysates with high titres were required the standard protocol to purify bacteriophage was used. This involved using the material obtained from the liquid lysate method before the final filtration step.

2.7.2.1 Standard protocol to purify bacteriophage

If the lysed culture had been refrigerated beforehand (**see 2.6.1**) it was brought to ambient temperature (minimum of 25°C and maximum of 35°C), before proceeding. NaCl was added to a final concentration of 1M to the lysed culture. The salt was dissolved by occasional swirling after which the conical flask was stored on crushed ice for 1 hour. Bacterial debris was removed by centrifugation at 11,000g for 10minutes at 4°C in a Sorvall RC-5B Refrigerated Superspeed Centrifuge (Du Pont Instruments). The

supernatant liquid was removed in a laminar flow hood and placed in sterile conical flasks. The precipitate was discarded and the centrifuge containers rinsed with sterile broth, in order to be reused for the second centrifugation step.

Polyethylene glycol (PEG 8,000) was added to a final concentration of 10% w/v, which was slowly dissolved by stirring on a magnetic stirrer at room temperature. The flask was then cooled on crushed ice for at least 1 hour or left overnight at 4°C, to allow the bacteriophage to form a precipitate. The precipitate was recovered by centrifugation at 11,000g for 10 minutes at 4°C. The supernatant was discarded and the remaining fluid drained away from the pellet by placing the centrifuge container in a tilted position. The pellet was re-suspended using 5 x 2ml lambda buffer. Using a 10ml pipette, the suspension was transferred into 25ml sterile IWAKI centrifugation containers. An additional 1ml of buffer was used to rinse the centrifugation container. 1ml of chloroform was added to the bacteriophage suspension and vortexed for 30 seconds. The organic and aqueous phases were separated by centrifugation at 3000g for 15 minutes at 4°C. The aqueous phase containing the phage was removed, made up to 50ml with lambda buffer and stored in a sterile glass container at 2-8°C. Aliquots of purified phage preparation were supplemented with 10% v/v Glycerol and stored at -80°C.

2.8 Phage enumeration method

2.8.1 Soft agar overlay method

5ml of previously prepared sterile soft agar (0.4% w/v technical agar No.3, in TSB, Oxoid) in 15ml bijoux bottles were melted at 100°C and then placed in a 50°C waterbath for at least one hour before use. 100µl of phage preparation were added, then vortexed for 2 seconds, after which 100µl of prepared overnight bacterial culture adjusted to approx. 1×10^8 cfu/ml were added and vortexed again for 5 seconds. The content of the bottle was then carefully poured onto a freshly prepared TSA plate and allowed to set. All plates were incubated overnight at optimum temperature in inverted position. After incubation plates with dilutions high enough to yield a countable number of plaques were selected (40-300) and plaque numbers enumerated.

5ml of previously prepared cooled sterile soft agar in 15ml bijoux flasks were melted at 100°C in a boiling waterbath for at least 5 minutes and placed in a 50°C waterbath for

at least one hour before use. 100µl of phage preparation was added, then vortexed for 3 seconds, 100µl of prepared overnight bacterial culture (adjusted to approx. 1×10^8 cfu/ml using TSB) were added and vortexed again for 5 seconds. The contents of the bottle were then carefully poured onto a TSA plate for use with soft agar overlays (see 2.1.5.4) and allowed to set. All plates were incubated overnight at optimum temperature in inverted position. After incubation plates with dilutions high enough to yield a countable number of plaques were selected (40-300 depending on plaque size) and plaque numbers enumerated.

2.9 Optical density measurements

To ensure repeatability and method standardisation it was important to correlate optical density readings at a specific wavelength to the number of cells present. Bacterial suspensions were required for different experiments to be prepared in different ways e.g. cells were washed and re-suspended in PBS for the revised surface spotting method, however diluted overnight suspensions were used for the soft agar overlay method. Due to different experimental requirements a graph relating optical density to viable counts was produced for each set of conditions (Hugo & Russell, 1992).

The Beer-Lambert Law $[X] = kA$ stipulates a linear relationship between the concentration of a suspension and the absorbance. The absorbance of a cell suspension is directly proportional to cell number after construction of a calibration plot. $[X]$ = concentration, k = proportionality constant, A = absorbance.

2.9.1 Relating Optical density measurements to viable counts at 600nm (cells washed and suspended in PBS)

100ml of sterile TSB were inoculated with 1-2 colonies of *Acinetobacter* W6492, maintained on TSA and incubated in an orbital shaker at 37°C, 120rpm, overnight. 30ml of the overnight culture was centrifuged in a Sorvall RT6000 B refrigerated centrifuge at 2500 x g, 21°C for 20minutes. The supernatant was discarded, the cell pellet was re-suspended, washed with 30ml PBS and vortexed for 60 seconds. This step was repeated twice. The cell pellet was finally suspended in 30ml PBS.

4ml of this cell suspension was diluted with 16ml PBS, the resulting cell suspension was considered the starting suspension (100%). From this suspension further dilutions were made in PBS to give concentrations of 80%, 60%, 40%, 20% and 10% of the original suspension. Optical density measurements were made of each of the suspensions using a Helios ϵ (Unicam) Spectrophotometer at 600nm.

For the viable count the original cell suspension was diluted in two hundredfold (1ml+99ml PBS) and three tenfold (1ml+9ml PBS) dilutions. 0.2ml of each of the three tenfold dilutions was surface spread in triplicate onto overdried TSA plates and incubated for 16-18 hours at 37°C. On the following day the colonies on each countable agar plate were counted. The mean results of the viable count were extrapolated and related to the mean optical density (**Figure 2.1**, n=3).

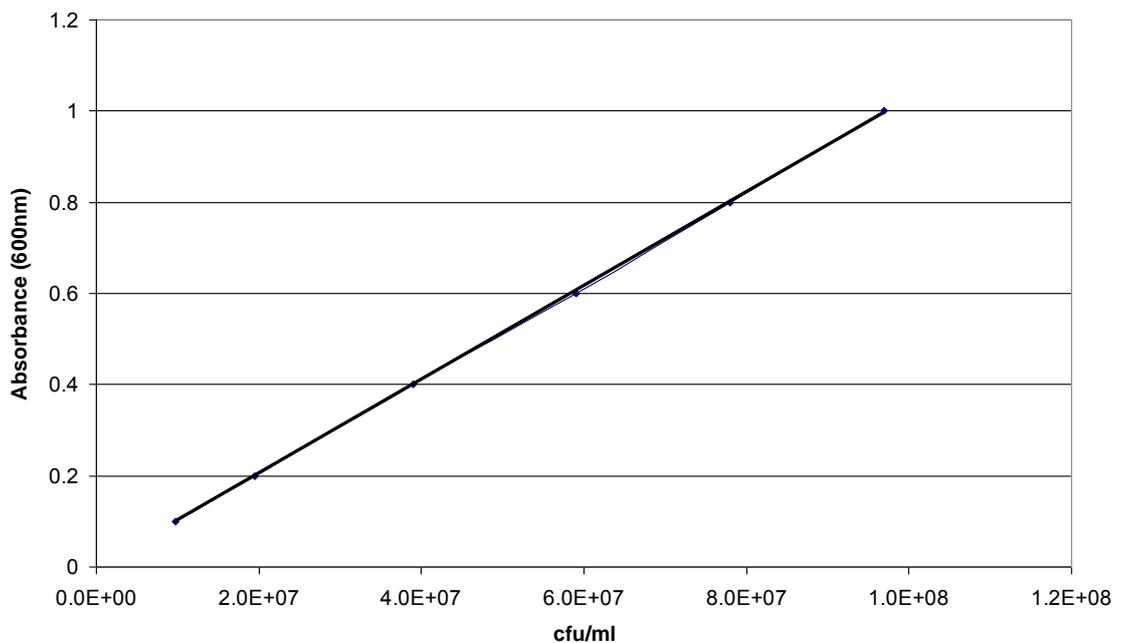


Figure 2.1 Absorbance at 600nm correlated with cfu/ml in PBS

2.9.2 Relating optical density measurements to viable counts at 600nm (overnight suspension)

100ml of sterile TSB was inoculated with 1-2 colonies of *Acinetobacter* HER1423 or *Acinetobacter* HER1404 respectively and incubated in an orbital shaker at 37°C, 120rpm, for 16-18h. Dilutions were made of the overnight suspension as described

above, using sterile TSB as diluent and the optical densities measured at 600nm in a Helios ϵ (Unicam) Spectrophotometer. A viable count was carried out and the results extrapolated and related to the optical density (**Figures 2.2 & 2.3**).

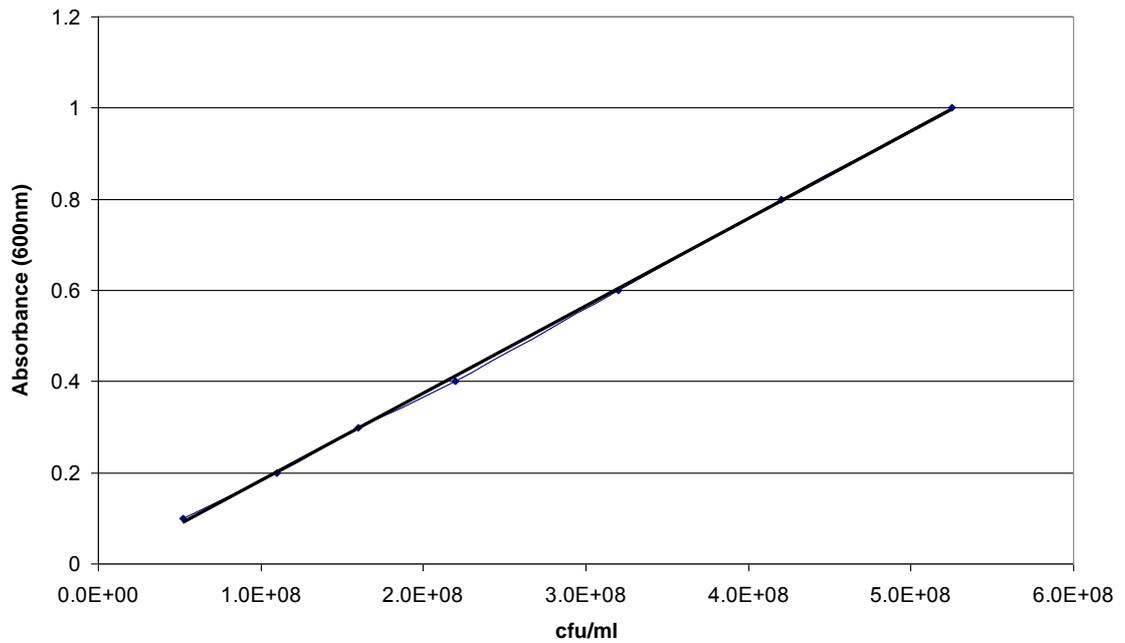


Figure 2.2 Absorbance at 600nm correlated with cfu/ml in an overnight suspension of *Acinetobacter* HER1423

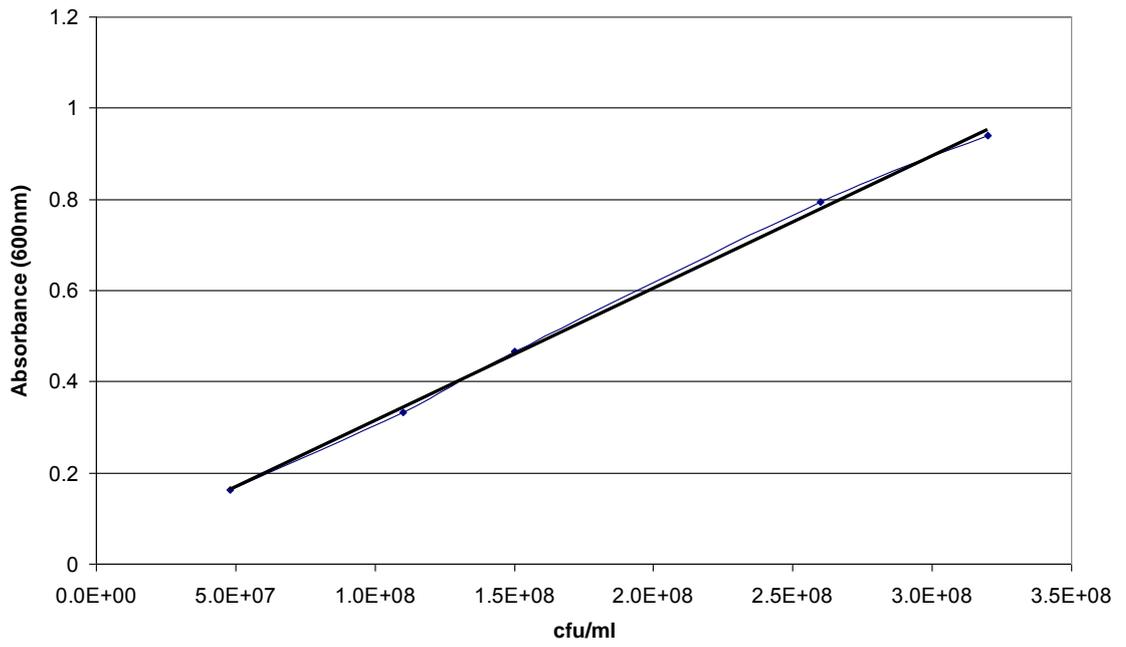


Figure 2.3 Absorbance at 600nm correlated with cfu/ml in an overnight suspension of *Acinetobacter* HER1401

Chapter THREE

Identification and characterisation of nine clinical „Sussex“ *Acinetobacter* spp. isolates

3.1 Introduction

Nine viable clinical *Acinetobacter* spp. isolates were donated by Dr. J. A. Child, Consultant Microbiologist at the Surrey and Sussex NHS Trust in December 2000, together with relevant antibiotic disc sensitivity data and some isolation history (**Appendix 2**). Some of the isolates were confirmed as multiply resistant to antibiotics, of which three represented an outbreak strain at the Queen Victoria Hospital (QVH), East Grinstead. Two other isolates, which were also multiply resistant, were confirmed to be a different strain (**Appendix 2**), one of which was responsible for infectious episodes at another Sussex Hospital. Dr. Child reported that most of the sensitive strains were almost incidental isolations, which could even be contaminants in blood cultures. The outbreak strains were reported to be more virulent and hardy and were named as the cause for bacteraemia and ventilator-associated pneumonia in ICU. In more rare cases these strains were responsible for severe soft-tissue infections in the QVH and Crawley hospital (Written communication Prof. G. Hanlon with Dr. J. Child, 6 December, 2000; **Appendix 2**).

The QVH has, in common with national and international reports, experienced problems with *Acinetobacter* strains which have rapidly acquired resistance to antibiotics and been responsible for outbreaks in hospitals with diminished treatment options. This small selection of isolates, referred to as “Sussex” isolates, were the start of a growing *Acinetobacter* spp. collection, which was begun with the intention of isolating and characterising bacteriophage for each isolate suitable for therapeutic purposes, which would eventually lead to a therapeutic phage library. It was important to use relevant

clinical isolates of *Acinetobacter* spp. for this purpose since culture collection strains may present an entirely different bacteriophage susceptibility profile.

STRAIN SHORT CODE	Strain isolation history	Out-break strain	Source	MRI
R1973	From patient with severe burns. Became amikacin resistant.	Yes	QVH	Yes
R2751	From wound of patient on surgical ward. Treated with IV colistin.	Yes	QVH	Yes
R3417	Representative of an outbreak strain at a Sussex Hospital, other than QVH.	No	-	Yes
R4474	Blood-culture isolate from patient with severe burns, who later died, <i>Acinetobacter</i> infection was contributory factor	Yes	QVH	-
R45502	From a patient with severe burns. Became amikacin resistant	Yes	QVH	Yes
R46383	From burns patient, not an endemic strain.	No	-	
W5211	From blood culture of patient with 80% burns, later died.	Yes	QVH	Yes
W6108	Blood culture isolate from patient on a general medical ward	-	-	No
W6492	Blood-culture isolate, medical patient	-	-	-

Table 3.1: Isolation history of Sussex strains, information as provided with samples

Legend Table 3.1:

Outbreak strain 'Yes' identifies a strain described as an 'outbreak strain' in the letter obtained with the samples

Source 'QVH' identifies strains clearly identified as isolated at the Queen Victoria Hospital, East Grinstead.

'- denotes where insufficient information was provided

MRI 'Yes' identifies a multiple resistant isolate

The information presented in **Table 3.1** was provided with the *Acinetobacter* spp. samples, the short strain codes used were originally assigned by the hospital pathology laboratory.

The original approach was to characterise the nine Sussex strains and search for suitable lytic bacteriophages from local environmental sources. Literature suggests that bacteriophages are available in abundance in the environment and can be isolated from sources such as sewage, surface and waste water (Holt et al., 1994). As no detailed protocol has yet been published specifically for potential therapeutic *Acinetobacter* phage, it was decided to use as many different approaches as possible for each sample.

The experimental protocol was designed to isolate strains of *Acinetobacter* alongside phages, with the rationale that phage and host are likely to be found in the same sample. Thus the detection of the host may serve as an indicator of potential presence of phages in the material. In order to avoid overgrowth by other microorganisms expected to be present in waste water and sewage samples, it was necessary to investigate the use of selective media for the isolation of clinical and environmental *Acinetobacter* strains.

3.2 Materials and Methods

3.2.1. Storage and routine subculture of Sussex strains

Strains obtained frozen, from Dr. J. Child, Consultant Microbiologist at the Surrey and Sussex NHS Trust were defrosted and one loopful streaked on TSA plates to obtain single colonies. 1-2 single colonies were vigorously dispersed in 100ml TSB in vented conical flasks, incubated for 16-18 hours at 37°C at 120 rpm. Liquid subcultures of each strain were directly cooled to -10°C and kept frozen at -80°C in 10% glycerol in TSB in cryovials. Stock cultures were made by defrosting a cryovial each month, using one loopful to streak on TSA plates, to isolate single colonies and to check the purity of the

culture. Plates were incubated in inverted position at 37°C for 16-18 hours and were kept refrigerated at 4°C. The stock cultures on agar plates were subcultured weekly.

3.2.2 Routine culture and growth of Sussex isolates

Bacterial inocula were prepared from one single isolated colony and were vigorously dispersed in 10ml TSB. Cells were grown for 16-18 hours in loosely capped glass universal bottles at 37°C on a shaking platform rotating at 120rpm.

3.2.3 Characterisation of Sussex isolates using microscopy

Stock cultures of all Sussex isolates were freshly subcultured on TSA. Appearance and shape of individual cells was observed using phase contrast microscopy (Section 2.4.1). Results are shown in Section 3.5.1.

3.2.4 Gram staining of Sussex isolates

Stock cultures of all Sussex isolates were freshly subcultured on TSA and the method described in Section 2.4.2 was followed. Results are shown in Section 3.5.2.

3.2.5 Oxidase test

Stock cultures of all Sussex isolates were freshly subcultured on TSA and the method described in Section 2.4.3 was followed. Results are shown in Section 3.5.3.

3.2.6 Catalase test

Stock cultures of all Sussex isolates were freshly subcultured on TSA and the method described in Section 2.4.4 was followed. Results are shown in Section 3.5.4.

3.2.7. Identification of nine clinical Sussex strains using the API20NE system

On receipt only three strains were described in terms of species: W6492, R45502 and R1973, were described as *A. baumannii* or as belonging to the *A. baumannii* complex. It was considered important to differentiate the strains on the basis of phenotype in as much detail as possible.

Although a preliminary identification of the present bacteria was possible, based on Gram stain and microscopy, further species identification required the identification of additional phenotypic features. There are various brands of identification systems on the market but the API system is considered to be the most reliable. Due to the specific design of API20NE for use with non-fermentative Gram negative bacteria and because of a comparative review by Towner & Chopade (1987), evaluating the usefulness of this test system on 122 separate strains this particular kit was chosen.

API20NE and API20E test kits (bioMérieux, Lyon, France) were used according to manufacturer's instructions for the identification of isolates. Freshly subcultured colonies on TSA were used to inoculate the test strips. According to the manufacturer's literature the Analytical Profile Index I.D. was not valid before 48 hour incubation for all strains, which warranted the maximum incubation time at 30°C. Results are shown in **Section 3.5.5.**

3.2.8 Growth of 9 clinical Sussex isolates at 42 and 44°C

In order to differentiate between *Acinetobacter baumannii* and *Acinetobacter calcoaceticus*, it was necessary to investigate the growth behaviour at 42 and 44°C. Strains not belonging to the *Acinetobacter baumannii/ calcoaceticus* complex were also included in this experiment, as growth temperatures were considered to be of general interest.

TSA plates were streaked from frozen stock and incubated for 16-18 hours at 37°C. From these plates 1-2 colonies were further sub-cultured onto TSA plates and incubated as before. 1 was colony used to inoculate 2ml and 1-2 colonies to inoculate 10ml of TSB which were incubated at 42 or 44°C for 16-18 hours. The liquids were shaken at 120rpm. After incubation plates and liquids were inspected visually. Optical densities (OD₆₀₀) above 0.1 but below 0.2 were counted as weak growth. Results are shown in **Section 3.5.6.**

3.2.9 Growth curves at 37°C, using Bioscreen

10ml of TSB were inoculated with 1-2 colonies of each host and incubated for 16-18 hours at 37°C in an incubator with a shaking platform set to 120rpm. After overnight

incubation the cell suspension was centrifuged at 2000g in a Centaur 2 (Sanyo) centrifuge for 10 minutes and resuspended in equal volume of PBS. The OD₆₀₀ was adjusted with PBS to read between 0.33-0.35 (equivalent to 1x10⁸ cfu/ml). 60µl of adjusted bacterial suspension were added to 240µl of prewarmed TSB in each well of a 100-well plate (1 in 5 dilution) 10 wells were filled with TSB only as blanks. The plate was maintained at 37°C and read with shaking every 10 minutes in a Bioscreen-C, (Growth curves USA, NJ, USA), set at 600nm for turbidity measurements. Results are shown in **Section 3.5.7**.

3.3 Antibiotic susceptibility of nine Sussex isolates using the BSAC disc diffusion method

The purpose of this experiment was to confirm the antibiotic sensitivity data provided with the samples and to determine if any of the strains had deviated or mutated to a non-resistant genotype or phenotype. Results are shown in **Section 3.5.8**.

3.3.1 The British Society for Antimicrobial Chemotherapy (BSAC) Disc Diffusion Method for Antimicrobial Susceptibility Testing

Antibiotic susceptibility testing is a method used routinely on bacterial isolates to ascertain resistance and susceptibility to antibiotics. It is based on using critical concentrations which allows a separation of organisms into resistant and susceptible, which is now widely known as the „breakpoint“ technique, a term first used by Ericsson & Sherris (1971). The disc diffusion method relies on phenotypic susceptibility testing, the outcome of which can be highly dependent on experimental conditions (Bergeron & Ouellette, 1998). The authors also claim that more than one method should be used to obtain accurate susceptibility profiles, however in clinical practice this is often not the case and for the purposes of this investigation was not done.

Examples of parameters affecting experimental outcome are thickness and type of agar, correct storage of test discs and density of bacterial lawns. To allow replication of results the experimental setup used in this investigation is described in detail. Pitfalls of the disc susceptibility method are the absence of internationally agreed break points and different bacterial species and strains have differing susceptibilities to the same

antibiotics. It could be argued that a DNA based approach may be more suitable to detect bacterial resistance genes. The drawback of genotypic testing is that the absence of a bacterial gene encoding for resistance does not guarantee susceptibility of a bacteria to a specific antibiotic. Similarly the detection of a resistance gene does not automatically mean the bacterium in question will show resistance to a specific antibiotic.

The BSAC Disc Diffusion Method for Antimicrobial Susceptibility Testing, Version 3, January 2004 was used to ascertain the antimicrobial susceptibility of 9 Sussex clinical isolates of *Acinetobacter* spp.

ISA (Oxoid, Basingstoke, UK) was prepared according to the manufacturer's instructions. Molten agar was poured into 90mm sterile Petri dishes to give a mean depth of 4.0mm± 0.5mm (25ml). The surface of the agar was allowed to dry for 10minutes in a fan-assisted drying cabinet. The plates were left in open plastic bags at ambient temperature overnight, the bags then sealed and stored at 4-8°C.

3.3.1.1 Establishing ideal inoculum density- method optimisation

The BSAC protocol recommends comparison of inoculum with 0.5 McFarland Standard, which is described as not „fool-proof“ by the BSAC (The development of the BSAC standardized method of disc diffusion testing, http://www.bsac.org.uk/_db/_documents/Chapter_4.pdf, visited December 2005).

In addition it requires an optical comparison between the standard and liquid culture by the naked eye, which can prove difficult. The BSAC was unable to provide details as to the optical density equivalents of a 0.5 McFarland standard (see **Appendix 2**, personal communication Jenny Andrews, BSAC, 5 March 2003). However the BSAC method allows any other method of obtaining semi-confluent growth if they are shown to be equivalent (see **Figure 3.1**).

Mahony et al. (1999) mentioned that an optical density of 0.09-0.10 (625nm) is equivalent to a 0.5 McFarland standard.

Lightest acceptable

Ideal

Heaviest acceptable

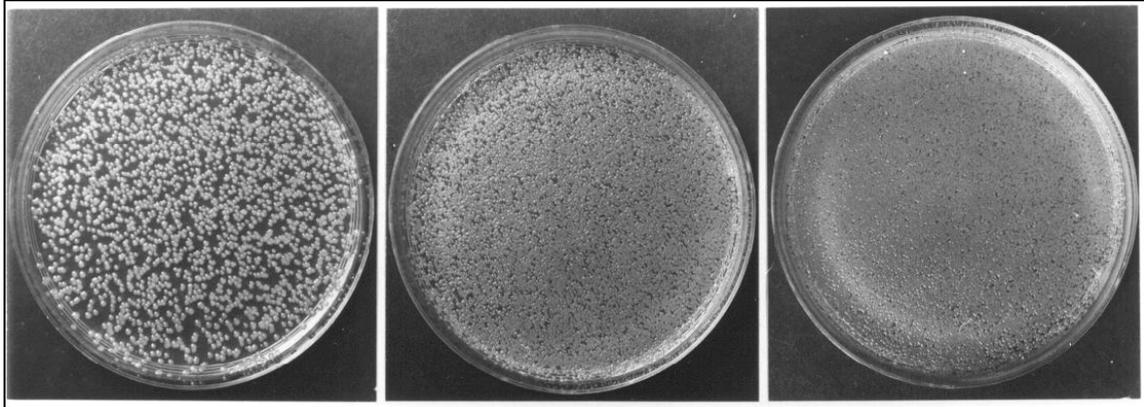


Figure 3.1 Acceptable ranges for inoculum densities (Picture source: BSAC Disc Diffusion Method for Antimicrobial Susceptibility Testing, Version 5, January 2006)

1-2 colonies of HER1424 (Section 2.2.2) were picked with a sterile loop from a TSA plate and used to inoculate 100ml of ISB, which was incubated for 16-18h at 25°C at 120rpm. The OD₆₂₅ of the overnight suspension was established (ISB as blank) and it was diluted with sterile water to match OD₆₂₅ between 0.08 and 0.15. For all remaining strains 1-2 colonies were processed using the same method and incubated for 16-18h at 37°C, at 120rpm. The adjusted broths were diluted in 3 tenfold dilutions using sterile water as diluent. The adjusted suspensions were used within 15 minutes. A sterile cotton swab was dipped into the suspension and swabbed over ISA plates in three directions (n=3). This produced a very even lawn. Plates were allowed to dry for 5 minutes and placed in the incubator within 20 minutes of swabbing. The plates were incubated at 25°C for 16-18 h. On the following day a selection of lawns was photographed and compared to the BSAC recommended density of lawn (**Figure 3.1**).

The method worked equally well when an overnight suspension was produced using TSB instead of ISB, with the respective broth used as blank. The BSAC recognizes that some problems with testing *Acinetobacter* spp. have been related to difficulties in achieving the correct inoculum, which emphasises the importance of this method optimisation. The BSAC recommends in cases where inoculum density is unacceptable to repeat the disc diffusion test (The BSAC Disc Diffusion Method for Antimicrobial Susceptibility Testing, Version 5, January 2006).

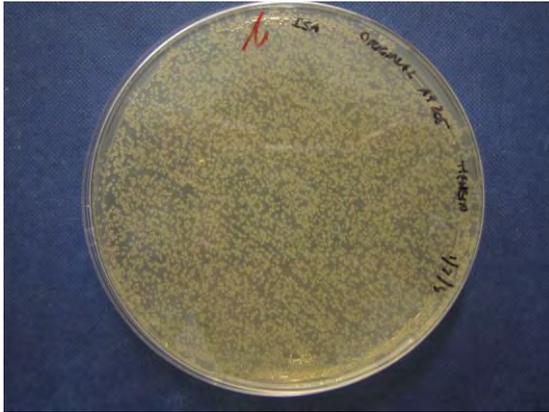


Figure 3.2 An *Acinetobacter* strain (HER1424) with ideal inoculum density

It was established that a lawn of suitable quality could be produced by adjusting the OD_{600} to 0.1-0.2 which was then diluted one hundred-fold in water and swabbed onto ISA plates (Figure 3.2).

3.3.1.2 Preparation of inoculum

1-2 morphologically similar colonies were transferred into 10ml sterile TSB and incubated at 37°C at 120rpm for 16-18h. The turbidities of the overnight suspensions were adjusted photometrically (as described in Section 3.3.1.1) and diluted one hundred fold using sterile water. The adjusted cell suspensions were used within 15 minutes of preparation. A sterile cotton-wool swab was dipped into the suspension, excess liquid removed by touching the inside of the boiling tube, swabbed over ISA plates in three directions and allowed to dry for 5 minutes, before applying 6 discs in equidistance on the plates using an Oxoid disc dispenser 9mm for 6 cartridges (Oxoid, Basingstoke, UK). Plates were incubated within 15 minutes of disc application in an inverted position at 37°C for 16-18hours. Diameters of zones of inhibition were measured using callipers. Average values of two readings were calculated and compared to published breakpoints for *Acinetobacter*. In cases where inoculum density was outside the acceptable range disc diffusion tests were repeated twice for all antibiotics.

Inhibition zones were interpreted using the BSAC Disc Diffusion Method for Antimicrobial Susceptibility Testing, Version 5, January 2006 and for selected antibiotics using „An NCCLS global information supplement“, NCCLS, Jan. 2002. Readings are expressed as resistant (R) or sensitive (S), and intermediate (I). Wherever

the different NCCLS breakpoints led to a different interpretation of results in comparison to BSAC breakpoints they were stated in brackets.

The manufacturer provides several strengths of antibiotic for the disc diffusion method, in line with either the BSAC, the SRGA (The Swedish Reference Group for Antibiotics) or NCCLS, now CLSI (Clinical and Laboratory Standards Institute).

For ciprofloxacin 5µg discs were used, rather than 1µg discs employed by the BSAC. The higher strength discs were used in this investigation, as the majority of isolates were considered multiply resistant, NCCLS provides breakpoints for the higher strength discs.

The BSAC allows amoxicillin/clavulanic acid to be reported the same as for ampicillin and recommends 10 µg, however 30µg discs were chosen as they represent the strength most likely to be effective.

3.4 LAM agar and liquid medium validation experiments

The clinical isolates described in **Section 3.1** were the first set of *Acinetobacter* strains to arrive at the University of Brighton. It was intended to increase this collection with *Acinetobacter* samples collected from environmental sources in the hospital vicinity.

At the same time it was decided to undertake a search for lytic phage active against *Acinetobacter* spp. This led to a search for a selective growth medium for *Acinetobacter*, which would be expected to exclude or at least reduce the overwhelming background contamination expected in sewage and waste water samples.

3.4.1 Selective media for *Acinetobacter*

Several selective and differential media for the isolation of *Acinetobacter* spp. have been reported. Garrison (1963) developed a selective medium for what was classified as *Bacterium anitratum* at the time, however it is not widely used now. Mandel et al. (1964) published a recipe for a medium simply designed to reduce the background contamination of *Enterobacteriaceae* in urethral and vaginal discharges. It was not selective enough to suppress the growth of *Pseudomonas*, *Proteus* or *Aerobacter aerogenes* (Mandel, et al., 1964). Mandel's medium contained agar, soya peptone,

pancreatic digest of casein, lactose, maltose, sodium chloride, bile salts and bromocresol purple and was subsequently improved to become the classic Herellea medium by replacing maltose with sucrose (MacFaddin,1985). Grehn & von Graevenitz (1978) developed a medium for the isolation of for *Acinetobacter calcoaceticus* subsp. *anitratum*, however it is also not widely used.

In 1983 Holton described a selective and differential medium suitable for the isolation of *Acinetobacter* spp from clinical sources, known as Holton's agar. It contained desiccated ox bile rather than bile salts and ampicillin, cefsulodin and vancomycin. Holton (1983) improved the Herellea agar by substituting lactose with fructose, mannitol and phenylalanine and replacing the original indicator with phenol red. Enrichment cultivation was a methodology used by microbiologists of the „Delft School“ (Van Niel, 1955; Bergogne-Berezin et al., 1996) but it is not widely used anymore and is more time consuming than use of selective agar (Jawad et al., 1994).

Jawad et al. (1994) observed that existing media designed for the selective isolation of *Acinetobacter* spp. are either not sufficiently selective (due to the absence of appropriate inhibitory agents for other organisms) or too inhibitory (particularly due to the presence of ampicillin). Jawad et al. (1994) developed Leeds *Acinetobacter* Medium (LAM) reportedly suitable for the isolation of *Acinetobacter* spp. of DNA groups 1, 2, 3 and 13 from clinical and environmental sources. The authors compared Herellea and Holton's agar using 134 strains of *Acinetobacter* spp., the majority of which were environmental samples (105) collected from the Leeds General Infirmary, Leeds, UK. LAM proved to be superior to the other two media.

According to Jawad et al. (1994) the agar colour of LAM is turned mauve by the growth of *Acinetobacter* spp. and colonies appear as pink. The colour change is due to the high alkalinity produced in the medium by the growth of this bacterium, caused by liberation of ammonium ions from complex nitrogenous materials present in the medium, which changes the indicator colour of phenol red from the original orange. Leeds *Acinetobacter* Medium contains sodium chloride to inhibit the growth of bacteria sensitive to salt, *Acinetobacter* being a skin commensal is salt tolerant. Ferric ammonium citrate is present as a H₂S indicator; the presence of a black inorganic

sulphide precipitate would imply the absence of *Acinetobacter*. Sucrose, D-fructose and mannitol are available for acid production, with phenol red as indicator.

Jawad et al. (1994) reported that *Acinetobacter* spp. isolated from clinical sources showed better growth than *Acinetobacter* species found in environmental samples. It proved unsuitable for growing *A. johnsonii* and *A. haemolyticus*, which are considered more susceptible to bile salts No. 3 and β -lactam antibiotics than other species.

Despite LAM agar already having been tested by Jawad et al. (1944) on a selection of isolates, it was important to ascertain which of the Sussex and other isolates would grow on this selective medium and whether it would prove a reliable indicator of the presence of *Acinetobacter* in sewage/waste water samples or simpler samples with considerable background contamination.

According to the literature LAM has only been used with the full combination of antibiotics (vancomycin HCl 10mg/l, cefsulodin 15mg/l and cephradine 50mg/l). It was of interest to produce the LAM base with only part-combinations or no antibiotic at all, and compare the results to LAM agar supplemented with the full set of antibiotics. The intention was to investigate if omission of one or more antibiotics could make this medium more suitable for the purpose of this investigation.

3.4.2 Growth characteristics of Sussex isolates on LAM

LAM agar was produced according to the protocol described previously (**Section 2.3.5.2**).

Each plate was divided into six sections, a nichrome loop with 4mm diameter was used to apply the inoculum to one section of each LAM agar and TSA plates, which were used as positive control. To produce the inocula a 200 μ l pipette tip was used to transfer one single colony from each TSA plate into 100 μ l PBS, which was vortexed to produce a smooth suspension.

For comparison *S. aureus* ATCC 6538, *P. aeruginosa* ATCC 9027, *Citrobacter freundii* ATCC 8090, *E. coli* TM3, *E. coli* ATCC 8739 were treated in the same way and photographs taken of plates where growth occurred.

All plates were incubated for 16-18 h at 37°C. Results are shown in **Section 3.5.9**.

3.4.3 Growth characteristics of Sussex isolates on LAM with varying antibiotic compositions

LAM agar was made following the procedure described in **Section 2.3.5.1**, without addition of antibiotics and aseptically divided into 50ml aliquots immediately after autoclaving.

One LAM agar aliquot was poured antibiotic-free. To the remainder, sterile filtered solutions of vancomycin 10mg/l cefsulodin 15 mg/l and cephadrine 50 mg/l were added in varying combinations once the agar had reached 50°C, after which the agar was mixed and poured into plates. *Acinetobacter* strains were streaked from frozen for single colonies on TSA and grown for 16-18 hours at 37°C and processed as described in **Section 3.4.2**. All plates were incubated for 16-18 hours at 37°C. Results are shown in **Section 3.5.10**.

3.4.4 Modifications to LAM

LAM did not appear to be ideal for the purpose of isolating *Acinetobacter* from a variety of samples, since it did not permit growth of three out of nine isolates.

As the same isolates showed different sensitivities to a selection of cephalosporins (**Section 3.5.8, 3.5.9**), it was attempted to improve the properties of LAM by developing a medium which could be made in-house, containing a different cephalosporin.

As part of this investigation MLAM was developed and tested, where due to availability of components neutralised soya peptone was replaced with peptone water and the addition of NaCl reduced accordingly, 10g of technical agar No.1 (Oxoid) were replaced with 12 g of technical agar No.3 (Oxoid). Most importantly cefsulodin was replaced with equal amounts of ceftazidime.

3.4.5 Growth characteristics of Sussex strains on MLAM

MLAM was prepared according to the protocol described in **Section 2.3.5.3**.

Acinetobacter strains were streaked from frozen for single colonies on TSA, grown for 16-18 h at 37°C and further processed as described in **Section 3.4.2**. Results are shown in **Section 3.5.10**.

3.4.6 Liquid Leeds *Acinetobacter* medium validation experiment

Due to the absence of any published liquid selective medium suitable for clinical *Acinetobacter* strains, LAM was trialled without the addition of any technical agar and its properties and shortcomings investigated (referred to as LAM liquid). Initially it was important to ascertain whether *Acinetobacter* species would grow in LAM liquid. It was also of interest to investigate detection limits of LAM liquid to avoid false negative results when attempting to isolate bacteria from various sources. A further question was whether LAM liquid was suitable for enrichment purposes and could be used to selectively support the growth of *Acinetobacter* spp. and hence phage material present in the sample, whilst suppressing faster growing microorganisms, which may interfere with the enrichment process.

3.4.7 Growth of clinical *Acinetobacter* isolates in LAM liquid

1 L of LAM was produced according to the formulation described in **Section 2.3.5.1** with omission of technical agar. The pH was adjusted to 7, the medium steamed to dissolve the components and 10ml dispensed into boiling tubes with loose metal caps before autoclaving. After cooling to ambient temperature 0.5ml of a filter sterilised solution containing vancomycin, cefsulodin and cephradine (to achieve a final concentration of 10mg/L, 15mg/L, 50mg/L respectively) was added to each boiling tube under aseptic conditions.

Four *Acinetobacter* strains were chosen which had successfully grown on LAM agar. The rationale was to determine the lowest inoculum to yield growth in LAM liquid. Additionally to check whether it would be possible to determine the presence of *Acinetobacter* in LAM broth by a colour change alone, indicative of pH shifts caused by metabolic activity (**Table 3.2**).

pH	Colour
2.3	Yellow
5.3	Yellow
6.8	Yellowish orange
7.3	Orange
7.8	Reddish orange
9.3	Red

Table 3.2 Colour of phenol red over a range of pH values

1-2 colonies of strains R45502, R2751, R4474 and R1973 were used to inoculate 100ml TSB each and incubated overnight at 37°C, 120rpm. The OD₆₀₀ of all strains was recorded and adjusted with TSB to read between 0.19-0.29, as this is equivalent to approximately 1×10^8 cfu/ml (**Section 2.8.1**). The adjusted suspensions were serially diluted in eight 10-fold increments with TSB. Viable counts were performed, as described in **Section 2.5**, the results were used to estimate the inoculum added to each 10ml of LAM. 100µl of 5 dilutions of the serially diluted cell suspensions (containing approximately 10^{-5} cfu/ml) were used to inoculate 10ml of LAM liquid, which were incubated at 37°C with shaking at 120rpm for 16-18 hours. 2 boiling tubes containing sterile LAM were incubated alongside the samples to serve as blank.

After incubation all samples were inspected visually, OD₆₀₀ measurements were taken using the sterile incubated LAM liquid as blank. One loopful of each LAM liquid was taken and spread over TSA agar plates to confirm viability of the overnight cultures. The rationale behind this was to check whether viable bacteria could still be isolated after overnight incubation in LAM liquid, as this may prove useful if LAM liquid was to be employed as part of an enrichment protocol for the isolation of bacteriophage.

Results are shown in **Section 3.5.11**

3.4.8 Growth of clinical *Acinetobacter* isolates with artificial background contamination in LAM liquid

In results of **Section 3.4.7** it was shown that LAM liquid supports the growth of *Acinetobacter* spp., however it is unclear if it limits the growth of other organisms. It would also be of interest if *Acinetobacter* spp. could outgrow other organisms present in the medium at low inoculum densities, despite heavy background contamination. Provided the LAM liquid permitted phage propagation, these attributes would make it an ideal enrichment medium.

3.4.8.1 Growth of *Acinetobacter* spp. in LAM liquid with and without background contamination found in waste water

Experimental setup as described in **Section 3.4.7** with the following alterations was followed: For Sample A the adjusted suspensions were serially diluted in eight 10-fold increments with sterile PBS. For Sample B the adjusted suspensions were serially diluted in eight 10-fold increments with pooled waste water, obtained from two different toilets within the University of Brighton (U-tube bends) to provide background contamination. The pooled waste water sample was serially diluted with PBS to produce viable counts according to the protocol described in **Section 2.5**.

100µl of each of the serially diluted *Acinetobacter* cell suspensions (Samples A and B) were used to inoculate 10ml of LAM liquid, which were incubated at 37°C 120rpm for 16-18 hours. A viable count was produced for each *Acinetobacter* dilution with PBS (see **Section 2.5**) the results were used to estimate the inoculum added to each of the boiling tubes containing 10ml of LAM. 2 boiling tubes containing sterile LAM were incubated alongside the samples to serve as blank.

After incubation all samples were inspected visually, OD₆₀₀ measurements were taken using the sterile incubated LAM liquid as blank. Results are shown in **Section 3.5.12**.

3.4.8.2 Growth of waste water samples on TSA and LAM agar

100µl of the undiluted wastewater sample were surface spread onto overdried TSA and LAM agar plates and incubated at 37°C for 16-18 hours, to check for growth on both media. Results are shown in **Section 3.5.12**.

3.4.8.3 Membrane filtration

Membrane filtration was included in case the waste water samples (**Section 3.4.8.1**) viable counts were very low. Dilutions of overnight suspensions of samples A and B were produced to get a variety of concentrations and to investigate if it was still possible to detect small numbers of *Acinetobacter* colonies when background contamination was present.

0.5ml of the LAM sample inoculated with the highest dilution of strain R2751 (A and B) was removed and serially diluted in 4.5ml of sterile PBS. The resulting dilutions were membrane filtered under vacuum using 0.45µm Sartorius cellulose nitrate filters. The filters were placed on LAM agar plates and incubated at 37°C for 16-18h. In addition viable counts were performed on the overnight suspensions of isolates R2751, R4408 and R4471 inoculated with the highest two dilutions of A. Results are shown in **Section 3.5.12**.

3.5 Results

3.5.1 Characterisation of Sussex isolates using microscopy

Microscopic appearance: Intact short rods could be seen, some giving the appearance of being spherical or egg shaped (**Figure 3.3**). Most cells gave the appearance of coccobacilli, which can usually be observed when *Acinetobacter* specimens are prepared from agar cultures (Koneman et al., 1994). No motility was observed.

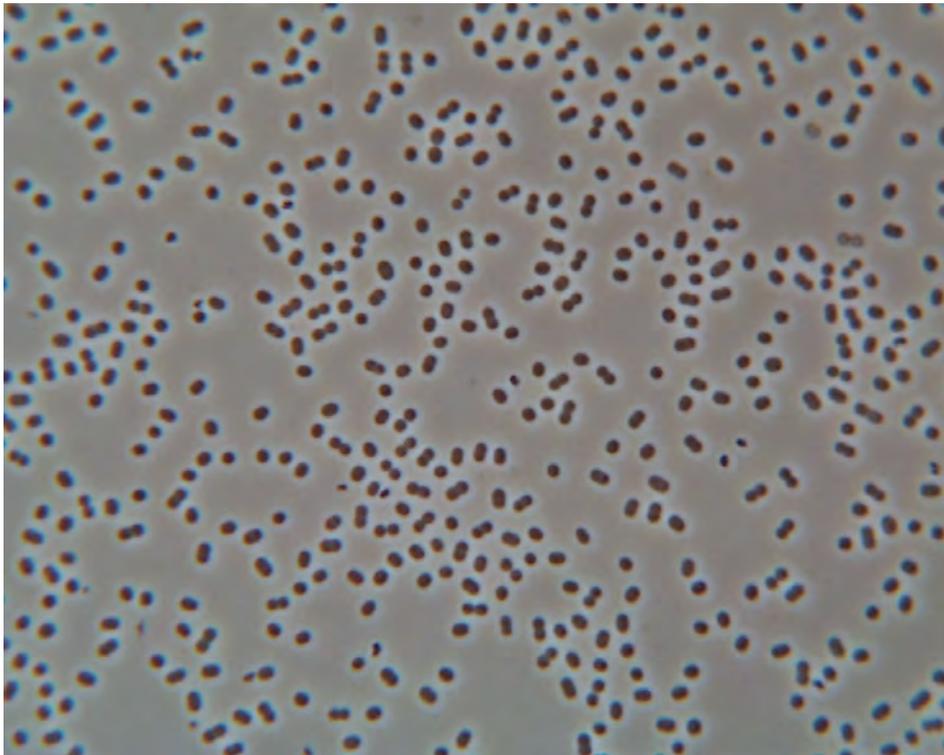


Figure 3.3 Photomicrograph of R2751 using a Leitz Wetzler microscope

3.5.2 Gram staining of Sussex isolates

The presence of pink stained bacteria indicated a Gram-negative identity (**Table 3.3**).

3.5.3 Oxidase test

As typical for *Acinetobacter* spp. the oxidase test was negative (**Table 3.3**)

3.5.4 Catalase test

Rapid effervescence for all isolates indicated a positive result, which is typical for *Acinetobacter* spp (Table 3.3).

Test	Outcome
Gram stain	Negative
Microscopic appearance	Short rods (coccobacilli in stationary phase)
Oxidase	Negative
Catalase	Positive
Motility	Non-motile
Growth requirement	Aerobic

Table 3.3 Phenotypic properties of Sussex isolates

3.5.5 Identification of nine clinical Sussex strains using the API20NE system

The 48 hour Analytical Profile Indices as per API20NE 6th edition (1997) were presented in Table 3.4 for reference purposes, allowing the results to be reinterpreted in case of a design change of the API20NE test strips or change of taxonomic status of individual strains.

Legend Table 3.4:

* Strains marked were in the *Acinetobacter baumannii/ calcoaceticus* complex and could be assigned to the correct species on grounds of growth behaviour at 42 and 44°C.

** Strain W6492 was further tested using the API20E system, according to API20NE literature recommendation. The 24 hour reading profile was: 0204042, which was interpreted as *Acinetobacter calcoaceticus* var. *anitratu*s (good identification).

STRAIN SHORT CODE	API20NE READING (48 hours)	SPECIES
R1973	1041073	<i>A calcoaceticus</i> *
R2751	0041073	<i>A calcoaceticus</i> *
R3417	0001073	<i>A baumannii</i> *
R4474	4041073	<i>A calcoaceticus</i>
R45502	1041073	<i>A baumannii</i> *
R46383	0000012	<i>A lwoffii</i>
W5211	0041072	<i>A baumannii</i> *
W6108	0000012	<i>A lwoffii</i>
W6492	0001071	<i>A calcoaceticus</i> **

Table 3.4 Identification of Sussex strains using the API20NE system (n= 3)

3.5.6 Growth of 9 clinical Sussex isolates at 42 and 44°C

W6492 was the only strain to show weak growth (*) in 2ml broth (Table 3.5), $OD_{600}=0.142$. It also showed weak growth on TSA, and where the inoculum was applied heavily a discoloration occurred, changing the colour of the colonies to caramel from the usual cream-white.

Colonies from both areas were subcultured onto TSA plates and incubated at 37°C for 16-18 hours. Growth in both cases appeared as opaque colonies, without discoloration.

Strain short code	2ml	10ml	TSA	2ml	10ml	TSA
	42°C			44°C		
R1973	+	+	+	+	+	+
R2751	+	+	+	-	-	-
R3417	+	+	+	+	+	+
R4474	+	+	+	-	-	-
R45502	+	+	+	+	+	+
R46383	+	-	-	-	-	-
W5211	+	+	+	+	-	+
W6108	-	-	-	-	-	-
W6492	*	-	*	-	-	-

Table 3.5 Growth of Sussex isolates at 42 and 44°C (n=2)

Legend Table 3.5: *=*weak growth*

3.5.7 Growth curves at 37°C, using Bioscreen-C

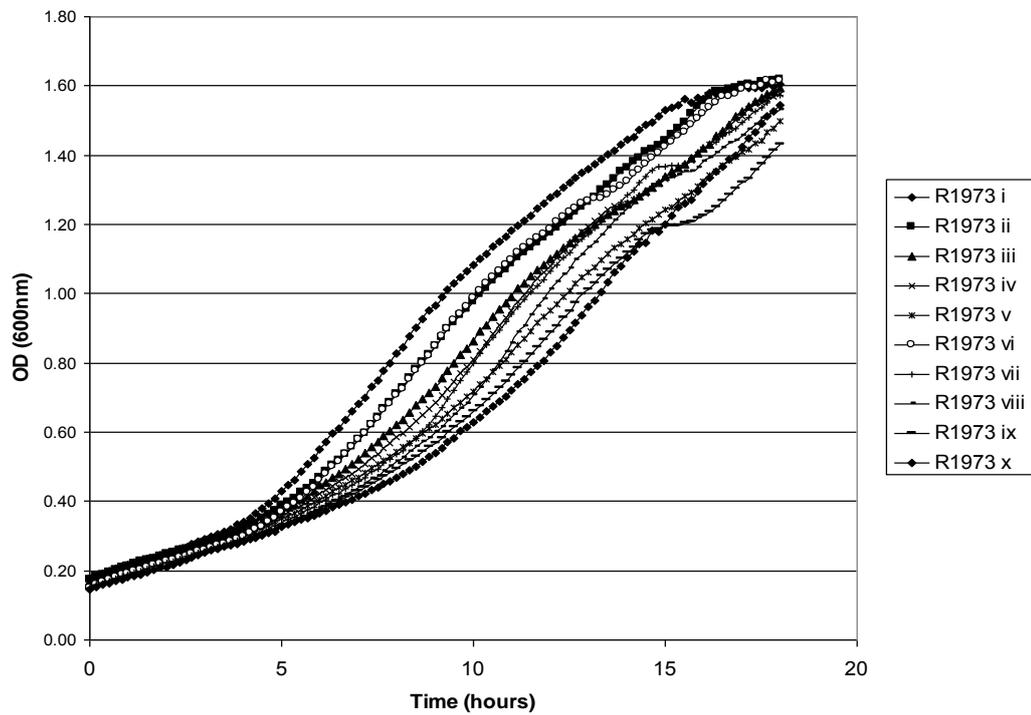


Figure 3.4 Growth curves of R1973 at 37°C (n=10)

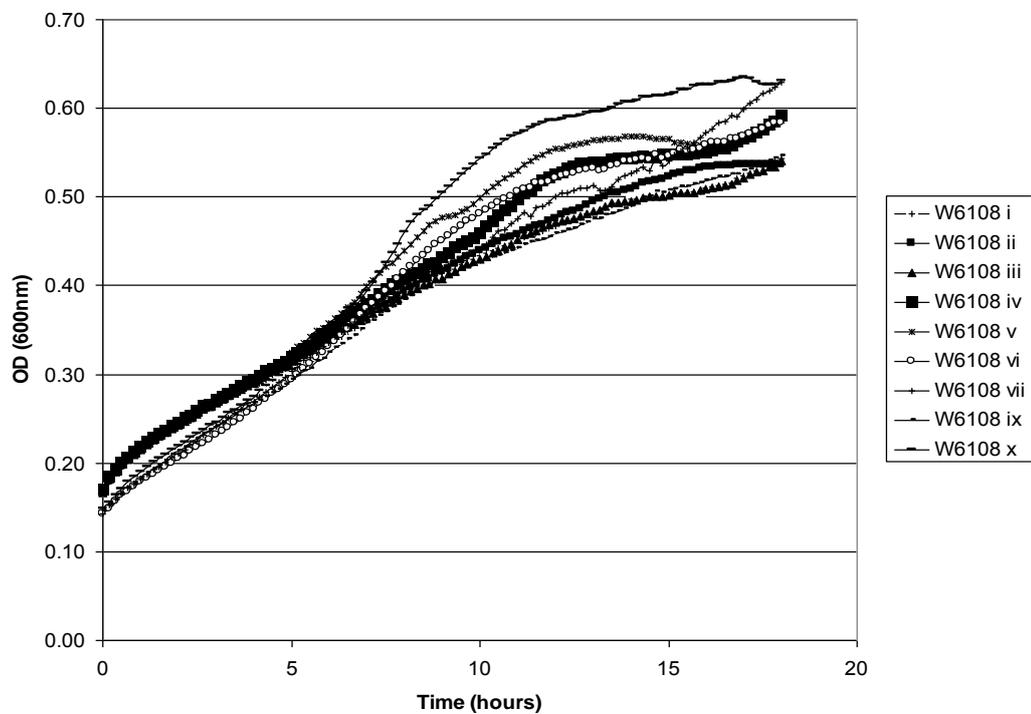


Figure 3.5 Growth curves of W6108 at 37°C (n=10)

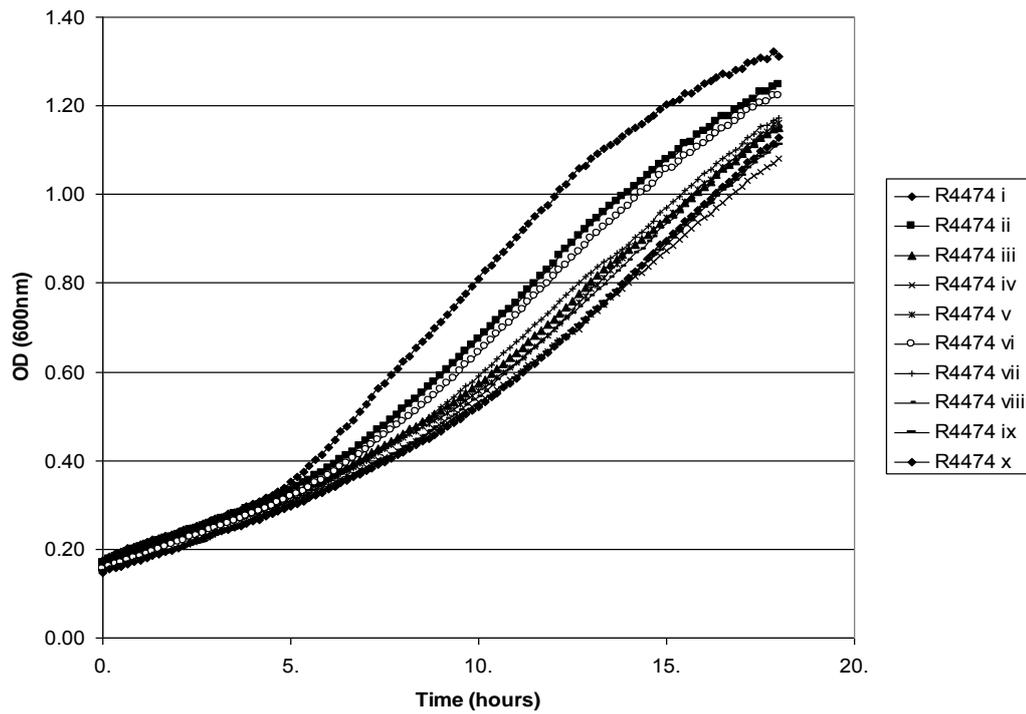


Figure 3.6 Growth curves of R4474 at 37°C (n=10)

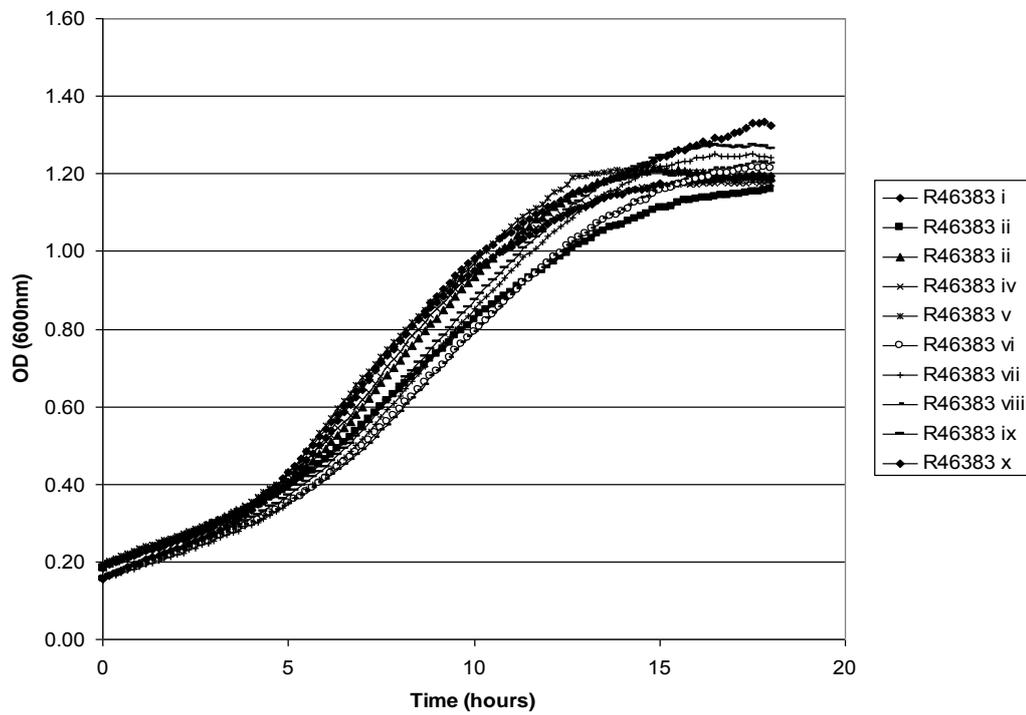


Figure 3.7 Growth curves of R46383 at 37°C (n=10)

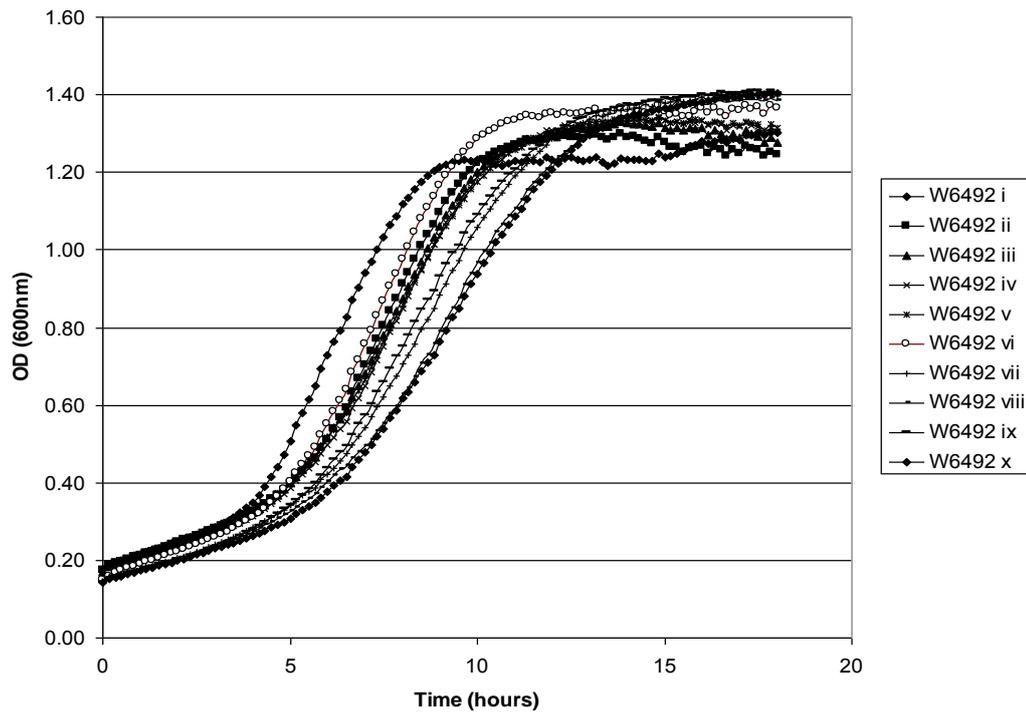


Figure 3.8 Growth curves of W6492 at 37°C (n=10)

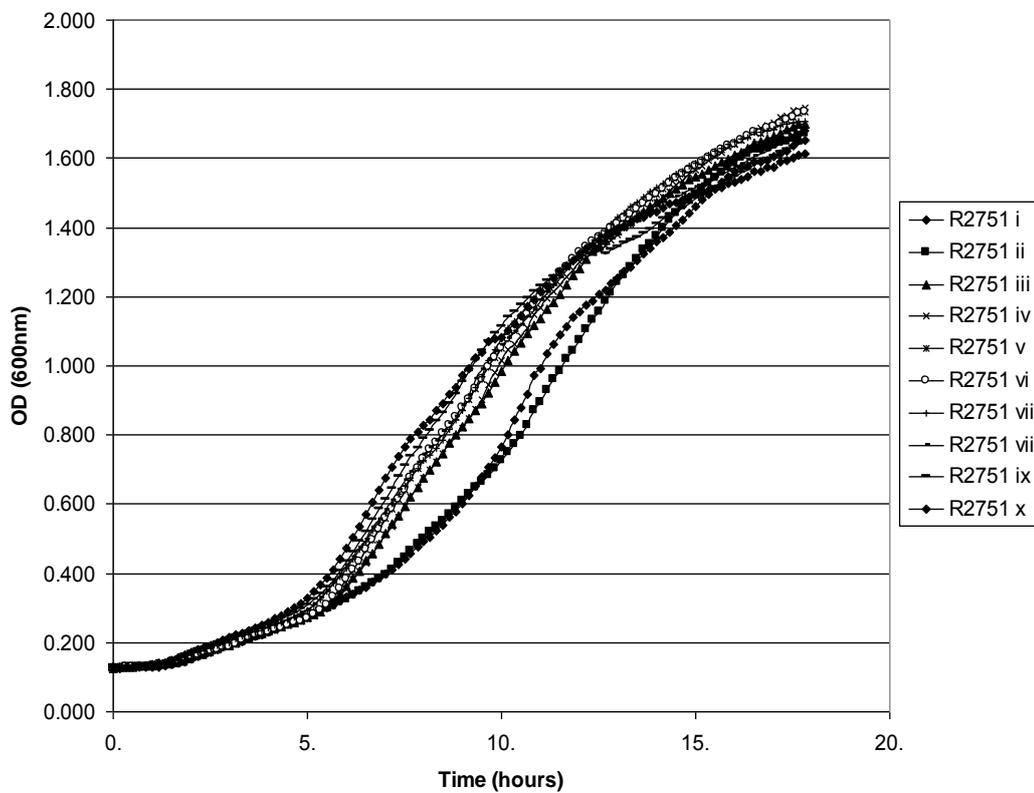


Figure 3.9 Growth curves of R3417 at 37°C (n=10)

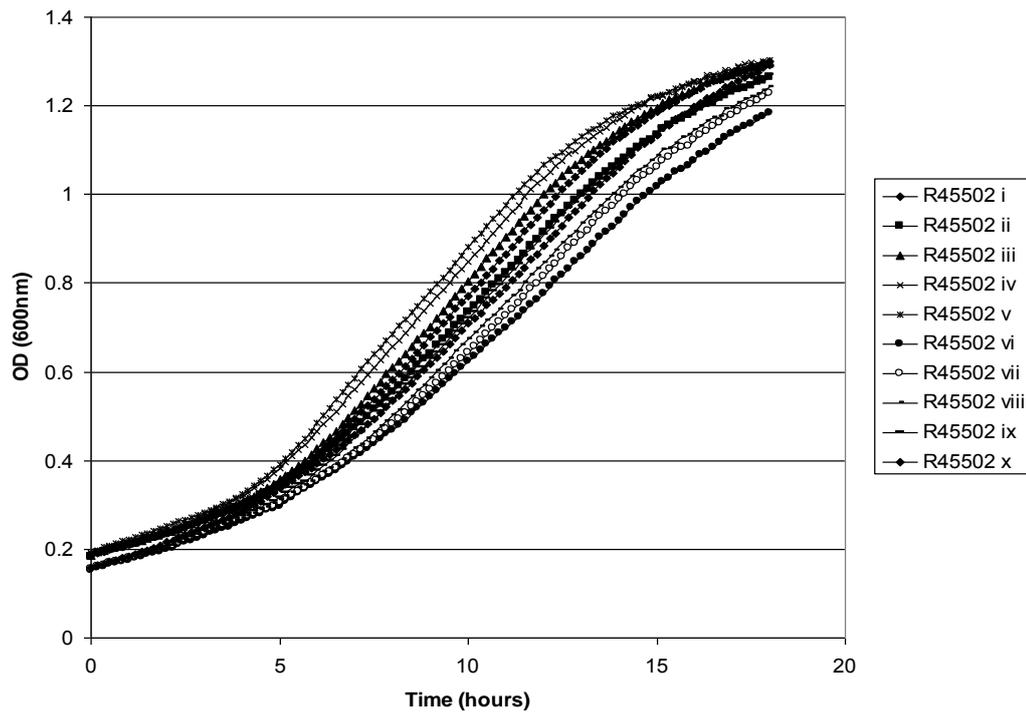


Figure 3.10 Growth curves of R45502 at 37°C (n=10)

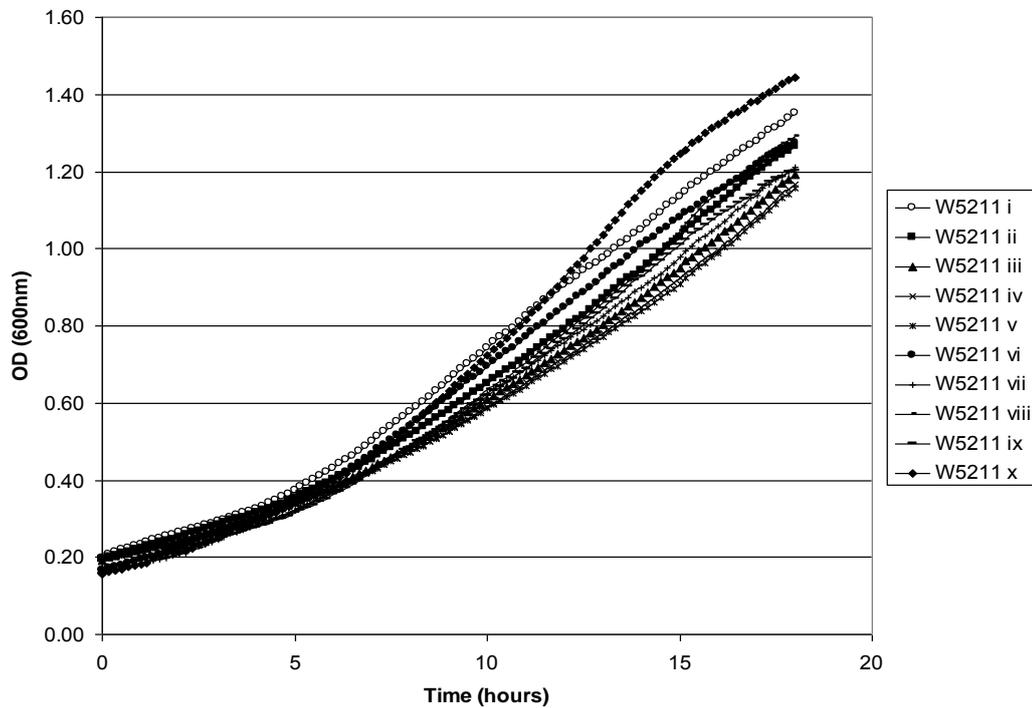


Figure 3.11 Growth curves of W5211 at 37°C (n=10)

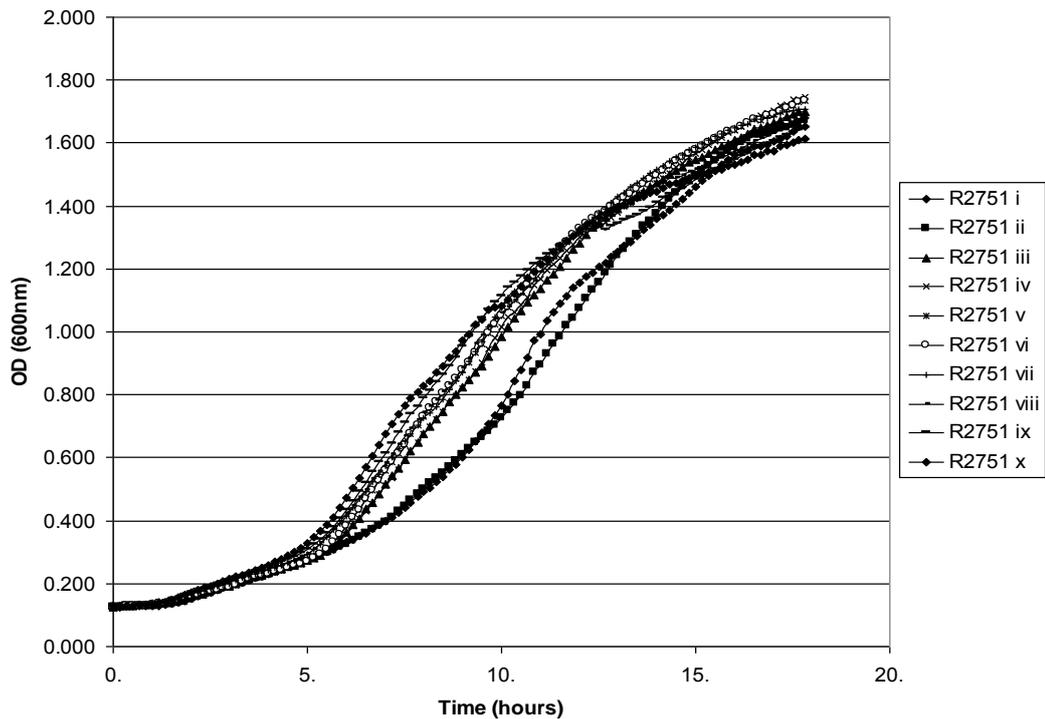


Figure 3.12 Growth curves of R2751 at 37°C (n=10)

As can be seen from **Figures 3.4-3.12** all isolates grew at 37°C. Isolate W6108, *Acinetobacter lwoffii* (**Figure 3.5**), a strain usually considered a cool growing, environmental isolate (Bergogne-Berezin, 1996) successfully grew at 37°C, however only to an average optical density of 0.5-0.6. This may be an indication that this isolate could be an isolate growing more vigorously at temperatures below the physiological range.

3.5.8 Antibiotic susceptibility of Sussex isolates using the BSAC method

Overall the findings presented in **Table 3.6** correlate with the sensitivity data provided with the samples (**Appendix 2**). The exceptions are the ciprofloxacin results as the BSAC employs 1µg discs whilst NCCLS recommends 5µg. The higher strength discs were used in this investigation, as the majority of isolates were considered multiply resistant. Despite the differences in strength for each method the interpretation of results varies only for strain R1973, which becomes intermediate when NCCLS breakpoints

are employed. Results for amikacin differ from Dr. Child's findings probably due to the fact that different breakpoints were used to interpret the data.

In three cases (R1973, W5211, R1973) the isolates produced zones of inhibition which were very close to being considered intermediate. Some strains reported as sensitive in 2000, would be considered resistant under more recent BSAC guidelines. It needs to be borne in mind that breakpoints are an arbitrary figure arrived at by evaluating the available evidence at that time. For the purpose of comparison NCCLS breakpoints from 2002 were also included. The BSAC *Acinetobacter* spp. breakpoints have not changed for the antibiotics listed above since 2001, apart from amikacin which had its breakpoint between 19 and 20mm in 2001, which was consequently changed to a wider spectrum of sensitivity (15-19, 2006) and gentamicin (19/20, 2001 to 16-20, 2006).

Despite using test discs containing 30 µg of amoxicillin/clavulanic acid, rather than the 10µg recommended by the BSAC, the majority of strains remained resistant to this commonly used combination of antimicrobials. NCCLS break points are given for trimethoprim-sulfamethoxazole rather than trimethoprim alone as used by the BSAC, NCCLS breakpoints for trimethoprim alone are not available. Results where NCCLS breakpoints were used to interpret the trimethoprim-sulfamethoxazole inhibition zones need to be interpreted with caution, as some isolates are reported as intermediate and one even as sensitive (NCCLS), when the same results were interpreted as resistant using BSAC break points.

Tetracycline, neomycin and vancomycin were chosen to be included in the investigation, despite the fact that current breakpoints values are not available for *Acinetobacter*. Aminoglycosides are used for treatment of *Acinetobacter* infections but increasing numbers of resistant strains have emerged since the late 1970s (Bergogne-Berezin et al., 1996). The aminoglycosides used in this investigation showed in-vitro activity against some strains tested. Neomycin would not be suitable for therapy of *Acinetobacter* infection, despite a similar spectrum of activity to gentamicin, due to its systemic toxicity, however it may be a clinical option for topical application. In the absence of breakpoints it remains of interest whether sensitivity to gentamicin correlates with zones of inhibitions above a certain size for neomycin.

Antimicrobial	Strength	NCCLS	BSAC	R1973	R2751	R3417	R4474	R45502	R46383	W5211	W6108	W6492
Amikacin	30mcg	14-17	15-19	R	R	S	R	R	S	R	S	S
Amoxy/ clav.acid	30mcg	N/A	17/18	R	R	R	R	R	S	R	S	R
Aztreonam	30mcg	15-22	23/24	R(S)	R	R	R	R	R	R	R(I)	R
Cefixime	5mcg	N/A	19/20	R	R	R	R	R	R	R	R	R
Ceftazidime	30mcg	14-18	27/28	R	R	R	R	R	R	R	R	R
Cefuroxime	30mcg	N/A	19/20	R	R	R	R	R	S	R	S	R
Ciprofloxacin	5mcg	15-21	17/18	S(I)	R	R	R	R	S	R	S	S
Gentamicin	10mcg	12-15	16-20	R	R	R	R	R	S	R(I)	S	S
Imipenem	10mcg	13-16	22/23	R	R(S)	S	R(S)	R(S)	R(I)	S	R(I)	S
Neomycin	10mcg	N/A	N/A	0	10	15	12	12	20	12	20	19
Piperacillin	100mcg	17-21	23/24	R	R	R	R	R	S	R	S	R(I)
Tetracycline	50mcg	14-19	33/34	R	R	R	R(I)	R(S)	R(S)	R(S)	R(S)	R(S)
Trimethoprim	1.25mcg	10-16	14-20	S	R	R	R(I)	R(I)	R(I)	S	R(I)	R(I)
Vancomycin	30mcg	N/A	N/A	6	0	0	6	0	14	0	14	0

Table 3.6 Antibiotic susceptibility of nine Sussex isolates using the BSAC disc diffusion method (n=2)

Legend for Table 3.6:

R – Resistant

S- Sensitive

I- Intermediate

(R) – Sensitive according to the NCCLS guidelines, but resistant or intermediate according to the BSAC cut-off points

(S) –Resistant according to the NCCLS guidelines, but sensitive or intermediate according to the BSAC cut-off points

(I) – Intermediate according to the NCCLS guidelines, but sensitive or resistant according to the BSAC cut-off points

Amoxy/clav.acid reads as amoxicillin plus clavulanic acid

Cefuroxime as cefuroxime sodium

Tetracycline is not routinely used for *Acinetobacter* infections, it has been used in reported early cases of native valve endocarditis and urinary tract infections caused by *Acinetobacter* spp. (Bergogne-Berezin et al., 1996), however tigecycline, which is structurally related has been praised for its in-vitro activity against multiresistant *Acinetobacter baumannii* (Livermore, 2005). Sensitivities for tetracycline, as a representative of a drug group may be a helpful indicator for the usefulness of tigecycline in a clinical setting. The BSAC removed breakpoints of tetracycline in version 4, 2005, to allow a review of breakpoints for this group of agents. As the BSAC Disc Diffusion Method for Antimicrobial Susceptibility Testing version 5, 2006 did not contain breakpoints for this drug, breakpoints from version 3, Jan 2004 at 33/34 mm were used to interpret the data.

Colistin was not included despite the fact that it remains a commonly used drug in multiple-resistant *Acinetobacter* infections at QVH and UK hospitals, despite its renotoxicity. It was excluded from this investigation as Oxoid colistin discs were not

available, because the molecular size of this drug makes agar diffusion erratic and results would not have been useful.

3.5.9 LAM agar and liquid medium validation experiments

Strain short code	Ceph	Vanc	Cefs	Ceph +Cefs	Vanc+ Cefs	Vanc+ Ceph	No Antibiotic	LAM
R1973	+	+	+	+	+	+	+	+
R2751	+	+	+	+	+	+	+	+
R3417	+	+	+	+	+	+	+	+
R4474	+	+	+	+	+	+	+	+
R45502	+	+	+	+	+	+	+	+
R46383	+*	-	-	-	-	-	+	-
W5211	-	+	+	-	+	-	+	-
W6108	-	-	-	-	-	-	+	-
W6492	+	+	+	+	+	+	+	+

Table 3.7 Growth of Sussex isolates on LAM with varying antibiotic composition (n=2)

Three out of nine isolates failed to grow on LAM agar (**Table 3.7**), despite it being promoted as being the best existing selective and differential medium, in terms of recovery of isolates (Jawad et al., 1994). Susceptibility to LAM of all control strains (**Table 3.8**) was as described by Jawad et al. (1994). The reason why environmental isolates grow less readily on LAM agar than clinical isolates is considered to be the presence of bile Salts No. 3 and β -lactam antibiotics in the medium (Jawad et al., 1994). However, as all the isolates grew on LAM agar without antibiotics this is not the reason

for failure of growth in this instance. The presence of cephalosporins and vancomycin are responsible for the inhibition of growth in those cases.

Strain short code	Ceph	Vanc	Cefs	Ceph +Cefs	Vanc+ Cefs	Vanc+ Ceph	No Antibiotic	LAM
<i>E. coli</i> TM3	-	-	-	-	-	-	-	-
<i>E. coli</i> 8739	-	-	-	-	-	-	-	-
<i>S. aureus</i> 6538	-	-	-	-	-	-	-	-
<i>P. aeruginosa</i> 9027	-	-	-	-	-	-	-	-
<i>Citrobacter freundii</i> ATCC 8090	*1	*1	*1	*1	*1	*1	*1	*1
<i>Serratia marcescens</i>	*2	*2	*2	*2	*2	*2	*2	*2
<i>E. coli</i> TM3	-	-	-	-	-	-	-	-
<i>E. coli</i> 8739	-	-	-	-	-	-	-	-
<i>S. aureus</i> 6538	-	-	-	-	-	-	-	-

Table 3.8 Growth of control strains on LAM with varying antibiotic composition (n=2)

Legend for Tables 3.7 and 3.8:

Ceph: cephadrine 50mg/l, *Vanc*: vancomycin HCl 10mg/l, *Cefs*: cefsulodin 15mg/l

No Antibiotic: LAM base without antibiotic addition; *LAM*: contains all three antibiotics: cephadrine 50mg/l, vancomycin HCl 10mg/l and cefsulodin 15mg/l

+ Clearly visible growth of pink colonies, change of agar colour to mauve (**Figure 3.14**).

- No visible growth, agar colour remains orange (**Figure 3.14**).

+* R46383: Weak growth only, pink colonies, agar area around growth changed to mauve

*1 *Citrobacter freundii* gave rise to yellow colonies on yellow background (**Figure 3.13**)

*2 Pink colonies on orange background

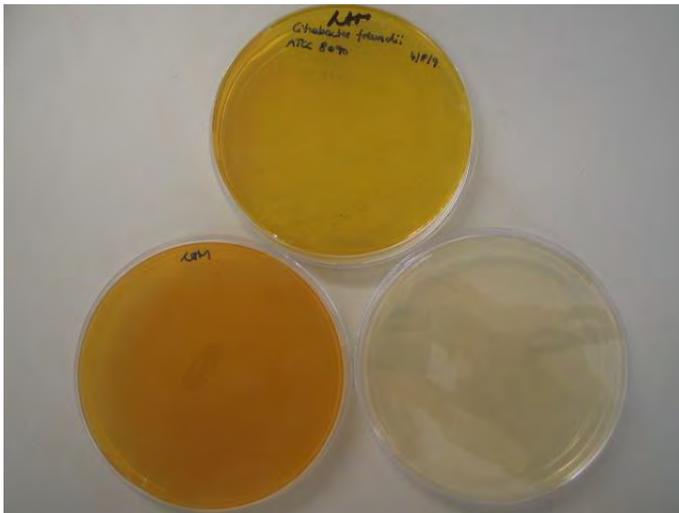


Fig 3.13 Top: *Citrobacter freundii* ATCC 8090 on LAM agar, Bottom left: LAM agar, Bottom right: TSA plate

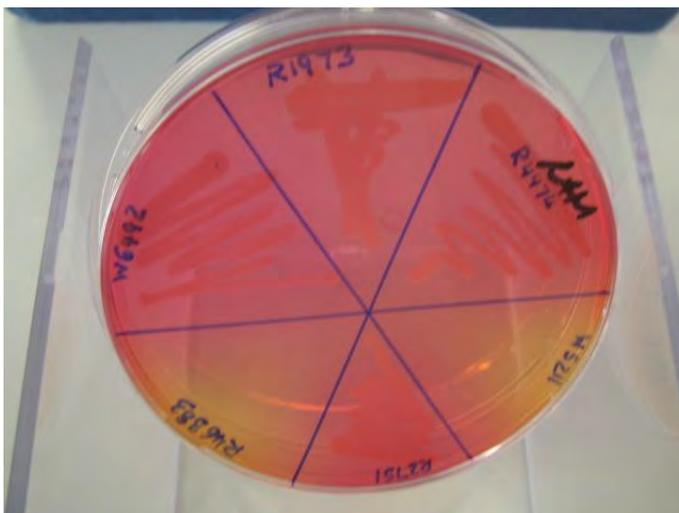


Fig 3.14 Typical appearance of LAM plate

All isolates apart from W6108 and R46383 grew on LAM agar supplemented with Vancomycin only. This correlates with BSAC disc sensitivity data where both isolates gave a zone of inhibition of 14mm, whilst the remaining strains yielded zones of inhibition between 0 and 6mm. There are no BSAC/NCCLS breakpoints available for this antibiotic. Apart from R46383 and W5211 all isolates gave the same response to both cephalosporins when grown on LAM- they were either resistant or in the case of W6108 sensitive to both. R46383 grew sparingly in the presence of cephradine, however failed to grow when the agar was supplemented with cefsulodin. In the case of W5211 this phenomenon was even more pronounced, it grew vigorously in the presence of cefsulodin, but did not when cephradine was present. This was reflected in the BSAC disc diffusion results where R46383 and W6018 were sensitive to cefuroxime, but resistant to ceftazidime and cefixime.

A possible explanation could be that *Acinetobacter* isolates display subtle differences in susceptibility to different cephalosporins, which are not expressed in disc diffusion breakpoints and are clinically mostly irrelevant. However for the purpose of investigating the usefulness of a selective medium containing antibiotics this is important. This idea is supported by the BSAC reporting problems with cefuroxime during the design of the disc diffusion method, as the MIC distribution for *Acinetobacter* spp. straddles the MIC breakpoint concentrations (Andrews, 2001). This was similarly observed by the BSAC with ceftazidime, where over 30% of *Acinetobacter* spp. were reported as false-resistant. This implies that isolates which would be susceptible to this antibiotic *in-vivo* failed to grow under certain laboratory conditions, similar to the phenomenon observed when grown on LAM agar. Jawad et al (1994) accounted for this by reducing the concentration of cefsulodin 30mg/l to 15mg/l in the final version of LAM, which inhibited the growth of 8% of *Acinetobacter* strains rather than 35%. The authors considered piperacillin as a component of LAM agar, however it was deemed unsuitable on grounds of MIC data. This is not reflected in the BSAC disc diffusion results in **Section 3.5.8**, where the resistance profile of all strains to piperacillin is the same as for cefuroxime with the exception of W6492, which is intermediate rather than resistant when NCCLS criteria are applied. It could be argued that the sample size used (9) is modest in comparison to the 86 strains investigated by Jawad et al. (1994).

Acinetobacter spp. owe some of their intrinsic resistance to β -lactams to effective efflux pumps, a combination of making Penicillin binding proteins less susceptible to binding and β -lactamases and an impermeability of their outer membrane to these agents (McGowan, 2006; Bergogne-Berezin et al., 1996). It could be claimed that the subtle differences seen in cephalosporin sensitivity were caused by varying degrees of lipophilicity of different agents within this group or simply variations in effectiveness of efflux pumps.

3.5.10 Growth characteristics of Sussex isolates on LAM with varying antibiotic compositions

Strain short code	Growth	Strain short code	Growth
R1973	-	R46383	-
R2751	+	W5211	-
R3417	+	W6108	-
R4474	+	W6492	-*
R45502	+		

Table 3.9 Growth of Sussex strains on MLAM (n=2)

* W6492 (**Table 3.9**) showed weak discoloration of MLAM where the inoculum was applied heavily, this was classified as „no growth“, as individual colonies could not be seen with the naked eye.

MLAM proved to be difficult to work with and apart from freshly poured plates taking disproportionately longer than TSA plates to set, the agar was significantly softer than LAM or TSA plates. This made inoculating the plates with metal loops difficult and caused large quantities of waste as many plates were not suitable for use. Another potential problem was that the colony size and viscosity of colonies varied between isolates, which made it difficult to deliver exactly equal inocula using wire loops. After several unsuccessful attempts to produce MLAM of satisfactory quality, it was decided

to use technical agar No. 1 instead, which produced plates with very similar rheological properties to TSA.

The theory that *Acinetobacter* spp. display different sensitivities to cephalosporins is further supported by the results of this experiment (**Table 3.7**). On MLAM (where cefsulodin was replaced with ceftazidime) W6492 produced only weak discoloration of agar rather than luxurious growth, however on LAM isolate W6492 grew vigorously. This finding did not correlate with disc diffusion data (**Section 3.5.8**), where W6492 was reported as resistant to ceftazidime. All isolates were reported as resistant to ceftazidime, however five isolates failed to grow on MLAM, whilst only three isolates did not grow on LAM agar. R1973 and W6492 did not grow on MLAM, yet grew vigorously on LAM, with the only difference in terms of antibiotics being the replacement of cefsulodin with ceftazidime. This cannot be accounted for apart from a possible synergy of antibiotics inhibiting growth of some isolates. MLAM permitted growth of even a smaller number of isolates than LAM, which led to this medium being abandoned.

3.5.11 Growth of clinical *Acinetobacter* isolates in LAM liquid

Inoculum (cfu/ml)	R1973	OD ₆₀₀	R4474	OD ₆₀₀	R45502	OD ₆₀₀	R4751	OD ₆₀₀
10 ¹	O-R	++	Y	+	Y	+	R	++
10 ³	Y	+	O-R	++	Y	+	R	++
10 ⁵	Y	+	Y	+	Y	+	Y	+

Table 3.10 Growth of Sussex isolates with varying inoculum concentration in LAM

Legend Table 3.10:

O-R orange-red (reddish orange)

Y yellowish orange

R red

++ *Heavy growth (OD₆₀₀ above 1.0)*

+ *Growth*

It is apparent that a change of the original colour of the medium from orange (like LAM agar) to red correlates with heavy growth and OD₆₀₀ readings above 1.0. At lower cell densities a change in colour was not as apparent (**Table 3.10**). It is also of interest that under the experimental conditions LAM inoculated with larger cell numbers did not necessarily yield heavier growth. This may be due to differences in aeration during incubation. It appears that after overnight incubation higher cell densities (reddish orange) were associated with alkaline and lower densities with mildly acidic conditions (yellowish orange), corresponding to estimated pH of 7.8 and 6.8 respectively.

It was observed that the blank turned yellow-orange after overnight incubation, in the same way as the samples yielding low growth. It was possible to recover viable bacteria from all samples with one exception after incubation. This may be of importance if LAM liquid was employed concurrently as enrichment and isolation medium.

3.5.12 Growth of *Acinetobacter* spp. in LAM liquid with and without background contamination found in waste water

As waste water was not further diluted, but used as a diluent for samples labelled as B the viable count was equal in all these samples, whilst the *Acinetobacter* count varied.

Viable *Acinetobacter* spp. could be recovered from all LAM samples containing *Acinetobacter* at concentrations between 2-6x10⁸ cfu/ml, with the exception of R1973 (see **Table 3.11**). This demonstrates that LAM liquid does not kill *Acinetobacter* isolate cells.

Samples containing *Acinetobacter* spp. and waste water resulted in higher optical densities after overnight incubation, hence it appears that LAM liquid also supports the growth of background contamination. Another reason for more vigorous growth occurring could be that the metabolic products of the background contamination provide favourable conditions for the proliferation of *Acinetobacter* spp.

<i>Acinetobacter</i>	Strain R1973				Strain R2751			
	Sample A		Sample B		Sample A		Sample B	
	OD ₆₀₀	colour						
10 ⁴	0.048	O	1.295	Y	0.736	O-R	1.220	Y
10 ³	0.019	O	1.301	Y	0.629	O-R	1.184	Y
10 ²	0.021	O	1.246	Y	0.553	O-R	1.344	Y
10 ¹	0.013	O	1.278	Y	0.502	O-R	1.213	Y
<10 ¹	0.014	O	1.250	Y	0.410	O-R	1.259	Y
Strain R45502								
<i>Acinetobacter</i>	Strain R45502				Strain R4478			
	Sample A		Sample B		Sample A		Sample B	
	OD ₆₀₀	colour						
10 ⁴	0.857	O-R	1.256	Y	0.759	O-R	1.256	Y
10 ³	0.697	*1	1.278	Y	0.695	O-R	1.280	Y
10 ²	0.613	O	1.233	Y	0.694	O-R	1.272	Y
10 ¹	0.551	O	1.249	Y	0.597	O-R	1.250	Y
<10 ¹	0.310	O	1.311	Y	0.014	O-R	1.175	Y

Table 3.11 Optical density reading OD₆₀₀ of samples A and B with varying concentrations of *Acinetobacter* spp. inocula.

Legend Tables 3.11 & 3.12: Colours of LAM liquid after incubation: O-R: orange-red, Y: (lemon) yellow, O: orange,

*1 Colour of top of sample: orange-red, bottom: orange

Viable count of waste water sample: 4×10^4 cfu/ml of unidentified colonies.

TNTC: too numerous to count

Dilution	A	Colony appearance on filter	Surrounding agar	B	Colony appearance on filter	Surrounding agar
1:10 ¹	TNTC	Pink	Mauve	TNTC	Red/yellow	Yellow
1: 10 ²	TNTC	Pink	Mauve	TNTC	Red/opaque	Yellow
1: 10 ³	TNTC	Pink	Orange	TNTC	Opaque	Yellow
1: 10 ⁴	TNTC	Faint pink	Orange	TNTC	Opaque	Yellow
1: 10 ⁵	TNTC	Faint pink	Orange	TNTC	Opaque	Yellow
1: 10 ⁶	TNTC	Faint pink	Orange	TNTC	Opaque	Yellow
1: 10 ⁷	TNTC	Faint pink	Orange	TNTC	Opaque	Yellow
1: 10 ⁸	TNTC	Faint pink	Orange	41	Opaque	Yellow
1: 10 ⁹	TNTC	Faint pink	Orange	5	Opaque	Yellow

Table 3.12 Appearance of colonies after membrane filtration of dilutions of samples A and B (n=3)

Waste water alone incubated in LAM turned the indicator colour to yellow, so did samples which had also been inoculated with considerable quantities of *Acinetobacter*. This implies that when background contamination is present judging by indicator colour alone is insufficient for establishing whether *Acinetobacter* is present. This may make LAM less suitable for selective enrichment of water samples.

The surface spread undiluted wastewater on TSA and LAM agar yielded vigorous growth after incubation. On LAM plates semi-confluent growth of opaque colonies

could be observed. On TSA plates approximately 150 cfu/plate various colonies of different colour size and shape could be counted, ranging from orange, pink to opaque. Most colony margins were not distinct, which made an exact colony count impossible.

Samples labelled as A, containing only various concentrations of *Acinetobacter* gave different indicator colours than samples labelled as B, which contained additional wastewater (**Table 3.12**). It appears that the presence of background contamination changes the indicator colour to yellow. It is of interest that *Acinetobacter* spp. on membrane filters grown on LAM resulted in typical pink colonies with mauve agar background only when growth was vigorous. Despite the fact that all samples labelled as A were marked TNTC, dilutions 1:10¹ to 1: 10³ showed entirely confluent growth, whilst higher dilutions resulted in separate colonies which were too numerous to count. Hence when growth on the membrane filter reaches subconfluent levels the surrounding agar remains the typical original LAM orange colour. Membrane filtration may be a suitable method of isolating and identifying *Acinetobacter* when large volumes of clear liquid samples are available, which are expected to have a low overall cell count but with a high proportion of *Acinetobacter* spp.

3.6 Discussion

3.6.1 Identification of 9 clinical Sussex strains using the API20NE system and growth at various temperatures of 9 clinical Sussex isolates

A variety of clinical *Acinetobacter* strains were investigated, the majority involved in outbreaks at various local hospitals. Despite the small sample size the trend reported in the literature appears to be confirmed that *Acinetobacter* strains involved in outbreaks are generally multiply-resistant (Bergogne-Berezin et al., 1996). *A. calcoaceticus* is frequently reported as the main species indicated in outbreaks, however the collection of Sussex strains contained four *A. calcoaceticus*, three *A. baumannii* and two *A. lwoffii*. It could be argued that the taxonomic status of some strains may be debatable, due to only one identification system being used and *Acinetobacter* taxonomy overall remaining an object of discussion (Bergogne-Berezin et al., 1996); However the diversity of nutritional preferences of all strains and differences in permissive growth temperatures show that it may be clinically short sighted to focus only on finding therapeutic phage

for *A. baumannii* infections. Strain W6492 *Acinetobacter calcoaceticus* var. *anitratus* deserves particular attention. It changed the colour of colonies from opaque to caramel, possibly indicating a change in sugar metabolism. The majority of *Acinetobacter* strains do not have the capacity to utilise glucose, Van Schie et al., (1989) reported a phenotypic change in *Acinetobacter calcoaceticus* where it mutated to utilisation of glucose as its sole carbon source. The metabolic adaptability of *Acinetobacter* is not new, it has been reported by Juni in 1972. This is of importance as mutants like this can outcompete other strains by their improved capacity to utilise different carbon sources. The capability of switching metabolic pathways can make identification on the basis of phenotype more difficult.

3.6.2 Antibiotic susceptibility of 9 Sussex isolates using the BSAC disc diffusion method

Assessing the antibiotic susceptibility of isolates donated to the University of Brighton 6 years previously proved to be difficult, simply because the breakpoints used to interpret zones of inhibitions were not supplied. For this reason NCCLS and BSAC breakpoints published near the time of isolation and current BSAC breakpoints were used to interpret the findings, which mirror the resistance patterns reported by Dr. J. Child. Three antibiotics which are not routinely included in disc susceptibility testing, reflected in the absence of published breakpoints were included in the investigation. Vancomycin was included because it is an essential component of MLAM and LAM media, therefore it was of interest to see if isolates produced zones of inhibitions at all. Tetracycline was included as a representative of a drug group, particularly as tigecycline has been praised in literature as the saviour in the fight against multiresistant *Acinetobacter* (Livermore, 2005). Neomycin, despite being unsuitable for systemic use was included to investigate if it reflected the patterns of zones of clearance seen in gentamicin. Two out of nine isolates were sensitive to gentamicin (R46383, W6108), the zones of inhibition for neomycin were 20mm in both cases, as compared to between 0 and 15mm for the other isolates which were resistant to gentamicin. In order to prove any correlation between resistance to one aminoglycoside and zones of inhibition exceeding certain limits in another would require more data. Two isolates (R4474,

R45502) were resistant to all antibiotics used in this investigation, if interpreted using current BSAC standards.

Apart from W5211 and R3417 all isolates were resistant to imipenem (considering current BSAC breakpoints), which reflects a worrying trend since carbapenems are a drug group with increasing reports of resistance of *Acinetobacter* spp. isolates worldwide (Brown & Amyes, 2006). Results for imipenem were repeated four times, because NCCLS (13-16) and BSAC (22/23) breakpoints are considerably different. *Acinetobacter* resistance to cephalosporin is well documented in the literature, it is well established that a combination of β -lactamases, efflux pumps and an impermeability of their outer membrane to these agents are responsible for this phenomenon (Bergogne-Berezin et al., 1996). However it is of interest that two isolates (R46383, W6108) were resistant to cefixime and ceftazidime, yet not cefuroxime sodium. This is probably due to the different side chains, resulting in different spectra of activity. Ironically cefuroxime a second generation cephalosporin showed higher levels of activity against *Acinetobacter* than the 3rd generation agents, which are generally considered to be more active against *Acinetobacter* (Bergogne-Berezin et al., 1996). This may be of relevance in laboratories where a representative cephalosporin may be selected to establish resistance to cephalosporins in general. Caution is advised where resistance to one agent of this drug group is extrapolated, as it should not be assumed that 3rd generation cephalosporins are more effective against *Acinetobacter* spp. than cefuroxime sodium.

3.6.3 Growth characteristics of Sussex isolates on LAM with varying antibiotic composition and MLAM

The fact that *Acinetobacter* spp. show different susceptibilities to various cephalosporins was reflected in the disc susceptibility results and in the growth characteristics on LAM with varying antibiotic composition. The growth of W5211 was inhibited by cephradine 50mg/l, however grew when cefsulodin 15mg/l was present in the medium. Jawad et al. (1994) observed a similar phenomenon where 5% of all *Acinetobacter* strains tested were inhibited by cephradine 50mg/l, yet cefsulodin 15mg/l inhibited the growth of 8% of strains. Four out of nine clinical Sussex isolates failed to grow on LAM agar and five did not grow on MLAM.

Considering LAM is the most widely used selective medium for *Acinetobacter* spp. and apparently better than the only two agars which are at all useful for the isolation of clinical isolates these results were not promising. LAM remains the medium of choice simply because of the lack of alternatives rather than its ability to selectively grow *Acinetobacter* spp. and suppress proliferation of other microorganisms. Attempts to modify the medium (MLAM) to improve its performance were unsuccessful, MLAM proved to be more restrictive towards *Acinetobacter* growth than LAM. Technical agar No. 3 also proved unsuitable for large scale production of MLAM plates, despite dissolution before autoclaving, as it required long periods to solidify and a large number of plates were unsuitable for use as the agar did not provide the gel properties expected of an agar at ambient temperatures.

Despite the fact that LAM did not support growth of almost 50% of the Sussex isolates, it appeared to be a good indicator for the presence of *Acinetobacter* spp., as it allowed visual differentiation between expected contaminants of clinical and other samples. LAM also suppressed growth of two *E. coli* strains, which may make it a useful tool in excluding coliform background contamination when used in conjunction with sewage and waste water samples.

3.6.4. Growth of Sussex isolates in LAM liquid without background contamination

All selected *Acinetobacter* spp. which had grown on LAM agar also grew in LAM liquid. When LAM was inoculated with low numbers of *Acinetobacter* spp. they generally grew vigorously. In the single example where growth was not vigorous, the number of cells used to inoculate the sample was not directly proportional to the optical density after incubation, which implies that inappropriate aeration due to insufficient agitation may have been the cause. Optical density readings above 1.0 correlated with a change of pH indicator to red, which is distinct from the original orange of the medium. At lower cell densities, however it was not possible to rely on the colour change alone to differentiate between samples containing cell counts below 1.0 and those not viable. This means LAM liquid does not give a straightforward optical indication of whether growth occurred or not, but optical density measurements need to be included, which makes this approach more impractical particularly with large numbers of samples.

It was observed that the blank turned yellow-orange after incubation, in a similar manner to those samples yielding low growth, which is an indication, that mild acidification of medium took place.

3.6.5 Growth of Sussex isolates in LAM liquid with background contamination

The optical density of cultures after incubation was proportional to the initial *Acinetobacter* inoculum, which implies that reasons other than the quality of the medium were responsible for disproportionate cell growth in the previous experiment. One sample only changed to the typical orange-red indicative of vigorous growth in the top half of the boiling tube, yet remained orange in the bottom, an indication that metabolism of sugars was an aerobic process. This is a further indication that using boiling tubes as incubation vessels is less than ideal as the samples may not be thoroughly agitated.

In cases where an artificial background contamination was introduced, even where initial cell counts of the waste water were equal to the *Acinetobacter* inoculum, a colour change to yellow was seen, which is typical for the presence of species other than *Acinetobacter* on LAM agar, potentially a large number of enteric bacteria, which may have fermented the sugar in LAM, causing subsequent acidification.

Overgrowth of LAM liquid by contaminants in the presence of *Acinetobacter* spp. would pose a distinct disadvantage in cases where waste water and sewage are used to isolate *Acinetobacter* or for enrichment, as it is probable that *Acinetobacter* will be present as a small percentage of the total bacterial bioburden. Samples which underwent membrane filtration produced results which were equally unpromising. When *Acinetobacter* was present on its own in large quantities it was simple to identify it by the pink colonies and the mauve background. When cell numbers dropped, yet were still too numerous to be countable, the colouring of the agar was barely distinguishable from the original orange of LAM. Despite the colonies being described as faint pink, it would be necessary to include further methods to truly identify an isolate as *Acinetobacter* spp. When background contamination was present it was impossible to detect *Acinetobacter* colonies on the filter, despite viable counts of

Acinetobacter and waste water being equal before overnight incubation in LAM. Not one single *Acinetobacter* colony could be identified as such, despite sample B being of low enough density to identify individual colonies. This implies that *Acinetobacter* colonies may either have been outgrown by the contaminants or could not be detected in the experimental setup used or more acid was produced by contaminants than alkali produced by *Acinetobacter* spp.

3.7 Conclusion

MLAM is less suitable for the isolation of *Acinetobacter* than LAM, partly due to the choice of cephalosporin and due to the properties of technical agar No. 3. LAM however remains the selective medium of choice, rather for lack of a better selective and differentiating medium suitable for the isolation of a wide spectrum of *Acinetobacter* spp. than its outstanding qualities.

LAM liquid supports growth and allows recovery of *Acinetobacter* spp. and may be suitable for enrichment. A change of colour to red, indicative of growth only occurs at optical densities over 1.0, which implies the indicator change cannot be relied upon as indicator of growth alone, but must be supported by optical density measurements. As some indicators imply that agitation was not sufficient under the experimental setup used, it may be beneficial to use vessels allowing more efficient agitation during the incubation process. Glass vessels of a conical shape may be most beneficial, as they allow maximum agitation and visual inspection after incubation. For the identification of *Acinetobacter* in samples LAM liquid may have only limited usefulness. LAM liquid supports the growth of background contamination and may therefore be less useful in isolation and enrichment procedures. Using membrane filters on LAM is an insufficient method to be relied upon solely to identify *Acinetobacter*, however it may be useful in aiding the isolation and identification when low cell numbers and relatively low background contamination are expected.

The antibiotic sensitivity data obtained for the clinical isolates makes it even more apparent that resistance in *Acinetobacter* spp. is a severe clinical challenge and current choices of antibiotics insufficient. Phage therapy could offer a true clinical alternative in

Chapter Three: Identification and characterisation of nine clinical „Sussex“ *Acinetobacter* spp. isolates
cases of multi-drug or pan-drug resistant *Acinetobacter* causing infections and wound
colonisation.

Chapter FOUR

Attempted isolation of *Acinetobacter* spp. and phage from sewage and waste water

4.1 Introduction

The clinical isolates listed in **Section 3.1** were the beginning of an *Acinetobacter* spp. collection at the University of Brighton and it was decided to try to expand this collection to include isolates and associated lytic bacteriophages from the hospital environment and other environmental sources. At the time of the investigation no detailed current published protocols on the isolation of therapeutic *Acinetobacter* phage were available. Before 2005 specific bacteriophage isolation methods for the purpose of finding therapeutic phage had not been published.

Adams (1959) a regularly cited source and allegedly the only comprehensive textbook on all aspects of phage since 1926 (Adams, 1959 p. iv) dedicates a brief two pages on the isolation of a virus lysing *E. coli*. A recognized shortage of published phage isolation methods and lack of understanding of phage led to the writing of a series of textbooks on the subject (personal communication E. Kutter, 28 September 2004) ‘Bacteriophages, Biology and Application’ (2005) Kutter and Sulakvelidze (Eds.); ‘Bacteriophages: 2 Methods in Molecular Biology (2009), Clokie and Kropinski (Eds); ‘Bacteriophage Ecology’ (2008) Abedon (Ed): Edition 2, Cambridge University Press. While some of these textbooks are now available they were not published at the time the work described in this Chapter was being conducted.

Acinetobacter species are prevalent in the environment but these strains do not represent those most likely to cause disease. In order to acquire relevant isolates it was decided to

sample waste water from the Queen Victoria Hospital (QVH) sewers, sinks and toilets whilst a patient colonised with confirmed *Acinetobacter* was present on the ward.

The rationale was that if *Acinetobacter* spp. were present on the patient, the hands of the nursing staff and on equipment in the vicinity, there would be a higher likelihood of *Acinetobacter* being found in the waste water system, which would make it more likely for *Acinetobacter* phage to be isolated. It is generally understood that phage (considered by some the most abundant life form on earth), are present in a large variety in environmental sources (Kutter & Sulakvelidze, 2005). Bergey's Manual of Systematic Bacteriology (Holt, 1984) states that 'Lytic phages for acinetobacter [sic] are isolated readily from sewage', using Twarog & Blouse (1968) and Herman & Juni (1974) as the basis of this judgement. It is recognised that phages are more likely to be present if the conditions are favourable for the host and generally isolation procedures depend upon lytic growth of the phage (Kutter & Sulakvelidze, 2005).

Before starting isolation of phage its bacterial host needs to be available and grown in pure culture, preferably in exponential growth phase. Growth conditions should be designed to accommodate the preference of the host bacterium and as many different approaches as possible to isolate phage should be attempted (Kutter & Sulakvelidze, 2005). Mandilara et al. (2006) reported a two year study during which they built a phage library isolated from sewage and sludge and found that certain types of phage routinely occurred at higher densities than others in the same samples. Concurring with other investigators somatic coliphages were most abundant in samples, while phages for *Bacteroides fragilis* generally occurred at concentrations several magnitudes lower than other phages, as measured in plaque forming units (pfu) per gram sample. It was also demonstrated that the type of sample (raw wastewater, raw sludge and digested sludge), sampling location and how the material had been processed in the sewage treatment plant directly affected the number of viable phage particles present.

This establishes that from a statistical viewpoint some types of phage are naturally present in significantly lower densities than others and may therefore be harder to detect. It also confirms that sampling location and conditions are critical to the successful recovery of desired phage. The same research team reported an existing

correlation between the number of bacterial indicators and the presence of bacteriophage. Mandilara et al. (2006) found evidence that in some phage-host combinations large quantities of bacteria isolated were indicative of large numbers of matching phage particles in the sample. Presence of bacteria served as a predictor for the presence or absence of certain types of bacteriophages in wastewater. It is stated in the literature that phage can be relatively easily isolated from various sources (Kutter & Sulakvelidze, 2005) but know-how, sampling location, season and even luck (sic!) play an important role in successfully isolating lytic phage (personal communication E. Kutter, 28 September 2004; N. Chanishvili, 18 February 2005)

4.1.1 Specific isolation protocols for *Acinetobacter* phage

Twarog & Blouse (1968) reported that for the isolation of phage two techniques were used. In one method two litres of sewage were centrifuged twice (45 min at 590g and 60 min at 105,000g), the supernatant liquid was decanted and the pellet eluted with broth overnight at 4°C. In the second technique the sewage was centrifuged for 45 min at 590g and filtered through a series of membrane filters, with 0.01µm pore size as the last step. Phage was eluted from the 0.01 µm filter. In both cases 1ml of eluate and 1ml of log-phase cell suspension were added to 30ml broth, incubated overnight, bacteria were removed by low speed centrifugation and aliquots of the supernatant filtered through 0.45 µm filters. Serial dilutions of the filtrates were plated, incubated overnight, plaques cored and replated five times.

Herman & Juni (1974) described a phage isolation protocol where samples of activated sludge from a sewage processing plant were inoculated into flasks of Penassay broth and incubated with shaking overnight at 30°C. The contents of each flask were centrifuged and filtered through 0.2µm membrane filters. Filtrates were plated with *Acinetobacter* spp. using the agar overlay method. Plaques were picked, plated again with the host strain and finally used to inoculate exponential phase bacterial cultures, incubated with shaking until lysis was evident. The lysates were purified by centrifugation and membrane filtration.

Coffi (1995) reported that the majority of phages used as part of his research came mainly from two pre-existing collections, however 20 phages were isolated from sewage from Quebec. The report describes a successful method for the isolation of *Acinetobacter* phage, however the method was described in insufficient detail. Sewage was sampled from a waste treatment plant near Quebec, Canada and after sedimentation the samples were centrifuged for 10 minutes at 10,000 rpm.

The supernatant liquid was recovered, stored at 4°C and was used to determine the content of phage in the sewage and for enrichment. 5ml of the supernatant liquid was filtered through a Millipore HA 0.45µm membrane filter, 0.2ml of filtrate were applied to a bacterial lawn on TSA and incubated (no temperatures or time given). This was repeated using a range of *Acinetobacter* spp. For the enrichment 100ml of centrifuged sewage were added to 100ml Brain Heart Infusion broth with 1% (w/v) CaCl₂ plus 5ml of bacterial culture and the mixture incubated at 30°C for 18-24 hours. On the following day, 5 ml of the incubated mixture were filtered using a 0.45µm membrane filter and the presence of phage was confirmed by lysis.

Ackermann & Turcotte (1970) refer to the isolation of *Bacterium anitratum* phage with sparse details. 'The phage and its host originated from sewage. They were cultivated in broth and TSA at 30°C. The quantitative experiments were performed using double layers of 0.5% agar.'

Soothill (1992) reported that phage BS46 was isolated by an enrichment method using 15 litre batches of sewage. He claims to having used an upscaled isolation method after Adams (1959) without disclosing further details.

Adams (1959) described the isolation of *E. coli* phage, whereby the host was subcultured in broth and mixed with 1ml of supernatant obtained by centrifuging pooled sewage for a few minutes. After overnight incubation at 37°C the mixture was centrifuged to remove bacteria and filtered through a Corning ultrafine sintered glass filter, this filtrate was then tested for plaques using either of two methods. In the 'plating method' the filtrate was serially diluted (at least four 1 in 10 dilutions) in a broth culture of the host and 0.1ml aliquots of this dilution were directly spread over

agar plates, which were incubated overnight. Plaques or circular areas of lysis in the bacterial lawns were an indication of presence of phage. In the second method 10ml of broth were inoculated with 0.05ml of 'visibly turbid culture of host organism' and 0.1ml of filtrate to be tested for lytic phage. The mixture was incubated and examined hourly, the optical density measured, whilst comparing it to a control containing only bacteria. If the optical density did not rise significantly or even dropped the broth dilutions of culture may be tested further for phage using the 'plating method'.

Apart from Twarog & Blouse (1968) and Soothill (1992) the protocols specific for the isolation of *Acinetobacter* phage do not disclose information on the volume of sewage needed to achieve positive results. There is no information on the number of attempts required, whether the operator was skilled and experienced in phage isolation, the exact materials used and in some cases even incubation times and temperatures. None of the methods disclosed where the sewage was sourced from, whether it was from a municipal or hospital sewer, how it was stored after sampling, the ambient temperatures or season. In the case of Herman & Juni (1974) it is unclear whether the sewage had been treated or at which stage of treatment the sample was removed and if upper or lower layers of sludge have been sampled.

4.1.2 Aim

The aim of this study was to develop different approaches to isolate *Acinetobacter* strains and/or corresponding bacteriophages from hospital samples of varied origin and other waste water and sewage outlets. Any bacteriophage isolated during these procedures must be lytic at physiological temperature.

4.2 Material and Methods

4.2.1 Bacterial strains

Sussex bacterial isolates described in **Section 2.2.1** were used for experiments involving Queen Victoria Hospital (QVH) waste water samples (**Section 4.2.2**). For the assessment of sewage samples (**Section 4.2.5**) an additional isolate described in **Section 2.2.2** was included.

4.2.2 QVH Waste water samples

Sample Origin	Short code
ICU Room 1 sink	BU1
Burns unit Changing room men	BU2
ICU room 2 sluice sink	BU3
Burns unit open ward 4 Bed	BU4
ICU room 2 sink	BU5
ICU room 1 sluice sink	BU6
Main Drains (toilet and sink collector)	DR1

Table 4.1 Sample origins and corresponding short codes

QVH was visited on a single occasion for sample collection when the burns unit was treating a patient with confirmed *Acinetobacter* infection. Sinks and toilets within the unit were chosen as sampling sites. Short codes were assigned according to origin- BU for burns unit and DR for main drains, which was a collector drain for toilet and sink waste water for the entire QVH site (see **Table 4.1**). ICU stands for the specialist intensive care unit within the burns ward. Approximately 100-150ml of wastewater was withdrawn via suction from a u-tube from sites BU1-BU6 and collected in sterile screw

cap containers. For DR1 a container was dipped into the sewage collector and the wastewater decanted into a sterile glass bottle. It was observed during sampling of DR1 that large amounts of foam were visible in the drain and the sample. Samples were transported to the laboratory in a sealed box containing a refrigeration block and processed on the same day.

4.2.3 Sewage samples from Poynings, Scaynes Hill and other collection sites

All sewage samples (see **Table 4.2**) were provided by The School of the Environment, University of Brighton, from water samples taken as part of an unrelated project and their original identification numbers were retained.

Older samples were received as frozen at -70°C and thawed overnight at 4°C before experimentation. Fresh samples were refrigerated after collection and received within 48 hours of sampling, experimentation was commenced on receipt.

No information was provided on the exact sampling site and whether the samples had undergone any processing at the respective treatment plant, before sampling. All samples contained particulate matter, which was allowed to sediment on arrival.

Samples were used for experimentation without further processing see **Section 4.2.5**.

Sample origin	Short code	Sampling date
University Drain	P4HM01	05.11.2001
University Drain	P4HM02	12.11.2001
Poynings Water treatment plant	P4HM03	18.02.2002
Poynings Water treatment plant	P4HM04	26.02.2002
Poynings Water treatment plant	P4HM05	05.03.2002
Poynings Water treatment plant	P4HM06	12.03.2002
Poynings Water treatment plant	P4HM07	09.04.2002
Poynings Water treatment plant	P4HM08	07.05.2002
Poynings Water treatment plant	P4HM09	07.05.2002
Poynings Water treatment plant	P4HM10	13.05.2002
Poynings Water treatment plant	P4HM11	29.05.2002
Poynings Water treatment plant	P4HM12	29.05.2002
Scaynes Hill sewage treatment plant	P4HM14	03.03.2003
Scaynes Hill sewage treatment plant	P4HM15	03.03.2003
Scaynes Hill sewage treatment plant	P4HM16	03.03.2003
Scaynes Hill sewage treatment plant	P4HM17	03.03.2003
Scaynes Hill sewage treatment plant	P4HM18	03.03.2003

Table 4.2 Origin, short codes and sampling dates of sewage samples

4.2.4 Isolation of *Acinetobacter* spp and phage from Queen Victoria Hospital waste water samples

Nine 10ml aliquots of NB were inoculated with 1-2 colonies of clinical *Acinetobacter* isolates R4474, W6108, R2751, W5211, R46383, R3417, R45502, W6492 and R1973 (Sussex Strains, **Section 2.2.1**), all maintained on TSA plates. The suspensions were vortexed, 2ml of each suspension were removed and pooled in 7x100ml of NB or TSB.

The pooled *Acinetobacter* suspensions were incubated in an orbital shaker at 37°C at 120 rpm for approximately 60min. The collected waste water samples (BU1-DR1) were centrifuged in a Sorvall RC-5B refrigerated superspeed centrifuge (Du Pont Instruments, Delaware, USA) at 100g for 20min at 4°C to remove any large particles. 10-20ml of the supernatant liquid were added to each flask of the pooled *Acinetobacter* suspensions (enrichment step) and these suspensions were incubated overnight in an orbital shaker at 37°C at 120 rpm. The mixtures were centrifuged in a Sorvall RC-5B Refrigerated Superspeed Centrifuge (Du Pont Instruments, Delaware, USA) at 11,000 g for 20min. The supernatant liquids were filtered using 0.45-micrometer Nalgene syringe filters (Nalge) and BD Plastipak 10ml luer syringes (Becton Dickinson) in a laminar flow cabinet. All samples, supernatants and filtrates were stored at 4°C.

4.2.4.1 Surface spotting of filtered supernatant liquid ‘Direct plate selection’

20µl of the filtered supernatant liquids (BU1-BU6 and DR1) were spotted onto overdried TSA plates, which had previously been surface spread with 200µl of pure overnight *Acinetobacter* broth culture. Results **Section 4.3.1**

4.2.4.2 Overlay method using filtered supernatant liquid ‘Direct plate selection’

The filtered supernatant liquids (BU1-BU6 and DR1) were used for the overlay method: 2.5ml of molten soft agar maintained at 50°C were inoculated with 200 µl of one

overnight *Acinetobacter* culture (9 different Sussex isolates) and 0.5ml of the filtered supernatant liquid was added.

The samples containing filtered supernatant and overnight *Acinetobacter* culture were vortexed, poured over a set TSA plate and swirled gently to ensure an even overlay. The overlay plate was left to set for approx. 60 min and incubated overnight at 37°C.

Results are documented in **Section 4.3.2**.

4.2.4.3 Propagation of bacteria found in unfiltered supernatant liquid and pellet

200 µl of the original unfiltered wastewater supernatant liquid were surface spread onto 6 LAM agar plates and incubated for 48h at 37°C and 30°C for 12hours. A broth overnight culture of *Acinetobacter* R45502 was 4-way streaked for single colonies on LAM incubated at 37°C for 17hours to serve as a visual reference. The appearance of colonies on LAM was compared to the reference strain *Acinetobacter* R45502, colonies which resembled the appearance of *Acinetobacter* on LAM (pink colonies, mauve background) were subcultured on TSA and incubated for 16-18 hours at 37 °C. In cases where a range of colonies was present, 3 representative colonies were further investigated for each type. Individual colonies were used for oxidase tests, in cases where the outcome of the test was deemed insufficient to exclude the presence of *Acinetobacter* a Gram-stain was performed.

The pellets arising from centrifugation of the original water samples were 4-way streaked on LAM and incubated at 37°C and at 30°C for 36 hours. Further identification of colonies was achieved by visual comparison with reference strain *Acinetobacter* R45502 on LAM, oxidase and catalase tests and Gram-stain.

Results are shown in **Section 4.3.3**

4.2.4.4 Checking for growth of wastewater samples on TSA and subculture onto LAM

One test plate of TSA was surface-spotted with 20 µl of each of the wastewater samples in order to check for bacterial growth and incubated overnight at 37°C. Growth from the TSA plate was 4-way streaked on LAM plates and incubated for 16-18 hours at 37°C to obtain single colonies. Results are presented in **Section 4.3.4**

4.2.5 Isolation of *Acinetobacter* spp and corresponding phage from sewage samples

100ml of sterile LAM broth were inoculated separately with 10ml of sewage samples P4HM01- P4HM18 and incubated at 30°C for 48 hours on a shaking incubator operating at 110 rpm.

Controls of 9 Sussex strains and HER1424 (see **Chapter 5**) were prepared by re-suspending a single colony from a TSA plate into 10ml LAM broth and incubating alongside the sewage samples. Growth of controls was checked by removing a loopful of LAM and 4-way streaking onto LAM agar which was incubated for 16-18 hours at 37°C. Five 20µl samples were removed from the incubated sewage samples and spotted onto LAM agar plates, then incubated for 16-18 hours at 37°C. After incubation areas with uniform bacterial growth were chosen, subcultured on TSA and LAM and incubated for 16-18 hours at 37°C. Individual colonies on TSA were used for oxidase and catalase reactivity and selected isolates used for Gram stains and API20NE tests.

10ml aliquots of sewage samples were centrifuged in a Sorvall RC-5B refrigerated superspeed centrifuge (Du Pont Instruments, Delaware, USA) at 11,000 g for 20min. The supernatant liquid was filtered using 0.45-micrometer Nalgene syringe filters (Nalge) and BD Plastipak 10ml luer syringes (Becton Dickinson) in a laminar flow cabinet. All sample supernatant liquids and filtrates were stored at 4°C.

Supernatant liquids were used for 'Direct plate selection' as described in **Sections 4.2.4.1 & 4.2.4.2**. Results are shown in **Section 4.3.5**.

4.2.6 Attempts at obtaining bacteriophage from other sources

As the isolation of *Acinetobacter* phage from local environmental sources was unsuccessful a large range of culture collection catalogues were searched for *Acinetobacter* phage.

4.2.6.1 Culture collections

A search was made on the Internet for websites detailing information on *Acinetobacter* phages. One particularly useful resource was a site having links to culture collections throughout the world. The 523 culture collections registered were searched for relevant collections and each individual collection web site, where available visited, (<http://wdcm.nig.ac.jp/hpcc.html>, visited February, 2003).

In the rare cases where bacteriophages were listed, attempts were made to contact a correspondent at the collection for further enquiry. No *Acinetobacter* phages were listed on any of the linked websites.

DSMZ Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, German Collection of Microorganisms and Cell Cultures (http://www.dsmz.de/microorganisms/phage_catalogue.php, visited February 2003)

Outcome: No *Acinetobacter* phages listed on website.

ATCC American Type Culture Collection contacted via sales website.

Outcome: The Bacteriology Department explained that *Acinetobacter calcoaceticus* phages ATCC 9956-B1, 27738, 27738-B1, and 27738-B2 were lost, which had been listed on page 156 of the ATCC catalogue of strains 1, 12th Ed. (1976). They were unable to obtain replacements for these items therefore the item numbers were deaccessioned. (Personal communication Vasia Dekou, Product Specialist, Biomaterials, LGC Promochem via ATCC Technical Inquiries, 17 March 2003)

NCTC National Collection of Type Cultures, PHLS Central Public Health Laboratory (now Health Protection Agency).

No *Acinetobacter* phages were available in the collection. Bacteriophages are only accepted by NCTC where they are active against pathogenic bacterial strains. An additional e-mail was sent to Dr Barry Holmes, Head of NCTC, to enquire if it would be possible to obtain *Acinetobacter* phage from or via NCTC, but it was declined.

NCIMB The National Collection of Industrial, Marine and Food Bacteria (http://www.ncimb.co.uk/html/culture_collection.php, visited February 2003) Selection of *Acinetobacter* spp. but no *Acinetobacter* phage.

UKNCC The United Kingdom National Culture Collection (<http://www.ukncc.co.uk/html/Databases/search.asp>, visited February 2003) Wide selection of *Acinetobacter* strains, but no *Acinetobacter* phage.

Subsearches: CCAP, NCYC, NCPPP, NCWRF, ECACC, NCPV all negative.

4.2.6.2 Contact with individuals in the phage community

The next step was to contact researchers in phage biology or phage therapy for either a protocol which had been successfully used to isolate *Acinetobacter* phage suitable for therapeutic purposes or for samples of *Acinetobacter* phage and host.

The bacteriophage ecology group (<http://www.mansfield.ohio-state.edu/~sabedon/>, visited February 2003) (**S T Abedon**), Associate Professor of Microbiology, Ohio State University, Mansfield, OH, USA.

Outcome: On this website popular with many phage researchers a petition was posted to respond if the reader knew about any *Acinetobacter* phage in circulation.

At the point of response Prof. Ackermann at Felix d'Hérelle Reference Center for Bacterial Viruses, Laval University had already been contacted, Prof. Abedon recommended contacting H.-W. Ackermann.

A request for *Acinetobacter* phage for the purpose of this investigation was posted on the ASM online bulletin board by Prof.S. Denyer.

Outcome: No replies

E. Kutter (Personal Communication, 08 March 2004, 28 September 2004, and via <http://www.evergreen.edu/phage/> (visited February 2003), The Evergreen State College, Olympia, USA, despite long experience of phage isolation had no experience of *Acinetobacter* phage isolation, referral to S. T. Abedon and Eliava Institute.

P Barrow (Personal Communication, 21 January 2003), Institute for Animal Health, Compton Laboratory, Compton, Berkshire RG20 7NN, United Kingdom, no *Acinetobacter* phage. Currently works in conjunction with Eliava Institute.

J Soothill, (e-mail) contacted at the Department of Infection, Medical School, Edgbaston, Birmingham, currently Honorary Senior Lecturer for Great Ormond Street Hospital for Children, NHS Trust.

Dr. Soothill started up a company (Biocontrol Limited) to exploit phage therapy of *Pseudomonas aeruginosa* infections and is no longer working with *Acinetobacter*.

M O Santos Ferreira, Associate Professor of Microbiology, Fac. Farm., Universidade de Lisboa. **J F Vieu**, Laboratory Director, Service des Entérobactéries and **B Klein**, both Pasteur Institute, Paris, France

Source: Phage-Types and susceptibility of 26 antibiotics of nosocomial strains of *Acinetobacter* Isolated in Portugal, J Int Med Res (1984) 12, 364, Santos Ferreira et al. (1984). At least 21 bacteriophages, used for phage typing of *Acinetobacter* strains were used.

Letters to Santos Ferreira were written in Spanish (personal communication, 17 February 2003 & 26 February 2003) and English. Follow-up via e-mails and phone calls were unsuccessful.

Vieu developed a successful phage isolation method (Santos Ferreira, et al., 1984; Giammanco et al., 1989; Vieu, Minck and Bergogne-Berezin, 1979) but neither Vieu nor Klein could be contacted at the Pasteur Institute, Paris. Bouvet, however, responded after several attempts.

P JM Bouvet, S Jeanjean, J F Vieu, Unité des Entérobactéries, Pasteur Institute, Paris, France. **L DijksHoorn**, Department of Clinical Microbiology, Erasmus University Rotterdam, Netherlands.

Source: Species, Biotype and Bacteriophage type determinations compared with cell envelope protein profiles for typing *Acinetobacter* strains, Journal of Clinical Microbiology (1990), 170-176

Bouvet replied after a letter and separate e-mails were sent in French. It transpired that Dr. Vieu, who had worked exclusively on phages at the Pasteur Institute, had retired. This was confirmed by the findings of a PubMed search, where the last publications by this author were in 1990. It was recommended to contact Francine Grimont at the Pasteur Institute and Dr. Ackermann (who had already been contacted at that point).

Francine Grimont was contacted and a meeting at the Pasteur Institute, Paris arranged with A. Henein and Professor G. Hanlon and Dr J-Y Maillard (Collection Nationale de Cultures de Microorganismes, Institut Pasteur, 25 rue du Docteur Roux, Paris F-75724 Cedex 15, France).

Outcome: A selection of phage lysates and bacterial strains was presented. Due to lack of documentation it was unclear which phage belonged to each host, from where they originated and to which species they belonged. All presented lysates and a strictly limited number of randomly picked strains of *Acinetobacter* were transferred to the University of Brighton. See **Section 4.2.7-** Pasteur Material.

Dr. Jan Van Duin and **S. Van den Worm**, at Department of Biochemistry, Gorlaeus Laboratories, Leiden University, The Netherlands who had collaborated with Prof. H.-W. Ackermann, could be contacted and provided HER1424 and HER424 (AP205) and corresponding host. See **Chapter 5**.

Prof. H.-W. Ackermann, Felix d'Hérelle Reference Center for Bacterial Viruses, GREB, Faculté de médecine dentaire, Université Laval, *Acinetobacter* phage and hosts found, first contact was made with Collection Phages (collection.phages@bcm.ulaval.ca, 19 March 2003, who were prepared to send *Acinetobacter* strains and phage against a charge for each phage and host under strict conditions forbidding further transfer of the material. After 18 months of negotiations the material arrived at the University of Brighton in August 2004. Results are presented in **Chapter 6**.

Interestingly there appears to have been a link between the Pasteur Institute in Paris and the Felix d'Hérelle Reference Center, Canada. Klovinis et al., (2002) reported that phage HER424 was originally isolated from Quebec sewage by the enrichment technique (Coffi, 1995). The host was described as genospecies 16 (Bouvet & Jeanjean, 1989) and was claimed to be a urine isolate, obtained through P.J.M. Bouvet (Pasteur Institute, Paris, France). The authors reported that phage and host were held in the Felix d'Hérelle Centre, Canada under the accession numbers HER424 and HER1424. **Coffi (1995)** MSc project, 'Lysotypie des *Acinetobacter*' (French), University Laval, with H.-W.

Ackermann as project supervisor, reported that the phages used as part of his research came mainly from two collections, Reference Center 'Felix d'Hérelle', Laval University and The Pasteur Institute in Paris (F. Grimont, Enterobacteriaceae Unit). One phage was sent by Dr. **E. Juni**, Ann Arbor, MI, USA. The University of Michigan website reports on the 18th September 1997, that Prof. Elliot Juni had retired. (<http://www.umich.edu/news/index.html?Releases/1997/Sep97/chr091897e>, visited 20 November 2006)

Nina Chanishvili, Head of Laboratory for Genetics of Microorganisms and Bacteriophages., The Eliava IBMV, Tbilisi, Georgia, during personal communication (18 February 2005) it transpired that *Acinetobacter* phage had been isolated at the Eliava Institute, however due to problems with Intellectual Property Rights it could not be provided.

4.2.7 Material obtained from the Pasteur Institute, Paris

This material consisted of a selection of *Acinetobacter* strains and unmatched phage preparations obtained from the Collection Nationale de Cultures de Microorganismes, Institut Pasteur, France (**Section 4.2.6.2**). The intention was to find at least one lytic phage and host pair.

12 strains of *Acinetobacter* spp. and 19 different *Acinetobacter* phage preparations which were previously part of Dr Vieu's collection were obtained from the Pasteur Institute, Paris in the form of cryovials. Upon arrival in the laboratory the phage and bacterial samples were stored at 4-8°C.

4.2.7.1 Investigating viability of Pasteur isolates

One loopful of surface growth was removed from the cryovials containing bacterial isolates and used to inoculate 100ml TSB in 250ml conical flasks, which were incubated at 120 rpm for 18 hours at either 37°C or 30°C (Holt et al., 1994; Bergogne-Berezin & Towner, 1996). In addition TSA plates were 4-way streaked with the isolates and incubated for 18 hours at either 37°C or 30°C. If no growth could be detected on the

first attempt, the experiment was repeated five times, if after the fifth attempt still no growth occurred it was considered 'Non Viable'. For long-term storage of viable strains see **Section 2.2.5**. Results are shown in **Section 4.3.6**.

4.2.7.2 Method optimisation for bacteriophage host screening using multiple application devices

With a total of 20 viable bacterial isolates (Sussex, Pasteur and Leiden isolates) to screen against 20 different phages (including HER1424) over 400 pipetting steps were required to apply phage alone, excluding repeats of the experiment. In order to streamline the screening process two different methods were trialled, both with a similar principle whereby 20 different bacterial lawns were produced in triplicate utilising all *Acinetobacter* isolates, then adding one or several rows of different phage droplets and PBS as blank in one manual step.

4.2.7.3 Production of bacterial lawns for phage screening

Overnight cell suspensions were produced by inoculating 10ml TSB with a single colony from a TSA plate and incubated for 16-18 hours on a shaking platform set at 120rpm. Incubation temperatures were 37°C except for Ac 76.426 E20 1989, which was incubated at 30°C and AP205 which was grown at 25 °C.

The overnight suspension was centrifuged in a Sorvall centrifuge at 2500xg for 10 minutes. The supernatant liquid was decanted and the cell-pellet was re-suspended in 10ml PBS. The OD₆₀₀ was adjusted to yield a reading between 0.3 and 0.4 with PBS.

100 µl of adjusted overnight cell suspension were applied to overdried TSA plates and a lawn was produced using plastic spreaders, after surface drying for 15 minutes phage droplets were applied using respective methods.

4.2.7.4 Method optimisation using a multipoint inoculator for phage screening.

A Multipoint inoculator, A400, Applied Quality Services, Horsham, West Sussex was used, with autoclaved solid metal rods to deliver the phage and metal wells as a

reservoir for phage lysate, applying 1µl droplets onto bacterial lawns in a laminar flow hood. A modified method commonly used to phage type *Salmonella* was used, in accordance with methods by Public Health Laboratory Service, Colindale, London, UK (Anderson et al., 1977; Daly et al., 2000).

As only small quantities of lysates were supplied 100µl of lysates were diluted with 900 µl lambda buffer, 0.5ml of each dilution was used to fill each well of the multipoint inoculator. One well was filled with lambda buffer only as blank. The basic principle of this multipoint inoculator was that the solid metal rods were dipped into the wells serving as phage reservoirs and then touched the agar surface containing the bacterial lawn to deliver the phage droplet. The rods were dipped back into the phage lysates for delivery onto the next plate. After application of phage the plates were allowed to dry at ambient temperature for 60 minutes and incubated in inverted position at the respective incubation temperatures for 16-18 hours. Results are presented in **Section 4.3.7**.

4.2.7.5 Method optimisation using a multipoint pipettor for phage screening

Bacterial lawns were produced on TSA as described in **Section 4.2.7.3**.

Several attempts were made to deliver phage droplets simultaneously with a multipoint pipettor, varying the number of pipette tips loaded into the pipettor, which determines the distance between phage droplets and the volume of drops delivered. Volumes which were too small did not allow uniform delivery, sometimes air bubbles and consequently phage aerosols and in some cases no phage droplet was delivered at all. When phage droplets were too large the droplets merged, making it impossible to use the plate for phage screening. In cases where each of the pipettor ports was loaded with a pipette tip, droplets of acceptable size were too close together. As reduction in droplet size was not considered for the reasons mentioned above, the distance between droplets was modified by loading only every second pipettor port with a tip.

Pipette tip boxes were filled with 100µl tips in every other cavity and sterilized. A template was made which allowed marking of the agar plate at the exact spot where the

phage droplet was to be applied, with sufficient distance to avoid merger of phage droplets. This allowed the application of three different phage preparations in each row and a total of 9 phage samples or blank per 9 cm TSA plate. A sterile 96-well plate was filled with sample in the relevant wells to allow pipetting of three phage spots at once. After each delivery of three 10µl of phage suspension in triplicate the tips were expelled and a new set loaded. After phage application the TSA plates were left at ambient temperature for 15 minutes and incubated in inverted position at 37°C (except for AP205- 25°C and AC 76.426 E20 1989-30°C).

The results are presented in **Section 4.3.8**

4.3 Results

4.3.1 Surface spotting of filtered supernatant liquid from burns unit (QVH) samples

On all plates a very dense lawn had formed (BU1-BU6 & DR1). In areas where filtered supernatants had been applied the lawn was no different from the surrounding area, when observed with the naked eye and a magnifying glass. Clearance zones or plaques indicative of presence of lytic phage were absent.

4.3.2 Overlay method using filtered supernatant liquid-QVH

No plaques or clearance zones could be observed on any of the plates.

4.3.3 Bacteria found in unfiltered supernatant liquids and pellets-QVH

Sample	Appearance of colonies	Size (mm)	Oxidase test	Gram-stain
<i>Acinetobacter</i> R45502	Circular, pink colonies, mauve background	1-2	Negative	Negative
BU1	25 yellow colonies, pink- orange background	2-3	Positive	-
BU2	Mould growth	-	-	-
BU3	No growth	-	-	-
BU4	No growth	-	-	-
BU5	11 pink circular colonies, orange background	1-2	Positive	-
BU6	Circular yellow colonies with mauve background	1-2	Positive	Negative, rod shaped
DR1	>50 opaque colonies, yellow background	1-3	Positive	-

Table 4.3 Surface spread unfiltered supernatant liquid, appearance on LAM and further tests

Sample BU1 gave rise to 25 colonies were counted, some circular, with raised centres, some with irregularly shaped borders, 2-3mm diameter on average, with pink-orange background, all subcultures were oxidase positive (**Table 4.3**). Only samples BU1, BU5, BU6 and DR1 yielded growth on LAM agar, initial appearance on this selective agar did not resemble *Acinetobacter*. Further tests confirmed that the isolates in question were not *Acinetobacter* spp. (see **Table 4.3**)

BU1	BU2	BU3	BU4	BU5	BU6	DR1
No growth	Mould growth	No growth	No growth	No growth	Growth	No growth

Table 4.4 Bacteria found in pellet, cultured on LAM

As was found with the supernatant liquids (see **Table 4.3**), not all pellets obtained from centrifugation yielded growth (see **Table 4.4**). No further attempts were made to subculture or identify the microorganisms present in these samples containing mould. BU6 showed circular yellow-pink colonies with mauve background, subcultured colonies on TSA were oxidase positive, which indicates the microorganism present was not *Acinetobacter* spp.

4.3.4 Checking for growth of wastewater samples on TSA and subculture onto LAM -QVH samples

Five wastewater samples from the burns unit QVH did not yield any growth when 20µl were surface spotted onto a TSA plate. Two samples yielded growth and were subcultured onto LAM, BU6 gave rise to circular pink colonies with orange background, which was investigated further. Despite the organism in question being Gram-negative, a positive oxidase test confirmed that the microorganism was not *Acinetobacter* spp. Similarly sample BU6 gave rise to circular yellow colonies with yellow background, a clear indicator that the bacteria in question would not be

Acinetobacter spp., which was further confirmed with a positive oxidase test (**Table 4.5**).

Sample	Appearance of colonies	Diameter (mm)	Oxidase test	Gram-stain
BU1	No growth	-	-	-
BU2	No growth	-	-	-
BU3	No growth	-	-	-
BU4	No growth	-	-	-
BU5	No growth	-	-	-
BU6	circular pink colonies, orange background	1-2	Positive	Negative
DR1	circular yellow colonies, yellow background	1-3	Positive	-

Table 4.5 Appearance of 20µl surface spots when subcultured on LAM, including further tests

4.3.5 Sewage samples on LAM agar

All 18 sewage samples yielded growth on LAM agar which appeared as cream-white confluent colonies, approx. 2.5cm in diameter, with raised rims and white mottling in the centre. Two LAM plates grew mould and therefore care was taken to avoid the areas containing fungal growth when samples were removed for subculture. None of the subcultured TSA or LAM plates showed any carryover fungal growth.

4.3.5.1 Appearance of subcultured colonies on LAM

All isolates appeared as opaque colonies approx. 1-2 mm in diameter, the appearance of the surrounding agar ranged from yellow to peach, however did not resemble the mauve as seen with *Acinetobacter* isolates (See **Table 4.6**).

Short code	Growth	Colony appearance	Appearance of surrounding agar	Microscopic appearance of cells
P4HM01	++	Opaque	Dark yellow	Motile, long rods
P4HM02	+	Opaque	Dark yellow	Short rods
P4HM03	+++	Opaque	Peach	Long rods
P4HM04	+	Opaque	Dark yellow	Twitching, long rods
P4HM05	+	Opaque	Dark yellow	Short rods
P4HM06	+	Opaque	Dark yellow	Long rods
P4HM07	+	Opaque	Yellow	Long rods
P4HM08	+	Opaque	Yellow	Long rods
P4HM09	++	Opaque	Dark yellow	Motile, long rods
P4HM10	+	Opaque	Dark yellow	Long rods
P4HM11	+++	Opaque	Dark yellow	Long rods
P4HM12	+	Opaque	Yellow	Long rods
P4HM14	++	Opaque	Yellow	Long rods
P4HM15	+++	Opaque	Peach	Long rods
P4HM16	+	Opaque	Peach	Short rods
P4HM17	+	Opaque	Yellow	Long rods
P4HM18	+++	Opaque	Dark yellow	Long rods

Table 4.6 Growth of subcultured sewage isolates on LAM

4.3.5.2 Controls

All control strains grew on LAM agar except R46383, W5211 and W6108. Strains AP205 and W64992 showed only weak growth.

4.3.5.3 Testing of sewage isolates subcultured on TSA

Short code	Catalase test	Oxidase test	Gram-stain
P4HM01	Positive	Negative	Negative
P4HM02	Positive	Negative	Negative
P4HM03	Positive	positive	Negative
P4HM04	Positive	positive	Negative
P4HM05	Positive	positive	Negative
P4HM06	Positive	positive	Negative
P4HM07	Positive	negative	Positive
P4HM08	Positive	Positive	Negative
P4HM09	Positive	Positive	Negative
P4HM10	Positive	Positive	Negative
P4HM11	Positive	Positive	Negative
P4HM12	Positive	Positive	Negative
P4HM14	Positive	Positive	Negative
P4HM15	Positive	Positive	Negative
P4HM16	Positive	Positive	Negative
P4HM17	Positive	Positive	Positive
P4HM18	Positive	Positive	Negative

Table 4.7 Results of catalase and oxidase test and Gram- stain

A combination of catalase and oxidase tests and Gram-stain were chosen to quickly identify potential *Acinetobacter* isolates present (**Table 4.7**).

As only P4HM01 and P4HM02 matched the phenotypic profile of *Acinetobacter* sufficiently to warrant additional investigation, only these two isolates were further tested using the API20NE kit (**Table 4.8**).

Sample short code	Possible species
P4HM01	<i>Agrobacterium radiobacter</i>
P4HM02	<i>Aeromonas hydrophila</i>
P4HM02	<i>Aeromonas caviae</i>
P4HM02	<i>Vibrio fluvialis</i>

Table 4.8 Outcome of API20NE test for selected isolates

4.3.5.4 Phage isolation from sewage samples

No plaques or zones of clearance could be seen when the ‘direct plate selection’ method was used.

4.3.6 Viability of Pasteur isolates

Not all Pasteur isolates proved to be viable, despite several attempts to revive them. The original identification of the isolates shown in **Table 4.9** was retained, as none of them could be matched as host to any of the phage material they were not further identified or processed.

All viable isolates except Ac 74.97 E4 1989 and Ac 76.426 E20 1989 grew at 30°C and 37°C, the colony size varied significantly between different isolates (**Table 4.10**).

Short codes	Status	Short codes	Status
Ac 74.98 E5 1989	Non Viable	E2/Ac 74-94(2) 1995	Viable
Ac 76.426 E20 1989	Viable	Ac 75.37 E11 1989	Viable
Ac 74.97 E4 1989	Viable	E1/Ac 74-93(41) 1995	Viable
E15 Mx. 75.127 2.78	Non Viable	Ac. 74.107(phE7) 1995	Viable
Ac 75.53 E13 1989	Viable	Ac. 74.110 E8 1989	Viable
3 B Ac 75.126 E14 1986	Viable	Ac 74.101(phE6) 1995	Viable

Table 4.9 Viability of Pasteur isolates

Pasteur bacterial strains	37°C			30°C	
	(mm)	TSB	TSA	TSB	TSA
Ac 76.426 E20 1989	-	-	-	-	+
Ac 74.97 E4 1989	0.7	+	+	-	-
Ac 75.53 E13 1989	0.6	+	+	+	+
3 B Ac 75.126 E14 1986	0.7	+	+	+	+
E2/Ac 74-94(2) 1995	0.4	+	+	+	+
Ac 75.37 E11 1989	1.2	+	+	+	+
E1/Ac 74-93(41) 1995	1.5	+	+	+	+
Ac. 74.107(phE7) 1995	0.83	+	+	+	+
Ac. 74.110 E8 1989	0.55	+	+	+	+

Table 4.10 Growth of viable strains at 30°C and 37°C, colony size (mm) when grown at 37°C

4.3.7 Results of multipoint inoculator for phage screening

All plates showed heavy bacterial growth where the phage inoculum was applied, including blank. In fact the bacterial growth appeared denser where the phage droplet had been applied than on the surrounding lawn. The method was repeated whilst attempting to sterilise the rods between each phage delivery and before placing them back into the phage reservoir, however after a few attempts it was considered too labour intensive and the method was abandoned.

4.3.8 Results of multipoint pipettor used for phage screening

No areas of clearance could be observed on any plates, the bacterial lawn on the plates was uniform throughout.

4.4 Discussion

4.4.1 Isolation of *Acinetobacter* spp. and phage for waste water and sewage samples

Two main aims were the driving force for this range of experiments: Firstly, an attempt to acquire more clinical isolates of *Acinetobacter* spp., (the presence of these Gram-negative bacteria was considered to have been very likely, as a patient infected with *Acinetobacter* was present at the burns unit at the time of investigation). Direct removal of isolates from the patient for further investigation was not feasible due to ethical and legal reasons.

The second aim was to attempt to isolate lytic phage matching the Sussex isolates from the various samples collected.

The textbook edited by Kutter & Sulakvelidze (2005) which was published after completion of this investigation describes similar methods to the protocols used here, where the host bacteria are added to the original unfiltered samples and a basic enrichment protocol is followed. Kutter & Sulakvelidze (2005) termed the methods described in **Section 4.2.4.1, 4.2.4.2** 'Direct Plate Selection'. As the number of potential phage particles present could not be estimated an enrichment step was included (**Section**

4.2.4) which is also recommended by Kutter & Sulakvelidze (2005) for dilute samples containing a low number of phage particles.

A variety of microorganisms were isolated from the waste water samples, including moulds, however none of the bacteria proved to be *Acinetobacter*. Choosing LAM as part of the investigation appeared to reduce the number of likely *Acinetobacter* candidates from the outset. The visual appearance of some isolates on LAM was an instant marker that they were not *Acinetobacter*. None of the colonies resembled the typical pink colonies of *Acinetobacter* on a mauve background. The closest matches were isolated from sample BU5, the colonies were pink but on orange background and BU6 with yellow-pink colonies on mauve background.

On comparison with the reference strain *Acinetobacter* R45502, it was apparent that none of the colonies resembled *Acinetobacter* even remotely. To further confirm that the organisms in question were not *Acinetobacter* spp. the oxidase test was chosen as it quickly eliminated a range of potentially confounding bacteria.

LAM proved to be useful in aiding the identification of the isolates in question, as it limited the growth of background contamination. However it was not selective enough to only allow the growth of *Acinetobacter* spp. as discussed in **Section 3.6**. LAM agar also inhibits the growth of some *Acinetobacter* spp. particularly *A. johnsonii* and *A. haemolyticus*, and in the specific experiment described in **Section 3.4.2** three out of nine *Acinetobacter* isolates failed to grow on LAM entirely. To avoid missing an *Acinetobacter* isolate which may not grow on LAM, the experiment described in **Section 4.2.4.3** was devised, involving TSA plates.

Samples BU1 and BU5 failed to grow on TSA, most likely due to the small sample size of 20 µl (compared to 200 µl used for surface spreading on LAM in (**Section 4.2.4.3**)) and an estimated low cell count. Repeats of the experiment led to the same results i.e. no growth. Overall, samples BU2, BU3, BU4 failed to grow and produce visible bacterial colonies in all experimental setups, regardless of the medium used. BU1 supported growth only when the supernatant liquid was surface spread onto LAM, but not when a smaller volume was surface spotted onto TSA. This may be due to the fact

that a small volume of a sample with a low cell count was used. However the cell pellet, which would be expected to contain higher cell numbers, did not support any growth either. It is possible that in the case of BU1 the isolate favoured the growth conditions provided by LAM to TSA, hence processing of the pellet did not give rise to colonies.

One plausible explanation for samples BU2-BU4 not giving rise to any isolates, could be that the intrinsic viable cell count of the waste water samples was low, which may be due to large amounts of cleaning products and disinfectants decanted into the canalisation, indicated by excessive foaming when drawing sample DR1.

None of the 17 sewage samples gave rise to any plaques using the Direct Plate Selection. It could be argued that samples P4HM01-P4HM12 did not harbour viable phage due to being frozen and stored at -70°C for months and in two cases for years.

However samples P4HM14-P4HM18 which had been freshly drawn were refrigerated after collection for 48 hours and were processed immediately after receipt. It is unknown if a surface layer of sewage was removed and from which part of the treatment plant the samples had been drawn. *Acinetobacter* is an aerobic Gram-negative bacterium and unlikely to survive in lower layers of sewage or activated sludge for long periods of time. The theory of phage isolation relies on the premise that phage are most likely to be found where favourable conditions for the host prevail (Adams, 1959). It is possible that storage for 48 hours without agitation may not have been beneficial for the recovery of viable *Acinetobacter* spp. The location of where the samples were removed is important as bacterial and phage densities may vary at different stages of sewage treatment, certain phage types are more sensitive than others to various treatment steps (Mandilara et al., 2006).

4.4.2 Phage isolation methods

Phages are present in a large variety and numbers in environmental and other sources (Kutter & Sulakvelidze, 2005). The question arises why *Acinetobacter* phage could not be isolated in this range of experiments. Despite the assertion in Bergey's Manual of Systematic Bacteriology (Holt, 1984) that 'Lytic phages for acinetobacters are isolated readily from sewage' citing Twarog & Blouse (1968) and Herman & Juni (1974) as the

basis of this judgement, the results of this investigation do not correlate with this statement. The phrase ‘isolated readily’ in conjunction with the account of Twarog and Blouse, who apparently used a mere 2 litres of raw sewage for the isolation of *Bacterium anitratum* (now known as *Acinetobacter anitratus*) may imply that finding phage could be undertaken without prior specialist knowledge or skills, extreme efforts or even large sample volumes or numbers. Soothill (1992) reports successful isolation of *Acinetobacter* phage using a scaled-up version of the enrichment method by Adams (1959) from 15 litre batches of sewage. As the author referred to batches, it could be inferred that the sample throughput was rather large. Unfortunately none of the authors provided further information on the origin of sewage (treated/untreated), whether it was freshly drawn or previously frozen, how it was stored before it reached the laboratory, during which season the experiment was undertaken and the ambient temperatures at the sampling site. It is also unclear whether the investigator was experienced in phage isolation, where the sample was drawn and how many previous unsuccessful attempts preceded the finding of the *Bacterium anitratum* phage. Scientists who have successfully isolated phage, report that successful isolation depends on trial and error and is affected by multiple factors. Chanishvili has reported that entire teams with experienced members overlooking phage isolation go on phage ‘hunts’ at the Eliava Institute in Tbilisi and spring and summer are the best seasons for phage hunting. It is also possible to isolate several phages per week, subject to know-how (E. Kutter, N. Chanishvili, personal communications 8 March 2004 & 18 February 2005)

Isolation of specific microorganisms can be a time-consuming task requiring considerable resources. Payan, et al. (2005) who used phage as an indicator for the isolation of new *Bacteroides* isolates, reported that at least one host strain was isolated per attempt, estimating a total cost of 1,000 Euros including consumables and labour for each attempt.

From Twarog and Blouse’s (1968) account it appears that by the correct treatment of only 2 litres of sewage phage could be found. It raises the question why 17 sewage samples from different sources and a range of waste water samples, using a broad range of approaches did not yield *Acinetobacter* spp. or a corresponding phage. It could be

argued that the frozen sewage samples were not fresh enough and during the storage at lower temperatures phage material may have been damaged. It is plausible to expect the presence of cleaning products which finally find their way into canalisation or improved sewage treatment methods to affect the presence of bacteria and phage in samples. Soothill (1992) who reported experimentation requiring 15 Litre batches of sewage may provide a further indication why the experiment was unsuccessful; it may simply have been that sample volumes were too low. It is plausible that unagitated storage of the freshly drawn sewage samples for 48 hours before receipt decreased any chances of isolating *Acinetobacter* spp., however it should not have affected the viability of phage.

Mandilara et al. (2006) reported that a correlation exists between the number of bacterial indicators detected and the presence of bacteriophage in sewage and sludge. This implies that as *Acinetobacter* spp. could not be isolated in this investigation the chances of finding corresponding phage in the same samples were also remote. Grehn & von Graevenitz (1978) reported that their research team was unable to isolate *Acinetobacter anitratus* from faeces during an experimental period of 2 years. The team found that *A. anitratus* could not be isolated from the faeces even in the same patients where *A. anitratus* was found in wounds and sputum. This raises the question whether this situation applies to other *Acinetobacter* species. As phage are most likely to be found where favourable conditions for the host prevail, it is rather unlikely for *Acinetobacter anitratus* phage to be present in faeces/sewage hence this may be used to challenge the statement in Bergey's Manual of Systematic Bacteriology (Holt, 1984). It may be prudent to approach this rather general statement and interpret it as 'some lytic phages of *Acinetobacter* have been isolated readily from sewage'.

The method used by Soothill (1992), where 15 litre batches of sewage were used for the isolation of *Acinetobacter* phage make it apparent that even successful phage isolation setups may require large sample volumes, several attempts and scaled-up experimentation. It may be a simple statistical issue, larger numbers of samples and volumes of sewage batches increase the chances of isolating phage.

Based on the findings of Grehn & von Graevenitz (1978) it is questionable whether *Acinetobacter anitratus* and other *Acinetobacter* species are simply less commonly

isolated from stools/sewage than other sites, however it is intriguing that some investigators have succeeded in *Acinetobacter* phage isolation from sewage.

The challenge to isolate a suitable phage for each host is in principle similar to phage typing, where attempts are made to complement each bacterial strain with phage. Santos Ferreira et al. (1984) reported that out of sixty-two *Acinetobacter calcoaceticus* strains 20% could not be typed at all (80% typable) despite using two complementary phage-typing systems; Joly-Guillou et al. (1990) reported out of 117 clinical *Acinetobacter* isolates only 78 were typable (66.7%), while Giammanco et al. (1989) reported that 37.7% of *Acinetobacter* isolates could be correctly typed, the rest were untypable or resistant. These findings imply that even when large phage libraries are available it is not always possible to find a suitable phage for each bacterial isolate. Interestingly the latter authors attribute the inability to match all isolates with phage (100% typability) to the fact that the set of phage used originated from France, however the bacterial isolates were obtained in Palermo (Italy).

Similarly, Payan et al. (2005) observed that a *Bacteroides fragilis* strain detected a number of phages in different areas of the Mediterranean region and South Africa, however failed to detect significant numbers in Northern Europe and the United States. It appears that where and possibly when phage were isolated is critical in finding a suitable host. This line of reasoning may be inverted and it could be stipulated that the timing and location of the host isolation may affect the success of isolating corresponding phage. It appears sensible to attempt to co-isolate phage and corresponding host simultaneously from samples, however investigators have reported this is not always necessary (Kutter & Sulakvelidze, 2005). It may be possible that apart from the low cell count in the waste water samples, which reduced the chances of isolating *Acinetobacter* host and corresponding phage the fact that the Sussex isolates were chronologically sampled years before the waste water may have complicated the attempted isolation further.

4.4.3 Difficulties encountered obtaining *Acinetobacter* phage with matching host

A major issue arising from this investigation was why it was so difficult to source *Acinetobacter* phage and matched hosts from the scientific community. The answer may be held partly in the reasons for scientists to keep *Acinetobacter* phage stocks with matched hosts in their respective laboratories. *Acinetobacter* has in recent years not been as ‘fashionable’ in the wider research community as for example *Pseudomonas aeruginosa* or *Staphylococcus aureus*, possibly due to a smaller number of patients being affected by this bacterium. Until recently *Acinetobacter* spp. was still considered a common skin commensal. The Health Protection Agency describes it on its website as ‘a harmless coloniser on the skin of healthy people and [which] usually poses very few risks’, in another paragraph it is acknowledged that ‘a few species, particularly *Acinetobacter baumannii*, can cause serious infections – mainly in very ill hospital patients.’ ‘Hospital-adapted’ strains of *Acinetobacter* are sometimes resistant to antibiotics and are increasingly difficult to treat (http://www.hpa.org.uk/infections/topics_az/acinetobacter_b/default.htm visited January 2007).

This information is correct and may be targeted at the general public, however it does not reflect the urgency of the need for research into alternative treatment options.

A combination of these factors may have affected the resources allocated to research in this field and limited the number of investigators researching *Acinetobacter* and consequently phage material. It is only relatively recently that *Acinetobacter* spp have become of serious clinical importance. Another reason why *Acinetobacter* phage are in limited supply may lie in the fact that phage and host isolation can be time-consuming, unpleasant (especially when isolated from sewage), and expensive (Payan et al., 2005).

Interestingly the reasons for investigators to hold stocks of *Acinetobacter* phage and isolates were varied. Vieu at the Pasteur institute and his collaborators focussed on phage typing of clinical isolates (Santos Ferreira et al., 1984; Giammanco et al., 1989). Coffi, under the supervision of Ackermann, studied the material for the main purpose of taxonomy (Coffi, 1995) and Klovins and his group investigated the genetic makeup of

Acinetobacter phage (Klovins et al., 2002). Only Soothill used *Acinetobacter* phage with the intention of potential human phage therapy (Soothill, 1992). However obtaining material or exact phage isolation protocols from these sources proved unsuccessful. It became apparent during this investigation that only two investigators within Europe have published their success in isolating *Acinetobacter* phage (Vieu et al., 1979; Soothill, 1992).

4.4.4 Pasteur material phage screening

Of the 12 bacterial isolates obtained from the Pasteur Institute in Paris, ten were successfully revived but two were non-viable. This was not surprising as the isolates had apparently been stored for many years at room temperature (personal communication Grimont, 07 April 2004). No records were provided as to the origin or isolation history of these isolates. The phage preparations had previously been the property of Dr. Vieu who had retired before the start of this investigation (Personal communication with Bouvet, P., 18 February 2003). He stated his correspondence address in a scientific paper as ‘Center of Phage-typing, Pasteur Institute, Paris, France’ (Joly-Guillou et al., 1990). Vieu co-wrote papers with Joly-Guillou and Bergogne-Berezin who had worked at the Bichat Hospital, 46 rue Henri Huchard, Paris, France, from where ‘one hundred and seventeen outbreak *Acinetobacter* isolates, from 85 hospitalized patients in 1987 and 1988’ originated (Joly-Guillou et al., 1990). It is possible that the isolates revived in this study had originated from that source although this cannot be stated with any certainty.

From the chronological appearance of references it appears that *Acinetobacter* spp. related research at the Pasteur Institute was initiated by Vieu who published a paper in collaboration with Bergogne-Berezin and others (Vieu et al., 1979), followed by Bouvet & Grimont who probably further utilised the contacts and material established by Vieu (Bouvet & Grimont, 1986; Bouvet & Grimont, 1987). The lack of information in the literature makes it difficult to establish whether the *Acinetobacter* isolates provided by the Pasteur Institute were originally used by Vieu or had been added by Bouvet & Grimont and whether they had ever been matched to any phage preparation in the possession of the Pasteur Institute; Although a personal communication (07 April 2004)

from F. Grimont indicates both phage and isolates were materials used by Vieu. It is clear that Vieu was a key figure in establishing work with *Acinetobacter* phage at the Pasteur Institute, as he is credited for developing phage-typing systems at the Institute Pasteur (Giammanco et al., 1989). Giammanco et al. (1989) mention 21 bacteriophages which originated from France plus a collection introduced in 1982 as described by Santos Ferreira et al. (1984). Both papers included Vieu as a collaborator and it may be speculated as he was experienced in working with and isolating phage, his role may have been to provide expertise on handling phage. From Klovins et al. (2002) is it known that at least one bacterial isolate (HER1424, *Acinetobacter haemolyticus*, see **Chapter 5**) made its way via Bouvet at the Pasteur Institute, Paris to Quebec, Canada to be matched with phage HER424, which implies that even if there was no direct collaboration at least material transfer took place.

4.4.5 Lack of suitability of multipoint inoculator for phage screening

As all plates showed heavy bacterial growth after application of phage droplets using the multipoint inoculator (see **Section 4.3.7**), including blanks, the method was considered unsuitable for phage screening. Applied Quality Services have been unable to provide a protocol suitable for phage screening, despite the multipoint inoculator being advertised as suitable for this purpose.

Several possible causes for the increased density of lawn at the place of phage delivery compared to the surrounding areas could be identified: by applying phage droplets, the growth conditions for bacteria were improved, resulting in a denser lawn in that area. This is rather unlikely as the lysates were diluted in PBS, which should not lead to enhanced bacterial growth. Another possibility is that the multipoint tips had picked up contamination from the bacterial lawns, when the rods were dipped into the phage preparation and then applied to the TSA plates. As the rods were dipped back into the phage preparation, bacterial contamination picked up from the previous TSA plate was introduced into the phage reservoir. By delivering the next phage droplet onto a different TSA plate it could have been inadvertently contaminated with bacteria picked up from the previous plate. It is very unlikely that the lysates themselves contained contamination.

4.5 Conclusion

Despite a number of investigators having reported isolating *Acinetobacter* phage from sewage in the literature, attempts at repeating this exercise from the same source proved unsuccessful. Factors contributing to the successful isolation have been identified as isolating phage chronologically and spatially near the origin of the host bacterium, large volume throughputs, choosing the correct sampling time and possibly luck. There are no precisely detailed protocols for the isolation of *Acinetobacter* phage published.

Due to the absence of plaques or any indication of clearance zones it can be concluded that lytic phage were not present or not present in quantities sufficient to give rise to lysis to *Acinetobacter* in any of the samples. This does not exclude the potential presence of temperate *Acinetobacter* phage in the samples. However Kutter et al. (2005) described that it is extremely difficult to isolate temperate phage with standard methods. Temperate phages are not useful in phage therapy, and hence are of lesser interest in this investigation.

The approach of choosing a variety of different methods to process the samples as described in phage isolation protocols did not further the outcome of the investigation.

A range of microorganisms could be isolated from some samples, but after further investigation it transpired that none of the isolates were *Acinetobacter*. As the host bacteria did not appear to be present in the samples it is unsurprising that corresponding phage could not be isolated.

Attempts at obtaining existing *Acinetobacter* phage stocks were of limited success, in part due to small numbers of laboratories holding suitable material, loss of material, migration or retirement of investigators and intellectual property issues; However the overall number of phage isolates held was modestly enhanced.

The phage lysates and *Acinetobacter* isolates obtained from the Pasteur Institute, Paris were investigated but efforts to match the large numbers of phage lysates provided with suitable hosts remained unsuccessful.

Chapter FIVE

Investigation of temperature dependent lytic relationship of host and phage HER1424 and HER424

5.1 Introduction

Bacterial host *Acinetobacter* spp. and phage, HER1424 and HER424 were obtained from Dr. Jan van Duin at Leiden University. The phage is also referred to as *Acinetobacter* phage 205 or AP205 by Klovin's et al., (2002). The material may also be referred to as „Leiden“ in the text to avoid confusion with HER1423 or HER1425 obtained at a later date from Laval, Canada.

Klovin's et al. (2002) reported that phage AP205 was originally isolated from Quebec sewage by the enrichment technique citing Coffi (1995). The host was described as belonging to genospecies 16 (Bouvet & Jeanjean, 1989) and was claimed to be a urine isolate, obtained through P.J.M. Bouvet (Pasteur Institute, Paris, France). The authors stated that phage and host were held in the Félix d'Hérelle Centre, Canada under the accession numbers HER424 and HER1424 respectively. Phage and host were reported as having been grown at 28°C in brain-heart infusion broth or trypticase soya agar (Difco). High-titre lysates were reported as having been prepared from plates with confluent lysis. Interestingly Klovin's et al. (2002) report that during their own investigation, cell lysis did not always occur when HER1424 was infected with AP205 at $OD_{650}=0.2$ at 28°C. HER424 (AP205) is a ssRNA (single stranded) bacteriophage, with a short genome and belongs to the group of Leviviridae, which are capable of infecting a range of Gram-negative bacteria. Provided the bacteria express suitable pili

on the cell surface, the Leviviridae are capable of injecting the viral RNA into the cytoplasm (Klovins et al., 2002). Phage titres of 10^{14} pfu/ml have been reported to be reached (Joshi et al., 1982; Lohr 2005; McKay et al., 1993); Klovins et al. (2002) were primarily interested in the diversity of ssRNA phages, which reportedly cause lytic infections (Klovins et al., 2002), ssRNA coliphages are divided into two genera Levivirus and Allolevirus, with the latter missing a lysis gene.

Phage species belonging to the same genus show very little diversity, for example strains MS2, R17, f2, M12 and JP501 show more than 95% identity in RNA sequence (Klovins et al., 2002). AP205 is 27-30nm in size and of spherical or hexagonal shape, similar to coliphages. This phage is known to attach to the sides of *Acinetobacter* pili which are 6nm in diameter (Klovins et al., 2002). Coffi (1995) considered AP205 the first ssRNA phage with an affinity for *Acinetobacter* spp.

5.1.1 Genetic profile of AP205 (HER424)

AP205 is a RNA phage, with a similar length genome to MS2 (species I) and GA (species II) (Klovins et al., 2002). The coat protein of HER424 contains 130-133 amino acids and the actual genome is only 9 nucleotides shorter than that of SP (species IV), which is the longest ssRNA phage currently known. This phage possesses two reading frames which are not found in Q β , MS2 or PP7, and in common with other phages it has a maturation gene, and a minor capsid component which is essential for attachment to bacterial pili and consequently infection (Klovins et al., 2002). AP205 is longer than Q β due to two reading frames (ORF1 & ORF2), larger intergenic regions, and a longer maturation protein.

Klovins made the discovery that HER424 has two extra reading frames (open reading frames ORF), which makes this the first published ssRNA phage with a different genetic map to other phages of this type. Despite three major genes occupying identical positions to those found in ssRNA coliphages, which are related and better studied, the lysis gene is in an unexpected position and two small open reading frames are found ahead of the maturation gene (Klovins et al., 2002).

The aim of this chapter was to characterise bacterial host and phage. A range of experiments was devised to validate and optimise results and to explore the seemingly temperature dependent lytic relationship between the phage and its host.

5.2 Methods

Phage AP205 (HER424) and corresponding host (HER1424) were kindly provided by Dr. Jan van Duin, (Department of Biochemistry, Gorlaeus Laboratories, Leiden University, PO Box 9502, 2300 RA Leiden, The Netherlands). During transit neither the phage nor the bacterial host had been refrigerated. The phage was supplied as liquid lysate and the bacteria had been inoculated onto transport agar both in sealed cryovials. Upon arrival the material was stored at 2-8°C until required for use.

The instructions provided with the material recommended growth of HER1424 on rich medium. For infection with HER424 the recommendation was to start a bacterial culture in rich medium with supplementation of 100mM CaCl₂ (personal communication, Dr. van Duin, 24 January 2003). Some phages require divalent cations to adsorb to the host (Kutter & Sulakvelidze, 2005). Once the culture had reached an OD₆₀₀ of approximately 0.3, the shaking of the culture was stopped and phage was added at a multiplicity of infection of 5-10. The titre of the phage was unknown by the originator at the point of sending, however a ratio of 50µl lysates to 20ml culture was recommended. The infected culture was then incubated without agitation for 15 minutes to allow phage attachment and then incubated at 28°C with agitation for approximately 3 hours. According to the originators, the culture apparently did not lyse, despite the presence of a large number of phage particles. During communication with S. van den Worm it transpired that during his investigation, despite several attempts the phage had never produced any plaques.

5.2.1 Propagation on arrival and long-term storage

1 loopful of transport agar containing the bacteria was streaked onto TSA plates for single colonies and grown for 16-18 hours at 28°C in an Innova 4230 Refrigerated Incubator shaker, New Brunswick Scientific, NJ, USA. An overnight suspension of

HER1424 was then prepared by suspending 1-3 colonies from one of these TSA plates into 100ml TSB. After incubation at 28°C for 16-18 hours in a refrigerated orbital shaker at 100rpm the suspension was supplemented with 10%(v/v) sterile glycerol and dispensed aseptically in 1ml volumes into 1.2ml Corning cryogenic vials. The vials were frozen initially at -20°C and transferred after 24 hours to a -80°C freezer.

5.2.2 Routine subculture of *Acinetobacter* HER1424

A cell suspension of HER1424 was kept frozen at -80°C (10% Glycerol in TSB) in cryovials. Stock cultures were made by defrosting one cryovial each month, using one loopful to streak on TSA plates for single colonies and incubating at the chosen temperature for 16-18 hours. The resulting cultures were sealed and kept refrigerated at 4°C. The stock cultures on agar plates were subcultured weekly. Despite Klovin's reporting 28°C as the routine incubation temperature, temperatures of 37°C, 30°C and 25°C were used for reasons of practicality. In addition TSA plates were 4-way streaked with the isolates and incubated for 18 hours at 37°C, 30°C or 25°C. Incubation at 25°C was routinely undertaken in an Innova 4230 Refrigerated Incubator shaker, New Brunswick Scientific, NJ, USA.

5.2.3 Phenotypic properties, identification of HER1424 with API20NE test system, microscopic examination and digital imaging using a bright-field light microscope

1 loopful of transport agar was 4-way streaked on TSA and incubated for 16-18 hours at 28°C. This was repeated using freshly defrosted stock cultures of HER1424. Only freshly grown cultures were used for this experiment. Gram stain, microscopic appearance and growth requirement were investigated and oxidase and catalase tests performed. For methods see **Section 2.4**. The results are shown in **Section 5.3.1**.

For the identification of HER1424 test instructions for the use of the API20NE test kit were followed, (bioMérieux, Lyon, France) and the results were interpreted in conjunction with the oxidase test results. This procedure was repeated each month using freshly defrosted stock cultures of HER1424. For Method see **Section 3.2.7**.

For the microscopic examination 1 loopful of transport agar was 4-way streaked on TSA and incubated for 16-18 hours at 28 °C. One colony was removed from the freshly grown culture and mixed with a drop of purified, sterile water on a glass microscope slide. A coverslip was added and the specimen examined under phase contrast microscopy using a 100x oil immersion objective and an Olympus DP10 microscope digital camera system.

5.2.4 Antibiotic susceptibility of HER1424 using BSAC disc diffusion method

This experiment was designed to further characterise HER1424, investigate its antimicrobial sensitivity spectrum and to facilitate comparison of resistance profiles with other *Acinetobacter* spp.

The BSAC Disc Diffusion Method for Antimicrobial Susceptibility Testing version 5, 2006 was used to ascertain the antimicrobial susceptibility of HER1424. (For Method see **Section 3.3.1**) All results were interpreted using BSAC break points and are given in **Section 5.3.2**.

5.2.5 Growth characteristics of HER1424 on Leeds Acinetobacter Medium (LAM)

The rationale of this experiment was to characterise HER1424 and confirm its identity by its growth pattern on LAM agar.

1-3 colonies of an overnight culture of *Acinetobacter* HER1424 grown on TSA were used to inoculate LAM plates in duplicate and incubated for 16-18 hours at 37°C. (For method see **Section 3.4.1- 3.4.2**; Results **Section 5.3.3**).

5.2.6 Growth characteristics of HER1424 at 20, 25 and 30°C

This experiment was aimed at establishing if growth occurred at temperatures ranging from 20 to 30°C in solid and liquid medium, reporting average colony sizes in the absence of phage.

The recommended optimum growth temperature for HER1424 was reported by Klovins et al. (2002) to be 28°C. Average growth temperature optima were reported in the region of 33-35°C for most *Acinetobacter* strains (Holt et al., 1994) and 37°C, or higher for some clinical strains (Bergogne-Berezin & Towner, 1996). Glucose non-fermenting Gram-negative bacteria may be incubated at 30°C according to Gilardi (1978), as it “represents a compromise between lower and higher temperatures”.

In order to determine if the recommended temperature by Klovins et al. (2002) was a specific requirement, a range of different incubation temperatures were investigated and the growth characteristics monitored.

A freshly thawed stock culture of HER1424 was 4-way streaked on TSA plates and used to inoculate several flasks containing 100ml of TSB. Each set of plates and broths were incubated at 20, 25 and 30°C for 16-18 hours, broths were agitated by an orbital shaker set at 120rpm. For temperatures below ambient the cultures were always incubated in an Innova 4230 Refrigerated Incubator shaker, New Brunswick Scientific, NJ, USA. The results are shown in **Section 5.3.4**.

5.2.7 Growth curves of HER1424 at 25, 30 and 37°C

This range of experiments was designed to obtain growth curves, calculate doubling times and to determine after which time-points log phase was reached at the respective temperatures, which is important for choosing optimum time-points for infection with phage.

1-3 colonies of an overnight culture of *Acinetobacter* HER1424 grown at 25°C, for 16-18 hours on TSA were used to inoculate 10ml of TSB. The broth was incubated for 16-18 hours at 25°C in an orbital shaker set at 120rpm. One ml of the resulting overnight bacterial suspension was used to inoculate each of 3x100ml sterile TSB in 250ml

conical flasks. The flasks were incubated at 25°C (refrigerated incubator), 30 and 37°C with agitation at 120rpm in an orbital shaking motion for 16-18 hours.

The baseline OD₆₀₀ of a blank broth prepared from the same batch as above was set to zero. Optical density readings of all three broths were taken at 30 minute intervals after inoculation, using Fisherbrand semi-micro cuvettes 1.6ml, and a Unicam Helios ε Optical density reader for all readings. All cuvettes were filled with 0.9ml of sample. After each reading the liquid was transferred back into the conical flask.

The results are shown in **Section 5.3.5**.

5.2.8 Log phase experiment at 37°C, using a Bioscreen-C

This experiment using an automated system measuring optical density was designed to obtain several growth curves simultaneously, to confirm when log phase was reached.

10ml of TSB were inoculated with 1-3 colonies of HER1424 and incubated for 16-18 hours at 25°C in a refrigerated incubator on a shaking platform set to 120rpm. After incubation the cell suspension was centrifuged at 2,200 x g in a Centaur 2 (Sanyo) centrifuge for 10 minutes and resuspended in an equal volume of PBS. The OD₆₀₀ was adjusted with PBS to read between 0.33-0.35 (equivalent to 1x10⁸ cfu/ml). 60µl of adjusted bacterial suspension were added to 240µl of prewarmed TSB in each well of a 100-well plate (1 in 5 dilution); 10 wells were filled with TSB only as blanks. The plate was maintained at 37°C and read every 10 minutes in a Bioscreen-C, Growth curves USA, NJ, USA, set at 600nm for turbidity measurements. The results are shown in **Section 5.3.6**.

5.2.9 Growth characteristics of HER1424 at 4°C ± 1°C

It was observed during preliminary experimentation that colonies grown on TSA increased in size over a period of several weeks storage in the refrigerator. In order to investigate this further TSA plates were inoculated using 1-3 colonies of stock culture, cling-film wrapped to avoid loss of moisture and stored in a refrigerator maintained at

approximately 4°C. Plates were optically checked for appearance of colonies on a twice weekly basis. Results are shown in **Section 5.3.7**.

5.2.10 Propagation of phage HER424 using host HER1424

As only a limited amount of the bacteriophage HER424 was initially supplied, there was a need to propagate the phage material and produce sufficient quantities of purified lysate for future experiments. Van den Worm, co-author of Klovins et al. (2002) provided a detailed propagation protocol with the phage and host material (personal communication, 9 January 2003 & 24 January 2003, **Appendix 2**). It was recommended that the host HER1424 (also referred to as sp186 by the authors) be grown on minimal medium, then rich medium supplemented with 100mM CaCl₂, (which was claimed to be necessary for phage attachment) at 28°C. The advice was that phage should be added at a multiplicity of infection of 5-10 once the culture reaches an OD₆₅₀ of approximately 0.3, however the phage titre was unknown. It was recommended to add 50µl of lysate to 20ml of culture, incubating the infected bacterial suspension at 28°C without agitation and then continuing incubation at the same temperature for approximately 3 hours.

Van den Worm recommended growing the cells first on minimal medium (M9 Minimum Medium Salts, Maniatis Formulation supplemented with trace elements) to stimulate pilus production. Then to switch to LC medium (a rich medium and modification of Luria broth containing 10g Bactotryptone, Difco, 8g NaCl, 5g yeast extract, 1g MgSO₄·7H₂O, 20mg thymine and 1ml Tris·Cl pH 7.9), before infecting with phage (personal communication, 9 January 2003, **Appendix 2**).

5.2.10.1 Production of CaCl₂ solutions- Solution 1

12.2g of dried CaCl₂·6H₂O were added to four flasks containing 100ml TSB, the crystals were dissolved and two samples boiled for two minutes to dissolve the ingredients. All samples were autoclaved and checked for precipitation. This was repeated with NB and Isosensitest broth, to investigate if dissolved calcium would precipitate in various broths. The results are shown in **Section 5.3.8.1**

5.2.10.2 Production of CaCl₂ solutions- Solution 2

12.2g of CaCl₂*6H₂O were dissolved in 100ml demineralised water. TSB powder was dissolved in the calcium solution under constant stirring and whilst heating on a Bunsen flame. The supplemented broth was autoclaved and checked for precipitation. The results are shown in **Section 5.3.8.2**

5.2.10.3 Production of CaCl₂ solutions- Solution 3

12.2g of CaCl₂*6H₂O were dissolved in 100ml demineralised water, the crystals were dissolved, the solution autoclaved and optically checked for precipitation.

Before inoculation of TSB sterile CaCl₂*6H₂O solution was added to produce a 100mM solution. Immediately after inoculation and during incubation the supplemented broths were optically checked for precipitation. A blank containing only sterile supplemented TSB was incubated alongside samples.

The results are shown in **Section 5.3.8.3**

5.2.10.4 Phage propagation attempt 1

100ml of TSB were inoculated with 1-3 colonies of HER1424 *Acinetobacter haemolyticus* and incubated in an orbital shaker at 37°C, 120rpm for 16-18 hours. 50µl of lysate were added to 20ml of bacterial suspension, incubated at 30 °C for 15 minutes without shaking, then incubated in a refrigerated incubator with orbital shaker at 28°C at 110rpm for 3 hours.

The infected cell suspension was centrifuged in a Sorvall RC-5B Refrigerated Superspeed Centrifuge (Du Pont Instruments) in a GSA rotor at 16,319 x g for 20 min at 20°C.

The supernatant liquid was filtered using 0.45micrometer Nalgene syringe filters (Nalge) and BD Plastipak 10ml Luer syringe (Becton Dickinson) in a laminar flow cabinet. Sample supernatant (lysate) and pellet were stored at 4°C. The filtered

supernatant liquid was diluted 10-fold to produce 10 dilutions and each dilution was used for the soft agar overlay method n=3 (see **Section 2.3.4**).

Additionally TSA plates were surface spread with 100µl of overnight suspension of 9 Sussex strains (see **Section 2.2.1**) and HER1424. The excess liquid was discarded, the lawn dried for 10 minutes and 20 µl of droplets of filtered lysates applied in quintuplicate. All plates were incubated at 25°C for 16-18 hours. The results are shown in **Section 5.3.8.4**.

5.2.10.5 Phage propagation attempt 2

100ml of TSB containing 100mM CaCl₂·6H₂O were inoculated with 1-3 colonies of *Acinetobacter haemolyticus* HER1424 and incubated in an orbital shaker at 28°C, with shaking at 100rpm for 16-18 hours. 50µl of the original „Leiden“ lysate were added to 20ml of overnight bacterial suspension and at the same time 2ml of lysate from Phage propagation attempt 1 (**Section 5.3.8.4**) were added to a further 20ml of overnight bacterial suspension. Both infected cell suspensions were incubated at 28°C for 15 minutes without shaking, then incubated on an orbital shaker at 28°C shaking at 100 rpm for 3 hours. The infected cell suspensions were centrifuged in a Sorvall RC-5B Refrigerated Superspeed Centrifuge (Du Pont Instruments) in a GSA rotor at 16,319 x g for 20 min at 20°C.

The supernatant liquids were used for the soft agar overlay method (see **Section 2.3.4**). TSA plates were surface spread with 100µl of overnight suspension of 9 Sussex strains (see **Section 2.2.1**) and HER1424. The excess liquid was discarded, the lawn dried for 10 minutes and 20µl of droplets of filtered lysates applied in quintuplicate. All plates were incubated for 16-18 hours at 25°C in a refrigerated incubator. The results are shown in **Section 5.3.8.5**.

5.2.11 Temperature shifts affecting plaque formation of HER1424 and HER424

1 loopful of defrosted stocks of HER1424 each was used to inoculate three flasks containing 100ml TSB. 100ml were incubated at 20°C (Sample A) in a refrigerated incubator shaker with constant shaking at 120rpm. 100ml incubated at 30°C (Sample B) in a standard incubator without refrigeration function on a shaking platform set to 120rpm. 100ml were incubated for 16-18 hours in an incubator set to 20°C without refrigeration function, however due to ambient temperature shifts the actual temperature fluctuated between 19 and 24°C, with rare peaks at 29°C during incubation (Sample C). The actual temperature was measured using the digital display on each incubator and a calibrated thermometer placed on the bottom of the incubator which could be read without opening the incubation chamber.

All broths were incubated concurrently for 16-18 hours. After overnight incubation 100µl of each of the cell suspensions were surface spread on overdried TSA plates in duplicate and allowed to dry for 10 minutes. A sterile loop was dipped into the transport lysates originally received from Leiden University and randomly streaked across the bacterial lawn. This was repeated using lambda buffer as blank.

The resulting plates were incubated at the same temperature as the overnight cell suspensions, with the exception of the plate produced using the cell suspension Sample C, which was incubated for 3 hours at 30°C and then transferred to a refrigerated incubator set to 20°C. After incubation all plates were investigated for plaques or zones of clearance and the results are given in **Section 5.3.9**.

5.2.12 Production of a primary lysate using HER1424 and HER424

A modified method after Kutter & Sulakvelidze (2004) was used.

2ml of sterile Lambda buffer were added to plates with semi-confluent plaques containing Sample A (**Section 5.2.13**), superficial layers of the bacterial lawn and plaques were manually scraped off the plate and aseptically transferred into 100ml TSB. The broth containing phage and bacteria was incubated overnight at 25°C in a

refrigerated incubator, using a shaking platform set to 120rpm for 16-18 hours. On visual examination the culture was clearly turbid with white curdling and strings in the suspension, a phenomenon which is indicative of successful phage multiplication.

10ml Chloroform were added, the flask covered and placed back in the incubator for 15 minutes at 20 °C. The suspension was then centrifuged using a Sorvall RC-5B Refrigerated Superspeed Centrifuge (Du Pont Instruments) at 4000g for 20 min at 20°C. The supernatant liquid was separated from the cell pellet, syringe filtered and stored at 4°C, this preparation was referred to as AEH019(4/6/3)3.

5.2.13 Apparent relationship between temperature and ability to form plaques of HER1424 and HER424 using crude phage preparation AEH019(4/6/3)3

Before embarking on a scale-up procedure to produce large volumes of lysates, it was deemed necessary to further investigate the fragile, seemingly temperature-dependent lytic relationship between phage and host.

100ml of TSB were inoculated with one loop of HER1424 from frozen stock, and incubated at 120rpm at the respective temperatures: Sample A, 20°C (refrigerated incubator); Sample B, 22°C and Sample C, 30°C ± 2. On the following day the OD₆₀₀ of each broth was measured. 100µl of each bacterial suspension were surface spread on overdried TSA plates in duplicate and allowed to be absorbed into the agar for approx. 60 minutes. 10µl of a filtered suspension of lysate AEH019(4/6/3)3 were surface spotted in triplicate onto the agar surface and incubated at the respective temperature with one exception.

In one case (Sample C) the overnight culture was grown at 22°C, but the plate with phage droplets was incubated at 25°C. Additionally a 10% v/v Chloroform and broth mixture was prepared by adding chloroform to sterile broth and vigorously shaking the container. 10µl of this mixture was used on all plates (Blank1), in order to be able to differentiate a potential change in lawn due to the Chloroform contained in

AEH019(4/6/3)3 from a plaque. For comparison (Blank2) 10µl of Lambda buffer was also used on all plates.

Additionally soft agar overlays were performed (**Section 2.3.4**) where the phage stock AEH019(4/6/3)3 was used undiluted and overnight cell suspensions from Samples A, B & C were used. Incubation took place at the respective temperatures as before, with one exception. For sample C the overnight bacterial culture was grown at 22°C and the plate was incubated at 25°C.

On the following day all clearance zones were measured with callipers and recorded.

Both broths containing the bacterial inocula were 10 and 100fold serially diluted in PBS. 20µl of each dilution was surface spotted in triplicate onto overdried TSA plates for viable counts and incubated for 16-18 hours at 37 °C. The results are given in **Section 5.3.10**.

5.2.14 Production of purified phage suspension of HER1424 (secondary lysates)

A modified method after Kutter & Sulakvelidze (2005) was used.

100ml of TSB, contained in a 250ml conical flask were inoculated with one loopful of freshly thawed stock of HER1424 and incubated on a shaking platform at 20°C in a refrigerated shaking incubator (120rpm) for 20 hours. 5x1ml aliquots of the overnight bacterial suspension were subcultured in 5x100ml TSB and incubated at 20°C in a refrigerated incubator shaker set to 120rpm until the optical density of OD₆₀₀ reached 0.5. 1ml of phage preparation AEH019(4/6/3)3 (see **Section 5.2.15**) was added to each of the bacterial suspensions. The flasks containing bacteria and phage were placed in a static incubator for 15 minutes at 20° C to facilitate infection of bacteria. Afterwards the flasks were transferred to a shaking platform and incubated for 36 hours at 120rpm at 20° C. After incubation the macroscopic appearance of lysates had changed, with stringy clumps sedimented at the bottom of the flask and the broth appearing to be clear, quite in contrast to the appearance of an uninfected overnight bacterial suspension. 10ml of Chloroform were added to each of the conical flasks in a fume hood and incubated

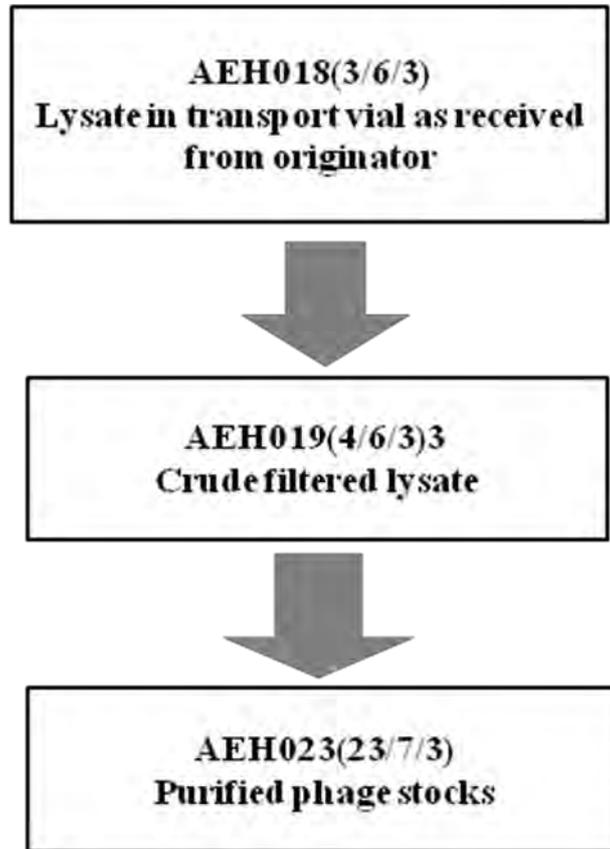
for 10 minutes at 120rpm at 20°C. In order to obtain a crude lysate the bacterial debris was removed from each flask by centrifugation at 2500g for 10minutes and the supernatant liquids filtered using a Millipore 0.45 µm syringe filter. All filtrates were pooled in a sterile 1000ml flask; NaCl was added to a final concentration of 1M to the lysed culture. The salt was dissolved by occasional swirling whilst the conical flask was stored on crushed ice for 1 hour.

Bacterial debris was removed by centrifugation at 11,000g for 10minutes at 4°C in a Sorvall RC-5B Refrigerated Superspeed Centrifuge (Du Pont Instruments). The supernatant liquid was removed in a laminar flow hood and placed in sterile conical flasks. The precipitate was discarded and the centrifuge containers rinsed with sterile broth, in order to be reused for the second centrifugation step. Polyethylene glycol (PEG 8,000) was added to the supernatant liquid to give a final concentration of 10% w/v. This was slowly dissolved by stirring with a magnetic stirrer at room temperature. The flask was then cooled on crushed ice for at least 1 hour or left for 16-18 hours at 4°C, to allow the bacteriophage to form a precipitate.

The precipitate was recovered by centrifugation at 11,000g for 10 minutes at 4°C. The supernatant liquid was discarded and the remaining fluid drained away from the pellet by placing the centrifuge container in a tilted position. The pellet was re-suspended using 5 x 2ml lambda buffer. Using a 10ml pipette, the suspension was transferred into 25ml sterile Chloroform resistant centrifugation containers. An additional 1ml of buffer was used to rinse the centrifugation container.

1ml of chloroform was added to the bacteriophage suspension and vortexed for 30 seconds. The organic and aqueous phases were separated by centrifugation at 3000 x g for 15 minutes at 4°C. The concentrated aqueous phase containing the phage was removed, made up to 50ml with lambda buffer and stored in a sterile clear glass container at 2-8°C. Five 1ml aliquots of purified phage preparation were supplemented with 10% v/v glycerol and stored at -80°C. The remainder of the highly concentrated and purified phage stock suspension produced using this protocol was referred to as AEH023(23/7/3), and was stored in a sterile clear screw cap glass container at 2-8°C.

5.2.15 Diagram to show propagation of phage HER1424 after receipt



5.2.16 Investigating viability, sensitivity to chloroform and quantitative titration of purified phage suspension

After production and purification of AEH023(23/7/3) its concentration was unknown. To allow any quantitative work with phage the number of plaque forming units per ml (pfu/ml) had to be determined. A variety of methods are available to allow determinations of phage concentrations, a classic, easily repeatable one being the soft agar overlay method. Some phages are reported to be sensitive to Chloroform. An absence of ability to bring about host cell infection after purification can be due to a variety of reasons and since Chloroform is used regularly in phage purification protocols, it is important to establish if it can affect viability. The stock bacteriophage suspension AEH023(23/7/3) was removed from the refrigerator and allowed to equilibrate to ambient temperature for 2 hours before use. If the ambient temperature was below 19°C it was placed for a short while in an incubator set to 25°C. To calculate

the phage titre 100µl AEH023(23/7/3) were diluted in 10-fold stages in lambda buffer, and dilutions 10^{-5} to 10^{-8} were plated in duplicate using the soft agar overlay method. This procedure was repeated on 7 different days. The results are shown in **Section 5.3.11**.

5.2.17 Validation of quantitative phage estimation using the soft agar overlay method

A range of 10-fold dilutions of AEH023(23/7/3) were prepared using lambda buffer as diluent. The dilutions that were predicted from previous estimations to give countable plaque numbers were plated using the soft agar overlay method (**Section 2.3.4**) on five different days in quintuplicate. An overnight cell suspension of HER1424 grown at 25°C in a refrigerated incubator for 16-18 hours at 120rpm was used as the host. The optical density of the overnight suspension was adjusted with sterile TSB to $OD_{600} = 0.45-0.5$ (n=5) prior to use. The results are given in **Section 5.3.12**.

5.2.18 Determination of loss of phage after filtration with 0.45µm syringe filter

10ml of TSB were inoculated with a single colony of HER1424 and incubated at 25°C in a refrigerated incubator for 16-18 hours to produce a broth suspension. On the following day the OD_{600} was adjusted to read approximately 0.45 using sterile TSB.

100µl of phage preparation AEH023(23/7/3) were diluted with lambda buffer to yield an approximate plaque count of 100-400 pfu/plate. All dilutions and diluents were allowed to reach ambient temperature before use. The ready diluted phage suspension was divided into two increments of 2ml. One 2ml aliquot was used to produce soft agar overlays n=8, the second aliquot of 2ml was filtered using a Minisart (Sartorius, Epsom, UK) syringe filter, 0.45µm, Plastipak (BD, NJ, USA) syringe and Neolus (Terumo, Surrey, UK) green needle. The filtrate was collected in sterile glass boiling tubes and used to produce soft agar overlays n=7. The experiment was performed in duplicate.

Both sets of overlays were incubated at 25°C for 16-18 hours and the number of plaques counted. The results are shown in **Section 5.3.13**.

5.2.19 Method optimisation for optimum optical density of inocula to yield best bacterial lawns for plaque counts

One loopful of frozen *Acinetobacter haemolyticus* HER1424 was subcultured on TSA and incubated at 25°C for 16-18 hours in a refrigerated incubator. One colony of the freshly grow culture was subcultured into 10ml TSB and incubated at 25°C on a shaking platform at 120rpm for 16-18 hours in a refrigerated incubator. The OD₆₀₀ of the overnight suspension was measured and recorded as 0.9. Aliquots of the overnight suspension were removed and the optical density adjusted with sterile TSB as closely as possible to read optical densities of 0.5, 0.4 and 0.3.

100ml of each of the respective adjusted overnight suspensions were used in the soft agar overlay method, n=10 (**Section 2.3.4**) and incubated for 16-18 hours at 25°C in an Innova 4230 Refrigerated Incubator shaker, new Brunswick Scientific, NJ, USA. Average numbers of pfu/plate were counted and average plaque sizes in mm were recorded. The overall shape and ease of counting of all plates was assessed using backlighting and a magnification glass. The results are given in **Section 5.3.14**.

5.2.20 Impact of heat on phage activity

Three sets of 1ml of a phage suspension of HER424 with a known titer, yielding approximately 150-200 pfu/plate were maintained at 60°C, 30°C and 25°C respectively for 2 hours. As a control 1ml of the same phage preparation was kept at ambient temperature (21°C) for 2 hours. All phage preparations were plated (n=5) using the optimized soft agar overlay method described above.

HER1424 yielded plaques only within a specific temperature range when infected with AP205 (**5.3.15 & 5.3.16**), and so it was important to know whether the phage would be inactivated if heated to the respective incubation temperature. This would confirm whether the phage would be permanently inactivated due to temperatures above 25°C or

whether the mechanism whereby the phage did not yield plaques when infected at temperatures above 25°C was more complex. The results are shown in **Section 5.3.15**

5.2.21 Temperature dependent relationship between *Acinetobacter haemolyticus* HER1424 and phage AP205 (HER 424)

Preliminary experiments undertaken with unpurified phage lysates indicated that the conditions, particularly the temperature at which the bacteria were infected were critical for the formation of distinct plaques (**Section 5.2.13**).

The aim of this experiment was to investigate the apparent temperature-dependent lytic relationship between *Acinetobacter* phage and host and determine the permissive temperature for lysis and potential reasons for this unusual occurrence.

1-3 colonies of HER1424, grown on TSA were subcultured into 10ml TSB and incubated for 18 hours at 25°C, in an Innova 4230 Refrigerated Incubator shaker, New Brunswick Scientific, NJ, USA at 120rpm. After incubation the OD₆₀₀ was adjusted to 0.46 and the soft agar overlay method was employed (**Section 2.3.4**) using a purified phage suspension diluted in lambda buffer yielding approximately 100-200 pfu/plate of highly purified phage preparation AEH23(23/7/3).

Plates were incubated at a variety of temperatures (22, 25, 30, 35 and 37°C) for 16-18hours and plaques counted (n=5). The results are shown in **Section 5.3.16**.

5.2.22 Temperature switches affecting ability to form plaques

These experiments were designed to investigate whether the original incubation temperature or the infection temperature were critical for the formation of plaques. 10ml TSB were inoculated with 1-3colonies of HER1424 and incubated for 16-18 hours at 22, 25 or 37 °C on a shaking platform set to 120rpm. Incubation at 22°C took place in a refrigerated incubator, samples were incubated at 25°C and 37 °C in two standard, non-refrigerated incubators.

Soft agar overlay plates were produced using this overnight suspension and the phage suspension diluted to a concentration to yield approximately 150-300 pfu/plate. Plates

were incubated for 16-18 hours at 22, 25 or 37°C and the results recorded as plaques or absences of plaques, all plates were produced in duplicates. The results are shown in **Section 5.3.17**

5.2.23 Downshifting of temperature after infection

1-3 colonies of HER1424 were subcultured in 100ml of TSB and incubated for 16-18 hours at 37°C on a rotating platform set to 120rpm. The optical density at 600nm was measured and recorded (Subculture 1). 10ml of the overnight cell suspension were subcultured into 100ml TSB and incubated for 16 hours at 37°C on a rotating platform set to 120rpm. The general appearance of the broth and optical density were observed and recorded (Subculture 2).

100µl increments of this overnight suspension (Subculture 2) were used to produce soft agar overlays without addition of phage, plates were incubated at 25°C in a refrigerated incubator shaker for 16-18 hours.

30ml of the overnight suspension (Subculture 2) were infected with 100µl of phage suspension (1×10^8 pfu/ml) and placed in an incubator at 37°C for 10minutes. The infected cell suspension was centrifuged for 10minutes at $2,200 \times g$ resuspended and washed 3 times and finally resuspended in 20ml TSB (Infected S 2). 100µl increments of the infected and washed cell suspension were used to produce soft agar overlays without addition of phage, plates were incubated at 25°C for 16-18 hours. One ml of this cell suspension was subcultured into 100ml TSB (Subculture 3) and incubated at 37°C at 120rpm for 16-18 hours. 100µl increments of cell suspension (Subculture 3) after overnight incubation were used to produce soft agar overlays without addition of phage, plates were incubated at 37°C for 16-18 hours.

1ml of (Subculture 3) cell suspension was removed, syringe filtered to remove viable bacteria and 100µl increments of filtered cell suspension were used to produce soft agar overlays without addition of phage. The plates were incubated at 25°C for 16-18 hours (Subculture 3, F). The results are given in **Section 5.3.18**

5.2.24 Attempts to artificially create temperate phage

The rationale behind this experiment was to investigate if purely temperate phage progeny could be created by changing basic experimental parameters.

1-3 colonies of HER1424 were subcultured in 100ml of TSB and incubated for 16-18 hours at 37°C, at 120rpm (37°C Starter). A second 100ml flask of TSB was inoculated and incubated at 25°C (25°C Starter). The optical density OD₆₀₀ was measured to estimate the number of cells/ml present. The cell number and phage number in a known phage suspension were adjusted using TSB and lambda buffer respectively to yield 1:1 and 1:10 ratios of phage to bacteria, 1×10^8 and 1×10^7 pfu/ml respectively. The phage suspension was added to the overnight culture and left on the bench for 10 minutes, then put onto a shaking platform for 20 minutes at 37°C. 20ml of the suspension were then spun down at 2,200 x g for 10 minutes, the supernatant liquid discarded, while the pellet was washed with broth and resuspended in 20ml TSB. The cells were washed three times and the cell pellet suspended in 20ml broth. Finally 1ml of the resuspended suspension was used to inoculate 100ml of TSB, which was incubated for 16-18h at 37°C, and a shaking rate of 120rpm. 100µl syringe filtered aliquots of the bacterial suspension were used to produce soft agar overlays, without addition of phage, plates were incubated at 25°C, n=5 (Filtrate 1). This process of subculturing 1ml of infected cell suspension was repeated twice to yield Filtrates 2 and 3, which were processed equally to Filtrate 1 to produce soft agar overlays.

The results are shown in **Section 5.3.19**.

5.2.25 Investigating the possibility of clonal differences between plaques

The rationale behind this experiment was to investigate if heterogeneous plaque sizes were due to clonal differences between plaques, or an intrinsic feature of AP205 phage.

An overnight cell suspension was produced by inoculating 10ml of TSB with 1-3 colonies of HER1424 and incubating for 16-18 hours at 25°C at 120rpm. A phage preparation to yield approximately 100pfu/plate was used in the soft agar overlay

method (**Section 2.3.4**) and incubated at 25°C for 16-18 hours. The actual plaque count was 111 pfu/plate, the two largest plaques were established (6.5mm each) and the two smallest plaques (0.4mm each), using callipers.

A sterile cork borer was used to remove the two largest plaques, which were both placed in universal bottles containing 2ml Lambda buffer and 40 µl of Chloroform each. The two smallest plaques were removed using a sterile glass pipette and prepared in the same way as the large plaques. All four preparations were vortexed, left at ambient temperature for 2 hours and then stored in a refrigerator at 2-8°C.

100µl of each undiluted phage preparation obtained by plaque picking were used to prepare soft agar overlays to establish viability. All four plates showed no bacterial lawns, which indicated presence of large number of phage.

As a next step one preparation derived from the largest and smallest plaque respectively was serially diluted in 10-fold dilutions using lambda buffer. Soft agar overlays were produced in duplicate with dilutions 10^{-5} to 10^{-8} of the phage suspension and the size and appearance of plaques established.

The same preparations were used to create a next generation of plaques: 1-3 colonies of HER1424 were used to inoculate 10ml of TSB, which was incubated at 25°C for 16-18 hours. 2x2ml of the overnight cell suspension were used to inoculate 2x100ml of TSB and incubated at 25°C for approx. 4 hours, until an $OD_{600}=0.5$ was reached. 1ml of the phage preparation from the largest plaques and 1ml of the phage preparation from the smallest plaque were used to infect each broth and incubated for 16-18 hours at 25°C. The flasks containing bacteria and phage were placed in a static incubator at 25°C for 15minutes after which they were incubated for a further 18 hours at 25°C with shaking at 120rpm. After incubation 10ml of Chloroform were added to the flask in a fume hood and incubated for 10 minutes at 25°C with shaking at 120rpm. The preparation was filtered using Nalgene 0.45 µm methyl cellulose syringe filters, serially diluted with lambda buffer and used to produce soft agar overlays. The preparation propagated from the smallest plaque was termed ProgenyS and from the largest ProgenyL. Plates with

unmerged plaques with distinctive edges were chosen, plaques measured using callipers and the data statistically evaluated. The results are shown in **Section 5.3.20**.

5.2.26 Adsorption experiments at 25°C and 37°C

The aim of this experiment was to investigate the extent at which phage particles adsorb to bacterial cells at different temperatures by quantitatively measuring free phage in suspension.

A culture of HER1424 was grown in TSB at 25°C for 18 hours with shaking at 120 rpm. 10ml of this suspension were used to inoculate 100ml of sterile TSB in a 250ml Erlenmeyer flask and the estimated cell concentration established using optical density measurements. Viable counts were performed to calculate the actual number of viable bacteria in the reaction vessel. 1ml of the original overnight cell suspension grown at 25°C was also used to inoculate 100ml of TSB in a 250ml Erlenmeyer flask and incubated at 37°C for 16-18 hours at 120rpm. After overnight incubation 2ml were removed for viable counts and optical density measurements.

A purified suspension of HER424 was diluted to yield a specific phage titre and 1ml was added to the 250ml conical flask containing 100ml of the bacterial suspension to achieve an approximate phage to bacteria ratio of 1:1. The flask was manually agitated to ensure even mixing and placed in a static incubator for 20 minutes. Further incubation took place in a waterbath set to 25°C or 37°C shaken at 120rpm and 1.5ml samples were withdrawn at times 3 minutes and in 20 minute intervals after infection and syringe filtered. Filtrates were serially diluted with lambda buffer to produce dilutions which were used with the soft agar method to determine phage titres in each sample (n=5). The results are shown in **Section 5.3.21**

5.2.27 Screening Sussex isolates as potential hosts for AP205 at various temperatures.

5.2.27.1 Screening Sussex isolates as potential hosts for AP205 using the surface spotting method

The aim of this investigation was to attempt to match as many clinical isolates with a lytic phage. The 9 Sussex isolates (**Section 2.2.1**) were screened against phage AP205, to investigate its host range. The method used was an adaptation of Modified Method 1601 by the US Environmental Protection Agency (2001).

Freshly thawed stocks of Sussex isolates and HER1424, were each inoculated into 5 separate 10ml volumes of TSB using wire loops and incubated for 18 hours at 20, 22, 25, 30 and 37°C, on shaking platforms set to 120rpm. The optical densities of the overnight suspensions were checked to be between OD₆₀₀ 0.1 and 0.5, which corresponds to log phase growth (see **Section 3.5.7**).

100µl of the undiluted overnight bacterial suspensions were surface spread onto overdried TSA plates and 10 µl spots of purified phage suspension AEH23(23/7/3), at a concentration of approximately 100-200pfu/10 µl droplet were applied in triplicate alongside 10 µl spots of TSB as blank 1 and 10%v/v Chloroform in TSB as blank 2.

The surface spread plates with the spots applied were left at ambient temperature for one hour and then placed in an incubator at the same incubation temperature as the overnight suspension (20, 22, 25, 30 and 37 °C respectively) for 16-18 hours. On the following day the plates were checked for clearance zones, overall quality of lawn and other markings indicative of lysis. The results are shown in **Section 5.3.22**

5.2.27.2 Screening Sussex isolates as potential hosts for AP205 using the soft agar overlay method

Single colonies of 9 Sussex strains and HER1424 grown on TSA were subcultured into 10x10ml of TSB and incubated for 16-18hours at 37 °C. These cultures were used as inocula in the soft agar overlay method (**Section 2.3.4**) together with a phage

Chapter Five: Investigation of temperature dependent lytic relationship of host and phage HER1424 and HER424

concentration of approximately 100 pfu/ml (AEH23(23/7/3) n=2). The plates were incubated at 25°C or 37°C and the experiment was repeated using a higher concentration of phage, with approximately 1000pfu/ml.

The results are shown in **Section 5.3.23**.

5.3 Results

5.3.1 Phenotypic properties, identification of HER1424 with API20NE test system, microscopic examination and digital imaging using a bright-field light microscope

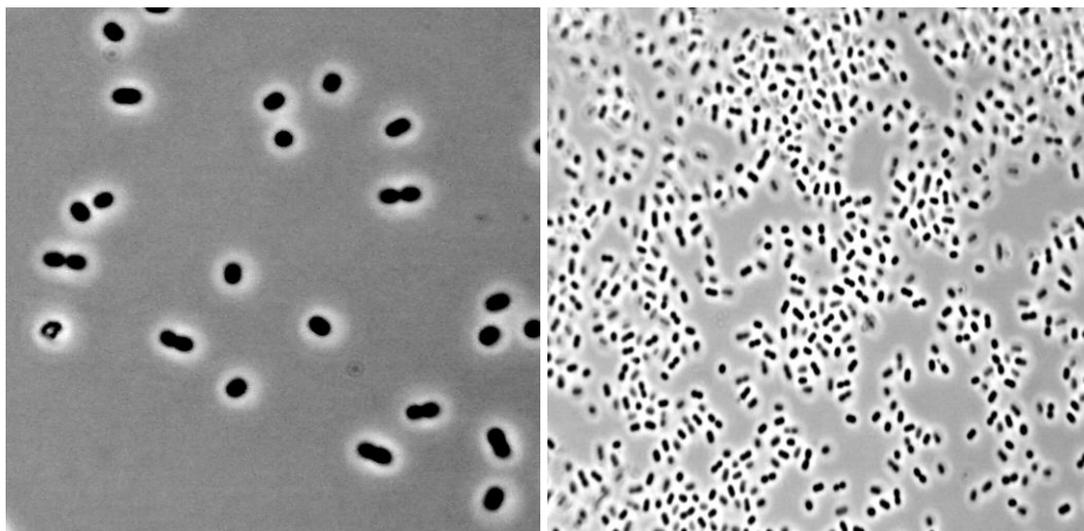
Test	Outcome
Gram stain	Negative
Microscopic appearance	Rods (Coccobacilli in stationary phase)
Oxidase	Negative
Catalase	Positive
Growth requirement	Strictly aerobic
Motility	Non motile

Table 5.1 Phenotypic properties of HER1424

The test kit API20NE, (bioMérieux, Lyon, France) yielded the following positive reactions: GEL, CAP, MLT, and CIT, which interpreted in conjunction with the oxidase test result (**Table 5.1**) translates to a profile of: 0010051, which matched *Acinetobacter haemolyticus* (Excellent identification).

The results are shown in **Figures 5.1** and **5.2** and indicate that the microscopic appearance of the cells was intact short rods, some appearing to be spherical or oval shaped.

Some cells gave the appearance of coccobacilli, which can usually be observed when *Acinetobacter* specimens are prepared from agar cultures (Koneman et al., 1994). No motility was observed.



Figures 5.1 & 5.2: HER1424 photographed using a phase contrast microscope

5.3.2 Antibiotic susceptibility of HER1424 using BSAC disc diffusion method

HER1424 was resistant to all cephalosporins and penicillins *in vitro*, apart from the ureidopenicillin piperacillin, which is considered an antipseudomonal penicillin.

HER1424 was sensitive to ciprofloxacin and imipenem, both bactericidal antibiotics, which act by inhibition of DNA gyrase and synthesis of the bacterial wall respectively.

It needs to be borne in mind that for this investigation 5 μ g test discs were used, however the cut-off point published by BSAC were published for 1 μ g discs, it is therefore possible that the result for ciprofloxacin is false positive. HER1424 also proved to be sensitive to gentamicin and amikacin both aminoglycosides having similar mechanisms of action, which inhibit protein synthesis by acting on the 30s ribosomal subunits. Neomycin and streptomycin aminoglycosides without published BSAC break-off points inhibited growth of HER1424. Cut-off points for penicillin G and vancomycin for *Acinetobacter* were also not published, however it appeared that penicillin G disks did not affect the growth of HER1424 at all. It is unsurprising as this bacterium was resistant to all other penicillins tested, with the exception of piperacillin.

Antibiotic	Disc content	BSAC	Results
Amikacin	30mcg	15-19	S
Amoxi/clav.acid*	30mcg	17/18	R
Aztreonam	30mcg	23/24	R
Cefixime	5mcg	19/20	R
Ceftazidime	30mcg	27/28	R
Cefuroxime sodium	30mcg	19/20	R
Ciprofloxacin	5mcg*	17/18	S
Gentamycin	10mcg	16-20	S
Imipenem	10mcg	22/23	S
Neomycin	10mcg	N/A	18
Penicillin G	10Units	N/A	0
Piperacillin	100mcg	23/24	S
Streptomycin	10mcg	N/A	17
Tetracycline	50mcg	33/34	R
Trimethoprim	1.25mcg	14-20	R
Vancomycin	30mcg	N/A	0

Table 5.2 Antibiotic susceptibility of HER1424 using BSAC disc diffusion method (n=4)

Legend for Table 5.2 :

**Amoxy/clav.acid reads as amoxicillin plus clavulanic acid*

Readings are expressed as R= Resistant, S=Sensitive or I=Intermediate

In cases where no cut-off points were published the result is given as inhibition zone diameter in mm.

Vancomycin, a glycopeptide antibiotic did not affect the growth of HER1424, this is unsurprising as the main therapeutic spectrum includes streptococci and *Staph. aureus*.

BSAC removed breakpoints of tetracycline in version 4 (2005), to allow a review of breakpoints for this group of agents. As the BSAC Disc Diffusion Method for Antimicrobial Susceptibility Testing version 5, (2006) did not contain breakpoints for this drug, breakpoints from version 3, Jan 2004 at 33/34 mm were used to interpret the data.

5.3.3 Growth characteristics of HER1424 on Leeds Acinetobacter Medium (LAM)

The organism failed to grow on the selective medium, regardless of the incubation temperature used. The experiment was repeated and incubated at 37°C and 30°C for 16 hours. One plate incubated at 30°C showed a mauve area where the inoculum was applied particularly heavily, however no single colonies could be seen on other areas of the agar plate. Several attempts to subculture the discoloured area failed to produce evidence of growth.

Jawad et al. (1994) reported that *Acinetobacter johnsonii* and *Acinetobacter haemolyticus* failed to grow on LAM, due to the properties of this medium. As expected HER1424 failed to grow on LAM, this further supports the result of the API20NE test and confirms the identity of this isolate.

5.3.4 Growth characteristics of HER1424 at 20, 25 and 30°C

A. haemolyticus HER1424 grew at all three temperatures on TSA and in TSB. For all broths the OD₆₀₀ reading was greater than 0.4. At 20°C the growth on TSA plates was vigorous (**Table 5.3**), however all colonies were (less than 0.5mm in diameter and too small to measure with callipers, at 25°C growth was equally vigorous, the average colony size was larger at 0.5mm. At 30°C growth was again healthy and the average colony size was twice that found at 25°C at 1-2 mm.

	20 °C	25 °C	30 °C
Overall Growth on TSA	++	++	++
Overall growth TSB	+	+	+
Average colony size on TSA	<0.5mm	0.5mm	1-2mm

Table 5.3 Growth characteristics of HER1424 at 20, 25 and 30 °C

Legend Table 5.3: + growth, ++ vigorous growth

5.3.5 Growth curves of HER1424 at 25, 30 and 37°C

A. haemolyticus HER1424 grew readily at 25, 30 and 37°C in liquid culture under the conditions stated. It appeared that when colonies grown on TSA were subcultured into TSB, log phase was entered after 0.5 hours regardless of incubation temperature (**Figure 5.3**).

Differences were observed in the maximum optical densities reached at different temperatures. It appeared that the maximum OD₆₀₀ reached at 37°C and 25 °C were 0.40 and 0.42 respectively, at 30°C OD₆₀₀ of 1.2 was reached. Interestingly HER1424 appeared to grow more vigorously at 30°C than at 25°C, yet less vigorously at 37°C. Doubling times (**Table 5.4**) were calculated using optical density readings recorded between 0.5 and 4 hours.

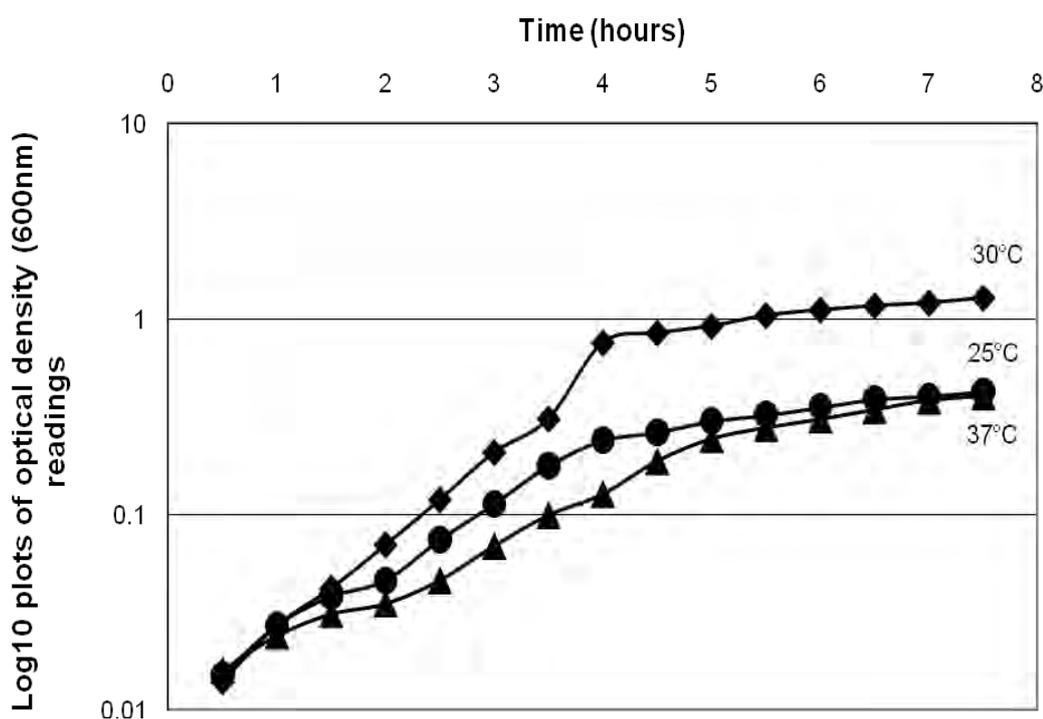


Figure 5.3 log₁₀ growth curves of HER1424 at different temperatures

Legend Figure 5.3:

Incubation temperatures: Squares- 30°C, circles- 25°C, triangles-37°C

It is clear from the doubling times that HER1424 grew fastest at 30°C, hence the optimum growth temperature for HER1424 appeared to be 30°C in liquid culture. Cell suspensions grown at 37°C routinely had low optical densities after overnight culture.

Temperature	Calculated doubling times (h)
25°C	0.90
30°C	0.59
37°C	1.17

Table 5.4 Doubling times of HER1424 grown in TSB at various temperatures

5.3.6 Log phase experiment at 37°C, using a Bioscreen-C

In contrast to the Sussex isolates HER1424 yielded an $OD_{600}=0.7$, below 1.0 even after incubation for 20 hours (**Figure 5.4**).

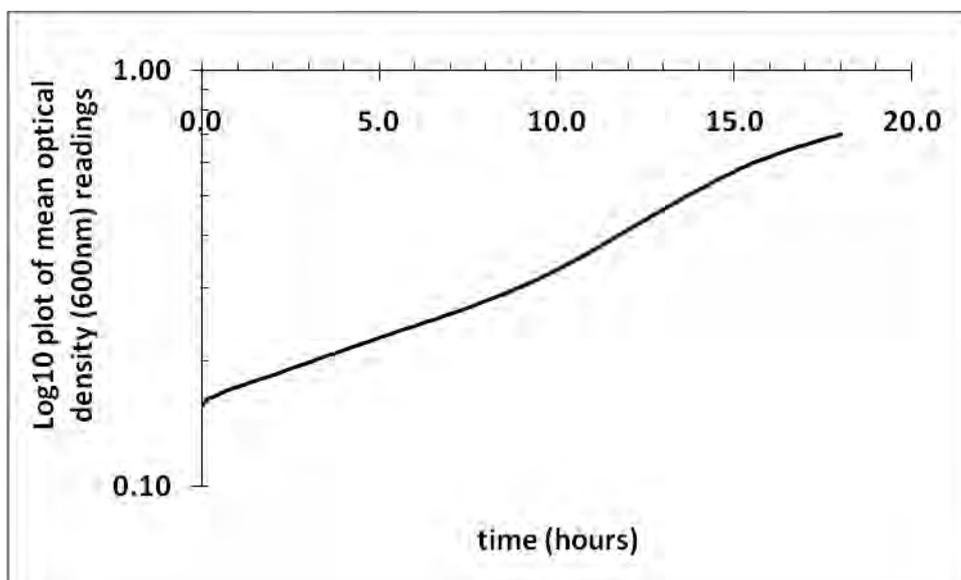


Figure 5.4 Mean growth curve of HER1424 at 37°C (n=9)

The data used in **Figure 5.4** also demonstrates that HER1424 was in log phase when the OD_{600} can be read as 0.5 after at least 14 hours of incubation. This information is essential in determining when phage can be added to the bacterial suspension to ensure the host is in log phase and most susceptible to phage.

Despite the different experimental setup, this experiment in combination with **Section 5.2.7** demonstrates that HER1424 reaches log phase, a prerequisite to infection with phage, even when grown at 37°C.

The phage lysate protocol (**Section 2.7**) was designed to add phage when an optical density of 0.5 (600 nm) was reached. From these experiments it can be concluded that the protocol used was appropriate as the bacterial growth curve for each strain was in the exponential phase at point of exposure to phage.

5.3.7 Growth characteristics of HER1424 at 4°C +/- 1°C

After storage for 8 weeks in the refrigerator the colonies had reached an average diameter of 1-2mm, suggesting that growth did occur at 4°C, however at a much slower rate than at routine temperatures. The experiment was repeated three times to exclude false positives.

5.3.8.1 Production of CaCl₂ solutions- Solution 1

Supplemented TSB showed precipitation after boiling for 2 minutes and heavy precipitation after being autoclaved. NB showed no precipitation after CaCl₂ was dissolved concurrently with the broth powder, however when samples were boiled for 2 minutes and autoclaved, samples showed slight precipitation immediately. After storage for 10 days precipitation and re-crystallisation of large flakes was observed.

Supplemented Isosensitest broth showed immediate precipitation after addition of CaCl₂, which increased after boiling for 2 minutes and autoclaving.

5.3.8.2 Production of CaCl₂ solutions- Solution 2

After addition of TSB powder the CaCl₂ turned cloudy and precipitated on boiling and after autoclaving.

5.3.8.3 Production of CaCl₂ solutions- Solution 3

Calcium chloride hexahydrate showed no signs of precipitation after dissolution in water or after autoclaving. On addition of autoclaved calcium chloride hexahydrate solution to sterile TSB no precipitation occurred. After autoclaving the inoculated supplemented broth remained clear, however some calcium crystals had formed on the bottom.

Change in salt concentration, as could be caused by precipitation, can change the titre of coliphage T2 preparations approximately 1000 fold (Kutter & Sulakvelidze, 2005). By investigating other phage propagation protocols it soon became clear that a range of calcium concentrations were acceptable in phage breeding. Other investigators have

used concentrations ranging from 5mM to 50mM (Daehnel, et al., 2005; Mugdal et al., 2006; Nilsson, et al. 2000) compared to the 100mM CaCl₂ recommended by van den Worm-personal communication, 14 January 2003.

This clearly makes solutions 1 and 2 (**5.2.10.1-.5.2.11.3**) less suitable than solution 3, as they appear to easily precipitate and would make any attempts at quantitative work with phage difficult to manage.

5.3.8.4 Phage propagation attempt 1

None of the 10 dilutions used to produce soft agar overlay plates with host HER1424 yielded any plaques. The additional plates where bacterial lawns had been produced using 9 Sussex strains and phage droplets had been applied did not show any thinning of lawn or areas of clearance. These results imply that the phage could not be either successfully propagated under the experimental conditions used or phage did not produce clearance zones or plaques because it was not lytic under the experimental conditions. Klovins et al. (2002) had reported that cell lysis did not always occur when HER1424 was infected with AP205, when cells had been grown at 28°C to an OD₆₅₀=0.2.

In a personal communication one co-author J. van Duin reported that he had never been able to produce plaques with this phage and host combination (14 February 2003, **Appendix 3**). The fact that phage and host did not show any signs of lysis was hardly surprising, considering the information provided. What seemed puzzling was that the authors claimed that ssRNA phages cause lytic infection. AP205 being a confirmed RNA phage closely related to ssRNA phages was a likely candidate for lysis. Evidence by the authors suggests that AP205 contains an ORF (ORF1) which may encode a lysis protein. When ORF1 was cloned into an expression vector its induction in *E. coli* halted cell growth. The authors suggested that ORF1 translates into a peptide which has lysis function. As only lytic phage would be useful in phage therapy, it was of interest to see if slight changes in experimental conditions like addition of calcium ions or temperature shifts would lead to bacterial lysis. A second attempt (**Section 5.2.10.5**) was devised,

providing the experimental conditions recommended by Klovins et al. (2002) for phage propagation.

5.3.8.5 Phage propagation attempt 2

The outcome of this range of experiments did not lead to any plaques or clearance zones. The addition of calcium ions as recommended by Van Duin „to aid attachment“ of phage to host did not lead to lysis. Neither did the recommended incubation temperature of 28°C. The addition of original lysate to a host suspension did not lead to lysis, neither did 2ml of putative lysate which was obtained by the first phage propagation attempt (5.2.10.4).

None of the 10 dilutions used to produce soft agar overlay plates yielded any plaques and none of the lawns with phage droplets applied showed any areas of clearance or even thinning of lawns. In the same way as **Section 5.2.13** this outcome was a little surprising as the team including Klovins and Van Duin had used the recommended protocol to propagate phage, yet this did not involve plaque formation.

During further correspondence with one of the investigators (personal correspondence S. van den Worm, 14.02.2003, **Appendix 2**) it transpired that despite the complex infection protocol being used, plaques were never produced. In van den Worm's paper co-authored by Klovins et al. (2002) it was reported that when HER1424 was infected with HER424 (AP205) at 28°C lysis did not always occur, despite the fact that AP205 would be considered a lytic phage on grounds of genetic mapping, especially due to the extra reading frames ORF1 and ORF2 and previous reports by Coffi (1995).

The main interest of Klovins et al. (2002) in AP205 was to study this phage genetically. In the context of their work it was irrelevant if AP205 formed plaques or not, at most it may have been inconvenient as phage are more difficult to titrate without being able to use the soft agar overlay method, which depends on plaque formation. Phage can still be propagated without the formation of plaques or clearance zones, however counting or detecting the actual presence of phage particles is more difficult. The authors resorted to electron microscopy to resolve this issue. In order for this material to be meaningful in the context of phage therapy and this research the phage needed to form plaques.

This was necessary for the detection, confirmation of successful propagation and quantification.

Phage propagation attempts 1 and 2 differed in the presence or absence of calcium ions and incubation temperature. A further 2ml of putative lysate from experiment described in **Section 5.3.8.4** were added to the bacterial culture in addition to 50µl of „Leiden“ lysate to ensure large amounts of phage would be present. The lower incubation temperature of bacterial suspension after infection of 28°C (2°C below the temperature used in the phage propagation attempt 1 (**5.3.8.4**)) did not change the experimental outcome significantly.

On the basis of these preliminary experiments it was decided to instigate another range of experiments with slightly different infection and growth conditions to establish if HER1424 with HER424 could be induced to form plaques (**5.2.13**).

5.3.9 Temperature shifts affecting plaque formation of HER1424 and HER424

	Sample A	Sample B	Sample C
Incubation temp. cell suspension	20 °C	30 °C	20-29 °C
Incubation temp. petri dish	20 °C	30 °C	30°C (3h) 20°C (15 h)
Appearance sample	Plaques TNTC	No plaques	Some plaques
Appearance blank	No plaques	No plaques	No plaques

Table 5.5 Incubation temperature affecting plaque formation

Legend Table 5.5: *Sample A, B, C- 100ml HER1424 samples with various incubation temperatures (see Section 5.2.11)*

This experiment included three different setups in one experiment. The wide temperature range to which Sample C was exposed to (20-29 °C) was due to shifting temperatures inside a non-refrigerated incubator, caused by ambient temperature changes. It was discovered by chance that lower or fluctuating incubation temperatures would lead to plaque formation.

Samples incubated at 30°C did not yield any plaques (**Table 5.5**), the lawn was scraped where the inoculation loop had been used to apply phage preparation, however this was distinctively different to the plaques seen on Sample A. Sample C showed some individual plaques, where the inoculation loop had touched the bacterial lawn, most ranging from 2-4mm in diameter. Sample A produced so many plaques that areas of clearance were confluent in some parts of the plate (**Figure 5.5**). In all experiments the blank did not give rise to any plaques.



Figure 5.5 Sample A with areas of confluent plaques

This demonstrates that when infecting bacteria with phage, changing incubation parameters such as temperature can affect the outcome of the experiment. As most basic incubators do not have integrated refrigeration or fan-assisted heating units, the temperature in standard incubators is not always constant. In most cases mild temperature fluctuations or the effects of ambient temperature, season or heat emitting equipment are negligible, possibly irrelevant and remain unnoticed, however in this case it had a profound effect on the outcome of this experiment. It could be speculated that Coffi (1995) had access to a refrigerated incubator and a temperature controlled

laboratory, which could explain why he found the phage to be lytic, despite growth temperatures of 28°C.

Van den Worm may have either worked with an incubator displaying 28°C, however actually incubating material closer to 30°C, which may have explained the absence of lytic behaviour. Assuming that the team collaborating with Klovin, van Duin and van den Worm always used the same experimental setup, slight fluctuations due to changing season or heat emitting equipment may have caused the rare shift to lytic behaviour referred to in the publication (Klovin et al. 2002).

This chance discovery proved that phage AP205 (HER424) gives rise to plaques under certain experimental conditions, it was important to explore these parameters and pinpoint to the exact conditions which would give rise to plaques. To achieve this more phage material needed to be produced as only a small quantity had been provided by the investigators. A primary lysate with the assignment code AEH019(4/6/3)3 was prepared (5.2.13) to embark on a range of experiments establishing if plaque formation could be repeatedly achieved under certain conditions (5.2.20-5.2.23). It was also important to ascertain if a temperature higher than 20°C was suitable for routine incubation of HER424, as cell densities achieved at this temperature were significantly lower than at 30°C. Results in Section 5.3.5 established that the optimum growth temperature for HER1424 was 30°C.

5.3.10 Apparent relationship between temperature and ability to form plaques of HER1424 and HER424 using crude phage preparation AEH019 (4/6/3)3

The plates produced using the soft agar method and incubated at 20°C were clear after incubation. No lawn or individual colonies could be observed (Table 5.6). There could have been a variety of reasons for this phenomenon. It could be argued that in the case of the sample incubated at 20°C the density of the optical suspension, hence the cell count to start with was lower than for the other samples, however it is unlikely that this was the only reason for the absence of a lawn. The overnight suspension used to produce the soft agar overlays had an optical density OD₆₀₀ of 0.48, which corresponds

to approximately 1×10^8 cfu/ml. This cell concentration should have been sufficient to create a dense lawn (**Table 5.7**) after overnight incubation. The observation that at 22 °C and 22-25°C the overlay agar had turned opaque, yet on the agar surface individual colonies could be seen may give a further clue as to the reasons for the absence of a lawn

Temperature	Overlay	Lawn density
20°C	Clear overlay	No individual colonies on agar surface
22°C	Clear overlay	Individual colonies on agar surface
22/25°C	Opaque overlay	Density of individual colonies increased
30°C	Dense bacterial lawn	Dense lawn

Table 5.6 Results of soft agar overlay method

Temperature	Optical Density (600nm)
20°C	0.483
22°C	0.841
30°C	0.754

Table 5.7 Optical density of overnight cell suspensions grown at different temperatures

Temperature incubated	Phage Spots	Size of Clearance zones (cm)			Chloroform spots (blank 1)	Lambda buffer spots (blank 2)
20°C	Clearance zones	1.0	1.05	0.9	Lawn thinning	Undisturbed lawn
20°C	Clearance zones	1.0	1.17	1.6	Lawn thinning	Undisturbed lawn
22°C	Clearance zones	0.9	0.9	1.1	Lawn thinning	Undisturbed lawn
22°C	Clearance zones	1.0	1.0	1.0	Lawn thinning	Undisturbed lawn
22/25°C	Clearance zones	1.26	1	1.03	Lawn thinning	Undisturbed lawn
22/25°C	Clearance zones	0.9	0.9	1.1	Lawn thinning	Undisturbed lawn
30°C	Shadow areas, no clearance	0.9	0.9	1.0	Lawn thinning	Undisturbed lawn
30°C	Shadow areas, no clearance	0.8	0.9	1.0	Lawn thinning	Undisturbed lawn

Table 5.8 Results for surface spotting method at a range of incubation temperatures

It should be borne in mind that the crude phage preparation AEH019(4/6/3)3 was used undiluted and the phage count was unknown. It is very likely that the large number of phage particles at the low incubation temperature suppressed bacterial growth or even

lysed cells, hence there was no lawn to be observed. The aim of this experiment was not to enumerate the phage, but to investigate whether the preparation was viable and whether plaques could be produced with it. No specific plaques were produced, however it provided important clues as to the nature of the phage and host relationship, which appeared to be somewhat unusual.

The optical densities of the overnight cell suspension grown at 22 °C and 30°C were not dissimilar to the overnight suspension grown at 20°C. A thick bacterial lawn was produced at 30°C , yet when a similar cell count was incubated at 22°C and 25°C there was no lawn, only a varying number of individual colonies. Even more importantly the same bacterial suspension (grown at 22°C) was used for plates incubated at 22°C and 25°C, at 22°C the overlay remained clear with individual colonies, yet at 25°C the overlay was opaque and the surface of the agar showed only individual colonies. It could be argued that the bacterium grows slower at lower temperatures (see **Section 5.3.5**) and low incubation temperatures can affect the colony size, (see **Section 5.3.4**), but lower temperatures are not inhibitory to growth as such (**Section 5.3.7**). Yet growth at a lower rate cannot simply explain why at 25°C the overlay agar surface grew some individual colonies, yet at an incubation temperature of 5°C higher, the plate was covered with a thick bacterial lawn.

The reason for this phenomenon is most likely to lie with the phage. A possible explanation could be that the phage somehow lyses or inhibits the growth of the host at lower temperatures but once the temperatures reach 30°C this effect is lost.

As the phage titre was unknown it is possible that large numbers of phage may have inhibited the growth of bacteria at incubation temperatures of 20°C and 22°C, the colonies growing on the top of the overlay agar may have simply been colonies arising from mutants resistant to the lytic phage.

The reason why the number of colonies was so low may be because the bacterial growth on the overlay agar surface is not a „normal lawn“ of bacteria per se, but a sparse number of „survivors“, which have not encountered the effects of the lytic phage. Investigating the results of the surface spotting method further supports this argument.

The same bacterial suspensions which were used for the soft agar overlays and resulted in barren TSA plates with individual colonies, were used to surface spread onto TSA plates and resulted in dense bacterial lawns (**Table 5.8**). Chloroform as blank yielded a localised thinning of the lawn, however this was distinctively different in appearance to the plaques produced. The area where the chloroform in broth droplets had a confluent, thick lawn, however it appeared marginally thinner than the surrounding lawn. This is most probably due to the chemical killing the bacteria present on the agar surface, however when the chloroform evaporated the surrounding lawn re-grew into the chloroform spot.

It is likely that a temperature dependent relationship exists between the phage and host. At temperatures of 30°C the phage may switch into its temperate state and HER1424 can grow uninhibited, explaining the dense lawn with absence of plaques. At temperatures at or near 25°C a larger number of resistant mutants may have arisen, possibly explaining the opacity of the overlay agar, yet overall the phage was still capable of exerting its lytic effect. When the incubation temperature reached 30°C AP205 did not appear to exert its lytic action. As the phage appeared to be capable of two distinct life-cycles (lytic at lower temperatures and temperate at higher) it is likely to be temperate and not lytic as described by (Klovins et al., 2002).

5.3.11 Investigating viability, sensitivity to chloroform and quantitative titration of purified phage suspension

The phage concentration of AEH023(23/7/3) was calculated as 2×10^{10} pfu/ml additionally demonstrating that the purified preparation was viable (**Table 5.9**). As chloroform had been used during the purification of phage preparation AEH023 (23/7/3) it can be concluded that phage AP205 was not sensitive to chloroform. Interestingly plaques produced by AP205 looked different to the classic honeycomb appearance of most other phages.

Plaque size was heterogeneous and ranged from 1mm to 6mm in diameter, plaque edges were not always smooth and plaques did not always have the classical appearance of a bullet hole, but were mostly round with irregular, jagged edges.

Mean pfu/ml calculated
7.2×10^{10}
1.4×10^{10}
1.8×10^{10}
1.1×10^{10}
1.76×10^{10}
1.55×10^{10}
1.8×10^{10}
Overall mean: 2.3×10^{10} pfu/ml approx. 2×10^{10}

Table 5.9 Average phage titres of AEH023 (23/7/3) (n=7)

No calcium was added at the point of infection, either for the production of the primary or the secondary lysates. This was mainly because all attempts at adding CaCl_2 to various broths had resulted in some flocculation or crystallisation (see Section 5.3.8.1-5.3.8.3). Changes in salt concentration have been reported to change the titre of coliphage T2 preparations approximately 1000-fold (Kutter & Sulakvelidze, 2005). It was decided that any risk of precipitation or changes in ion levels due to crystallisation of calcium and consequently an ever changing phage titre could not be outweighed by the potential benefit of breeding larger amounts of phage. The satisfactory phage titre confirmed that calcium ions were not necessary for producing infection of HER1424 with AP205.

5.3.12 Validation of quantitative phage estimation using the soft agar overlay method

A one-way repeated measures ANOVA was conducted for both sets of data (Tables 5.10 & 5.11), to compare plaque counts obtained on different days, using different cell suspensions treated in a standard way. The mean and standard deviations are presented in Tables 5.12& 5.13.

Count 1	207	196	186	220	221
Count 2	156	234	210	222	205
Count 3	154	184	209	211	217
Count 4	182	222	186	248	222
Count 5	185	221	182	209	248

Table 5.10 Plaque counts dilution 1, n=5

Count 1	24	28	13	30	35
Count 2	20	16	18	23	32
Count 3	31	34	16	23	30
Count 4	21	28	24	26	22
Count 5	26	37	14	29	28

Table 5.11 Plaque counts dilution 2, n=5

Descriptive Statistics dilution 1			
	Mean	Std. Dev.	N
Count 1	206	15.182	5
Count 2	205.4	29.812	5
Count 3	195	26.163	5
Count 4	212	27.713	5
Count 5	209	27.249	5

Table 5.12 Mean and standard deviation for dilution 1

Descriptive Statistics dilution 2			
	Mean	Std. Dev.	N
Count 1	26	8.276	5
Count 2	21.8	6.260	5
Count 3	27.2	7.529	5
Count 4	24.2	2.863	5
Count 5	26.8	8.288	5

Table 5.13 Mean and standard deviation for dilution 2

The results of the one-way repeated measures ANOVA indicated there was no statistically significant difference between plaque counts on different days for both data sets. For dilution 1 Wilks' Lambda=0.056, $p>0.0005$ and dilution 2 Wilks' Lambda=

0.002, $p > 0.0005$. The p values are greater than 0.0005 which implies that the method used is repeatable (**Tables 5.12&5.13**).

The quantitative method developed to establish phage titres was statistically proven to be repeatable, however it is essential to discuss factors affecting the actual number of plaques formed on the agar surface.

Experience during this investigation has highlighted a range of factors which have repeatedly been proven to affect the phage count, the outcome of many attempts at improving the process of producing soft agar overlays is summarised in Materials and Methods **Section 2.3.4**. The reproducible quality of TSA plates was essential to achieve repeatable results. Important parameters included agar thickness, moisture content, (which in turn was dependent on storage conditions and temperature) whether the plate was poured in a fan-assisted cabinet and for how long the plates were allowed to dry before being packaged in plastic bags (**Section 2.3.2**). Plates stored in the refrigerator contained condensation which had an effect on the plaque count. Overdried plates gave a lower plaque count than plates poured following the optimised method as described in **Section 2.3.2**. Consequently all plates for each set of experiments were prepared on the same day.

The volume of overlay agar had an influence on the final plaque count, as the 100 μ l volume of phage suspension routinely used was diluted in the volume of the overlay agar. Low Volumes (3ml) caused the overlay surface to become too dry during overnight incubation. Consequently the bacterial lawn may not have been as luxurious as expected and the plaque count was found to be too low. Excessive volumes of overlay agar (above 5ml) did lead to inaccurately low plaque counts, possibly due to slower diffusion of phage leading to reduced plaque growth (Kutter & Sulakvelidze, 2005). Adding large volumes of phage sample to the overlay agar diluted the total percentage of agar and led to the overlays remaining liquid. The percentage of agar contained in soft agar overlays affected the diffusion of the phage particles through soft agar and was critical for repeatable plaque counts. Factors affecting the volume of the soft agar overlay were accuracy of pipetting, loss of moisture or collection of condensation in the bijoux tubes during autoclaving or melting of agar in a waterbath.

Even types of storage container for the soft agar and type of lids affected the volume of liquid evaporated during the storage and experimentation, which consequently had an influence on the final volume and consistency of the agar. Metal lids with rubber lining gave rise to condensation after autoclaving and lower levels of evaporation during storage, compared to plastic lids. Correct storage of phage played an integral part in achieving accurate and repeatable plaque counts. The phage preparation was stored in Eppendorf tubes, protected from light in the refrigerator, however this posed a problem on winter days. When ambient temperature was lower than 17°C plaque counts became inaccurate and plaques appeared to be unevenly distributed on the plates. To overcome this problem the phage preparation to be used on that day was placed in an incubator set to 37°C, before adding to the molten soft agar which was kept at 50°C.

A number of factors had an influence on the number of plaques arising on a plate, following the plaque enumeration method in particular cell concentration, incubation temperature of overnight cell suspension and infection temperature. The use of purified phage lysates at ambient temperatures below 19°C led to lower plaque counts, which may be due to an aggregation of phage particles and resulted in lower numbers of distinct plaques. As phage lysates were stored at 4°C, protected from light to improve shelf life, warming the working suspension to a suitable temperature before experimentation, without contamination or exposure to UV light or heat was routinely achieved by pre-warming the phage preparation in an incubator set to 25°C and optional brief warming to 37°C.

Flaming of the glassware necks during the soft agar overlay method proved essential in achieving consistent phage counts, excessive foam formation after vigorous vortexing of the soft agar overlays led to larger variations in plaque counts.

5.3.13 Determination of loss of phage after filtration with 0.45µm syringe filter

Part of the phage purification protocol (Section 2.7.2) involved syringe filtration, to avoid spoilage through bacteria. It was important to know whether the syringe filtration would affect the number of phage particles in suspension or whether phage would be adsorbed onto the filter membrane and the phage titre would drop after each filtration step. This was of relevance since syringe filtration was an important method to separate phage from bacteria and was used in a range of experiments.

Unfiltered 1	295	294	249	450	168	183	212
Unfiltered 2	138	131	170	148	155	133	145
Filtered 1	135	175	205	416	407	358	297
Filtered 2	141	126	153	127	157	129	135

Table 5.14 Plaque counts before and after syringe filtration of phage lysates

A paired-samples t-test was conducted to evaluate the impact of filtration on the number of plaques counted, with the raw data depicted in **Table 5.14**. There was no statistically significant change in plaque numbers in unfiltered ($\bar{M}=203.2$, $SD=87.15$) or after filtration ($\bar{M}=217.87$, $SD=107.91$; $t(30)=0.544$, $p>0.05$ of phage preparation AEH023 (23/7/3). Degrees freedom $df=14$, significance 0.595

The same data (**Table 5.14**) were processed using a one-way repeated measures ANOVA to compare plaque numbers before (pre-intervention) and after filtration (post-intervention). There was no significant effect for filtration, Wilks' Lambda=0.595, $p>0.05$, multivariate eta squared=0.021

There was no statistically significant difference in numbers of counted plaques before or after filtration of the phage sample. This implies that the effect of filtration on the

number of plaques was negligible and any differences between plaques numbers before and after filtration are not statistically significant.

5.3.14 Method optimisation for optimum optical density of inocula to yield best bacterial lawns for plaque counts

Despite the fact that the soft agar overlay method has been used for many years and was even used by d'Hérelle (d'Hérelle, 1926) shortly after the discovery of bacteriophage, little information has been published on the ideal bacterial concentration to achieve optimum plaques for counting. This may be due to the fact that different bacteria have a variety of growth requirements with plaque size being primarily determined by the nature and size of the phage, thickness of top and bottom agar, type of medium, concentration of agar in the soft agar overlay (which in turn controls the diffusion of phage in this layer), plating cell density, burst size and distribution of absorption times (Kutter & Sulakvelidze, 2005).

OD₆₀₀	Average plaque size (mm)	Standard Deviation
0.3	1.77	0.32
0.4	1.75	0.55
0.5	3.02	1.17

Table 5.15 Correlation of optical density of cell suspension to average plaque size

From data presented in **Table 5.15** it becomes apparent that cell suspensions with optical densities below 0.5 produce very small plaques, which increases the difficulty for the operator to count them and to clearly distinguish them from the background lawn, even with magnification. However plates produced with the cell suspension adjusted to OD₆₀₀ = 0.5 showed a wider size distribution of plaques demonstrated by the standard deviation.

This implies that plaques do not simply become bigger, resulting in a larger homogenous overall size but only some plaques remain rather small. The reason for this is unknown, however it demonstrates the need for using the same protocol consistently. The optimum OD₆₀₀ for the production of bacterial lawns for the purpose of use with the soft agar overlay method, employing HER1424 and HER424 is between 0.45 and 0.5.

This series of experiments led to the development of a standardised soft overlay protocol (**Section 2.3.4**).

5.3.15 Impact of heat on phage activity

The phage preparation kept at 60°C yielded no plaques, and it is possible that at this temperature the phage preparation was actually inactivated. Plaque counts of the sample heated to 60 °C were significantly lower than all other samples and control ($p < 0.01$, $F = 562$). Preparations heated to 37°C and 25°C yielded plaque counts which were not statistically significantly different from the control titer ($p > 0.5$), which had been maintained at 21°C.

This experiment provides further evidence that phage preparation AP205 (HER424) is not inactivated when heated up to 37°C, yet it remains unclear why incubation at this temperature does not lead to plaque formation.

5.3.16 Temperature dependent relationship between *Acinetobacter haemolyticus* HER1424 and phage AP205 (HER 424)

Plates incubated at 22°C, 25°C and 30°C all showed the presence of plaques of heterogeneous size distribution (**Figure 5.6**). Using Tukey's and Gabriel's tests ($p < 0.05$) the number of plaques was significantly lower at 30°C compared to 22°C and 25°C and at 30°C the number of plaques was more variable and they appeared less distinct.

When the experiment was repeated with incubation temperatures at 35°C and 37°C ($n = 3$), no plaques were evident even though it was demonstrated in **Section 5.3.15** that the phage was not inactivated at these higher temperatures.

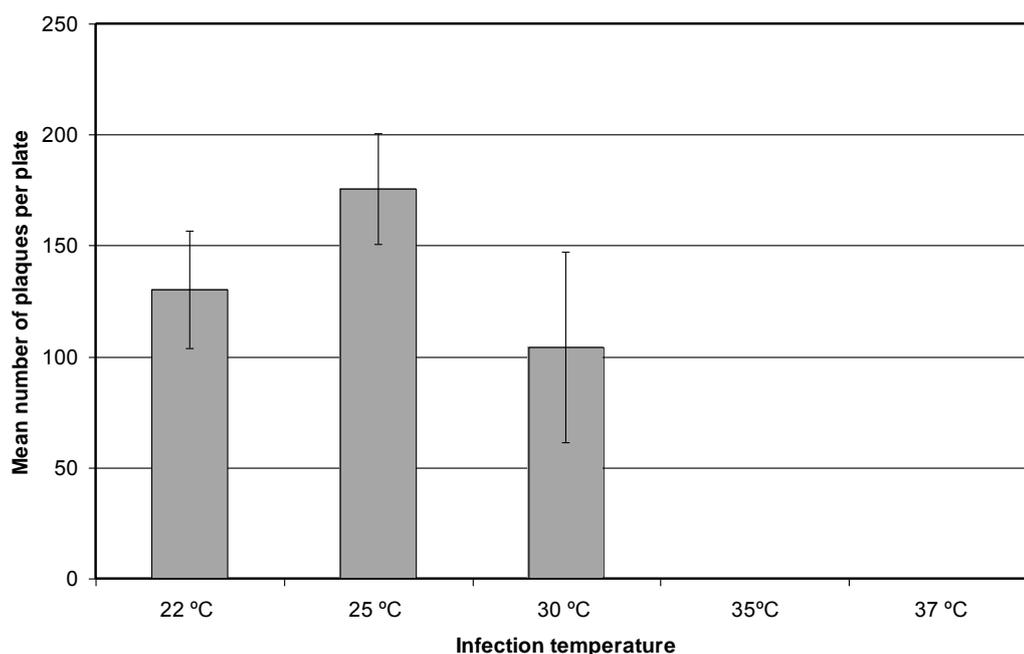


Figure 5.6 Temperature dependent plaque formation of HER1424 and HER425

Temperature	Average pfu/ml	Plaque appearance
22°C	130	Clearly defined plaque edges
25°C	176	Clearly defined plaques edges
30°C	104	Fuzzy plaque edges, blend into surrounding bacterial lawn
35°C	0	No plaques seen
37°C	0	No plaques seen

Table 5.16 Plaque appearance and average phage titre at different temperatures

Results for the viable counts for the bacterial suspensions grown at 22, 25 or 30 °C, which were subsequently used for infection with phage, did not statistically significantly differ from each other $p > 0.05$.

The optimum growth temperature for fastest growth of *Acinetobacter haemolyticus* HER1424 was 30°C, however for the purpose of producing well defined plaques incubation and infection at 25°C seemed more suitable.

The number of plaques was significantly lower $p < 0.05$, $F = 54$, more variable and plaque edges appeared less defined at 30°C compared to 22°C and 25°C (**Table 5.16**). At 35 and 37°C no plaques were evident at all. This outcome correlated with previous experiments, where an infection at 30°C did not result in clearly defined areas of lysis (**Section 5.3.10**). This raises the question why this may be the case.

Klovins et al. (2002) reported the presence of two open reading frames (ORF) in HER424, ORF1 is believed to encode a lysis protein (Klovins et al. 2002). A clustering of positively charged residues at the N terminus, grouping of hydrophobic amino acids near the C terminus and a sufficient length of 35 amino acids are indicators that this phage is likely to cause bacteriolysis. Additionally when ORF1 was cloned into an expression vector behind the P_L promoter, its induction in *E. coli* halted cell growth. Other findings by Klovins et al. suggest that ORF1 translates into a peptide that has a lysis function. The authors report that cell lysis did not always occur when *Acinetobacter* HER1424 was grown and infected with phage at 28°C. It appears that no temperature dependent lytic relationship between *Acinetobacter* phage and host has been described previously in literature. The only reports of temperature-sensitive bacteriophage were published by Hava and Camilli (2001) using a generalized transducing bacteriophage for *Vibrio cholerae*.

When the infection temperature reached 30°C or above AP205 did not appear to exert its lytic action, under the experimental conditions given. As the phage appeared to be capable of two distinct life-cycles (lytic at lower temperatures and temperate at higher) it is likely to be temperate.

It could be speculated that the reasons for the temperate nature of the phage at higher temperatures is that the three-dimensional open reading frame arrangement is different at lower temperatures, hence operating similarly to a temperature-dependent switch, where once a certain temperature is reached the phage goes into the lysogenic cycle. At

lower temperatures the open reading frames, which are deemed to be partly responsible for lysis are read correctly and lysis occurs.

Another possibility may be that the host does not form pili at higher temperatures in the same way as it does when temperatures are lower, hence making phage attachment and consequently infection more difficult. Baron et al. (2001) discovered that relatively minor temperature differences of 2°C affected pilus component assembly, T-pilus formation and virulence protein accumulation in *Agrobacterium tumefaciens*.

As pilus formation has been linked to bacterial virulence (Russell & Herwald, 2005), temperature dependent interaction with phage may indirectly be useful in studying pilus formation and infectivity in *Acinetobacter* spp. This theory does not account for instances where cells were grown at 25°C, a temperature most likely to be permissive of pilus formation, yet were not lysed when the temperature was switched to 37 °C.

Apart from specifying the diameter of the pili as 6nm, Klovins et al. (2002) did not specify which type of pilus phage AP205 attaches to. It is possible that the cellular appendage necessary for attachment of AP205 to HER1424 are F-pili, which are generally 8-9nm in diameter. F-pili are used by Gram-negative bacteria to exchange DNA, as an evolutionary tool for bacteria to share genetic information and possibly to increase resistance to antimicrobial agents (Daehnel et al., 2005). However, on the basis of diameter the pili in question are more likely to be Type IV also referred to as Tfp, which are considered to be 6nm in diameter and several micrometers in length and have been found in *Acinetobacter calcoaceticus* (Wall & Kaiser, 1999). These pili are polar and are implicated in some form of gliding motility and twitching. Lautrop introduced the term twitching to describe bacterial surface-motility, which did not depend on flagella in *Bacterium antitratum* now commonly known as *Acinetobacter calcoaceticus* (Lautrop, 1961).

Twitching motility can range from movements barely distinguishable from Brownian motion to extensive displacements, seen as small, intermittent jerks, which change direction of movement (Henrichsen, 1983). It is speculated that bacteria commonly found in soil have developed a more functional form of Tfp motility, to increase their

spatial range (Wall & Kaiser, 1999). Twitching is believed to be explained by the retraction model, with the mechanical basis being simple pilus extension and retraction (Bradley, 1980), a hypothesis which has been generally accepted without further investigation.

During microscopy of HER1424 motility was not observed, however the presence of pili does not necessarily have to conclusively result in twitching. It is generally an accepted fact that in cases where phages which attach to the host via pili, bacterial mutants which prove to be resistant to the phage generally lack the pili necessary for attachment and consequently infection (Bradley, 1973). However the relationship cannot be reduced to correlating a simple presence of pili to phage sensitivity of the host. Bradley distinguished between hyperpilated, nonpilated and wild-type cells. He found that both hyperpilated and nonpilated cell types of *Pseudomonas aeruginosa* were phage-resistant. Hyperpilated mutants were capable of absorbing phage particles, yet nonpilated cells were not. Furthermore hyperpilated cells had phage particles attached along the pili, yet rarely on the cell surface itself, yet wild-type cells had phage attachment on the cell surface (Bradley 1972a, 1972b, 1972c, 1972d, 1974).

To explain these differences observed by electron microscopy Bradley proposed the retraction theory, whereby phage bound at the pilus. If the pilus retracted the phage particles would be transported to the cell surface and concluded that phage-resistant hyperpilated mutants were simply unable to retract pili. On the basis of this retraction theory it could be speculated that the pili of HER1424 cannot retract at higher temperatures, hence the phage cannot exert its lytic potential on the host. Bradley's theory of pilus retraction, which due to experimental limitations has never been actually proven, cannot conclusively be linked to infection consequently an alternative theory is proposed here.

Alternative theory

Bordetella spp. are capable of switching between two distinct phases, modulating an adhesion protein, pertactin (a receptor for phage BPP-1) to escape infection with phage.

The bacterial phase switch is also linked to expression of virulence genes and ability to colonize the respiratory tract of mammalian hosts (Hatfull, 2002). This is one explored example where bacterial hosts seek refuge from phage infection by altering phage receptors.

In HER 1424 the pili found in hyperpiliated cells may have different surface structures compared to normal forms of bacteria, and are designed to act as „decoy structures“ allowing adhesion of phage to the pilus, yet protecting the cell surface from phage attachment and further progression to the lytic cycle. However in a situation analogous to the receptor lock and key principle (Morrison et al., 2006), the surface proteins of the bacteria may be designed to allow attachment, yet do not permit infection and further lysis of the bacteria.

Appropriate positioning of the phage tail on the cellular surface is essential for leading to delivery of viral genomes into the bacteria. Once the exact position has been achieved a signal is conveyed to the phage head to initiate cellular infection, yet little is known about the precise mechanics of this process (Kutter & Sulakvelidze, 2005). In an environment with low phage density where phage adheres to the host, without infection taking place, significant numbers of virulent phage particles may be prevented from attaching to susceptible cells, by pili with altered structure „mopping up“ the phage particles and hence avoiding progression to a full lytic cycle whereby significant numbers of phage particles are released.

5.3.17 Temperature switches affecting ability to form plaques

The results of this experiment imply that the actual infection temperature may play a critical role in whether plaques form or not on soft agar. It could be argued that the infecting concentration of $1.5-3 \times 10^3$ pfu/ml (150-300 pfu/plate) seemed rather low, however it was still high enough to yield plates with plaque numbers too high to be counted, yet at incubation temperatures of 37°C no plaques could be seen (**Table 5.17**). Repeating the experiment with higher plaque numbers seemed unrealistic, at higher phage titres lysis from without may have occurred (Delbrück, 1940), which may have been mistaken for infection by the phage under the experimental conditions.

Incubation temperature cell suspension	Incubation temperature soft overlay plates	Presence of plaques	Appearance of bacterial lawn (phage)	Appearance of bacterial lawn (blank)
22°C	25°C	YES	very dense	very dense
37°C	25°C	NO	Sparse	very dense
25 °C	25 °C	YES	very dense	very dense
25 °C	22 °C	YES	very dense	very dense
37 °C	37 °C	NO	Sparse	Sparse

Table 5.17 Effect of temperature switches on plaque formation

5.3.18 Downshifting of temperature after infection

This experiment was designed to investigate whether low temperatures are critical at the point of infection of bacteria with phage or at a later stage in order for plaques to form. Initial high phage titres were used for infection to ensure a statistically high chance of all cells being infected. The cells were centrifuged after a set contact time to ensure removal of excess phage particles in the suspension; however infected cells would harbour phage. Subcultures were employed to avoid phage originating from the infection step being carried over into the next step of the experiment and being included in the plaque counts.

Sample	Optical density OD ₆₀₀
Subculture S1	0.130
Subculture S2	0.401

Table 5.18 Optical densities of subcultures after overnight incubation

The overnight cell suspension S1 (**Table 5.18**) had an unusual appearance after incubation. The suspension displayed a coagulated mass at the bottom of the flask, the suspension was not turbid as expected, but appeared clear.

Sample	Incubation temperature soft agar overly plates	Plaques visible
Subculture 2	25°C	No
Infected S 2	25°C	No
Subculture 3	37°C	No
Subculture 3 F	25°C	3 plaques

Table 5.19 Incubation temperature and plaque visibility of samples

This experiment highlights a range of occurrences which may require further investigation. The overnight suspension (Subculture 1) produced what appeared to be a coagulated precipitate at the bottom of the flask and the optical density of the supernatant liquid was very low ($OD_{600}=0.130$), consequently this result was ignored.

More interesting is that despite the fact that the overall incubation temperature was maintained at 37°C over several subcultures and the infection temperature was 37°C (**Table 5.19**), the contact time between the phage and bacteria was only 10minutes, and a small number of plaques were formed (Subculture 3, F). The contact time between phage and bacteria was reduced to a short period and the cell suspension centrifuged after 10minutes to try to understand whether phages were capable of attaching to the host bacterium at 37°C.

It could be argued that the ambient temperature to which the infected cell suspension (Infected S2) would have been exposed during centrifugation was certainly below 37°C. This may have contributed to the formation of plaques, however the exact mechanism is not clear.

It is unsurprising that samples drawn from Subculture 1 and 2 did not yield plaques, as no phages were added. It is of interest that the filtered suspension (Subculture 3, F) yielded plaques, whether this may be due to intact phage being trapped inside the bacterial cells, which may have been burst open by the shear force induced during syringe filtration, releasing the phage can only be speculated. There are two distinct possibilities why the unfiltered suspension resulted in a dense lawn. One is that there were not enough lytic phage particles to produce plaques present; the other option could be that bacterial mutants resistant to phage may have overgrown any lytic plaques. It is clear from this experiment that further investigation is needed to elucidate the unusual relationship between phage AP205 and HER1424.

5.3.19 Attempts to artificially create temperate phage

The starter culture grown at 25°C (25°C Starter) virtually disintegrated after the second wash and spin step. Viscous foam could be observed on top of the liquid and no cell pellet was left after the third spin. Repeats of this experiment led to the same outcome.

It is possible that the experimental conditions which involved 1:1 and 1:10 ratios of bacteria to phage caused the bacteria to „lyse from without“. Lysis from without usually results from adsorption of large multiplicities of phage particles onto the host cells, but can occur even when equivalent amounts of phage and bacteria were mixed, as demonstrated by Delbrück (1940).

No replication of phage takes place within the cells which burst simply as a result of the extensive puncturing of the cell wall caused by multiple phage attack. It leads to a drastic reduction in optical density of a bacterial culture and release of cellular internal proteins into the extracellular environment. Additional shear forces may have facilitated the cellular disintegration brought about by lysis from without.

The starter culture grown at 37°C (37°C Starter) resulted in plaques when Filtrate 1 was plated. The cell suspension infected with 1:1 cells: phage resulted in a mean number of 270 plaques per plate, which corresponds to a phage concentration of approximately 2.7×10^4 pfu/ml. The cell suspension containing 1:10 cells:phage resulted in 30 plaques per plate on average, correlating with 3×10^3 pfu/ml. This correlates approximately with

the number of phage expected to be present in the final cell suspension as residue, assuming an even distribution of phage particles throughout the suspension.

Filtrate 2 resulted in less than 10 pfu/plate for the 1:1 cells: phage arm of the experiment and no plaques for the other part.

Filtrate 3 resulted in no plaques at all.

The falling numbers of pfu/ml on each subculture imply that phage particles may not be produced at 37°C; however they remain viable throughout each subculture and the incubation process. When the bacterial suspension was incubated at 37°C it did not affect the overall viability of the phage, as the filtered and infected cell culture was incubated at 25°C in the form of soft agar overlays plaques formed. This is further evidence that the temperature at which the cell suspension was grown may be less critical than the incubation temperature of the soft agar overlay plates. This attempt to breed temperate phage was therefore unsuccessful, as all samples containing sufficient quantities of residual phage after dilution led to plaques. It may however, provide further clues to the infectivity of phage AP205.

It remains interesting however why the starter culture at 25°C spontaneously disintegrated, which implies they lysed, yet the cells grown at 37°C did not. A feasible answer would be that the phage particles, present in high numbers may have replicated quickly and caused spontaneous lysis „from within“. „Lysis from within“ is caused by infection of a bacterium by a single (or few) phage particles and multiplication of this particle up to a threshold value. This is often simply referred to as lysis in literature. Cell contents are liberated after this lysis without deformation of the cell wall, simply by rupturing the cytoplasmic membrane (Delbrück, 1940).

It could be claimed that the cells grown at 25°C were „lysed from without“. „Lysis from without“ is caused by adsorption of phage particles above a threshold value. The cell shape changes and cell contents are liberated by a distension and final rupture of cell wall. No new phage is formed in this process, the bacteria simply accumulates the phage particles on its surface and then lyses (Delbrück, 1940). The question remains why the cells grown at 37°C were not affected in the same fashion. The answer may be

that phage do not attach to the cells grown at higher temperatures in the same way as to the cells grown at 25°C.

Another theory applicable to coli-phage T2 is that cells infected by T2 express a „resistance to lysis from without“ which allows adsorption onto the cells of hundreds of secondary phage without a significant decline in optical density, as demonstrated by Visconti (1953). Secondary phages in this context are viable phage particles which adsorb to the host cell after the primary phage (the first phage to infect the cell) has already infected the bacteria. There are no exact biochemical surface structural explanations in literature as to the exact mechanism of „resistance to lysis from without“.

5.3.20 Investigating the possibility of clonal differences between plaques

HER1424 and AP205 plaques did not appear to have the classical appearance of entirely circular „bullet holes“. Plaques appeared to have serrated edges and were heterogeneous in size (**Figure 5.7**), even when incubated at 25°C. It was of importance to understand whether there were clonal differences between plaques, and whether small plaques would always produce a progeny of small plaques and large plaques only result in large plaques.

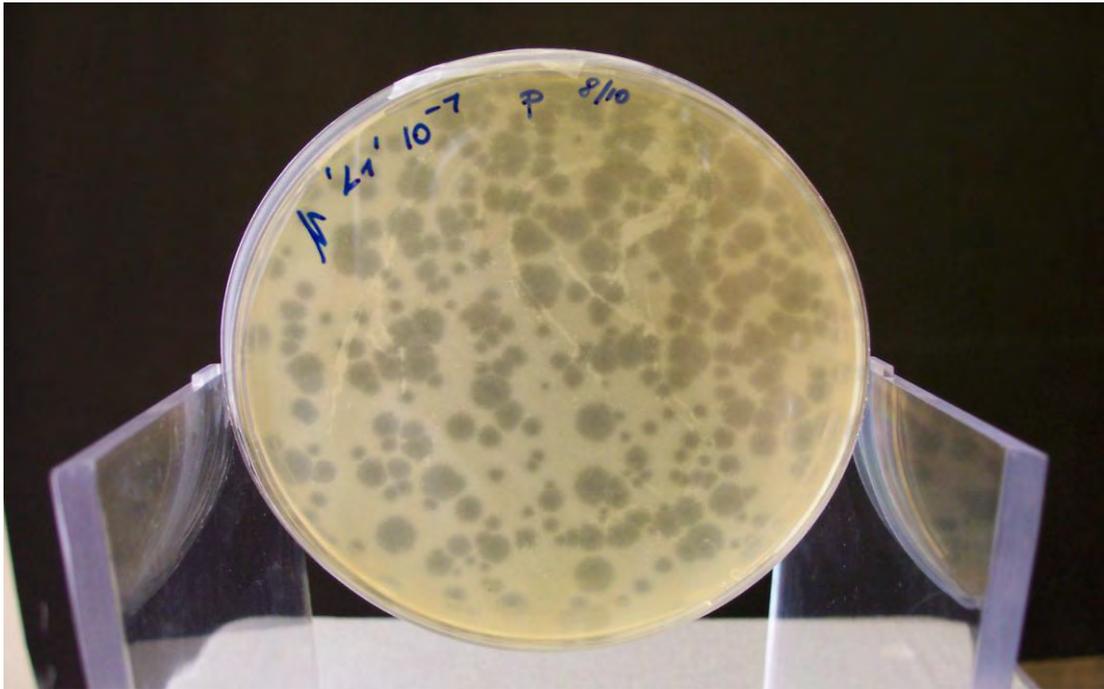


Figure 5.7 Typical appearance of heterogeneous plaques of AP205

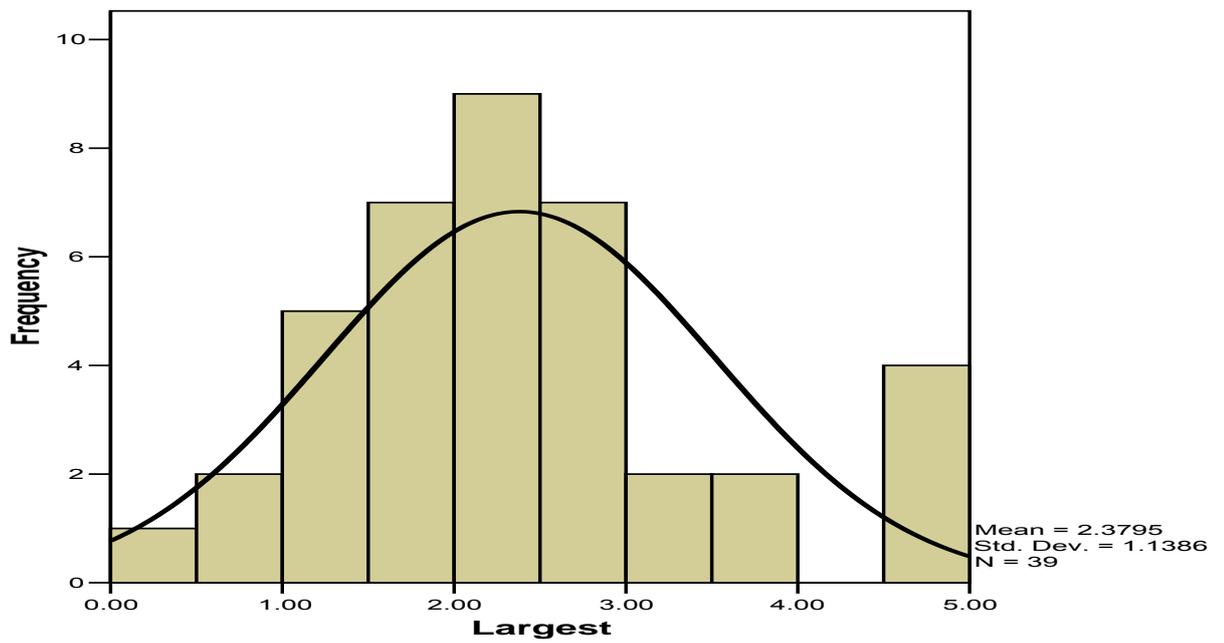


Figure 5.8 Plaque size distributions of viruses from the largest plaque

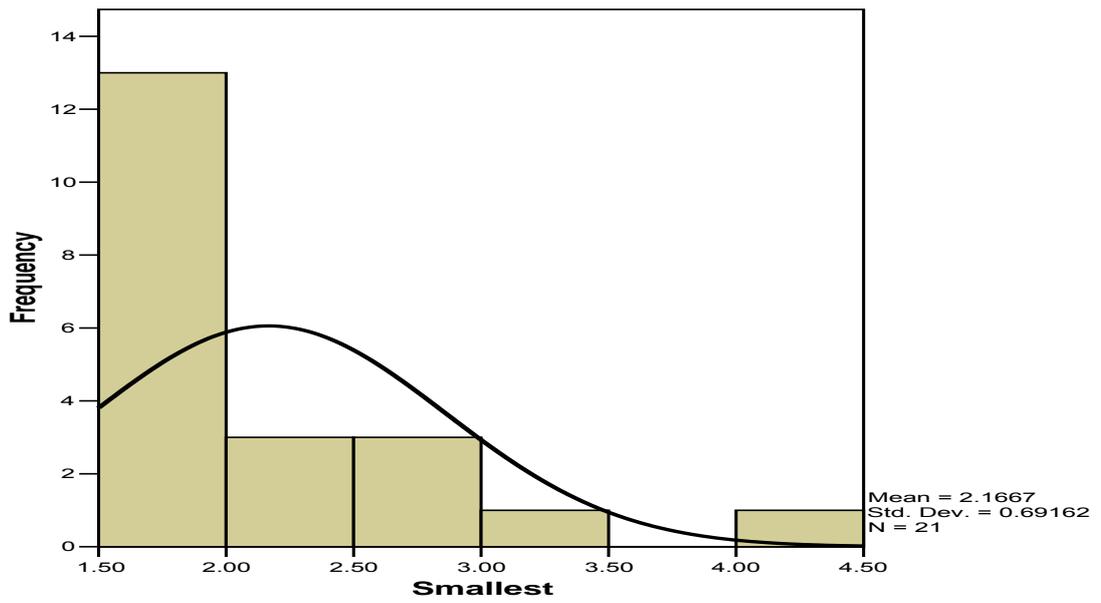


Figure 5.9 Plaque size distributions of progeny of the smallest phage

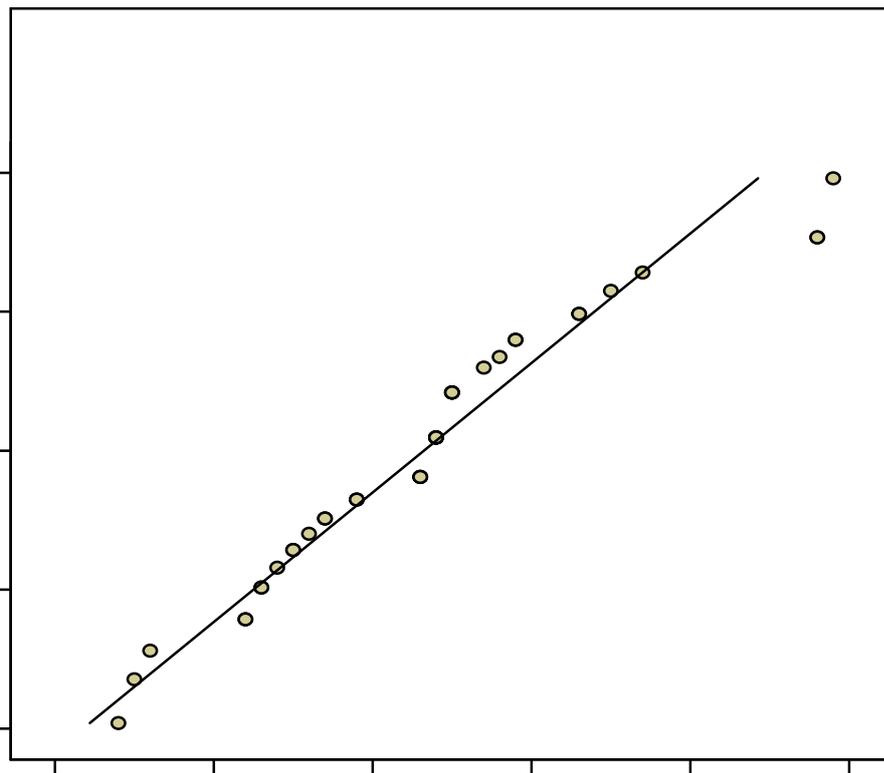


Figure 5.10 Q-Q plot of plaque size of viruses from the largest plaque (ProgenyL)

Both the smallest and largest plaque preparations yielded plaques of heterogeneous size, regardless of whether they were directly plated from the original extracts or from the progeny.

Descriptive statistics were used to analyse size differences of plaque progeny generated from the smallest and largest plaque „ProgenyS“ (n=21) and „ProgenyL“ (n=39). Normality of data was assessed using Kolmogorov-Smirnov (significance value ProgenyL = 0.023, sig. ProgenyS =0.000) and Shapiro-Wilk statistics (sig. =0.045 ProgenyL, sig=0.000 ProgenyS).

Histograms (**Figures 5.8&5.9**) are depicted to demonstrate the size distribution of plaques.

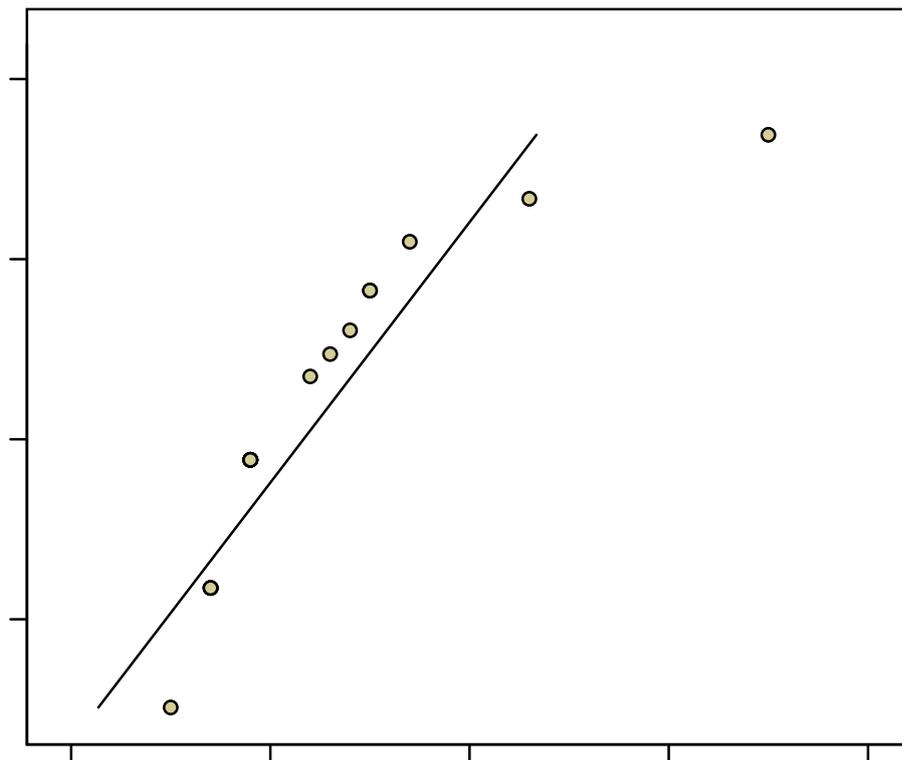


Figure 5.11 Q-Q plot plaque sizes of viruses from the smallest plaque (ProgenyS)

In **Figure 5.9** the data in the histogram are clearly skewed towards small size plaques. The histogram in **Figure 5.8** may give the superficial impression that the data for ProgenyL may be normally distributed, however this is not the case.

The significance values are less than 0.05 for progeny of the largest and smallest plaque, this suggests that the distribution in question is significantly different from a normal distribution. This implies the data are not normally distributed. Normal Q-Q plots confirm the deviation from normality as the data points do not fit a straight line (**Figures 5.10&5.11**).

Skewness and Kurtosis values for largest plaque progeny were 0.207 and 0.913 respectively, which indicates the distribution is near normal with a clustering of data at the lower end of plaque sizes. For smallest plaque progeny Skewness and Kurtosis values were 2.216 and 5.944, which clearly shows a clustering towards the lower end of plaque sizes. It appears that phages extracted from smaller plaques are more likely to give rise to progeny which are themselves small. These data are evidence that large plaques will give rise to small and large plaques, equally the same is true of small plaques, however small plaques are more likely to produce small plaques than large plaques.

It could be argued that upscaling the experiment may have resulted in more data; however the number of data points was sufficient to allow meaningful statistical analysis of the results. Another point of criticism could be that only one generation of propagated phage was used, however this experiment was only designed to elucidate the possibility of clonal differences of plaques. Repeated propagation may have also lost the original character of the „wild-type“ phage (Lu et al., 2003).

5.3.21 Adsorption experiments at 25°C and 37°C

Experiment parameters	A	B	C
Concentration of viable bacteria when infected in 100ml	7.3×10^7 cfu/ml	2.5×10^7 cfu/ml	6.2×10^5 cfu/ml
Phage titre in 100ml total volume	6×10^5 pfu/ml	1.8×10^7 pfu/ml	1.8×10^6 pfu/ml
Incubation temperature	25°C	25°C	37°C
Infection temperature	25°C	25°C	37°C
Graph symbols	White circles	black squares	grey triangles

Table 5.20 Parameters of adsorption experiments

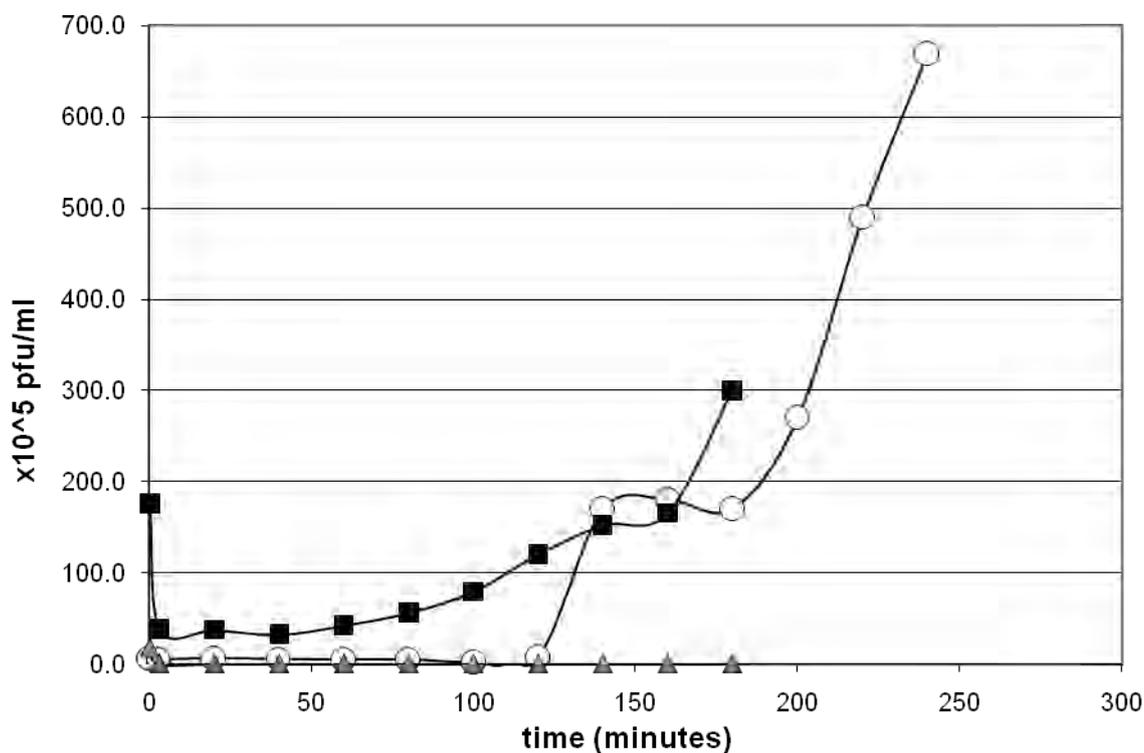


Figure 5.12 Adsorption of phage HER424 to HER1424 at 25°C and 37°C

At 37°C no increase in detectable free phage could be seen after 200 minutes, however for experiment B a 10-fold (180 minutes) and for experiment A a 100-fold increase (240 minutes) in pfu/ml could be seen (**Figure 5.7**). In all three experiments a drop in recoverable phage could be seen after 3 minutes, which implies that phage attach to bacterial cells at 25°C and 37°C within that time period. At 37°C however no viable, recoverable phage seemed to be produced under the experimental conditions. The phage particles added to the host suspension at 37°C were shown to have adhered to the bacterial cells as the phage titre of recovered phage was significantly lower than the calculated number of phage in suspension C (One tailed p-value $p < 0.01$ using t-test). Phage particles in experiment B showed a drop of almost one magnitude in free phage after 3 minutes. This reduction in numbers of recoverable phage immediately after infection can be routinely observed when T4-like phages infect *E. coli* (Kutter & Sulakvelidze, 2005). After infection of host with phage usually a further slight drop in phage numbers can be observed, followed by an increase and followed by a second plateau in phage numbers, which then leads to a significant increase in plaque numbers. This phenomenon can be observed with suspensions A and B; the drop in phage numbers was not as dramatic in sample A as in sample B, possibly due to sampling being slower than adsorption.

5.3.22 Screening Sussex isolates as potential hosts for AP205 using the surface spotting method

None of the plates showed clearance areas which could be indicative of phage infection (**Tables 5.21-5.24**). A few plates had irregularities in the lawn surface where the phage spot had been applied, however these were not proper clearance zones. After repeating all results for plates noted as „UI“ (unidentified) in duplicate, it transpired that none of the disturbances of the lawn were due to phage activity. Some chloroform blanks yielded areas of clearance and some broth blanks gave rise to disturbances in lawn confluence; however this was optically very different to clearance zones caused by lytic plaques.

22°C	CCI4	Broth	Lys1	Lys2	Lys3	Quality of lawn
R1973	0	0	0	0	0	Very dense lawn
R2751	0	0	0	0	0	Very dense lawn
R3417	0	0	0	0	0	Very dense lawn
R4474	0	0	0	0	0	Very dense lawn
R45502	0	0	0	0	0	Dense lawn
R46363	CL	UI	UI	UI	UI	Thin lawn
W5211	0	0	0	0	0	Dense lawn
W6108	0	0	0	0	0	Dense lawn
W6492	0	0	0	0	0	Very dense lawn

Table 5.21 Screening of Sussex isolates with AP205 at 22°C

25°C	CCI4	Broth	Lys1	Lys2	Lys3	Quality of lawn
R1973	CL	0	0	0	0	Very dense lawn
R2751	0	0	0	0	0	Very dense lawn
R3417	CL	0	0	0	0	Very dense lawn
R4474	CL	0	0	0	0	Very dense lawn
R45502	CL	0	0	0	0	Very dense lawn
R46363	0	0	0	0	0	Dense lawn
W5211	0	0	0	0	0	Very dense lawn
W6108	CL	UI	UI	UI	UI	Very thin lawn
W6492	CL	0	0	0	0	Very dense lawn

Table 5.22 Screening of Sussex isolates with AP205 at 25 °C

30°C	CCI4	Broth	Lys1	Lys2	Lys3	Quality of lawn
R1973	0	0	0	0	0	Very dense lawn
R2751	CL	0	0	0	0	Very dense lawn
R3417	CL	0	0	0	0	Very dense lawn
R4474	CL	0	0	0	0	Very dense lawn
R45502	0	0	0	0	0	Very dense lawn
R46363	0	0	0	0	0	Very dense lawn
W5211	0	0	0	0	0	Very dense lawn
W6108	CL	UI	UI	UI	UI	Single colonies only
W6492	0	0	0	0	0	Very dense lawn

Table 5.23 Screening of Sussex isolates with AP205 at 30°C

37°C	CCI4	Broth	Lys1	Lys2	Lys3	Quality of lawn
R1973	0	0	0	0	0	Very dense lawn
R2751	0	0	0	0	0	Very dense lawn
R3417	0	0	0	0	0	Very dense lawn
R4474	0	0	0	0	0	Very dense lawn
R45502	0	0	0	0	0	Very dense lawn
R46363	0	0	0	0	0	Very dense lawn
W5211	0	0	0	0	0	Very thin lawn
W6108	0	0	0	0	0	Very dense lawn,
W6492	0	0	0	0	0	Very dense lawn

Table 5.24 Screening of Sussex isolates with AP205 at 37°C

Legend to Tables 5.21-5.24:

0 -undisturbed lawn

CL-lawn different to surrounding area, potential clearance which may be indicative of presence of phage

UI- where irregularities in the lawn could be seen, all results were repeated in duplicate

Lys1, 2, 3-denotes phage lysate spots

5.3.23 Screening Sussex isolates as potential hosts for AP205 using the soft agar overlay method

Regardless of incubation temperature of plates and phage concentration used none of the Sussex isolates yielded any plaques when infected with a purified suspension of AP205 (Tables 5.25&5.26). This result correlates with the outcome of the surface spotting method (5.3.22).

Strain	Appearance of lawn	Pfu/plate
R1973	very dense lawn	0
R2751	very dense lawn	0
R3417	very dense lawn	0
R4474	very dense lawn	0
R45502	very dense lawn	0
R46363	dense lawn	0
W5211	very dense lawn	0
W6108	very dense lawn	0
W6492	very dense lawn	0

Table 5.25 Plates incubated at 25 °C

Strain	Appearance of lawn	Pfu/plate
R1973	very dense lawn	0
R2751	very dense lawn	0
R3417	very dense lawn	0
R4474	very dense lawn	0
R45502	very dense lawn	0
R46363	very dense lawn	0
W5211	very dense lawn	0
W6108	very dense lawn	0
W6492	very dense lawn	0

Table 5.26 Plates incubated at 37 °C

5.4 Discussion

The host organism HER1424 was a Gram-negative, oxidase negative, catalase positive, strictly aerobic and non-motile short rod. Phenotypic properties, microscopic appearance and identification with API20NE test system matched the characteristics of *Acinetobacter haemolyticus*. This was further substantiated when HER1424 failed to grow on Leeds Acinetobacter Medium, as Jawad et al. (1994) reported that *Acinetobacter haemolyticus* failed to grow on this medium. The largest colonies (1-2mm) on TSB were achieved when incubated at 30°C.

During an experiment investigating the growth characteristics of HER1424 at temperatures below 30°C it transpired that standard incubators without refrigeration units were subject to temperature fluctuations up to 6°C, depending on ambient temperatures and equipment use in the laboratory. The decision was made to subsequently incubate all material at temperatures below 30°C in an Innova 4230 Refrigerated incubator using an Innova 2000 Platform shaker (Brunswick Scientific, NJ,

USA) set at 120rpm for liquid cultures. HER1424 grew readily in liquid culture at 25, 30 and 37°C and logarithmic growth was already achieved 2-2.5 hours after subculture in TSB regardless of temperature. The optimum growth temperature of HER1424 was 30°C according to calculated doubling times and growth curves, growing faster than when incubated at 25°C and 37°C. It appeared that at 37°C near $OD_{600}=0.4$ the growth curve began to plateau, whereas at 30°C growth exceeded $OD_{600}=0.6$. Even after 18 hours incubation at 37°C with sufficient aeration in the Bioscreen, HER1424 OD_{600} failed to reach 0.80 (OD_{600}). This was very different from the growth behaviour of all the Sussex strains which reached turbidities of 1.2-1.6 in the Bioscreen experiments. The exception to this pattern was W6108, an isolate preferring lower optimum growth temperatures, and which, like HER1424, reached an $OD_{600} < 0.7$. It was demonstrated that HER1424 was in log phase growth when $OD_{600}=0.5$, which was important to host infection with phage and phage propagation. At lower temperatures of approximately 4°C HER1424 grew to an average colony size of 1-2mm over 8 weeks. It could be speculated that HER1424 was a psychrophile, as it showed similar growth curves to W6108 at 37°C, reluctantly grew at this temperature and even formed colonies at very low temperatures.

Tetracycline, neomycin and vancomycin were chosen to be included in the investigation, despite the fact that current breakpoints values are not available for *Acinetobacter*, as they would not usually be considered as antibiotics of choice to treat *Acinetobacter* infections. Streptomycin and neomycin yielded clearance zones of 17 & 18 mm respectively, which imply that these two aminoglycosides exert some activity on HER1424. Vancomycin and penicillin G on the other hand, did not appear to affect the growth of the Leiden isolate at all. HER1424 was resistant to all penicillins and cephalosporins used in this investigation, apart from piperacillin. This ureidopenicillin, which is usually considered an antipseudomonal agent, was the only agent with a penicillin structure to which HER1424 proved to be sensitive. Further investigation would be required to determine if this would apply to *in-vivo* situations.

Test discs containing 30 µg of amoxicillin/clavulanic acid were used rather than the 10µg recommended by BSAC, this may have given rise to false positives. Similarly

ciprofloxacin 5µg test discs were used, rather than the 1µg employed by BSAC. In both cases it would be recommended to use the appropriate strength of discs in future experiments. Piperacillin and amikacin appeared to display synergistic action on two occasions. Future experiments would be required to confirm this phenomenon.

Despite HER424 (AP205) being described as a lytic phage (Klovins et al., 2002), plaques did not routinely form at infection and incubation temperatures of 28°C or above, which correlates with the findings of Klovins et al. (2002).

As Klovins' team had recommended the addition of calcium ions to facilitate attachment, a range of combinations of calcium and broth mixtures were investigated. All preparations led to a certain degree of precipitation, which was deemed unfavourable, as small shifts in electrolyte levels in phage containing preparations can significantly affect phage titres. It was recognised that the presence of calcium was not the critical factor in plaque formation. For this reason and the instability of various calcium-containing broths no calcium was used in the preparation of any phage lysates.

Several attempts were made to propagate the phage using the host provided, once using the protocol of Klovins et al (2002) and in a second attempt utilising a modified version.

Klovins et al. (2002) reported that "After cell lysis (which did not always happen), cell debris was removed by centrifugation." The absence of plaques posed several problems in the context of this investigation. Plaques are indicative of lytic behaviour of phage, which is an essential prerequisite for suitability in phage therapy. However, even if HER1424 and HER424 were simply to be used as a model system, plaques allow for a relatively easy quantification of phage stocks and absence of plaques would require elaborate indirect methods such as electron microscopy to estimate phage particles present. For the team working at Leiden University reliable plaque formation was not necessary as it did not fall within the remit of their research. However for the purposes of the research in this thesis, the production of high titers of lytic phage which could be counted by simple enumeration methods, such as plaque formation, was very important. It was decided to modify the infection parameters and investigate whether this may have an impact on lytic behaviour.

After investigating a range of experimental parameters it was discovered that temperature played an important part in plaque formation of HER424. Temperature shifts led to plaque formation and it was found that a temperature-dependent lytic relationship existed between phage AP205 and host HER1424. A purified phage lysate was produced and was titrated seven times, with an average titre of 2×10^{10} pfu/ml. Phage AP205 proved to remain viable after exposure to chloroform. Phage plaques seemed unusual in their irregular appearance and heterogeneous size distribution. It also became evident that phage successfully attached to host and lysis occurred even without CaCl_2 addition, provided the appropriate growth and infection temperatures were used. The experimental results demonstrated that no specialist medium was required to propagate the host or phage and additional supplementation of TSA/TSB with bivalent ions was not a prerequisite for plaque formation. Future work could include the effect of changes in growth conditions such as pH or presence of metabolic products on plaque formation and attachment of phage to host. Identifying and studying the factors affecting pilus formation of HER1424 may be another point of interest, which may provide further insights into phage-host attachment and potentially virulence factors.

Various parameters were identified which affected plaque counts such as qualitative factors affecting overlay agar, TSA plates, methods of pipetting and properties of containers. It became apparent that slight changes in experimental conditions could potentially lead to dramatic changes in plaque numbers and as a result experimental design and material preparation followed meticulously prepared protocols. In this context the optimum optical density of cell suspension of HER1424 to form plaques using the soft agar overlay method was found to be $\text{OD}_{600}=0.5$. It is surprising that published protocols to produce plaques for quantitative purposes do not include a standardized cell suspension; most protocols refer simply to usage of overnight bacterial suspensions. It is likely that use of a non-standardized cell suspension with an unknown cell density could lead to large variations in plaque numbers. Future work could involve further exploration of factors which can affect plaque numbers and investigating means to minimise these variations. Proposed work could include several different phage and host pairs with variations in pH, bivalent ions, temperature, agar densities, cell and phage concentrations and operators.

Syringe filtration was proven to leave phage counts unaffected, using a paired-samples t-test and ANOVA. Heating phage preparations to 37°C for limited periods, a temperature at which no plaques were formed, did not affect the viability of phage. Raising temperatures to 60°C however, led to phage material not forming plaques, which is probably due to some proteins being denatured at this temperature. This demonstrated that at temperatures of 37°C phage remained viable and therefore the reason for the absence of plaques at this temperature needed to be further investigated.

The temperature-dependent relationship between HER1424 and AP205 was further investigated by generating plaques at temperatures ranging from 22 to 30°C. Statistical analysis showed that at lower temperatures (22 and 25°C) significantly more plaques were formed than at 30°C ($p < 0.05$). At 30°C the number of plaques was more variable and the appearance of plaques was less distinct than at the lower temperatures. It was established that for the purpose of producing clearly defined less variable numbers of plaques an incubation temperature of 25°C was optimal, despite the optimum growth temperature of the host being 30°C.

A paucity of factors affecting host- phage interaction is described in recent scientific papers and little has been published on phage switching from lytic to temperate life cycle. Since the aim of phage therapy is to administer lytic bacteriophage to humans, it is relevant to investigate all potential risk factors and ensure the therapeutic phage remains lytic throughout the treatment. The details of interactions between phage and host are not always fully understood and the unusual relationship between HER1424 and HER424 (AP205) may help elucidate unknown dynamics between phage and host.

Several hypotheses for the reason behind the temperature-dependent plaque formation of HER424 have been put forward in this thesis. Phage AP205 appears to lose its ability to infect the host cell at higher temperatures. One reason could be that the open reading frames predicted by Klovins et al. (2002) to be responsible for lysis may change their three dimensional structure depending on temperature. Then again they may be expressed or read differently depending on temperature. Different hypotheses focus on pilus formation; pili which can be utilized by phage for attachment and subsequently infection may be expressed differently or not at all at higher temperatures. The general

properties of pili such as surface structure, ability to retract or even simply number per cell may vary dependent on growth conditions. It is also possible that bacterial mutants (with pili to which phage cannot attach or even with no pili at all), outgrow the majority of other host cells at higher temperatures. Alternatively hyperpiliated cells may dominate at higher incubation temperatures, which may have more pili but with different surface proteins to which phage can attach yet are unable to infect. These „decoy“ pili may act as phage mops, forcing phage to attach to them, yet not leading to infection. It is generally accepted that pilus formation can be linked to bacterial virulence and therefore it can be speculated that some environmental strains have the capacity to become clinically significant due to increasing virulence by pilus formation, possibly due to temperature shifts. Further work could include a detailed investigation into pilus formation under different conditions and its effect on bacterial virulence. In addition the investigation of pilus retraction models and how the mechanism of infection with phage could be modified following the retraction could be of relevance as could identification of pilus phage attachment receptors, including structural changes due to temperature shifts.

The investigations in this thesis have highlighted that the infection temperature of host with phage was more important in achieving plaques than the original temperature at which the cell suspension was incubated. Future experiments could involve using a wider range of temperatures for incubation of cell suspension and soft agar overlay plates.

Experiments investigating viral and bacterial interaction after downshifting of temperature (5.2.23) highlighted a range of occurrences which may require further investigation. Improved experimental design could involve infecting one part of Subculture 2 with phage and incubating sample Subculture 2 with and without phage at a variety of temperatures. Varying incubation temperatures throughout the experiment and including syringe filtration steps at various points as well as addition of further phage increments, with variations on phage and cell concentrations may be beneficial for future experiments. Attempts were made to artificially create temperate phage,

which provided further evidence that phage virulence factors of AP205 were affected by temperature.

It could be claimed that the cells grown at 25°C were „lysed from without“. „Lysis from without“ is caused by adsorption of phage particles above a threshold value. The cell shape changes and cell contents are liberated by a distension and final rupture of cell wall. No new phage are formed in this process, the bacteria simply accumulates the phage particles and then „explodes“ (Delbrück, 1940). The question remains why the cells grown at 37°C were not affected in the same fashion. The answer may be that phage do not attach to the cells grown at higher temperatures in the same way as to the cells grown at 25°C.

Another theory applicable to coli-phage T2 is that cells infected by T2 express a „resistance to lysis from without“ which allows adsorption onto the cells of hundreds of secondary phage without a significant decline in optical density, as demonstrated by Visconti (1953). Secondary phages in this context are viable phage particles which adsorb to the host cell after the primary phage (the first phage to infect the cell) has already infected the bacteria. There are no exact biochemical surface structural explanations in literature as to the mechanism of „resistance to lysis from without“.

Further experiments could involve the following:

- Electron microscopy to investigate the actual cell shape before and after infection of different concentrations of lytic phage at various temperatures.
- Investigation of the concepts of „lysis from within“ and „lysis from without“ including novel methods of cell and phage imaging, ideally with dynamic imaging.
- Investigation of biochemical and surface structural changes of bacterial cells investigating the exact mechanism of lysis.

It would also be of interest to create a range of experiments investigating if viable lytic phage are produced at 37°C but not released into suspension. This could be achieved by using a short infection period at 37°C, followed by removal of phage in suspension,

subculture and artificial lysis of cells using chloroform. As the phage are known to be resistant to chloroform phage titres higher than expected after infection and dilution by subculture would point towards 37°C being permissive of phage formation.

A criticism of this range of experiments is that the soft agar overlays produced with Filtrates 1, 2 and 3 were only incubated at 25°C. However, it is unlikely that plates incubated at higher temperatures would have yielded plaques. Future experiments should involve incubation of soft agar overlays at a range of temperatures. This experiment has demonstrated that despite incubation and infection of cells taking place at 37°C, plaques formed when soft overlays were incubated at 25°C. This evidence, taken in conjunction with results from previous experiments, is a further pointer that the incubation temperature of soft agar overlays is more critical than the temperature of infection. As the infected starter culture grown at 25°C repeatedly disintegrated after centrifugation, this part of the experiment could not be continued. The disintegration of one cell suspension at 25°C whilst the cell suspension grown at 37°C appearing to remain unaffected demonstrates the drastic disparity caused by the difference of temperature. Future experiments could involve the addition of lower phage numbers, a variety of infection and incubation temperatures, shorter infection intervals, reducing the static infection period and possibly even omitting the infection phase whilst the flask is incubated on a shaking platform, progressing directly to the centrifugation step.

Other means of removing unattached phage from suspension like phage antibodies or the use of agents that specifically inactivate phage, (Adams, 1959) would avoid exposing the cells to shear forces caused by centrifugation. This experiment further highlighted that phage numbers appeared to remain constant when infection took place at 37°C, as the numbers of recovered phage decreased over three subcultures, from Filtrate 1 to 3. The effort to artificially create phage, which would not form plaques when overlays were incubated at 25°C by infecting cells at 37°C failed. Filtrate 3 did not yield plaques, however this is probably due to the original phage being diluted beyond being able to form plaques over the course of the experiment and no further viable lytic phage being produced or released at 37°C. This experiment may require considerable

modification in the future, however it has provided further evidence that temperature plays an important part in virulence and propagation of AP205.

When investigating the possibility of clonal differences between plaques, both the smallest and largest plaque preparations yielded plaques of heterogeneous size, regardless of whether they were directly plated from the original extracts or from their progeny. However phage extracted from smaller plaques were statistically more likely to give rise to progeny producing plaques which were themselves small. The size range of plaques was not shown to be normally distributed, using two different statistical tests for normality. This is not unusual as described by Hava & Camilli (2001) who report the same phenomenon observed with *Vibrio cholerae* phages. A point of criticism could be that only one generation of phages originating from one plaque was investigated, however successive plaque isolations were deliberately avoided, as the original character of the „wild-type“ phage may be modified by repeated processing (Lu et al. 2003). This experiment was only designed to elucidate the possibility of clonal differences of plaques. It is consensus that one plaque usually arises as progeny of one phage particle, hence one plaque is expected to contain genetically homogenous phage particles. Future work could include attempting to determine whether one plaque is genetically homogenous and that large and small plaques arise from the same type of phage. It would be interesting to investigate if successive phage subcultures actually change the character of „wild-type“ phage and how this would be expressed on a genetic level.

The reasons for the unusual heterogeneous size distribution of plaques and the unusual plaque shapes remain unclear. It could be speculated that the lysate provided contained two different phage strains, one with a mutant which results in smaller and one with larger plaques, however this is improbable as phage preparations were derived from single plaques, hence should be clonally homogenous. As progeny of small and large plaques resulted in heterogeneous plaques it is unlikely that the phage lysate was somehow contaminated. The heterogeneous plaque size was prevalent throughout the entire investigation, independent of operator and incubation temperature (provided plaques formed at all).

A heterogeneous size distribution of plaques has been published previously in a temperature-sensitive phage for *Vibrio cholerae* (Hava & Camilli, 2001). In this instance the material provided had been genetically examined by the originators (Klovins et al. 2002), and from personal correspondence it can be concluded that the originators did not have access to any other *Acinetobacter* phage. As a result it is extremely unlikely that the material had been contaminated by a different strain of phage however, it is possible that a mutant had arisen at some point of the investigation. In addition the morphological appearance of plaques produced by AP205 are identical, apart from size, and this makes the presence of a mixture of different phage strains unlikely, as most phages give rise to plaques with distinctive morphological characteristics.

It could be argued that upscaling the experiment may have resulted in more data, however the number of data points was sufficient to allow meaningful statistical analysis.

None of the Sussex bacterial isolates gave distinctive areas of clearance on exposure to phage which implies that the only host currently known for AP205 remains HER1424. Most results were recorded as unidentified (UI) this is due to the fact that disturbances and unevenness in the lawn are not immediate proof of lytic activity.

All isolates marked UI needed to be repeated and correlated with the second method, the soft agar overlay method. All results marked UI and CL were duplicated, ensuring that the lawn was of satisfactory quality to allow interpretation of results, however no areas of clearance or distinctive disturbances in the lawn could be observed on the repetition batch. None of the 9 Sussex isolates when infected with phage HER424 gave either rise to plaques or clearance zones, regardless of incubation temperature after infection.

One of the problems associated with the surface spotting method was the partial unevenness of the lawn and disturbances in the homogeneity of the lawn were common occurrences. It could be claimed that using standardised bacterial suspensions for the surface spreading may have yielded plates which were easier to interpret, however most

phage screening protocols use a simple undiluted overnight cell suspension for phage screening (Kutter & Sulakvelidze, 2005). Modified Method 1601 by the US Environmental Protection Agency (Anon, 2001), which describes a protocol for phage spotting using far more detail than other references, recommends that the optical density of the overnight suspension be between 0.1 and 0.5 at a wavelength of 520nm, to ensure the bacterial suspension is in its log phase. However considering the outcome of this experiment and a range of lawns being too thin to allow correct assessment of the result, it may be of benefit to refine the surface spotting protocol by using standardised and washed overnight cell suspensions for future experiments. This would avoid the problem of lawns becoming too thin to allow interpretation or avoid false negatives. Future experiments would involve trialling a modification of the surface spotting method, with optimum bacterial lawns.

It appeared that chloroform blanks did not consistently yield an area of clearance (CL). In some cases the bacterial lawn grew in the area where the chemical was applied, albeit slightly thinner than in the surrounding area. This was probably due to chloroform killing the bacteria on application and regrowth of bacteria from the edges of the lawn occurring once the chloroform had evaporated. The 10% v/v chloroform in broth blank had been freshly prepared and was vortexed before each application. It is likely that samples containing larger fractions of chloroform than others or consisting entirely of chloroform may have been drawn into the pipette, due to distinct phase separation between chloroform and the aqueous phase (broth). This is the most probable reason for the circular thinning of the bacterial lawn, reminiscent of clearance zones. The reasons for inclusion of a chloroform blank was to investigate if the chloroform used in the purification process of the phage may have any impact on the results, so as to exclude any false positives. It has to be mentioned however that the phage preparation had been prepared far in advance of the freshly made chloroform blank and the content of this chemical in the lysates would have been far less due to evaporation. A more suitable blank would have been prepared at the same time as the lysates. It can be concluded therefore that the residual chloroform which may be present in phage lysates has not affected the overall results of the surface spotting method.

5.5 Conclusion

Taking the individual test results into account it can be claimed with certainty that the bacterium in question is *Acinetobacter* spp. and more specifically *Acinetobacter haemolyticus*.

HER1424 was sensitive to amikacin, gentamicin and imipenem and was resistant to all other antibiotics tested in this investigation with published BSAC break-points. The test results for ciprofloxacin and amoxicillin/clavulanic acid may be false positive and would require further investigation.

HER1424 successfully grew at 20, 25 and 30°C in liquid culture and on TSA, with the colony size increasing directly in proportion to the incubation temperature. It was observed that in standard non-refrigerated incubators precise temperature control was not possible and all samples below 30°C were subsequently incubated in an Innova 4230 Refrigerated Incubator shaker, New Brunswick Scientific, NJ, USA to ensure controlled incubation temperatures. In liquid culture temperatures of 25, 30 and 37°C were proven to be permissive of growth of *Acinetobacter* HER1424. The optimum temperature in TSB was 30°C, at 37°C growth was less vigorous; growth was weaker at 25°C than at 30°C. The Bioscreen profile at 37°C was comparable to W6108, a Sussex isolate preferring lower incubation temperatures. Evidence demonstrated that HER1424 was in log phase growth when $OD_{600}=0.5$, when incubated at 37°C. This could not explain why HER1424 and corresponding phage AP205 did not form plaques, despite being described as lytic, when infected and incubated at 37°C. A range of phage propagation attempts were made, using different protocols with addition of bivalent cations and modification of infection and incubation temperatures.

It was essential to this investigation to achieve plaque formation, as it is the prerequisite to phage titration and quantitative methods, without the need for specialist equipment and extensive resources. The discovery of temperature shifts affecting plaque formation solved the issue of quantitative work, it highlighted a previously unknown complex interaction between phage and host and raised a number of questions. A range of

experiments was designed to further elucidate this complex relationship; however it was not within the remit of this investigation to provide conclusive answers.

Phage AP205 was deemed resistant to chloroform, a chemical integral to the phage lysate purification process. Divalent ions were not added in the process of producing the phage lysates, as the risks of phage titres dropping during the investigation, due to potential precipitation could not be outweighed by the benefits. A purified phage preparation could be produced with a phage titre of 2×10^{10} pfu/ml, which was considered adequate for the purpose of the investigation.

The soft agar overlay method, a vital tool in quantitative work with phage, was validated using one-way repeated measures ANOVA. Various factors potentially affecting plaque counts were identified. In particular it was emphasised that exact descriptions of soft agar overlay methods were essential and routinely using the same method and equipment including seemingly minor details like glassware and caps were important. It could be demonstrated that syringe filtration using Minisart Sartorius syringe filters, $0.45 \mu\text{m}$ by utilising paired-samples t-test and one-way repeated measures ANOVA did not affect the overall plaque counts. The optimum OD_{600} for the production of bacterial lawns for use with the soft agar overlay method, employing HER1424 and HER424 was found to be between 0.45 and 0.5. A standardised soft agar overlay method was subsequently devised. It was also decided to ensure a constant incubation temperature of 25°C for samples to be routinely incubated in a refrigerated incubator with shaking platform.

It could be established that phage previously heated to 60°C yielded no plaques, which implied that at this temperature phage were inactivated. It could be demonstrated that when phage HER 424 was heated to 25°C and 37°C samples remained viable. Two different statistical tests confirmed that the number of plaques using the same phage concentration for all experiments was significantly higher at incubation temperatures of 22°C and 25°C compared to 30°C . Plates incubated at 35°C or 37°C did not yield any plaques. Plaques at all incubation temperatures were of heterogeneous size distribution, at 30°C the number of plaques was more variable and they appeared less distinct, with blurred plaque edges. Various reasons for this temperature dependent plaque formation

of AP205 were suggested. The Open Reading Frames may have different three dimensional structures at different temperatures or may be read differently at higher temperatures, functioning similarly to a temperature dependent switch. Another theory concerned the host, HER1424 may form pili differently or not at all at higher temperatures, which play a role in phage attachment and consequently the phage infection process.

It appeared from a range of different experiments that the infection temperature at which the phage and host assemble was more critical than the temperature at which the cells were incubated to produce a cell suspension. This makes the theory based on the three dimensional structure of the ORF dependent on temperature more plausible than others. Evidence was presented that phage attached to host cells at 37°C; however it did not prove to be replication competent. There is evidence that shear forces like centrifugation acting on infected bacterial cells may aid the bursting open and release of phage. Temperate phage, which would not form plaques at 25°C, could not be bred. Cell suspensions grown and infected at 25°C with high concentrations of phage disintegrated after repeated centrifugation. It was proposed that this occurrence would be due to „lysis from without“. Bacterial cells grown in the same fashion but incubated at 37°C remained intact, despite exposure to the same shear forces. It was speculated that dependent on temperature HER424 may express a „resistance to lysis from without“ as previously described in coli-phage T2.

Phage HER424 seemed unusual as the occurrence of plaques was not a classic bullet shape, but plaques of heterogeneous size distribution, with irregular serrated edges.

Due to the different sized plaques occurring the question arose whether there may be clonal differences between plaques which could be responsible for the differences in size. A single phage generation was studied, which provided data that large plaques gave rise to heterogeneous sized plaques, yet small plaques were more likely to give rise to small plaques. Plaque sizes of small and large plaques were not normally distributed however, sample sizes were large enough to allow statistical tests to be meaningful. It was deemed unlikely that the original phage preparation of HER424 was contaminated with a different strain of *Acinetobacter* specific phage. Only one phage generation was

studied, to avoid breeding a phage over several generations, which had lost its „wild-type“ character. Evidence was inconclusive and would require further experimentation, including several different multi-generation studies to determine if plaque size correlates with clonal and consequently genetic differences.

Data demonstrated that numbers of free phage HER424 in suspension dropped within several minutes of exposure to host cells HER1424 at 25°C and 37°C. This was considered verification that phages attach to bacterial cells regardless of incubation temperatures. At 37°C free phage did not increase, whilst phage titres a hundred times greater than the original concentration could be measured after prolonged incubation at 25°C. This was further evidence that phages were capable of attaching to host cells independent of incubation temperatures, however it was not replication competent at 37°C.

No signs of lytic activity could be detected when any of the tested nine Sussex *Acinetobacter sp.* isolates were screened as potential hosts for phage HER424, at any of the given temperatures using either the surface spotting or overlay technique. This suggests that phage HER424 is specific for its host *Acinetobacter haemolyticus* HER1424, however further work with a wider range of isolates needs to be undertaken.

A. E. Henein¹, G. W. Hanlon¹, J-Y. Maillard¹ and S. P. Denyer².¹School of Pharmacy and Biomolecular Sciences,University of Brighton, Brighton, UK., ²The Welsh School of Pharmacy, University of Cardiff, Wales.

ABSTRACT

Acinetobacter species are antibiotic-resistant bacterial pathogens with increasing clinical significance, particularly in infections of high-risk patients such as those with severe burns. These patients have limited treatment options and often require highly toxic antibiotics. The need for an alternate approach to therapy in these cases is thus evident. We report here some preliminary investigations into the use of bacteriophages for the treatment of *Acinetobacter* infections of severe burns patients.

AP205 (HER424) is a ssRNA phage whose genome has been fully sequenced and has as its host *Acinetobacter haemolyticus*. Previous work has shown that this phage caused lysis of the host only erratically at 28°C despite the presence of a gene translating into a peptide with lytic function. Purified phage suspensions were enumerated using the overlay technique whereby phage and host cells (100µl of each) were mixed with soft agar and poured over the surface of TSA plates. The plates were incubated at a variety of temperatures and plaques counted after incubation. Those plates incubated at 20°C, 22°C and 25°C all showed the presence of plaques of heterogeneous size distribution. (This heterogeneity was shown not to be due to clonal differences). The number of plaques was significantly lower at 30°C compared to 22°C and 25°C and at 30°C the number of plaques was more variable and they appeared less distinct. At 35°C and 37°C no plaques were evident even though the phage was not inactivated at these higher temperatures. Host cells infected at 37°C, washed and then subcultured each day over a 3-day period gave rise to extremely low frequency of lysis when the temperature was downshifted to 25°C. The host range of AP205 was tested using 9 clinical isolates of *Acinetobacter* species (primarily *A. baumannii/calcoaceticus* complex) and no evidence of lysis was seen even at the permissive temperature of 25°C.

The identity of the mediator of the temperature sensitivity is uncertain, however previous investigators have reported the presence of an open reading frame, which does not appear to present in other phages and is putatively involved in translation of the maturation factor.

INTRODUCTION

The therapeutic properties of bacteriophage have been known and utilised in clinical practice for decades, however phage therapy has never been part of established therapeutics in the Western World (5). Decreasing sensitivity to antibiotics and the emergence of pan-drug resistant strains have given rise to concern that we may move back to the pre-antibiotic age. Antimicrobial resistance is a major public health threat internationally, severely limiting treatment choice in clinical settings and consequently complicating therapy (3, 6).

In the UK the House of Lords Select Committee on Science and Technology published its report 'Resistance to Antibiotics and other Antimicrobial Agents' in March 1998, recognizing the severity of the situation (3). Following that, the Department of Health published 'UK Antimicrobial Resistance Strategy and Action Plan', which defines eight action areas, one of which is 'to encourage the development of new and novel agents/technologies to detect, prevent and treat infection to overcome resistance.'

Acinetobacter sp. is an opportunistic pathogen with increasing clinical importance particularly in intensive therapy units, causing infections as diverse as septicæmia, meningitis, eye and urinary tract infections, endocarditis and pneumonia, predominantly in immunocompromised patients. Due to improved survival for these vulnerable patient groups nosocomial *Acinetobacter* infections are likely to increase (6).

Multiple drug resistant *Acinetobacter* sp., have become a major therapeutic challenge in burns units, where colonisation of burn wounds can lead to rejection of skin grafts, systemic infections and subsequently increased mortality (2).

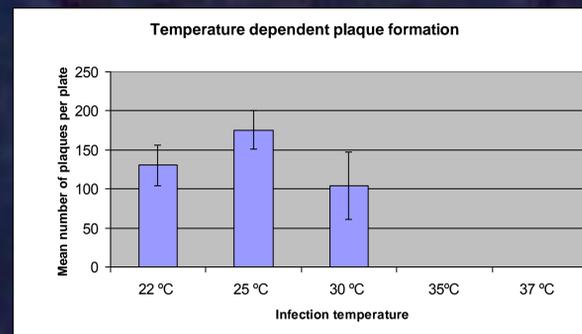
The aim of this study was to investigate the interaction of bacteriophage AP205 with *Acinetobacter* sp. in the context of phage therapy.

1. Temperature dependent plaque formation (Figure 1)

Purified phage suspension yielding a plaque count of approximately 100 pfu per plate were enumerated -using the overlay technique whereby 100µl of phage and host cells were mixed with soft agar (0.4% technical agar in TSB) and poured over the surface of TSA plates. Host cell cultures were incubated at 25 °C for 16 hours. Plates were incubated at a variety of temperatures for 16h and plaques counted.

Results:

The number of plaques was significantly lower, more variable and plaque edges appeared less defined at 30 compared to 22 and 25 °C. At 35 and 37 °C no plaques were evident at all.

2. Screening of phage AP205 against clinical isolates of the *Acinetobacter baumannii/calcoaceticus* complex

9 clinical isolates were used for the surface spotting method. 10µl drops were applied to bacterial lawns- clearance zones indicated lytic activity. Incubation temperatures of plates ranged from 20,22,25,30 to 37 °C. The same isolates were employed in the overlay technique, using phage preparation which would yield between 100 and 250 pfu per plate. Plates for the overlay technique were incubated at 37 and 25 °C respectively for 16-18 hours.

Results:

No signs of lytic activity could be found when any of the tested *Acinetobacter* sp. isolates were screened at any of the given temperature using either the surface spotting or overlay technique. This implies that AP205 is specific for its host *Acinetobacter haemolyticus*, however further work needs to be undertaken.

3. Phage host/cell adsorption at 25 °C and 37 °C

Overnight cell suspensions (16-18hrs) of *Acinetobacter haemolyticus* were incubated at 25 °C and 37 °C (120rpm). After adjustment of optical density they were infected with phage AP205 at a ratio of 1:1 and incubated in a static incubator at 25 or 37 °C for 20 minutes then agitated at 120rpm at constant temperature. 1ml samples were withdrawn after 5 minutes and every 20 minutes. Each sample was syringe filtered (0.45 µm pore size), serially diluted and phage numbers enumerated using the overlay technique described above.

METHODS & RESULTS

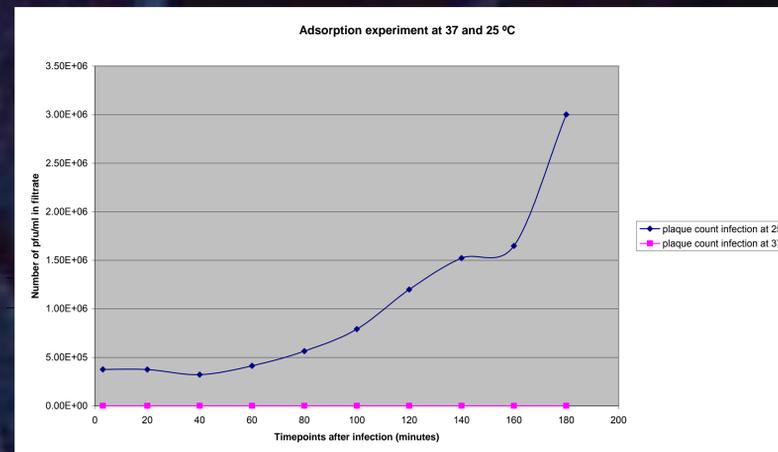


Figure 2:

Comparison of plaque counts of samples infected at 25 and 37 °C

Results: (Figure 2)

Samples drawn during the 25 °C experiment showed a 10-fold increase in pfu/ml (from 3.7E5 to 3E6) after 180 minutes. In contrast when the infection took place at 37 °C, no significant increase in phage count could be observed, it remained at approx. 2-4E4 pfu/ml. The results indicate that at 37 °C no viable lytic or insignificant amounts of phage are released from the host cells.

4. Phage adsorption and incubation at 37 °C with artificial lysis

A host cell suspension was grown for 24h at 37 °C and the optical density was adjusted. The cells were then infected with purified phage preparation of a known titre as described above. Following incubation at 37°C (120rpm) for 24 h, a 2ml sample was removed, syringe filtered and serially diluted. Chloroform was added to the reaction vessel to give a final concentration of 1% v/v and the culture incubated for a further 30minutes. After this time a further 2ml sample was withdrawn, syringe filtered and serially diluted. The phage present in the serial dilutions of both aliquots were enumerated using the overlay technique conducted at 25°C, and plaque counts compared.

Results:

There was no difference in plaque counts between samples drawn before and after chloroform addition. These results suggest that despite being able to adhere to the host cell at 37°C no replication of phage occurred within the cells.

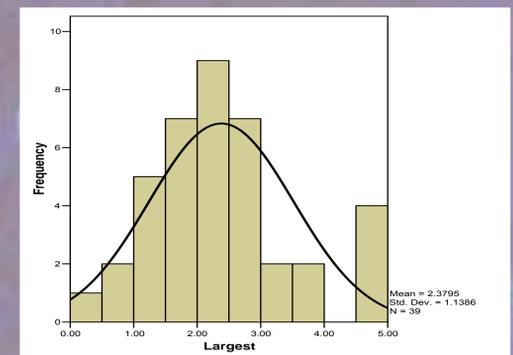
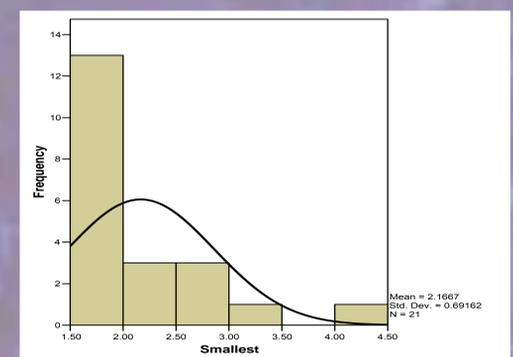
5. Assessment of clonal differences between plaques of heterogeneous size (Figures 3 & 4)

Plaques formed using the overlay technique were of heterogeneous size. In order to investigate if plaques of different size showed clonal differences the two largest (6.5mm) and two smallest (0.4mm) plaques on an overlay plate were removed with a corkborer and placed in lambda buffer and 1% v/v chloroform. The phage present were serially diluted and used to produce further plaques using the overlay technique. The purpose of this experiment was to determine if the phage from small plaques themselves produced small plaques (and vice versa). 100ml of host cell culture of known optical density was infected with 1ml of the phage suspension from the smallest plaque preparation and the process repeated for the largest plaque preparation. The cultures were incubated at 25°C for 24h.

Following incubation, chloroform was added to the reaction vessel (to give a final concentration of 1% v/v) in order to burst the cells, and aliquots were syringe filtered, serially diluted and plated using the overlay method.

Results:

Both the smallest and largest plaque preparations yielded plaques of heterogeneous size, regardless of whether they were directly plated from the original extracts or from their progeny. However, it appears that phage extracted from smaller plaques are more likely to give rise to progeny producing plaques which are themselves small. The size range of plaques was not shown to be normally distributed.

Figure 3:
Showing size distribution of progeny of the largest plaqueFigure 4:
Showing size distribution of progeny of the smallest plaque

DISCUSSION

The results shown here indicate that AP205 can produce a lytic infection at temperatures of 30°C and below. The adsorption experiment indicated that the phage were able to adhere to the host cells at both permissive and non-permissive temperatures, but that no subsequent release of phage occurred at 37°C. Artificial lysis of cells subjected to phage infection at 37°C showed that no viable progeny were produced. We have previously shown that host cells infected at 37°C and then downshifted to 25°C did not support viral replication (data not shown). The identity of the mediator of the temperature sensitivity is uncertain, however previous investigators have reported the presence of an open reading frame, which does not appear to be present in other phages and is putatively involved in translation of the maturation factor (4).

It was shown that AP205 yielded plaques of heterogeneous size, which was not due to clonal differences.

REFERENCES

- Department of Health 'UK Antimicrobial Resistance Strategy and Action Plan, June 2000. <http://www.publications.doh.gov.uk/pdfs/arbstrat.pdf>
- Edwards-Jones V, Greenwood JE, (2003) *Burns*, **29**, 15-24.
- House of Lords Select Committee on Science and Technology 'Resistance to Antibiotics and other Antimicrobial Agents', 17th March 1998, <http://www.parliament.the-stationeryoffice.co.uk/pa/ld199798/ldselect/ldscitech/081viii/st0701.htm>
- Klovins J, Overbeek GP, van den Worm SHE, Ackermann HW, van Duin J, (2002) *Journal of General Virology*, **83**, 1523-1533.
- Merrill CR, Scholl D, Adhya SL, (2003) *Nature Reviews*, **2**, 489-497.
- Soni S., cited in Meetings and Conferences, (2001) *The Pharmaceutical Journal*, **267**, 7168.

ACKNOWLEDGMENTS

The research was funded by the University of Brighton, Brighton, UK, and The Royal Pharmaceutical Society of Great Britain.

The authors thank Drs. van Duin and van den Worm for providing phage AP205 and corresponding host bacterium.

The potential of bacteriophage
therapy in *Acinetobacter* spp.
infections

Alexandra Elisabeth Henein

A thesis submitted in partial fulfilment
of the requirements of
the University of Brighton for the
degree of Doctor of Philosophy

Volume TWO

March 2009

University of Brighton

Declaration

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to this or any other university for a degree, and does not incorporate any material already submitted for a degree.

31st March 2009

Contents

Abstract	xxiii
List of Tables	xxiv
List of Figures	xxx
Acknowledgements	xxxiv
Abbreviations	xxxviii
Chapter ONE	1
Introduction	1
1.1 Functions and structure of human skin	1
1.1.1 Anatomy of normal skin	1
1.1.2 Host defence of intact human skin	3
1.1.3 Microflora	5
1.2 Burns	6
1.2.1 UK Burns statistics	6
1.2.2 Blond McIndoe Research Foundation	7
1.2.3 Quantification of Burn Injury	7
1.2.3.1 Total body surface area burn	8
1.2.3.2 Classification of burn depth	8
1.2.4 Prognosis	9
1.2.5 Pathophysiology of burn injuries	10
1.2.6 Histological and histochemical aspects of wound healing	11
1.2.6.1 Role of cytokines in wound healing	12
1.2.6.2 Modulation of wound healing	14
1.2.7 Management of burns wound of various depths	15
1.2.8 Burn wound cover and closure	16
1.2.8.1 Amniotic membrane	17

Contents

1.2.8.2 Autologous skin grafts	17
1.2.8.3 Human cadaver allografts and xenografts	18
1.2.8.4 Cultured human dermal keratinocyte sheets	18
1.2.8.5 Keratinocyte cell sheets on supports	19
1.2.8.6 Fibrin-glue and keratinocytes	20
1.2.8.7 Sprayed keratinocyte cell suspension	20
1.2.8.8 Skin substitutes	20
1.2.8.9 Combinations of fibroblasts and keratinocytes	22
1.3 Burn wound infection	23
1.3.1 Sources of burn wound contamination	23
1.3.2 Factors predisposing to microbial proliferation in burns	24
1.3.3 Significance of microorganisms present in the wound	25
1.3.4 Graft failure due to microbial contamination of the wound	26
1.3.5 Bacteria associated with wound infection	27
1.3.6 Burn wound infection management	29
1.3.6.1 Early closure of wounds	29
1.3.6.2 Topical antimicrobial agents	29
1.3.6.3 Regulatory molecules	29
1.3.6.4 Bacterial interference	30
1.3.6.5 Natural agents	30
1.3.6.6 Cytokines	30
1.3.6.7 Antibiotics	31
1.3.7 Pharmacokinetics of antibiotics in burn patients	31
1.4 Antibiotic resistance and resulting clinical problems	32
1.5 <i>Acinetobacter</i> species	32
1.5.1 Morphological, metabolic and cultural characteristics of <i>Acinetobacter</i> spp.	34
1.5.2 Growth temperatures	35
1.5.3 Growth media	35

Contents

1.5.4 Isolation from clinical samples/ Isolation of <i>Acinetobacter</i> spp. from various sources	36
1.5.5 <i>Acinetobacter</i> taxonomy	36
1.5.6 Mechanisms of resistance	39
1.5.6.1 β -lactam resistance	39
1.5.6.2 Imipenem (Carbapenem) resistance	39
1.5.6.3 Aminoglycoside resistance	40
1.5.6.4 Fluoroquinolone resistance	40
1.5.6.5 Phenotypic antimicrobial resistance patterns of <i>Acinetobacter</i> spp. reported by various centres	40
1.5.7 The role of <i>Acinetobacter</i> spp. in human disease	43
1.5.7.1 <i>Acinetobacter</i> spp. in wounds and burns	44
1.6 Alternatives to antibiotics	45
1.6.1 Reasons for a lack of new antibiotics	45
1.6.2 Photodynamic therapy (PDT)	46
1.6.3 Phytomedicines	47
1.6.4 Honey	48
1.6.5 Antibodies	48
1.6.6 Bacterial cell wall hydrolases (BCWH)	48
1.6.7 Antimicrobial peptides (AMP)	49
1.6.8 Bacteriophages	50
1.6.8.1 Phage display	51
1.6.8.2 Bacterial typing and detection	51
1.6.8.3 Phage-delivered medicinal substances	51
1.6.8.3.1 Vaccines	51
1.6.8.3.2 Antimicrobials	52
1.6.8.3.3 Genes	52
1.7 Bacteriophage therapy	52
1.7.1 History of phage therapy	53

1.7.2 Early Clinical application of phage therapy in scientific literature	53
1.7.3 Clinical applications of phage therapy	56
1.7.4 Animal experiments	57
1.7.5 Phage therapy in wounds- animal data	57
1.7.6 Phage therapy in wounds -human data	58
1.7.7 Safety of bacteriophage	61
1.7.8 Cytotoxicity testing	63
1.8 Bacteriophage isolation	63
1.9 Thesis Aim and Objectives	65
1.9.1 Aim	65
1.9.2 Objectives	65
Chapter TWO	67
Materials and Methods	67
2.1. Materials and Equipment	67
2.1.1 Chemicals and Microbiological Media	67
2.1.2 Equipment-Microbiology	68
2.1.3 Chemicals and Materials-Cell culture	69
2.1.3.1 Plasticware	71
2.1.4 Equipment- Cell culture	71
2.1.4.1 Microscopy	72
2.1.5 Molecular biology kits and bacterial identification kits	72
2.2 Bacterial strains and phage material	73
2.2.1 Clinical isolates of <i>Acinetobacter</i> species.	73
2.2.2 <i>Acinetobacter haemolyticus</i> (Leiden strain) and corresponding bacteriophage	74
2.2.3 Microbiological samples obtained from the Pasteur Institute, Paris.	75
2.2.4 Microbiological samples obtained from Laval University, Canada.	77
2.2.5 Long-term storage of <i>Acinetobacter</i> . spp	78
2.2.6 Routine subculture of <i>Acinetobacter</i> . spp	78
2.2.7 Routine culture of overnight suspensions of <i>Acinetobacter</i> spp.	78
2.3 Preparation of agar plates and overlay agar	79
2.3.1 Preparation of TSA plates for viable counts	79

Contents

2.3.2 Preparation of TSA plates for use with soft agar overlays (agar overlay method)	79
2.3.3 Soft agar	79
2.3.5 Selective Media	81
2.3.5.1 Leeds Acinetobacter Medium(LAM) (Jawad et al. 1994)	81
2.3.5.2 Leeds Acinetobacter Medium with varying antibiotic compositions	82
2.3.5.3 Modified Leeds Acinetobacter Medium (MLAM)	82
2.4 Identification methods for bacterial strains	83
2.4.1 Characterisation using microscopy	83
2.4.2 Motility	83
2.4.2 Gram staining	83
2.4.3 Oxidase test (including validation)	83
2.4.4 Catalase test	83
2.5 Viable count methods	84
2.6 Phage screening methods	84
2.6.1 Basic surface spotting method	85
2.6.2 Revised Surface spotting method	85
2.6.3 Soft agar spotting method	86
2.7 Production of crude filtered lysates	86
2.7.1 Liquid Lysate Method	86
2.7.2 Production of purified lysates	87
2.7.2.1 Standard protocol to purify bacteriophage	87
2.8 Phage enumeration method	88
2.8.1 Soft agar overlay method	88
2.9 Optical density measurements	89
2.9.1 Relating Optical density measurements to viable counts at 600nm (cells washed and suspended in PBS)	89
2.9.2 Relating optical density measurements to viable counts at 600nm (overnight suspension)	91

Chapter THREE	93
Identification and characterisation of nine clinical 'Sussex' <i>Acinetobacter</i> spp. isolates	93
3.1 Introduction	93
3.2 Materials and Methods	95
3.2.1. Storage and routine subculture of Sussex strains	95
3.2.2 Routine culture and growth of Sussex isolates	96
3.2.3 Characterisation of Sussex isolates using microscopy	96
3.2.4 Gram staining of Sussex isolates	96
3.2.5 Oxidase test	96
3.2.6 Catalase test	96
3.2.7. Identification of nine clinical Sussex strains using the API20NE system	96
3.2.8 Growth of 9 clinical Sussex isolates at 42 and 44°C	97
3.2.9 Growth curves at 37°C, using Bioscreen	97
3.3 Antibiotic susceptibility of nine Sussex isolates using the BSAC disc diffusion method	98
3.3.1 The British Society for Antimicrobial Chemotherapy (BSAC) Disc Diffusion Method for Antimicrobial Susceptibility Testing	98
3.3.1.1 Establishing ideal inoculum density- method optimisation	99
3.3.1.2 Preparation of inoculum	101
3.4 LAM agar and liquid medium validation experiments	102
3.4.1 Selective media for <i>Acinetobacter</i>	102
3.4.2 Growth characteristics of Sussex isolates on LAM	104
3.4.3 Growth characteristics of Sussex isolates on LAM with varying antibiotic compositions	105
3.4.4 Modifications to LAM	105
3.4.5 Growth characteristics of Sussex strains on MLAM	105
3.4.6 Liquid Leeds <i>Acinetobacter</i> medium validation experiment	106
3.4.7 Growth of clinical <i>Acinetobacter</i> isolates in LAM liquid	106

Contents

3.4.8 Growth of clinical <i>Acinetobacter</i> isolates with artificial background contamination in LAM liquid	108
3.4.8.1 Growth of <i>Acinetobacter</i> spp. in LAM liquid with and without background contamination found in waste water	108
3.4.8.2 Growth of waste water samples on TSA and LAM agar	109
3.4.8.3 Membrane filtration	109
3.5 Results	110
3.5.1 Characterisation of Sussex isolates using microscopy	110
3.5.2 Gram staining of Sussex isolates	110
3.5.3 Oxidase test	110
3.5.4 Catalase test	111
3.5.5 Identification of nine clinical Sussex strains using the API20NE system	111
3.5.6 Growth of 9 clinical Sussex isolates at 42 and 44°C	112
3.5.7 Growth curves at 37°C, using Bioscreen-C	114
3.5.8 Antibiotic susceptibility of Sussex isolates using the BSAC method	118
3.5.9 LAM agar and liquid medium validation experiments	122
3.5.10 Growth characteristics of Sussex isolates on LAM with varying antibiotic compositions	126
3.5.11 Growth of clinical <i>Acinetobacter</i> isolates in LAM liquid	127
3.5.12 Growth of <i>Acinetobacter</i> spp. in LAM liquid with and without background contamination found in waste water	128
3.6 Discussion	131
3.6.1 Identification of 9 clinical Sussex strains using the API20NE system and growth at various temperatures of 9 clinical Sussex isolates	131
3.6.2 Antibiotic susceptibility of 9 Sussex isolates using the BSAC disc diffusion method	132
3.6.3 Growth characteristics of Sussex isolates on LAM with varying antibiotic composition and MLAM	133
3.6.4. Growth of Sussex isolates in LAM liquid without background contamination	134
3.6.5 Growth of Sussex isolates in LAM liquid with background contamination	135

3.7 Conclusion	136
Chapter FOUR	138
Attempted isolation of <i>Acinetobacter</i> spp. and phage from sewage and waste water	138
4.1 Introduction	138
4.1.1 Specific isolation protocols for <i>Acinetobacter</i> phage	140
4.1.2 Aim	142
4.2 Material and Methods	143
4.2.1 Bacterial strains	143
4.2.2 QVH Waste water samples	143
4.2.3 Sewage samples from Poynings, Scaynes Hill and other collection sites	144
4.2.4 Isolation of <i>Acinetobacter</i> spp and phage from Queen Victoria Hospital waste water samples	146
4.2.4.1 Surface spotting of filtered supernatant liquid 'Direct plate selection'	146
4.2.4.2 Overlay method using filtered supernatant liquid 'Direct plate selection'	146
4.2.4.3 Propagation of bacteria found in unfiltered supernatant liquid and pellet	147
4.2.4.4 Checking for growth of wastewater samples on TSA and subculture onto LAM	148
4.2.5 Isolation of <i>Acinetobacter</i> spp and corresponding phage from sewage samples	148
4.2.6 Attempts at obtaining bacteriophage from other sources	149
4.2.6.1 Culture collections	149
4.2.6.2 Contact with individuals in the phage community	150
4.2.7 Material obtained from the Pasteur Institute, Paris.	154
4.2.7.1 Investigating viability of Pasteur isolates	155
4.2.7.2 Method optimisation for bacteriophage host screening using multiple application devices	155
4.2.7.3 Production of bacterial lawns for phage screening	155
4.2.7.4 Method optimisation using a multipoint inoculator for phage screening.	156
4.2.7.5 Method optimisation using a multipoint pipettor for phage screening	156
4.3 Results	158

Contents

4.3.1 Surface spotting of filtered supernatant liquid from burns unit (QVH) samples	158
4.3.2 Overlay method using filtered supernatant liquid-QVH	158
4.3.3 Bacteria found in unfiltered supernatant liquids and pellets-QVH	158
4.3.4 Checking for growth of wastewater samples on TSA and subculture onto LAM -QVH samples	159
4.3.5 Sewage samples on LAM agar	160
4.3.5.2 Controls	162
4.3.5.3 Testing of sewage isolates subcultured on TSA	162
4.3.5.4 Phage isolation from sewage samples	163
4.3.6 Viability of Pasteur isolates	163
4.3.7 Results of multipoint inoculator for phage screening	165
4.3.8 Results of multipoint pipettor used for phage screening	165
4.4 Discussion	165
4.4.1 Isolation of <i>Acinetobacter</i> spp. and phage for waste water and sewage samples	165
4.4.2 Phage isolation methods	167
4.4.3 Difficulties encountered obtaining <i>Acinetobacter</i> phage with matching host	171
4.4.4 Pasteur material phage screening	172
4.4.5 Lack of suitability of multipoint inoculator for phage screening	174
4.5 Conclusion	174
Chapter FIVE	175
Investigation of temperature dependent lytic relationship of host and phage HER1424 and HER424	175
5.1 Introduction	175
5.1.1 Genetic profile of AP205 (HER424)	176
5.2.1 Propagation on arrival and long-term storage	177
5.2.2 Routine subculture of <i>Acinetobacter</i> HER1424	178
5.2.3 Phenotypic properties, identification of HER1424 with API20NE test system, microscopic examination and digital imaging using a bright-field light microscope	178

Contents

5.2.4 Antibiotic susceptibility of HER1424 using BSAC disc diffusion method	179
5.2.5 Growth characteristics of HER1424 on Leeds Acinetobacter Medium (LAM)	179
5.2.6 Growth characteristics of HER1424 at 20, 25 and 30°C	180
5.2.7 Growth curves of HER1424 at 25, 30 and 37°C	180
5.2.8 Log phase experiment at 37°C, using a Bioscreen-C	181
5.2.9 Growth characteristics of HER1424 at 4°C ± 1°C	181
5.2.10 Propagation of phage HER424 using host HER1424	182
5.2.10.1 Production of CaCl ₂ solutions- Solution 1	182
5.2.10.2 Production of CaCl ₂ solutions- Solution 2	183
5.2.10.3 Production of CaCl ₂ solutions- Solution 3	183
5.2.10.4 Phage propagation attempt 1	183
5.2.10.5 Phage propagation attempt 2	184
5.2.11 Temperature shifts affecting plaque formation of HER1424 and HER424	185
5.2.12 Production of a primary lysate using HER1424 and HER424	185
5.2.13 Apparent relationship between temperature and ability to form plaques of HER1424 and HER424 using crude phage preparation AEH019(4/6/3)3	186
5.2.14 Production of purified phage suspension of HER1424 (secondary lysates)	187
5.2.15 Diagram to show propagation of phage HER1424 after receipt	189
5.2.16 Investigating viability, sensitivity to chloroform and quantitative titration of purified phage suspension	189
5.2.17 Validation of quantitative phage estimation using the soft agar overlay method	190
5.2.18 Determination of loss of phage after filtration with 0.45 µm syringe filter	190
5.2.19 Method optimisation for optimum optical density of inocula to yield best bacterial lawns for plaque counts	191
5.2.20 Impact of heat on phage activity	191
5.2.21 Temperature dependent relationship between <i>Acinetobacter haemolyticus</i> HER1424 and phage AP205 (HER 424)	192
5.2.22 Temperature switches affecting ability to form plaques	192

Contents

5.2.23 Downshifting of temperature after infection	193
5.2.24 Attempts to artificially create temperate phage	194
5.2.25 Investigating the possibility of clonal differences between plaques	194
5.2.26 Adsorption experiments at 25°C and 37°C	196
5.2.27 Screening Sussex isolates as potential hosts for AP205 at various temperatures.	197
5.2.27.1 Screening Sussex isolates as potential hosts for AP205 using the surface spotting method	197
5.2.27.2 Screening Sussex isolates as potential hosts for AP205 using the soft agar overlay method	197
5.3 Results	199
5.3.1 Phenotypic properties, identification of HER1424 with API20NE test system, microscopic examination and digital imaging using a bright-field light microscope	199
5.3.2 Antibiotic susceptibility of HER1424 using BSAC disc diffusion method	200
5.3.3 Growth characteristics of HER1424 on Leeds Acinetobacter Medium (LAM)	202
5.3.4 Growth characteristics of HER1424 at 20, 25 and 30°C	203
5.3.5 Growth curves of HER1424 at 25, 30 and 37°C	203
5.3.6 Log phase experiment at 37°C, using a Bioscreen-C	205
5.3.7 Growth characteristics of HER1424 at 4°C +/- 1°C	206
5.3.8.1 Production of CaCl ₂ solutions- Solution 1	206
5.3.8.2 Production of CaCl ₂ solutions- Solution 2	206
5.3.8.3 Production of CaCl ₂ solutions- Solution 3	206
5.3.8.4 Phage propagation attempt 1	207
5.3.8.5 Phage propagation attempt 2	208
5.3.9 Temperature shifts affecting plaque formation of HER1424 and HER424	209
5.3.10 Apparent relationship between temperature and ability to form plaques of HER1424 and HER424 using crude phage preparation AEH019 (4/6/3) ³	211
5.3.11 Investigating viability, sensitivity to chloroform and quantitative titration of purified phage suspension	215

Contents

5.3.12 Validation of quantitative phage estimation using the soft agar overlay method	217
5.3.13 Determination of loss of phage after filtration with 0.45 m syringe filter	221
5.3.14 Method optimisation for optimum optical density of inocula to yield best bacterial lawns for plaque counts	222
5.3.15 Impact of heat on phage activity	223
5.3.16 Temperature dependent relationship between <i>Acinetobacter haemolyticus</i> HER1424 and phage AP205 (HER 424)	223
5.3.17 Temperature switches affecting ability to form plaques	228
5.3.18 Downshifting of temperature after infection	229
5.3.19 Attempts to artificially create temperate phage	231
5.3.20 Investigating the possibility of clonal differences between plaques	233
5.3.21 Adsorption experiments at 25°C and 37°C	237
5.3.22 Screening Sussex isolates as potential hosts for AP205 using the surface spotting method	238
5.3.23 Screening Sussex isolates as potential hosts for AP205 using the soft agar overlay method	241
5.4 Discussion	242
5.5 Conclusion	253
Chapter SIX	258
Identification and characterization of Canadian bacteriophages and corresponding host bacteria	258
6.1 Introduction	258
6.2 Material and Methods	259
6.2.1 Propagation of host bacterial species	259
6.2.2 Long term storage of bacterial cultures	259
6.2.3 Routine culture and growth of bacterial cultures	259
6.2.4 Phenotypic properties of bacterial cultures	260
6.2.5 Identification of bacterial cultures using API20NE test system	260
6.2.6 Growth of bacterial cultures at 25, 30, 37, 42 and 44°C	260
6.2.7 Antibiotic susceptibility of bacterial cultures using the BSAC disc diffusion method	260

Contents

6.2.8 Growth characteristics of bacterial cultures on Leeds Acinetobacter Medium (LAM) with varying antibiotic compositions	261
6.2.9 Determination of host for each bacteriophage	262
6.2.9.1 Optimization of surface spotting method	262
6.2.9.2 Soft agar spotting method	263
6.2.9.3 Production of stock bacteriophages lysates	263
6.2.10 Selection criteria of suitable phage and host for further experiments	264
6.2.10.1 Reliable plaque formation	264
6.2.10.2 Crisp plaques seen at 37°C	264
6.2.10.3 Production of circular clearance zones	265
6.2.10.4 Formation of sufficient amounts of recoverable precipitate	265
6.2.10.5 Phage titre > 10 ¹⁰ pfu/ml	265
6.2.11 Analysis of infection cycle of phage HER1151 in host bacteria	266
6.3 Results	267
6.3.1 Phenotypic properties of bacterial cultures	267
6.3.2 Identification of bacterial cultures using API20NE test system	267
6.3.3 Growth of bacterial cultures at 25, 30, 37, 42 and 44°C	269
6.3.4 Antibiotic susceptibility of Canadian isolates using the BSAC disc diffusion method	271
6.3.5 Growth characteristics of Canadian isolates on Leeds Acinetobacter Medium (LAM) with varying antibiotic compositions	274
6.3.6 Determination of host for each bacteriophage	279
6.3.7 Selection criteria of suitable phage and host for further experiments	282
6.3.8 Analysis of infection cycle of phage HER1151 in host bacteria	284
6.4 Discussion	286
6.4.1 Phenotypic tests and identification	286
6.4.2 Antibiotic susceptibility of bacterial cultures	287
6.4.3 Determination of host for each bacteriophage	291
6.4.4 Comparison of host screening methods	293
6.4.5 Selection criteria of suitable phage and host for further experiments	294
6.5 Conclusion	298

Chapter SEVEN	301
Effects of phage on mammalian cells in vitro	301
7.1 Introduction	301
7.1.2 In vitro cultivation of cells and tissues (definitions)	302
7.1.3 Advantages of using cell culture compared to whole tissue or animals	303
7.1.4 Disadvantages of using cell culture compared to whole tissue or animals	304
7.1.5 Senescence, 'Normal cells' and transformed cell lines	305
7.1.6 Continuous cell line versus primary cell line	306
7.1.7 Embryonic Swiss Mouse Fibroblast cells (3T3 line)	306
7.1.8 Rationale for using cell culture	307
7.1.9 Cytotoxicity and viable cell quantification	307
7.1.10. Miniaturized colourimetric methods to determine cytotoxicity	307
7.1.10.1. Lactate dehydrogenase activity (LDH)	309
7.1.10.2 Assays using MTS tetrazolium compounds	309
7.1.11 Colony formation assay (clonal assay)	310
7.1.12 Cell mediators	310
7.2 Aims & Objectives	312
7.3 Material and Methods	312
7.3.1 3T3 Swiss mouse fibroblast cell line (3T3)	312
7.3.1.1 Standard growth vessels for 3T3 Swiss mouse fibroblast cells	312
7.3.1.2 Standard growth-medium for 3T3 Swiss mouse fibroblast cells	312
7.3.1.3 Standard incubation of 3T3 Swiss mouse fibroblast cells	313
7.3.1.4 Trypsinisation of 3T3 mouse cells grown in T25 flasks	313
7.3.1.5 Trypsinisation of 3T3 mouse cells grown in 24-well plates	313
7.3.1.6 Standard seeding and passaging of growth vessels	314
7.3.1.7 Counting of mammalian cells with Trypan Blue stain	314
7.3.1.8 Hoechst propidium iodide staining (HPI)	315
7.3.1.9 Cumulative population doublings of 3T3 Swiss fibroblast cell line in 24-well plates over 72 hours	315
7.3.1.10 Cumulative population doublings of 3T3 Swiss fibroblast cell line in T25 flasks over 96 hours	316

7.3.1.11 Validation of phage activity in tissue culture conditions without mammalian cells present	316
7.3.1.12 Validation of phage activity in tissue culture conditions with mammalian cells present	316
7.3.1.13 Cytotoxicity and cell proliferation assays for phage HER401 BS46 using a 3T3 mouse fibroblast cell line	317
7.3.1.13.1 Cytotoxicity assay using Trypan Blue and 3T3 cells	318
7.3.1.13.2 Cytotoxicity assay using HPI staining and 3T3 cells	319
7.3.1.13.3 Cytotoxicity assay measuring LDH release of 3T3 mouse fibroblast cells	319
7.3.1.13.4 Cell proliferation assay measuring MTS formation of 3T3 mouse fibroblast cells (I)	320
7.3.1.13.5 Cell proliferation assay measuring MTS formation of 3T3 mouse fibroblast cells (II)	321
7.3.1.13.6 Microscopy of 3T3 mouse fibroblast cells	322
7.3.2 Propagation of 3T3 Swiss mouse fibroblast cells for feeder layers	322
7.3.2.1 Standard growth-medium for 3T3 cells for feeder layers	323
7.3.2.2 Gamma irradiation of 3T3 Swiss mouse fibroblast cells for feeder layers	323
7.3.2.3 Standard growth medium for isolated keratinocytes	323
7.3.3 Isolation of epithelial cells from human biopsy-keratinocytes	324
7.3.4 Isolation of epithelial cells from human biopsy-fibroblasts	326
7.3.4.1 Human primary fibroblasts - transfer from Blond McIndoe Centre to the University of Brighton	327
7.3.4.2 Harvesting and freezing of human fibroblasts SKF276	328
7.3.4.3 Standard incubation of human fibroblasts SKF276	328
7.3.4.4 Standard growth vessels for human fibroblasts SKF276	329
7.3.4.5 Standard growth-medium for Fibroblasts SKF276	329
7.3.4.6 Seeding SKF276 cells from frozen stock	329
7.3.4.7 Trypsinizing of human fibroblasts SKF276 in T25 flasks	329
7.3.4.8 Trypsinizing of human fibroblasts SKF276 in T75 flasks	330
7.3.4.9 Trypsinizing of human fibroblasts SKF276 in 24-well plates	330

Contents

7.3.4.10 Mycoplasma screening of human fibroblasts SKF276	330
7.3.4.11 Doubling times of human primary fibroblasts SKF276 in 24-well plates	331
7.3.4.12 Cell proliferation assay of SKF276 cells (I)	331
7.3.4.12.1 MTS assay	332
7.3.4.13 Cytotoxicity assay using human fibroblasts SKF276 (I)	332
7.3.4.13.1 LDH method using SKF276 cells	333
7.3.5 Preparation of samples containing endotoxin	333
7.3.5.1 Bacterial cells disrupted using a sonicator	334
7.3.5.2 Bacterial cells disrupted by phage	334
7.3.5.3 Bacterial cells lysed by antibiotic	334
7.3.5.4 Bacterial cells disrupted using a Bead-Beater	335
7.3.5.5 Bacterial cells disrupted using an autoclave	336
7.3.5.6 Endotoxin test	336
7.3.5.7 Cytotoxicity assay using human fibroblasts SKF276 (II)	338
7.3.5.8 Correlation of cell numbers to MTS absorbance readings	339
7.3.5.9 Cell proliferation assay using SKF276 cells (II)	339
7.3.5.10 Cytokine release of human primary fibroblasts SKN276 challenged with phage	340
7.3.5.10.1 Wash buffer for cytokine experiments	340
7.3.5.10.2 Multi-well plates	340
7.3.5.10.3 Preparation of IL-1, IL-6, IL-8 and TNF- α standards	340
7.3.5.10.4 Sample treatment and dilution of SKN276 supernatant liquids for IL-1 assay- pilot experiment (I)	341
7.3.5.10.5 Cytokine assays for IL-1 for HDF (II)	341
7.3.5.10.6 Cytokine assays for IL-6, IL-8 and TNF- α for HDF	343
7.3.5.10.7 Sample treatment and dilution of SKN276 supernatant liquids for IL-8 assay- pilot experiment (I)	343
7.3.5.10.8 Sample treatment and dilution of SKN276 supernatant liquids for IL-8 assay (II)	344
7.3.5.10.9 Microscopy of SKN276 human fibroblast cells	344

Contents

7.3.6 Cytotoxicity assay for human keratinocytes SKN0106 with 3T3 mouse fibroblast feeder layers	344
7.3.6.1 LDH assay for human keratinocytes SKN0106 with 3T3 mouse fibroblast feeder layers	346
7.3.6.2 MTS assay for human keratinocytes SKN0106 with 3T3 mouse fibroblast feeder layers	346
7.3.7 Colony formation assay using V79 hamster cells	347
7.3.7.1 V79 hamster cells	348
7.3.7.2 Standard growth vessels for V79 hamster cells	348
7.3.7.3 Standard growth medium for V79 hamster cells	348
7.3.7.4 Standard seeding of V79 hamster cells	348
7.3.7.5 Standard incubation of V79 hamster cells	349
7.3.7.6 Trypsinisation of V79 hamster cells grown in T25 flasks	349
7.3.7.7 Colony formation assay to assess the cytotoxicity of phage and lambda buffer on V79 cells	349
7.3.7.8 Colony formation assay to assess the cytotoxicity of phage, diluents and cell extracts with varying endotoxin concentrations on V79 cells	351
7.4 Results	352
7.4.1 Cumulative population doublings of 3T3 Swiss fibroblast cell line	352
7.4.2 Determination of cumulative population doublings over 96 hours in T25 flasks	353
7.4.3 Validation of phage activity in tissue culture conditions without mammalian cells present	354
7.4.4 Validation of phage activity in tissue culture conditions with mammalian cells present	356
7.4.5 Cytotoxicity assay using Trypan Blue and 3T3 cells	360
7.4.6 Cytotoxicity assay using HPI staining and 3T3 cells	366
7.4.7 Cytotoxicity assay measuring LDH release of 3T3 mouse fibroblast cells	369
7.4.8 Cell proliferation assay measuring MTS formation of 3T3 mouse fibroblast cells (I)	371
7.4.9 Cell proliferation assay measuring MTS formation of 3T3 mouse fibroblast cells (II)	372

Contents

7.4.10 Microscopy of 3T3 mouse fibroblast cells	376
7.4.11 Mycoplasma screening of human fibroblasts SKF276	377
7.4.12 Population history of SKF276, after receipt from the Blond McIndoe centre	377
7.4.13 Doubling times of human primary fibroblasts SKF276 in 24-well plates	379
7.4.14 Cell proliferation assay of SKF276 cells (I)	379
7.4.15 Cytotoxicity assay using human fibroblasts SKF276 (I)	382
7.4.16 Viable counts for samples containing endotoxins	384
7.4.17 Multodisc clearance areas	384
7.4.18 Endotoxin levels of various cell extracts	385
7.4.19 Cytotoxicity assay using human fibroblasts SKF276 (II)	387
7.4.20 Correlation of cell numbers to MTS absorbance readings	390
7.4.21 Cell proliferation assay using SKF276 cells (II)	393
7.4.22 Cytokine assays for IL-1 fibroblasts SKF276	396
7.4.23 IL-6 release by fibroblasts SKF276-pilot experiment	397
7.4.24 IL-6 release by fibroblasts SKF276	397
7.4.25 IL-8 release by fibroblasts SKF276-pilot experiment (I)	399
7.4.26 IL-8 release by fibroblasts SKF276	400
7.4.27 TNF- α release by fibroblasts SKF276	402
7.4.28 Microscopy of SKN276 human fibroblast cells	402
7.4.29 LDH assay for human keratinocytes SKN0106 with 3T3 mouse fibroblast feeder layers	404
7.4.30 MTS assay for human keratinocytes SKN0106 with 3T3 mouse fibroblast feeder layers	406
7.4.31 Colony formation assay to assess the cytotoxicity of phage and lambda buffer on V79 cells	409
7.4.32 Colony formation assay to assess the cytotoxicity of phage, diluents and cell extracts with varying endotoxin concentrations on V79 cells	411
7.5 Discussion	419
7.5.1 Cytotoxicity tests	419
7.5.2 Cytotoxicity testing using 3T3 cells	420
7.5.2.1 Validation of phage activity in tissue culture conditions	421

Contents

7.5.2.2 Cytotoxicity assays using dyes	422
7.5.2.3 Cytotoxicity and cell proliferation assays (LDH and MTS assays)	423
7.5.3 Bacterial cell extracts with varying endotoxin concentration	424
7.5.4 Cytotoxicity testing using SKF276 human dermal fibroblasts	426
7.5.4.1 Cytotoxicity and cell proliferation assays (LDH and MTS assays)	427
7.5.6 Cytokine assays for IL-1 released from human dermal fibroblasts SKN276	428
7.5.7 Cytokine assays for IL-6 released from human dermal fibroblasts SKN276	429
7.5.8 Cytokine assays for IL-8 release from human dermal fibroblasts SKN276	430
7.5.9 Cytokine assays for TNF- α release from human dermal fibroblasts SKN276	430
7.5.10 Cytotoxicity testing using SKF276 human dermal keratinocytes	432
7.5.11 Colony formation assay using V79 hamster cells	433
7.6 Conclusion	436
Chapter EIGHT	439
General Discussion	439
8.1 Antibiotic resistance of Acinetobacter spp. strains	439
8.2 Identification, taxonomic status of Acinetobacter spp.	440
8.3 Selective media for the isolation of Acinetobacter spp.	441
8.4 Availability of Acinetobacter phage	442
8.5 Narrow host range	444
8.6 Resistance to bacteriophage	447
8.7 Phage cocktails	448
8.8 Restriction and modification	449
8.9 Phage behaviour in-vitro compared to in-vivo	450
8.10 Lytic nature of phages and transduction	450
8.11 Lack of interest of the pharmaceutical industry in phage	451
8.12 Regulatory agency approval	454
8.13 Intellectual property	455
8.14 Safety of phage- safety of phage preparations	455
8.15 Cytotoxicity- Nanotoxicity	457

Contents

8.16 Cytokine release	458
8.17 Immunogenicity	458
8.18 Ethics	459
8.19 Paradigm shifts	460
8.20 Absence of rigorous proof of efficacy	461
8.21 Conclusion	461
8.22 Suggestions for future work	462
Appendices	
Appendix 1	465
Appendix 2	467
References	472
Abstract & conference proceedings	523

Chapter SIX

Identification and characterization of Canadian bacteriophages and corresponding host bacteria

6.1 Introduction

Bacteriophages active against *Acinetobacter* species are not available in most commercial culture collections. Acquisition of such viruses therefore entailed searching the literature to request samples from groups who had recently been working with this material. The Felix d'Hérelle Reference Center for Bacterial Viruses, GREB, Faculté de Médecine Dentaire, Université Laval, Canada, agreed to a request for samples and provided 10 phage lysates and corresponding host bacteria (see **Section 4.2.6.2**). Information regarding exact origin and identity of the isolates was unclear as Coffi (1995), who had worked with at least one of the bacterial isolates and corresponding phage used different identifier codes to the accession numbers assigned by the Felix d'Hérelle Reference Center for Bacterial Viruses.

The primary objective of this study was therefore to purify the specimens and characterize both the bacteriophages and their host cells.

6.2 Material and Methods

6.2.1 Propagation of host bacterial species

10 isolates of *Acinetobacter* spp. and corresponding phages were obtained from Laval University, Canada. During transport all bacterial strains were stored on transport agar in 2ml cryovials while phage material was sent in the liquid lysate form in 1.5ml conical cryovials. The necks of all vials had been wrapped with plastic wrap. During shipment the material had not been refrigerated

On arrival all bacterial strains were streaked onto TSA plates and incubated at 37°C for 16-18 hours. All strains showed vigorous growth at this temperature except for strain HER1423 which failed to grow at temperatures above 30°C and consequently required incubation at 25°C.

6.2.2 Long term storage of bacterial cultures

3-4 colonies were picked from each TSA plate and used to inoculate 100ml of TSB, which was incubated at the optimum temperature (37°C for all strains except HER1423 which was incubated at 25°C). All inoculated broths were incubated for 16-18 hours on a shaking platform at 120rpm, and then supplemented with 10% v/v sterile glycerol, before pipetting into cryovials and freezing at -80°C.

6.2.3 Routine culture and growth of bacterial cultures

Stock cultures were produced by thawing the contents of one cryovial each month and using one loopful to streak on TSA plates for single colonies. The plates were incubated at 37°C (25°C for HER1423) for 16-18 hours. TSA plates containing stock cultures were kept refrigerated at 4°C and were sub-cultured weekly.

For liquid cultures a single colony was removed from the stock culture plate and vigorously dispersed in 10ml TSB. Cells were grown for 16-18 hours in loosely capped glass universal bottles at 37°C (25°C for HER1423) on a shaking platform rotating at 120rpm.

6.2.4 Phenotypic properties of bacterial cultures

Gram stain, microscopic appearance, motility and growth requirements were investigated and oxidase and catalase tests performed. For methods see **Section 2.4**.

Results are described in **Section 6.3.1**

6.2.5 Identification of bacterial cultures using API20NE test system

The bacterial cultures were prepared according to the manufacturer's instructions and analyzed using the API20NE, (bioMérieux, Lyon, France) test kits. The results were interpreted in conjunction with oxidase test results. The methods used are described in **Section 3.2.7**.

The results are shown in **Section 6.3.2**

6.2.6 Growth of bacterial cultures at 25, 30, 37, 42 and 44°C

TSA plates were routinely sub-cultured (see **Section 6.2.3**) and incubated for 16-18 hours at 37°C (25°C HER1423). 1-2 colonies were used to streak TSA plates, which were incubated at 25, 30, 37, 42 and 44°C respectively for 16-18 hours. 1 colony of each isolate was used to inoculate 2ml and 10ml of TSB which were incubated at 42°C or 44°C to allow differentiation of the *Acinetobacter calcoaceticus-baumannii* complex (Kämpfer, 1993) for 16-18 hours, the liquids were shaken at 120rpm.

After incubation the plates were assessed visually using a magnifying glass. Growth in TSB was assessed turbidimetrically.

The results are shown in **Section 6.3.3**

6.2.7 Antibiotic susceptibility of bacterial cultures using the BSAC disc diffusion method

The accession numbers assigned by Université Laval were different to the short codes used by Coffi (1995), who had worked with some if not all the provided isolates. Coffi (1995) had loosely described the isolates used for his research as partly from clinical sources. It was unclear if individual isolates were of clinical origin and what antibiotic resistance patterns they might exhibit. It was therefore important to identify antibiotic

resistance profiles of the Canadian isolates and compare them with *Acinetobacter* isolates obtained from other sources.

The method is described in Section 3.3.1, all results were interpreted using BSAC break points, (BSAC Disc Diffusion Method for Antimicrobial Susceptibility Testing version 5, 2006) to ascertain the antimicrobial susceptibility of the bacterial isolates.

The results are shown in Section 6.3.4

6.2.8 Growth characteristics of bacterial cultures on Leeds *Acinetobacter* Medium (LAM) with varying antibiotic compositions

LAM was produced according to instructions, without addition of antibiotics and aseptically divided into 50ml aliquots (see **Section 3.4.1 - 3.4.3.**). One LAM agar aliquot was poured antibiotic-free, to the rest vancomycin 10mg/l cefsulodin 15 mg/l and cephadrine 50 mg/l were added in varying combinations, mixed and poured into plates. One set of plates was produced containing all three antibiotics.

Various *Acinetobacter* strains were streaked from thawed frozen stocks to obtain single colonies on TSA and grown for 16-18 hours at 37°C, except for HER1423. Inocula were prepared by using a 200µl pipette tip to transfer one single colony from each TSA plate into 100µl PBS, which was triturated and vortexed to form a homogeneous suspension. Each plate was divided into six sections; a nichrome loop with 4mm diameter was used to apply the inoculum to one section of each LAM agar and TSA plates, which were used as positive control. For comparison *S. aureus* ATCC 6538, *P. aeruginosa* ATCC 9027, *Citrobacter freundii* ATCC 8090, *E. coli* TM3, and *E. coli* ATCC 8739 were treated in the same way. All plates were incubated for 16-18 hours at 37°C except HER1423 which was incubated at 25°C. After incubation the presence of growth and colony morphology was examined.

The results are shown in **Section 6.3.5**

6.2.9 Determination of host for each bacteriophage

As only a small volume of lysate had been provided it was necessary to identify a host suitable for propagation of each bacteriophage, to produce larger volumes of crude filtered lysate and subsequently purified phage preparations. Two different phage screening methods (surface spotting and soft agar spotting method) were chosen to match each phage to at least one host.

6.2.9.1 Optimization of surface spotting method

All samples were routinely incubated at 37°C, except for HER1423 which was always grown at 25°C. Universal bottles containing 10ml TSB were inoculated with 1-2 colonies of *Acinetobacter spp.* and incubated at their optimum temperature for 16-18 hours on a shaking platform set to 120rpm. This overnight suspension was centrifuged at 2011g for 10 minutes. The supernatant liquid was decanted and the cell pellet re-suspended in 10ml PBS. The optical density of the suspension was measured and adjusted with PBS to yield approximately 1×10^8 cfu/ml (see **Section 2.9**). This standardized cell suspension was used for experimentation within 15 minutes.

Sterile cotton buds were dipped into this cell suspension and streaked over the TSA plate in a 4 way fashion, to ensure the plate was evenly inoculated. The plates were incubated for 2 hours at the respective optimum growth temperatures to yield an even lawn. After two hours the position of phage droplets to be applied was marked on the back of the plates and 10-fold dilutions in lambda buffer of the original Canadian bacteriophages lysates were applied as 5 µl drops using a multipoint pipette, allowing delivery of 10 droplets per 9cm standard Petri dish.

The droplets were allowed to soak into the agar and all plates were incubated in inverted position for 16-18 hours. Circular areas containing no bacterial growth or individual colonies (Clearance zones) were regarded as evidence of lytic activity of phage. Thinning of the bacterial lawn implied some lytic activity or was seen as a sign of potential lysogeny.

The results are shown in **Section 6.3.6**.

6.2.9.2 Soft agar spotting method

Variations of surface spotting methods to detect phage are employed routinely by other investigators as described by Kutter & Sulakvelidze (2005).

Universal bottles containing 10ml TSB were inoculated with 1-2 colonies of *Acinetobacter spp.* at 37°C, except for HER1423 which was always grown and infected at 25°C. The bottles were incubated for 16-18 hours on a shaking platform rotating at 120rpm. Sterile 5ml aliquots of 0.4% w/v Oxoid Technical agar No.3 in TSB (soft agar) in 15ml bijoux glass bottles were placed in a boiling water bath for 5minutes. When the contents had melted the bottles were maintained at 50°C in a heated water bath for at least 90 minutes to allow the agar to cool but remain liquid.

100µl of the same cell suspension, as used for the optimised surface spotting method (**Section 6.2.9.1**) were used to inoculate 5ml aliquots of the soft agar, which were vortexed for 5 seconds and poured over a set plate of TSA. After letting the soft agar set for 60 minutes on the bench, the position of droplets was marked at the back of each plate and 5 µl drops of phage preparation were applied using a multipoint pipette.

The same criteria for interpretation of the results were adopted as described above.

The results are shown in **Section 6.3.6**.

6.2.9.3 Production of stock bacteriophages lysates

In order to produce a reasonable volume of phage stock the liquid lysate method was employed (see **Section 2.7**) to produce at least 50ml of crude filtered lysate for each phage.

Serial 10-fold dilutions of crude lysates were prepared using lambda buffer for viability testing. All crude lysates were tested for viability and presence of lytic phage (see **Section 2.3.4**) using the soft agar overlay method. In addition large volumes (400ml) of purified lysates were produced for all of those phages which yielded plaques with the crude filtered lysates (see **Sections 2.7- 2.7.2.1**).

6.2.10 Selection criteria of suitable phage and host for further experiments

In order to select the most suitable phage and host for further experiments a number of selection criteria were applied as follows:.

- Reliable plaque formation.
- Crisp plaques seen at 37°C.
- Production of circular clearance zones.
- Formation of sufficient amounts of recoverable precipitates.
- Phage titre $>10^{10}$ pfu/ml.

Most of the criteria relate to the ability of the phage in question to demonstrate lytic behaviour and hence should reflect their suitability for potential therapeutic use. Bacteriophages which fulfilled the first two conditions were used to produce large volumes of purified lysate, only bacteriophages which met all five criteria were considered for further experiments.

The results are shown in **Section 6.3.7**

6.2.10.1 Reliable plaque formation

Production of plaques must be repeatable using crude and purified lysates, at a range of dilutions using the soft agar method (see **Section 2.3.4**). This would avoid the potential inclusion of temperate phages in future experiments, as phage therapy requires the use of lytic phage.

6.2.10.2 Crisp plaques seen at 37°C

It is not only important to be able to produce plaques during experiments, but they must be obvious to the operator. When using the soft agar method, plaques should be circular, if not symmetric and easy to differentiate from the background lawn with the naked eye. Plaques with blurred or ‘fuzzy’ edges or turbid plaques which seem to blend in with the background bacterial lawn are an indication of potential lysogeny (Kutter &

Sulakvelidze, 2005). Since for therapeutic purposes it would be necessary for the phage to be lytic at 37°C it was deemed appropriate to only include phage which displayed lytic behaviour at this temperature.

6.2.10.3 Production of circular clearance zones

In addition to plaque formation, production of clearance zones with crude and purified lysates (using the revised surface spotting method, see **Section 6.2.9.1**) is a good indicator of lysis.

Discrete, circular clearance zones were considered an essential factor for visibility and ultimately allowing accurate quantitative work.

6.2.10.4 Formation of sufficient amounts of recoverable precipitate

When using the standard protocol to produce purified lysate (**Section 2.7.2**) normally a precipitate forms after PEG 8,000 addition. In some cases the volume of the precipitate was so small it was extremely difficult to recover. Even when greatest care was taken to re-suspend the precipitate in lambda buffer the yields of the purified preparation were low. For future scale-up it was therefore necessary to include this feature as a required characteristic.

6.2.10.5 Phage titre > 10¹⁰ pfu/ml

In most experiments it was necessary to use concentrated phage preparations as starting material. Purified lysates are likely to include low concentrations of endotoxin and small amounts of chemicals which were added to facilitate the purification steps e.g. chloroform, HCl, PEG or NaCl. By being able to include several serial dilutions before using the phage preparation these potentially toxic chemicals were considered to be present in such low concentrations that any effects on bacterial or mammalian cells would be negligible.

All phage and host pairs which gave rise to lysis in both screening methods were used to produce 2x200ml of lysates. Stringent selection criteria were applied (see **Table 6.13**) to identify the optimum candidates for the large scale production of purified

lysates. Each phage and host pair was assessed on five occasions using selection criteria introduced in **Section 6.2.10**.

6.2.11 Analysis of infection cycle of phage HER1151 in host bacteria

9.9ml of HER1151 suspension with an adjusted concentration of 5×10^8 cfu/ml was dispensed into 5 different 15ml PP Fisherbrand centrifuge tubes. Each sample was simultaneously infected with 0.1ml aliquots of purified phage preparation HER151 E13 (with a titre of 5×10^9 pfu/ml).

The final ratio of cell to phage was 10:1 in a total volume of 10ml, a Multiplicity of infection (MOI) of 0.1. The phage-cell suspensions were vortexed and incubated at 37°C in a stationary incubator. At time points 1, 5, 10, 15, 20, 25, 40, 60 and 120 minutes 0.1ml were removed and immediately placed in 1.5ml Eppendorf tubes containing 900 µl of lambda buffer and immediately syringe filtered, using 0.45 µm Millipore syringe filters. This was done to dilute the suspension 10-fold and to keep the volume removed from the reaction vessel to a minimum, yet producing sufficient volume for syringe filtration. After syringe filtration an aliquot from each filtrate was further diluted in 10-fold steps in sterile lambda buffer. Each dilution of every one of the four suspensions at each time point was plated using the soft overlay method. In addition a viable count of the infected bacterial suspension was performed to enumerate the actual number of cells used. Results **Section 6.3.8**

6.3 Results

6.3.1 Phenotypic properties of bacterial cultures

All isolates were Gram negative, oxidase negative and catalase positive (**Table 6.1**). The majority of isolates presented as short rods under the microscope, giving the appearance of coccobacilli in the stationary phase. None of the isolates appeared to be motile or displayed any twitching motility.

Test	Outcome
Gram stain	Negative
Microscopic appearance	Rods (Coccobacilli in stationary phase)
Oxidase	Negative
Catalase	Positive
Growth requirement	Strictly aerobic

Table 6.1 Phenotypic properties of Canadian isolates

6.3.2 Identification of bacterial cultures using API20NE test system

Table 6.2 gives the API20NE profiles of each strain after 24 and 48 hour incubation.

These profiles enabled the identity of all strains to be confirmed at least to genus level and in some cases to the species level (Towner & Chopade 1987; Begogne-Berezin & Towner 1996). However, it must be borne in mind that despite the acknowledged existence of at least 19 genomic species of *Acinetobacter* only 5 species can be found within the API20NE database (Begogne-Berezin & Towner 1996).

Strain short code	24h profile	48h profile
HER1032	0000473	0000473
HER1033	0001072	0001073
HER1050	0001063	0001063
HER1096	0041473	0041073
HER1151	0041451	0041051
HER1156	0000070	0000070
HER1162	0001073	0041473
HER1401	0001053	0001053
HER1423	0000040	0000040
HER1425	0000051	0000051

Table 6.2: 24 and 48 hour API20NE profile of *Acinetobacter* isolates

Table 6.3 links the strain short code assigned by the originators with the genus name. HER1156, HER1096, HER1425 were confirmed to be *Acinetobacter* spp., however could not be further identified using differentiation criteria such as growth at different temperatures, phenotypic properties and API20NE criteria.

The dilemma of reliably assigning an isolate to a species on grounds of phenotypic properties was recognized by Gerner-Smidt (1994) who identified that the phenotypic identification of *Acinetobacter* spp. is problematic. Despite claims by commercial manufacturers of identification systems that their product is able to discriminate between DNA groups, this has never been supported by published genotypic data.

HER1156	<i>A. lwoffii</i> or <i>A. junii/johnsonii</i>
HER1050	<i>A. baumannii</i>
HER1033	<i>A. baumannii</i>
HER1096	<i>A. baumannii/ calcoaceticus</i>
HER1423	<i>A. lwoffii</i>
HER1162	<i>A. baumannii</i>
HER1151	<i>A. baumannii</i>
HER1425	<i>A. junii/johnsonii</i>
HER1032	<i>A. baumannii</i>
HER1401	<i>A. baumannii</i>

Table 6.3 Identity of *Acinetobacter* strains by interpretation of API20NE profiles.

6.3.3 Growth of bacterial cultures at 25, 30, 37, 42 and 44°C

The growth of strains at various incubation temperatures on TSA is shown in **Table 6.4** and in TSB in **Table 6.5**.

The majority of strains grew vigorously at all temperatures ranging from 25 to 44°C.

The exceptions included HER1423, which could not be propagated at temperatures of 37°C or above. HER1156 grew at 37°C, yet failed to grow at the higher temperatures of 42°C or above. HER1050 showed a preference for higher temperatures, yet failed to grow at 25°C. HER1425 grew at higher temperatures up to 42°C, yet did not grow at 44°C.

Strain name	25°C	30°C	37°C	42°C	44°C
HER1032	+	+	+	+	+
HER1033	+	+	+	+	+
HER1050	-	+	+	+	+
HER1096	+	+	+	+	+
HER1151	+	+	+	+	+
HER1156	+	+	+	-	-
HER1162	+	+	+	+	+
HER1401	+	+	+	+	+
HER1423	+	+	-	-	-
HER1425	+	+	+	+	-

Table 6.4 Growth of *Acinetobacter* strains at various incubation temperatures on TSA

Legend Table 6.4 & 6.5: '+' growth, '-' no growth detected; OD_{600} values above 0.1 were counted as weak growth.

Strain short code	42°C		44°C	
	2ml	10ml	2ml	10ml
HER1032	+	+	+	+
HER1033	+	+	+	+
HER1050	+	+	+	+
HER1096	+	+	+	+
HER1151	+	+	+	+
HER1156	-	-	-	-
HER1162	+	+	+	+
HER1401	+	+	+	+
HER1423	-	-	-	-
HER1425	+	+	-	-

Table 6.5 Growth of bacterial cultures at higher temperatures in TSB

6.3.4 Antibiotic susceptibility of Canadian isolates using the BSAC disc diffusion method

All Canadian strains were sensitive to amikacin, amoxicillin & clavulanic acid, imipenem and ciprofloxacin (**Tables 6.6 and 6.7**).- this was in contrast to the 9 Sussex clinical isolates described in **Chapter 3**. Due to this difference it is unlikely that the Canadian strains were isolated during the course of an outbreak, however this does not automatically imply that this collection of isolates consists of only environmental isolates. The suppliers were not clear as to the origins of these isolates.

All isolates were resistant to aztreonam, except for HER1425; Only HER1151 and HER1033 were resistant to gentamicin. Test discs containing 30 µg of amoxicillin/ clavulanic acid were used, rather than the 10µg recommended by BSAC, all strains

were sensitive at this strength used. The same strains showed different susceptibility profiles to different cephalosporins. Only HER1032 and 1401 were sensitive to ceftazidime, however all strains were resistant to cefixime and four strains sensitive to cefuroxime.

Antibiotic	Code	HER1032	HER1033	HER1050	HER1096	HER1151
Amikacin	AK	S	S	S	S	S
Amoxicillin/clav	AMC	S	S	S	S	S
Aztreonam	ATM	R	R	R	R	R
Cefixime	CFM	R	R	R	R	R
Ceftazidime	CAZ	S	R	R	R	R
Cefuroxime sod.	CXM	R	R	S	R	S
Ciprofloxacin	CIP	S	S	S	S	S
Gentamicin	CN	S	R	S	S	R
Imipenem	IPM	S	S	S	S	S
Piperacillin	PRL	S	R	S	R	R
Tetracycline	TE	R	R	R	R	R
Trimethoprim	W	S	S	S	S	S

Table 6.6 Antibiotic sensitivities of Canadian isolates

Legend to Tables 6.6 & 6.7: *R – Resistant, S- Sensitive; Amoxicillin/clav.= amoxicillin with clavulanic acid*

Antibiotic	Code	HER1156	HER1162	HER1401	HER1423	HER1425
Amikacin	AK	S	S	S	S	S
Amoxicillin/clav	AMC	S	S	S	S	S
Aztreonam	ATM	R	R	R	R	S
Cefixime	CFM	R	R	R	R	R
Ceftazidime	CAZ	R	R	S	R	S
Cefuroxime sod.	CXM	R	R	R	S	S
Ciprofloxacin	CIP	S	S	S	S	S
Gentamicin	CN	S	S	S	S	S
Imipenem	IPM	S	S	S	S	S
Piperacillin	PRL	S	R	S	S	R
Tetracycline	TE	R	R	S	S	R
Trimethoprim	W	R	S	S	R	R

Table 6.7 Antibiotic sensitivities of Canadian isolates

Only two isolates were sensitive to tetracycline, which is in contrast to the 9 clinical Sussex isolates, which were all resistant. This agent was included as it is structurally related to tigecycline, which has been reported to show *in-vitro* activity against multi-resistant *Acinetobacter baumannii* (Livermore, 2005). BSAC removed breakpoints of tetracycline in version 4, 2005, to allow a review of breakpoints for this group of agents. As the BSAC Disc Diffusion Method for Antimicrobial Susceptibility Testing version 5, 2006 did not contain breakpoints for this drug, breakpoints from version 3, Jan 2004 at 33/34 mm were used to interpret the data.

6.3.5 Growth characteristics of Canadian isolates on Leeds *Acinetobacter* Medium (LAM) with varying antibiotic compositions

Strain	No AB	Cep	Van	Cef	Cep+ Cef	Van+ Cef	Van+ Cep	Cep+ Cef+ Van
HER1032	+	+	+	-	-	-	+	-
HER1033	+	+	+	-	+	+	+	-
HER1050	+	+	+	+	-	+	+	-
HER1151	+	+	+	-	-	-	+	-
HER1156	+	+	+	+	+	+	+	+
HER1162	+	+	+	+	+	+	+	+
HER1096	+	+	+	+	+	+	+	+
HER1401	+	+	+	+	+	+	+	+
HER1423	+	+	+	+	+	+	+	+
HER1425	+	+	+	-	-	-	+	-

Table 6.8 Growth of Canadian isolates on LAM with varying antibiotic components (n=2)

Legend to Tables 6.8 & 6.9: CEP- Cephadrine supplementation of LAM, CEF- Cefsulodin supplementation of LAM, VAN- Vancomycin supplementation of LAM, No AB- No Antibiotic supplementation, LAM with 3 AB- LAM supplemented with cephradine, cefsulodin and vancomycin; '+' growth, '-' no growth

The results shown in **Tables 6.8** and **6.9** indicate that all *Acinetobacter* spp. grew on LAM without antibiotic supplementation, which is in line with previous results (**Chapter 3**) and is further evidence that antibiotic free LAM can be a useful tool in aiding identification and isolation of *Acinetobacter* spp.

Strain	No AB	Cep	Van	Cef	Cep+ Cef	Van+ Cef	Van+ Cep	Cep+ Cef+ Van
<i>E.coli</i> TM3	-	-	-	-	-	-	-	-
<i>E. coli</i> 8739	-	-	-	-	-	-	-	-
<i>S. aureus</i> 6538	-	-	-	-	-	-	-	-
<i>P. aeruginosa</i> 9027	-	-	-	-	-	-	-	-
<i>Citrobacter freundii</i> ATCC 8090	+	+	+	+	+	+	+	+
<i>Serratia marcescens</i>	+	+	+	+	+	+	+	+

Table 6.9 Control strains on LAM agar with varying antibiotic components (n=2)

As LAM with different antibiotic supplementation or no antibiotic were not used by Jawad et al. (1994) it is difficult to compare the results with existing literature. As was shown previously with other *Acinetobacter* spp. strains (see **Chapters 3, 4 and 5**) susceptibility to different types of cephalosporins varied even within individual strains(see **Table 6.8**).

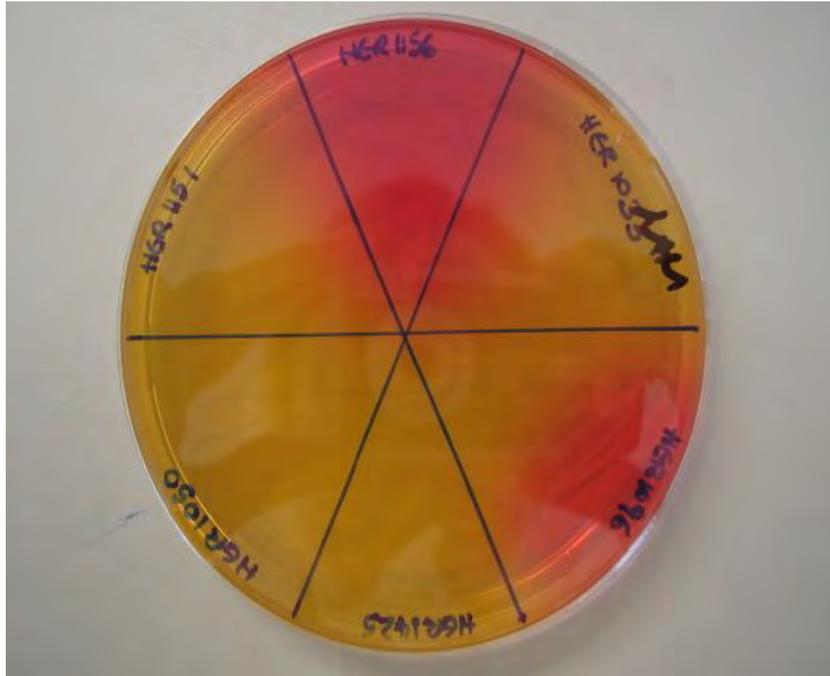


Figure 6.1 Growth behaviour of selected Canadian *Acinetobacter* species on LAM



Figure 6.2 Three Canadian bacterial species alongside three control species on LAM

This distinct sensitivity to different cephalosporins, which could be already demonstrated in **Section 6.2.7**, is reflected in the growth characteristics of these strains on LAM with varying antibiotic composition (**Table 6.8**), where HER1032 and

HER1033 grew on LAM supplemented with cephadrine alone but not LAM containing cefsulodin.

All *Acinetobacter spp.* with positive growth on any LAM agar, regardless of antibiotic composition displayed the typically clearly visible growth of pink colonies with a change of agar colour to mauve (**Figure 6.1**). On plates where no growth could be seen the agar colour remained orange.

STRAIN SHORT CODE	LAM	API20NE READING (48 hours)	Species
HER1032	-	0000473	<i>A. baumannii</i>
HER1033	-	0001073	<i>A. baumannii</i>
HER1050	-	0041073	<i>A. baumannii</i>
HER1096	+	0041073	<i>A. baumannii</i>
HER1151	-	0041051	<i>A. junii/ johnsonii or A. baumannii/calcoaceticus –</i>
HER1156	+	0000070	<i>A. lwoffii or A. junii/johnsonii</i>
HER1162	+	0041473	<i>A. baumannii</i>
HER1401	+	0001053	<i>A. baumannii</i>
HER1423	+	0000040	<i>A. lwoffii</i>
HER1425	-	0000051	<i>A junii/johnsonii or A. haemolyticus</i>

Table 6.10 Correlating growth on LAM agar with *Acinetobacter* species

In almost all cases all strains displayed either luxurious growth or none at all. One exception was strain HER1033 which grew only sparingly on LAM containing a combination of vancomycin and cefsulodin. It displayed weak growth with typical pink

colonies, only the agar area around the growth changed colour to mauve, the rest of the plate remained orange.

Only 5 out of 10 species successfully grew on LAM with the full complement of antibiotics, however all species grew successfully on the antibiotic free LAM. All isolates grew on LAM when only vancomycin or cephradine were present, however four proved sensitive to cefsulodin.

HER1050 was sensitive to a combination of cephradine and cefsulodin, despite successfully growing in the presence of each cephalosporin individually. Similar to previous experiments (**Chapters 3-5**) none of the control species grew on LAM, except *Citrobacter freundii* ATCC8090 with yellow colonies on yellow agar and *Serratia marcescens* with pink colonies on orange background (see **Figure 6.2**), which matches the findings of Jawad et al. (1994) (see **Table 6.9**).

Jawad et al. (1994) attempted to link growth on LAM with taxonomic identification, as reportedly *Acinetobacter johnsonii* and *Acinetobacter haemolyticus* failed to grow on LAM, due to the properties of this medium. A wider range of species failed to grow on LAM (**Table 6.10**) than reported by Jawad et al. (1994), which appear to make LAM unsuitable as a taxonomic tool. The correlation of API20NE reading to the respective species proved extremely difficult, despite each test being repeated three times. To allow comparison with other test kits entire 24 and 48 hour profiles were documented (**Section 6.3.2**). It is apparent that LAM agar is over-selective, as nearly 50% of selected isolates failed to grow on LAM, however all isolates grew on antibiotic-free LAM. Cultures of *E. coli*, *S. aureus* and *P. aeruginosa* failed to grow on antibiotic-free LAM and as a consequence on LAM itself. Three *A. baumannii* isolates grew on LAM, yet two failed to, which may be more telling of the antibiotic resistance patterns of the individual strains than the selective properties of LAM agar and is further evidence that LAM should not be relied upon for species identification.

Due to growth inhibition of common Gram negative and positive bacteria but permissive growth of *Acinetobacter* by antibiotic-free LAM, it may prove useful to use antibiotic-free LAM and LAM in parallel for future experiments. However due to the over-selectivity of LAM agar containing the full complement of antibiotics it would

appear to be unwise to rely solely upon this medium for isolation or assignment of isolates to specific species.

6.3.6 Determination of host for each bacteriophage

Bacterial strain	Phage	Surface spotting	Soft agar spotting
HER1032	HER32531	Clearance	Clearance
HER1033	HER33 A3/2	VDL	Shadow
HER1050	HER50 A10/A45	Clearance	Clearance
HER1151	HER151 E13	Clearance	Clearance
HER1156	HER156 E14	Shadow	VDL
HER1162	HER162 B9GP	Clearance	Clearance
HER1401	HER401 BS46	Clearance	Clearance
HER1423	HER423 133	Clearance	Clearance
HER1425	HER425 2213/73	Shadow	VDL

Table 6.11 Results of two phage screening methods of Canadian host and phage pairs

The basic concept of these optimized surface spotting methods is that each sample is spotted on a lawn of the potential host. None of the standard textbooks contain this method, yet it proved to be a fast method to screen potential hosts against large numbers of phage samples (Adams, 1959; Kutter & Sulakvelidze 2005). The advantage of this method over the more elaborate approach of Adams (1959) was that it allowed simultaneous screening of several phage preparations on one plate at the same time. Adams (1959) suggested pre-mixing bacterial culture with the phage preparation and then surface spreading it onto agar plates, which is material intensive when large numbers of host and potential phage preparations are to be processed.

To allow repeatable results the bacterial suspension was washed and standardized; a step omitted in customary protocols (Adams, 1959; Kutter & Sulakvelidze 2004). Swabbing of the bacterial suspension across the plate rather than spreading ensured a reproducible quality of bacterial lawn was achieved. This even and relatively thin lawn allowed easy detection of potential clearance zones. Even faint thinning could be observed when backlighting was used, and this proved particularly helpful in identifying phages which may not lyse the host, but only halt its growth. By careful observation phages which affected the growth behaviour of the host and may be potentially temperate, could be detected. This technique helped avoid false negatives where resistant mutants present in large numbers may overgrow the original isolate. This technique proved to be less labour intensive than the technique employed by Adams (1959), yet due to the enhanced properties of the bacterial lawn detection of phage material became uncomplicated.

The results of both phage screening methods (**Tables 6.11 & 6.12**) were combined to establish whether a particular phage could be lytic or may be potentially utilising more than one host (promiscuous). Such bacteriophages may be suitable for further experiments and potentially useful for phage therapy.

Bacterial strain	Phage	Surface spotting	Soft agar spotting
HER1162	HER96 B9PP	Clearance	Clearance
HER1401	HER50 A10/A45	Clearance	VDL

Table 6.12 Results of two screening methods of previously unmatched host and phage

Legend to Tables 6.11 & 6.12: *VDL- Very dense lawn*

The surface spotting and soft agar spotting methods allowed a high throughput whilst using low volumes of sample, compared to the multiple dilutions and soft agar overlays required for each of the bacterial hosts and phages for conventional screening.

Laval University had originally assigned one phage lysate to one bacterial host (**Table 6.11**), however during the screening procedure it transpired that phages HER96 B9PP and HER50 A10/A45 may have more than one host, which had not been explored by the originators (**Table 6.12**). The growth of strain HER1162 appeared to be affected by phages HER162 B9GP and HER96 B9PP, and HER1401 was susceptible to HER401 BS46 and HER50 A10/A45; the latter association in each case was not confirmed by the originators. HER96 B9PP gave rise to clear areas when grown with host HER1162, however the absence of plaque formation in this combination made this arrangement less interesting in the context of utilizing it for phage therapy. A similar scenario arose with phage HER50A10/A15 and HER1401 as host where the soft agar spotting method resulted in a very dense lawn, which led to the conclusion that lytic behaviour of this phage in combination with the host was unlikely. In the case of HER96 B9PP the combination with host HER1096 led to occasional plaque formation which did not occur with host HER1162, hence the combination of HER96 B9PP and HER1096 was pursued for the production of lysates.

The information provided by the originators did not disclose which phages would be lytic or temperate using the respective hosts, hence the combination of screening methods was essential before selecting phage and host pairs for a larger scale production of purified lysates for the purpose of potential phage therapy.

In **Tables 6.11 & 6.12** 'VDL' describes the presence of a 'very dense lawn', which indicates that the area where the phage droplet was applied was optically not different from the surrounding lawn, when observed with the naked eye or a magnifying glass with additional lighting. 'Shadow' indicated that the area where a phage droplet was applied showed less confluent growth than the surrounding areas of the bacterial lawn on one plate. When phages HER156 E14, HER425 2213/73 and HER33 A3/2 were screened against HER1156, HER1425 and HER1033 they gave rise to a 'shadow area', which according to Adams (1959) may be an indication that bacterial growth may be suppressed, however the phage may not be virulent enough to cause lysis. According to Adams (1959) the concept of virulence is not absolute, it can be increased by repeated subculture of phage in liquid culture, however this was not attempted in this instance as multiple subculture and purification cycles would have proved time consuming and other phage and host pairs which gave rise to lytic plaques were available.

Overall the surface spotting appeared to be easier to use than the soft agar spotting, due to the decreased number of stages and materials to produce a single plate. In all instances except in the case of HER1033 screened against HER33 A3/2 (**Table 6.11**) the surface spotting method seemed more sensitive than the soft agar spotting method. Due to the apparent sensitivity and the reduced strain on resources the surface spotting method appears more favourable,

6.3.7 Selection criteria of suitable phage and host for further experiments

Strain Identifier	Reliable Plaque formation	Crisp plaques seen at 37°C	Clearance zones at 37°C	Recoverable precipitate	Phage titre > 10 ¹⁰ pfu/ml
HER33 A3/2	No	No	No	Not done	Not done
HER42522 13/73	No	No	Yes	Not done	Not done
HER156 E14	No	No	Yes	Not done	Not done
HER401 BS46	Yes	Yes	Yes	Yes	1.3 x 10 ¹⁴
HER96 B9PP	No	No	Yes	No	Not done
HER32531	Yes	No	Yes	Yes	Not done
HER50 A10/A45	No	No	No	Not done	Not done
HER162 B9GP	No	No	No	Yes	Not done
HER151 E13	Yes	Yes	Yes	Yes	1.3 x 10 ⁹
HER423 133	Yes	No	Yes	Yes	2.8 x 10 ¹⁰

Table 6.13 Meeting of selection criteria of five bacteriophages for future work

Legend Table 6.13: *Phage titers as pfu/ml*

With regards to phage and host pairs meeting the selection criteria, the results of the surface spotting method and soft agar spotting method differed in individual cases

(**Tables 6.11 & 6.12**). The soft agar spotting method was technically more difficult, as the fresh layer of soft agar was moist and did not allow the phage droplets to be absorbed readily.

The phage and host combinations listed in **Table 6.12** (HER1162 & HER96 B9PP and HER1401 & HER50 A10/A45) were also assessed for their suitability for use in future experiments and included in **Table 6.13**. However, regardless of which host was used for propagation neither phage was suitable for further experimentation.

As a point of interest, the lysate of HER1096 & HER96 B9PP occasionally but unreliably lead to plaques. When the unpurified and filtered lysate was used for surface spotting to confirm activity of lysate it did not lead to clearance zones. Supplementation with chloroform led to the formation of clearance zones when compared to a blank. This was further supporting evidence that HER96 B9PP when grown with HER1096 was temperate.

HER401BS46 fulfilled all the criteria, except that when the titre was established it was noticed that each plaque was surrounded by a circular ‘halo’ (**Table 6.13**). This circular ‘halo’ area showed a confluent bacterial lawn which appeared to be less dense than the surrounding lawn, which may be considered a sign of potential lysogeny (Kutter & Sulakvelidze, 2005), however plaques with similar appearance published by Soothill (1992) proved to be highly active *in vitro* and *in-vivo*.

The unusually high titre of 1.3×10^{14} was confirmed several times, using freshly prepared dilutions on each occasion. Leviviridae have been reported to be concentrated to titres of 10^{14} pfu/ml (Joshi, 1982; Lohr 2005; McKay et al., 1993). The titre of HER401BS46 dropped after long term storage at 4°C with a visible precipitation forming. Ellis and Delbrück (1939) reported the same phenomenon in one phage preparation, where a drop in phage titre occurred, followed by precipitation which sedimented in the storage vessel. The preparation was filtered and re-titrated four times (n=5), the average titre was calculated as 8.7×10^9 pfu/ml. The drop in phage numbers was accounted for in experiments following the re-titration. HER32531 fulfilled all the criteria, except that plaques were very difficult to distinguish from the background bacterial lawn, even when a backlit plate counter and a magnifying glass were used. This disqualified it from being a suitable phage preparation for further experiments,

despite repeatable plaque formation at various concentrations at 37°C. HER423133 only gave rise to plaques when infected and incubated at 25°C; this made it an unsuitable candidate for experiments performed at physiological temperatures.

In summary HER151 E13 and HER401BS46 appeared to be the most suitable candidates for future experiments, as they reliably formed plaques at 37°C, which were easy to count. As the phage titre in the purified preparation of HER151 E13 was lower than other preparations, it was considered less suitable for experiments where concentrated samples were required.

6.3.8 Analysis of infection cycle of phage HER1151 in host bacteria

Figure 6.3 shows that the phage count dropped from 5×10^7 pfu/ml at t_0 to a mean recoverable phage count of 6×10^6 pfu/ml ($n=4$) after a one minute exposure to bacterial cells. According to (Kutter & Sulakvelidze, 2005) a reduction in recoverable phage numbers, even by two orders of magnitude immediately after infection can be routinely observed when T4-like phages infect *E. coli*. Starting from time point of t_1 after infection with phage there is usually a further slight drop in phage numbers, followed by an increase and then a second plateau in phage numbers leading on to a significant increase in plaque numbers.

Delbrück (1940) observed a similar phenomenon and attributed it to a large number of phage particles being bound to one bacterial cell, which then gives rise to only a single plaque.

A variety of different experimental approaches have been published under the collective name of 'single step' or 'one-step growth'. It could be argued that the experiments devised by Delbrück (1940) are so different from this experiment that they can hardly be compared.

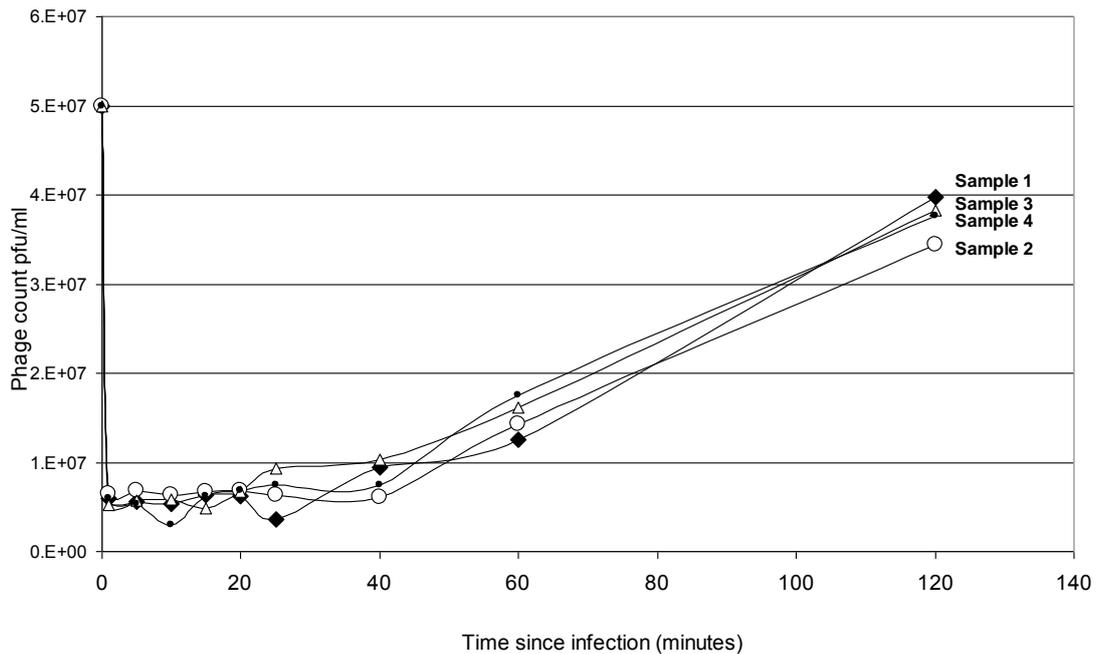


Figure 6.3 Single step growth of HER1156, 4 samples

Classic one-step growth curves use a multiplicity of infection (MOI) between 5 and 10, yet the MOI used here was 0.1. Kutter and Sulakvelidze (2005) use a MOI of 0.1 and use a volume of 100 μ l phage to infect 10ml of host at a concentration of approximately 2×10^8 cfu/ml, an experimental approach almost identical to the one used here.

Traditionally the infected cells are spun to a pellet, the supernatant liquid discarded and the pellet re-suspended 60 seconds after infection, to remove any free phage particles (Delbrück, 1940). In this way the infection is synchronized, which was not done in this experiment, however Kutter & Sulakvelidze (2005) also omit this step. The similarities however are striking, just like in the classical experiment a statistically significant decrease in phage numbers ($p < 0.01$) was followed by a statistically significant increase in plaques after timepoints 60 and 120 minutes ($p < 0.0.1$) using one-way ANOVA with Tukey's post-hoc test. Individual samples differ in terms of percentage change in plaque count and timing at which the decrease occurs. Slight differences in phage titre between the four samples can be attributed to the fact that plaque formation and attachment of phage to host in liquid medium occur following a Poisson distribution (Kutter & Sulakvelidze, 2005) and that samples were not synchronized. The mean plaque numbers of each sample before the first drop in plaque numbers were compared to the 'critical' concentration at the time point at which the reduction of phage particles occurred. In

Figure 6.3 Samples 1, 2 and 3 showed a decrease of 39%, 12% and 5% respectively at time point 25 minutes (compared to the average number of plaques from time points t_1 - t_{20} minutes). Sample 4 showed the most dramatic decrease in plaques numbers at different time points. These decreases are essentially fluctuations in plaque numbers and not statistically significant. It is likely that sample 4 showed a decrease in plaque numbers at time points 15 minutes and 10 minutes respectively, rather than at 25 minutes like the other samples, due to the absence of the synchronization step. Sample 4 showed a 13% decrease at time point t_{15} , the drop occurring earlier can be attributed to faster binding of phage particles to the host cells. A most likely reason for the different length of latent period and first point was the omission of the synchronisation step, by removing free phage from the suspension. The latent period was determined to finish after 25 minutes.

6.4 Discussion

6.4.1 Phenotypic tests and identification

All bacterial species obtained from Laval University were viable, could be successfully propagated and placed in long term storage. According to the phenotypic properties and by using an API20NE test system, all bacterial strains were identified as belonging to the genus *Acinetobacter*. Three isolates could not be identified to species level, despite using a recommended test kit (API20NE). Gerner-Smidt (1994) identified that it is very difficult to reliably assign isolates to one of the many officially unrecognized species, as the actual differences between species are so small. This was the case for HER1156, HER1096 and HER1425 where a combination of phenotypic properties and the results of the test kit were not sufficient to identify the bacterium to species level. Studies by Kämpfer, et al. (1993) and Gerner-Smidt et al. (1991) have shown that some genomic species are difficult to identify using phenotypic tests. A combination of nutritional characteristics and DNA-DNA hybridisation may be used to assign these three isolates to the current appropriate species. It was not within the remit of this research to go beyond considering phenotypic properties and the results of one test kit system, particularly considering the current taxonomic status of *Acinetobacter*.

Five different growth temperatures were chosen for investigation, four of which were determined by the parameters of the API20NE kit, in order to differentiate between

Acinetobacter species. All isolates were also incubated at 25°C, as it has been shown previously to be a critical temperature for lytic behaviour and it was important to establish if it was a temperature permissive of bacterial growth. Incubation at different temperatures demonstrated how diverse growth temperature requirements were. HER1050 grew at all temperatures, except at 25°C. HER1423 (*Acinetobacter lwoffii*), an isolate preferring lower temperatures for growth, failed to grow at or above physiological temperature. Consequently despite giving rise to plaques when infected with phage HER423133, this could only be achieved at sub-physiological temperatures. Similarly HER1156 did not grow at or above 42°C. HER1425 did not grow at 44°C. All other isolates successfully grew at all five temperature settings.

6.4.2 Antibiotic susceptibility of bacterial cultures

When using BSAC disc diffusion method all ten Canadian strains were sensitive to amikacin while two proved resistant to gentamicin, an aminoglycoside with a similar spectrum of activity.

It is possible that the two resistant isolates had acquired genes which could activate enzyme modification, an important resistance mechanism. Down-regulation of the oxygen-dependent active transport process of aminoglycosides into the cells is unlikely, as this would have resulted in cross-resistance to both aminoglycosides (Sweetman, 2006). All strains were identified as being sensitive to amoxicillin and clavulanic acid, it could be argued this is due to the discs being used containing 30 µg of amoxicillin/clavulanic acid, rather than 10µg recommended by BSAC. The used discs contained higher concentrations of the antibiotic combination than used by BSAC to calculate the respective breakpoints. This does not explain however why all Canadian strains were sensitive to the same strength of antibiotic, yet the majority of Sussex isolates were resistant when the same discs were used. It is possible that test discs containing only amoxicillin or amoxicillin/clavulanic acid at a lower dose would have lead to different resistance profiles regarding the Canadian isolates. Additionally clavulanic acid has been reported to enhance activity of amoxicillin even against species not generally considered sensitive to it e.g. *Moraxella catarrhalis*, *Burkholderia pseudomallei* (Sweetman, 2006). Future experiments could include using only amoxicillin or amoxicillin/clavulanic acid in combination at lower concentrations and

comparing the results. Apart from HER1425 all strains were resistant to aztreonam, which has a high affinity for penicillin-binding proteins of Gram-negative bacteria. It is possible that like *P. aeruginosa* it has some activity against *Acinetobacter* spp., but nine of the Canadian strains were insensitive to this antibiotic. In agreement with results in **Chapter 3** the Canadian strains displayed varying sensitivity to the three different cephalosporins used. All nine Canadian strains were resistant to cefixime, a bactericidal third generation cephalosporin. In the same way as *P. aeruginosa*, all *Acinetobacter* strains appeared to be intrinsically resistant to this antibiotic, which correlates with the results in **Chapter 3**. Ceftazidime, another third and cefuroxime a second generation cephalosporin on the other hand gave different results to cefixime. Three strains were sensitive to ceftazidime and four sensitive to cefuroxime sodium. Interestingly the isolates sensitive to either cephalosporin were not the same, apart from HER1425.

All ten isolates were sensitive to ciprofloxacin and imipenem. Ciprofloxacin acts by inhibiting the A subunit of DNA gyrase which is essential in the reproduction of bacterial DNA, while imipenem's mode of action is to inhibit the synthesis of the bacterial cell wall (Sweetman, 2006).

Both antibiotics are known to be active against *Acinetobacter* spp. (Bergogne-Berezin et al., 1996; Sweetman, 2006) but interestingly all the Canadian strains were sensitive to the two antibiotics, in contrast to sensitivity profiles of the Sussex isolates. This is evidence that the Canadian isolates were collectively sensitive to a wider range of antibiotics than the Sussex isolates. It is only speculative whether this is evidence supporting the theory that the Canadian isolates were unlikely to be hospital isolates.

Half of all the Canadian isolates were resistant to piperacillin, an ureidopenicillin with a similar spectrum of action to carbenicillin which is active against a wide range of Gram-negative organisms. This again is in contrast to the Sussex isolates where the majority of isolates proved to be resistant. Only two Canadian isolates were sensitive to tetracycline, a bacteriostatic antibiotic with a broad spectrum of activity against Gram-negative bacteria. The emergence of resistance, which is usually plasmid related and inducible, has reduced the value of this antibiotic. Resistance appears to be associated with the ability to prevent accumulation of the chemical inside the bacterial cell (Sweetman, 2006). *Acinetobacter* spp. may be resistant to tetracycline, most strains are

susceptible to doxycycline and minocycline (Bergogne-Berezin et al., 1996). It is interesting however that two of the Canadian isolates were sensitive to this drug, when none of the Sussex isolates were.

The sensitivity profile of the Sussex and Canadian isolates regarding trimethoprim, were dissimilar, three Canadian isolates were resistant, yet seven Sussex isolates were not sensitive to this drug. It is most likely that this is due to resistance, which can be plasmid mediated, due to overproduction of dihydrofolate reductase and changes in cell permeability (Sweetman, 2006). The absence of sensitivity could also be due to bacterial mutation which would render cells dependent on exogenous thymine and thymidine for growth.

All ten isolates successfully grew on LAM agar without antibiotic addition and LAM containing only vancomycin or cephradine. Every *Acinetobacter* isolate grew on LAM without antibiotic supplementation which correlated with previous results (**Chapter 3**). Growth, when present was clearly visible on all plates accompanied by a clear change of agar colour to mauve, with the only exception of HER1033, where growth was sparing on the antibiotic combination of vancomycin and cefsulodin.

Four isolates proved sensitive to cefsulodin. HER1050 seemed to be sensitive to a combination of both cephalosporins, yet it grew successfully when cultured on LAM supplemented with each individual antibiotic.

The sensitivities to the different cephalosporins varied, all strains successfully grew on LAM agar containing cephradine, however HER1032, HER1033 and HER1425 did not grow on agar supplemented with cefsulodin alone. This differed from the results in **Chapter 3**, where two isolates did not grow on plates supplemented with cefsulodin or cephradine on their own. As was reported in **Chapter 3**, individual strains have varying sensitivities to the two cephalosporins. HER1032, HER1151, HER 1425 and HER 1033 were sensitive to cefsulodin but not cephradine. HER1050 grew successfully in the presence of each cephalosporin alone, but when agar was supplemented with both growth was inhibited, which is possibly due to an additive effect of the two antibiotics. The weak growth of HER1033 on vancomycin and cefsulodin is difficult to explain as it did not grow on plates containing cefsulodin alone. This result may be due to insufficient diffusion of the cephalosporin or antagonism of the effects of one of the

antibiotics by the other. Factors which potentially could have affected the outcome of this experiment would be incomplete dissolution of technical agar, leading to different diffusion of antibiotics throughout the agar, varying concentrations of antibiotic throughout the agar due to inadequate mixing technique or inappropriate storage of LAM agar plates leading to the decay of chemicals. It is unlikely that the quality of agar plates may have affected the outcome of the experiment, as LAM agar was prepared in two separate batches and all results were read in quintuplicate.

All strains grew on plates supplemented with vancomycin, which is different to previous results (**Chapter 3**), where two out of nine isolates did not grow on plates containing this antibiotic. This may seem surprising, as the Sussex isolates are of confirmed clinical origins and in some cases were isolated from patients with a history of antimicrobial therapy. The Canadian isolates may be of clinical origin, however this is unconfirmed (Coffi, 1995). In the context of the BSAC results, three isolates did not grow in the vicinity of the BSAC test discs containing 30µg vancomycin, however LAM agar supplemented with 10µg/ml vancomycin permitted growth of all isolates.

Jawad et al. (1994) only used Leeds Acinetobacter Medium agar plates with three antibiotics for growth studies, as there are no published data on LAM with varying antibiotic composition or without antimicrobial supplementation the results cannot be compared with a range of data beyond this investigation.

Correlating the LAM results with each species proved difficult, partly because three isolates could not be clearly identified to species level. Jawad et al. (1994) report that *Acinetobacter johnsonii* and *Acinetobacter haemolyticus* failed to grow on LAM agar. Two isolates (HER1151 and HER1425) which may be *Acinetobacter johnsonii* failed to grow on LAM agar, however this is insufficient evidence on which to decide the exact species. Six of the ten isolates were *Acinetobacter baumannii*, four failed to grow on LAM, yet two grew successfully. Similarly, out of three *Acinetobacter baumannii* Sussex isolates (**Chapter 3**) only two grew on LAM. Strains HER1156 and HER1423, which are likely to be *Acinetobacter lwoffii* successfully grew on LAM, yet two Sussex isolates with the same species identification (R46383, W6108) did not grow on LAM, which is further compounding evidence that growth behaviour on LAM agar does not correlate with species identification. This does not however disprove Jawad's theory

that *Acinetobacter johnsonii* and *Acinetobacter haemolyticus* do not grow on LAM agar. It may seem unsurprising in clinical isolates that species identification and antibiotic sensitivity are not correlated, as antibiotic resistance is often acquired.

6.4.3 Determination of host for each bacteriophage

The optimized surface spotting method allowed a faster sample throughput and a more accurate screening of potential hosts for phage than existing methods. Due to the small volumes of lysate obtained a reliable way of identifying at least one host for each phage needed to be employed, to allow propagation of phage on a large scale. The short codes assigned by the Felix d'Hérelle Reference Center for Bacterial Viruses implied that phages and hosts with numerical similarities may be corresponding pairs. The information provided retrospectively (after the experiments were completed) confirmed that the correct phage and host pairs had been assigned (**Table 6.11**). The possibility that one phage may have more than one host was not communicated by the originators, however during the course of the investigation it became apparent that such a situation may exist (**Table 6.12**). This is of academic interest only as phage HER96 B9PP yielded only occasional plaques with host HER1096 and HER50 A10/A45 none at all regardless of which potential host was used.

The information provided by the originators did not identify whether particular phage and host combinations would be lytic or temperate. During a separate communication phages were divided into temperate, lytic and probably lytic by the originators, however the hosts were not confirmed. Categorically dividing phages into lytic and temperate may be arguable, as this can depend on the host, the critical influence over the switch between lysogeny and lysis are the presence and stability of protein CII, which the host can influence (Kutter & Sulakvelidze, 2005; Lewin, 2008). Bacterial cells are capable of influencing degradation of CII, for example by activating proteases degrading the critical enzyme by growth on rich medium. (Lewin, 2008), changes in growth media can also change intracellular ionic compositions in cells, which in turn can affect whether a phage becomes lytic or induces a state of lysogeny (Shkilnyj & Koudelka, 2007). It may be more constructive to relate to phage and host pairs as being in 'lytic interaction'. Additionally so called temperate phages may switch into a lytic cycle and vice-versa (Kutter & Sulakvelidze, 2005).

In this investigation an attempt was made to divide the interaction between the hosts and phage into lytic and temperate on the basis of the data presented in **Table 6.13**. The originators described phages HER33 A3/2, HER50 A10/A45, HER96 B9PP, HER162 B9GP and HER32531 as temperate, phage HER423 133 as lytic and the remainder as 'probably lytic'. Analyzing data presented in **Table 6.13** phage-host combinations were considered lytic on the basis of clearance zones obtained with the surface spotting method and soft agar spotting method plus repeatable plaque formation using the soft agar overlay method. Phages HER33 A3/2, HER50 A10/A45, HER96 B9PP, and HER162 B9GP were confirmed as temperate. With the unpurified lysate of HER96 B9PP a typical phenomenon previously described in conjunction with temperate phages was observed (Adams, 1959). The lysate without chloroform addition did not lead to any clearance zones using the surface spotting method, after chloroform addition and evaporation of the chemical, clearly visible clearance zones arose, compared to blank and all other lysates. It is well documented that chloroform can chemically induce lysis of cells infected by phage and liberate intracellular phage (Kutter & Sulakvelidze, 2005). This may be evidence that HER96 B9PP is a temperate phage, however alternatively as temperate phages would not necessarily give rise to progeny this phage may be lytic, but possessing a defective lysis gene.

Phages HER423 133, HER151 E13, HER401 BS46 were defined as lytic, plaques could be repeatedly produced and titres established. The information provided by the originators did not match the results in the case of HER425 2213/73 and HER156 E14 which were described as 'probably lytic' but did not yield plaques in this experiment. The surface spotting and soft agar spotting methods resulted in shadows or a dense bacterial lawn where lysates were applied; this makes these phages very unlikely to be lytic. HER32531 on the other hand was described as temperate by the Felix d'Hérelle Reference Center for Bacterial Viruses. It did however reliably form plaques remarkably not at 37°C, but when incubated and infected at 25°C and led to the formation of clearance zones using either screening technique. HER32531 may be more appropriately described as a temperature dependent lytic phage or 'probably lytic phage'. This highlights the importance of combining several techniques for phage screening and using incubation and infection temperatures lower than 37°C in addition to the standard incubation temperature.

6.4.4 Comparison of host screening methods

The surface spotting method applied in this investigation yielded outstanding visual results with clearly visible sharp edges of clearance zones. Even shadows, where bacterial growth was inhibited by phage, could be clearly seen. These shadows may be instrumental in detecting potential lysogeny of a phage, which may be suppressing growth but not be virulent enough to lyse the host (Adams, 1959). The surface spotting method proved to be a fast method compared to the agar overlay method, yet a combination with a second method seemed wise as demonstrated in the case of HER1033 & HER33 A3/2. Using the surface spotting method alone only a dense bacterial lawn could be seen, with the soft agar spotting method a shadow could be detected which was indicative that phage were present which displayed potential lysogeny. In two cases (HER1425 & HER425 2213173 and HER1401 & HER50 A10/A45) the soft agar spotting method was insufficient on its own to detect all potential lytic and lysogenic relationships between all phages and hosts. This demonstrates that a combination of both methods gave a more substantial insight into suitable host and phage combinations. Traditionally more basic soft agar spotting methods are used to screen large numbers of phage material against potential hosts, crude techniques where unwashed and unadjusted overnight suspensions of bacteria are used, and unknown quantities of liquid are applied using droppers or similar equipment (Kutter & Sulakvelidze, 2005).

The soft agar spotting method, despite being a modification of a classical method (Kutter & Sulakvelidze, 2005) was less practical and involved more manual steps than the surface spotting method. The surface agar needed to set before application of lysate droplets, which was time consuming, even after gelling of agar it remained moist which meant phage did not diffuse through the agar readily, as a result some clearance zones were smaller and more difficult to see than on surface spotting plates. The surface spotting method involved fewer steps and it proved less laborious to produce plates in such fashion. Less material was required and plates could be produced faster and placed in the incubator sooner as there was no additional layer of fresh agar which needed to set before further processing. The major drawback of the surface spotting method was that it proved overall less sensitive than the soft agar spotting method. It was insufficient in two cases in showing changes in bacterial growth, which could be

detected using the other method. The soft agar spotting method on the other hand led to more refined plate appearances, it was easier to detect shadows in the lawn which may be signs for lysogenic or potential lytic phages.

6.4.5 Selection criteria of suitable phage and host for further experiments

400ml of purified lysates were produced for phage and host combinations which yielded plaques with the crude filtered lysates. Five selection criteria were applied which qualified phage preparations to be used for tissue culture experiments (**Chapter 7**). Repeatable plaque formation with clearly visible plaques produced at 37°C in combination with the production of circular clearance zones (surface spotting and soft agar spotting methods) were considered good indicators for lytic behaviour. Repeatable plaque formation was essential for titration of phage counts for any further experiments, clear visibility of plaques made efficient counting of large quantities of soft agar overlay plates possible. The intention was to identify at least one *in-vitro* lytic phage-host model out of the ten candidates, which may have therapeutic potential and was useable under tissue culture conditions and which was reasonably easy to work with. Practical points like sufficient recoverable precipitate during the purification process to allow further processing and high phage titres were important in producing sufficient material for large scale tissue culture experiments. HER401BS46 yielded plaques which were surrounded by a symmetrical, circular 'halo', an area which showed decreased bacterial density compared to the surrounding bacterial lawn. This occurrence was not considered an issue as it improved the visibility of plaques and made counting easier. Soothill (1992) published a picture of plaques of phage BS46 grown on *A. baumannii* AC54A with a similar 'halo', this phage-host combination proved to be highly active *in vitro* and *in-vivo* in mice.

Purified phage lysate HER32531 yielded plaques, yet proved unsuitable for large scale experimentation, as the plaques were exceptionally difficult to see or count. Out of ten phage candidates only two appeared to be suitable for further experimentation: HER151 E13 and HER401BS46. HER423 133 was also a potential candidate, however infection and incubation temperatures had to be kept at 25°C, which was impractical and was not a reflection of physiological temperatures.

The analysis of the phage infection process experiment was an attempt to design an experiment which would help characterize a specific phage, in a similar way to the classic one-step experiment, without the need for expensive microscopic or genetic methods. Ellis and Delbrück described the one-step growth experiment (Adams, 1959) and developed a range of experiments to study the growth dynamics of bacteriophage (Delbrück, 1940; Ellis & Delbrück 1939). This technique allows the determination of the latent period and burst size in one experiment, it is however more complex than the experimental approach used here. The experiment leads to the identification of important parameters which can define a particular lytic infection cycle. The *latent period* is the minimum length of time elapsed from the adsorption of the phage particle to its host to lysis of the bacterial cell. *Burst size* is the average yield of phage particles from each infected host cell. The *eclipse period* is the time between infection and the appearance of mature virus within the cell, a time interval during which viral infectivity cannot be recovered.

Phage HER151E13 was purified to a concentration of 1.3×10^9 pfu/ml and yielded crisp plaques without halos, yet the phage titre remained stable over several months of storage. These features made this phage preparation an ideal candidate for this type of experiment, as a fairly small volume (0.1ml) was needed to contain sufficient phage particles to infect approximately $2-6 \times 10^8$ cells/ml at a MOI of 0.1 (Kutter & Sulakvelidze, 2005).

The 'single step' growth curve experiment described by Adams (1959) involved anti-phage serum obtained by injecting rabbits with phage and 'extreme dilution' whereby the drawn samples could be mixed with bacteria and plated directly without further dilutions.

Delbrück (1940) described his own experiment as 'less accurate both in respect to assay values and with respect to timing'. A modification was introduced in this experiment to improve the timing and involved removing extremely small volumes of the infected bacterial suspension, which could then be diluted in lambda buffer and immediately syringe filtered. In this way the adsorption of phage to bacteria would be stopped instantaneously possibly giving a more elegant approach than that used by Kutter & Sulakvelidze (2005). It should be pointed out that this modified experiment is quite

different from Delbrück's original one-step growth curve; however it is not dissimilar to the experimental setup of Kutter and Sulakvelidze (2005), whose book had not been published at the time of experimentation. No synchronization of infection took place which could have been achieved by infecting a small volume of cell suspension for one minute, followed by centrifugation, discarding the supernatant and resuspending the infected cells. This synchronized cell suspension would only have infectious intracellular phage particles present after centrifugation and could then be added to an exponentially growing culture of *Acinetobacter* spp. Another improvement would have been to expose one half of each sample to chloroform thereby allowing the measurement of free phage and any infectious intracellular phage particles (thus establishing the eclipse period). In the experimental setup used only free phage was measured.

This experiment established the latent period of phage (25minutes), a marker specific to each phage and can help differentiate between phages utilizing even the same hosts. The burst size cannot easily be determined with any other method but the soft agar overlay technique, which is an indirect method. The success of the experiment is dependent upon the accuracy of the soft agar overlay method, which in turn is dependent on the quality of the overlay agar, consistent moisture contents of the TSA plates and consistent inocula.

Differences in phage titre between the four samples can be attributed to plaque formation and attachment of phage to host occurring according to the Poisson distribution, more importantly to the fact that synchronization of infection did not take place. Absence of a synchronisation step contributed to the absence of a clearly determined eclipse period. Minor time delays in sample filtration, which may have lead to some host cells being exposed to phage for few seconds or minutes longer than others may have been another very minor contributing factor. It could be criticized that syringe filtration as a tool for dissociating phage from the host at set time points may affect phage counts by phage adsorbing to the filter membrane or clumps of phage being dissociated through filtration, leading to larger numbers of plaques, however results presented in **Section 5.2.18** make this scenario highly unlikely. Another argument could be that shear forces acting on the infected host due to the mechanical impact of filtration may have burst open infected hosts prematurely. The option of using centrifugation to

separate phage from host was explored, but dismissed on the grounds of inaccurate timing of host-phage separation. It would require further extensive experimentation to explore these options. It is likely that the small volume (100 μ l) removed from the reaction vessel at each time point may have statistically accounted for larger variations in plaque numbers than if larger samples would have been removed. This approach was necessary however due to the number of samples required and the necessity to keep the overall volume removed low.

In this research work an overnight cell suspension was diluted to produce a cell suspension with a given concentration, rather than growing the culture over several hours and infecting the suspension when that cell concentration had been reached. It could be argued that the cells were not in log phase immediately after dilution, unlike the cell suspension grown to the correct cell concentration as employed by Kutter & Sulakvelidze (2005), and this may affect the experimental outcome. However, if the dilution step used in this experiment significantly affected the experimental outcome, then samples diluted two- and four-fold in the protocols described by Kutter and Sulakvelidze (2005) may be equally affected. These authors recommend two- and four-fold dilutions of the infected bacterial cell suspension at predetermined time points to avoid infection of the host cells with more than one phage particle after the release of newly formed phage.

This was not done in this experiment, however, the omission of this step should not affect the number of phage particles released before the latent period had elapsed, as no new phage particles were available to re-infect the existing cells.

Future experiments could include diluting the infected cell suspension two and four-fold to avoid multiple infections of the host cells. Another approach could be to copy the experimental approach by Kutter and Sulakvelidze (2005) and include an additional experiment, where one half of the samples are prematurely lysed by chloroform. This would allow calculation of eclipse period, unadsorbed phage, efficiency of killing and efficiency of formation of infective centres. Burst size could not be calculated in this experiment, as synchronization did not take place and no plateau phase was observed. It is likely that multiple infections have taken place after the latent period had elapsed. Experimental setups with shorter sampling intervals and sampling over a longer period

of time may be beneficial for future experiments, but logistically very difficult to organize. For future experiments a multitude of different experimental approaches could be attempted and single-step experiments, a recognized tool to characterize phage and host interactions, could be performed for all plaque forming phage and bacteria pairs.

6.5 Conclusion

All bacterial strains obtained from external sources were successfully propagated and identified as belonging to the genus *Acinetobacter*. Seven strains could be identified to species level, however three could not by using phenotypic properties and API20NE test kits alone. This issue and the current taxonomic dilemma concerning *Acinetobacter* are recognized in literature. Future experiments may include DNA-DNA hybridization and further tests determining the nutritional preferences, to allow a more accurate species identification of the isolates.

Incubation at various temperature settings, as required by API20NE to allow identification to species levels, revealed that all but one isolate successfully grew at physiological temperature. Strain HER1423 required the lower temperatures of 25°C or 30°C for growth. One isolate did not grow at or above 42°C, another failed to grow at 44°C. All other isolates could be propagated at temperatures ranging from 25 to 44°C.

The antibiotic susceptibility profile of the Canadian strains was strikingly different to the Sussex isolates. It would be impossible to establish to which extent the Canadian strains had been exposed to antibiotics, unlike the Sussex strains.

Absence of current BSAC breakpoints for tetracycline and more importantly tigecycline, an antibiotic which has shown promising *in-vitro* activity against multi-resistant *Acinetobacter* spp, may warrant revisiting the original data once new breakpoints have been published. Publication of a repeatable assay regarding the resistance or susceptibility profiles of *Acinetobacter* spp. to colistin would be of benefit in the assessment of antibiotic resistance patterns. All isolates apart from one were resistant to aztreonam, only two isolates were resistant to gentamicin. Some strains showed different susceptibility profiles to different cephalosporins, which was reflected in the different growth behaviour on LAM with varied antibiotic supplementation.

Higher strength amoxicillin/clavulanic acid test discs than used by BSAC, future experiment could involve repeating the experiments using discs containing 10µg of the penicillin.

All strains grew on LAM agar without antibiotic supplementation or LAM plus either vancomycin or cephadrine. Growth behaviour on LAM could not be correlated with taxonomic classification; hence LAM did not prove to be suitable as a tool to facilitate identification to species level. Only five out of 10 isolates successfully grew on LAM with the full set of antibiotic supplementation, which considering it is recommended for isolation of *Acinetobacter* spp (Jawad et al., 1994) is disappointing. This is further evidence that LAM with antibiotics is over-selective. The fact that all 10 strains grew on antibiotic free LAM is promising, modification of this medium using a large range of different antimicrobials and trialling with statistically significant numbers of different isolates may be beneficial.

Recent evidence by the Working Party on the Control of multi-resistant *Acinetobacter* Outbreaks suggests that a medium published by Berlau et al. (1999) may be marginally superior in selectivity over the LAM medium by Jawad et al. (1994), however whether it may permit growth of a wider spectrum of *Acinetobacter* spp. strains than LAM is unclear.

For future experiments it would be interesting to collect a large number of isolates of documented different origins, combining different species identification methods including phenotypic properties, DNA-DNA hybridisation methods and defining parameters on which an assignment to a specific species was based, as current taxonomic rules are insufficient. Further it would be of interest to correlate antibiotic sensitivities and growth behaviour on selective media, particularly LAM. Provided a large number of isolates were included in the study a statistical correlation could be made between antibiotic sensitivities, ease of isolation on selective media, species and origin. This study could help establish prevalent international resistance patterns and correlate them with genetic data, origin and help establish to differentiate between intrinsic and acquired resistance.

In future experiments the possibility of synergistic interaction of two cephalosporins on HER1050 could be investigated, whether any potential clinical benefit could be derived

from this is a matter of speculation. The growth behaviour of HER1033 on agars supplemented with vancomycin and cefsulodin needed to be further explored, as the anomaly whereby it failed to grow in the presence of one antibiotic but successfully grew when two antibiotics were present needs further exploration.

The optimized surface spotting method was established to be at least an equally useful, yet less time consuming method than conventional phage screening protocols. This optimized method developed in the course of this investigation was superior over orthodox methods in that it involved a step whereby cell were washed and a known cell concentration was used to produce a lawn. This produced optimum conditions for reading results, even faint thinning of a lawn due to the presence of phage could be easily discerned, which was instrumental in detecting temperate phage. It was evident that both phage screening methods complemented each other and ideally should be used in combination.

All phage and host pairs originally matched by Laval University were confirmed via a combination of the optimized surface spotting and soft agar spotting methods. In addition two potential phage and host combinations were established. Future experiments may involve further exploration of this association; these phages may be ideal candidates for the research into virulence factors, dynamics affecting phage-host binding and may provide further data on how phages could be artificially modified to be promiscuous. The division of phages into lytic, probably lytic and temperate phage by the originators was not confirmed in all cases. The lytic potential of HER32531 was overlooked by Laval University, possibly because it required incubation and infection temperatures of 25°C to produce plaques. It was established that temperate phage may switch into a lytic cycle, or the same phage may form plaques in conjunction with one host, which highlights the categorical division of a phage into 'lytic' or 'temperate' as potentially too rigid. The description of the relationship between phage and host may be more fitting in terms of their lytic/lysogenic interaction. Rather than describing phage as lytic or temperate in the literature, the respective host should be mentioned, as a specific phage may exhibit a lytic interaction with one host but not another.

Chapter SEVEN

Effects of phage on mammalian cells *in vitro*

7.1 Introduction

Numerous reviews have promoted phage as a viable alternative to antibiotics or a treatment form, which can be used in conjunction with antimicrobials (Sulakvelidze et al., 2001; Summers, 2001; Weber-Dabrowska et al., 2001; Taylor et al., 2002). A range of animal experiments have provided supporting evidence that phage can be used *in vivo* to treat bacterial infections. Additionally numerous reports of human infections which were treated successfully with phage have been published, primarily in the former Soviet Union and Eastern Europe. Phage preparations have become commercially available and have received regulatory approval for use in food safety (U.S. Food and Drug Administration, 2006; Docket No. 2002F-0316 -formerly 02F-0316/). Human clinical trials are conducted or planned, published results remain unavailable in the public domain (Häusler, 2006; Matthey & Spencer, 2008; <http://www.biocontrol-ltd.com/>; visited, February 11, 2009) Despite this body of data, phages are not yet used as antibacterial therapy in the UK and no phage preparation for the purpose of treating human or animal infections has been licensed in the UK to date.

This caution is understandable given the experience of previous attempts to bring this form of therapy to the clinic. The regulatory authorities will need to be convinced of both efficacy and safety. To date little evidence has been published on the possible toxic effects of phage on human tissue or at a cellular level. It is the purpose of this chapter to investigate the cytotoxic effects of bacteriophages on cultured mammalian cells.

The series of tests described in this chapter is based on ISO 10993, which specifies the incubation of cultured cells either directly or through diffusion with extracts of a device or in contact with a device. The US Food and Drug Administration defines a medical device as ‘an instrument, apparatus, implement, machine, contrivance, implant, *in vitro* reagent, or other similar or related article, including a component part, or accessory which is: recognized in the official National Formulary, or the United States Pharmacopoeia or any supplement to them, intended for use in the diagnosis of disease or other conditions, or in the cure, mitigation, treatment, or prevention of disease, in man or other animals or intended to affect the structure or any function of the body of man or other animals, and which does not achieve any of its primary intended purposes through chemical action within or on the body of man or other animals and which is not dependent upon being metabolized for the achievement of any of its primary intended purposes’ For the purposes of this study bacteriophages are considered an extract of a medical device in the context of ISO 10993.

7.1.2 *In vitro* cultivation of cells and tissues (definitions)

The term cell culture indicates that the original tissue was dispersed into a cell suspension, which may be cultured in suspension or for cells with adhesive properties as a monolayer on the surface of a culture dish. In histotypic cultures cells have been reassembled to create a three-dimensional tissue-like structure, e.g. with the help of a matrix gel such as collagen, as used for some skin grafts. Organotypic cultures are a combination of different lineages of cells (e.g. fibroblasts and keratinocytes) to recreate a three-dimensional tissue-like structure mimicking some dynamic processes found *in vivo* of tissues made up of the same combination of cells (Freshney, 2004; Greenberg et al., 2005).

In tissue (explant) culture a slice of tissue is placed at a plastic-liquid interface, where the tissue is allowed to attach and cells migrate along the plastic surface. Primary tissue explants can be treated to derive cells for further cell culture e.g. to harvest keratinocytes from a skin sample. By mechanically and enzymatically breaking up the links between the cells in the primary explant a cell suspension can be produced, which is allowed to undergo several division cycles, which constitutes a primary culture. If this

cell culture is proliferative enough it can be subcultured into a fresh vessel, which is described as a passage, it then constitutes a secondary culture. After the next passage it becomes a tertiary culture, and so on. In the literature nomenclature is rarely used to describe a cell line beyond the third passage. Generally a primary culture is referred to as a cell line after the first subculture (Freshney, 2004).

7.1.3 Advantages of using cell culture compared to whole tissue or animals

The cell culture process allows single cells to act as individual units, similar to bacteria in liquid culture. Tissue samples or whole animals on the other hand are quite heterogeneous. Even replicates from the same tissue can vary in terms of cell types and stages of growth and viability of cells. After several passages cell lines assume a uniform or homogenous population (clonal), like microbial cultures the most vigorous cell type sustains the selective pressure and dominates the culture. At each subculture each replicate sample will be identical, retaining the characteristics of a cell line over long periods of time e.g. HeLa cells. Since replicates from the same sample taken at the same timepoint are identical, even when frozen in liquid nitrogen and used at a later timepoint, experiments become reproducible.

Whole animals are frequently used for toxicological testing, however using cell culture allows investigation of the effect of a particular compound on one specific cell type. It permits the study of biochemical changes in a particular cell type in finer detail, than *in vivo* (Freshney, 2004). Cell culture allows regulation of the physicochemical milieu in which the cells are grown. Control of physiological condition is also easily achieved, however most cell lines still require ill-defined constituents in their growth medium, such as serum (Ehrlich, 2004; Freshney, 2004; Knezevic et al., 2008). These supplements are prone to batch to batch variations, presence of hormones and other substances, which may affect the proliferation of cells. Gradual identification of essential components of serum has led to replacement with defined constituents and the development of serum-free alternatives. However serum has the advantage of prolonging the life-span of primary cultures, by up to 100%. During toxicity testing cultured cells are directly accessible to the reagent. This allows relatively small

quantities to be required for a range of screening tests. During *in vivo* testing large quantities of reagent may be lost due to absorption, distribution, metabolism and excretion and there is no guarantee that sufficient amounts will arrive at the target cells.

7.1.4 Disadvantages of using cell culture compared to whole tissue or animals

Apart from requiring specialised equipment and skill, a major limitation for the production of small amounts of tissue, is the expenditure in terms of time and cost. For human samples suitable donors need to be identified, appropriate paperwork has to be completed in fulfilment of the Human Tissue Act, 2004, which can be a time-consuming task, before the actual isolation of cells can begin.

Preparing tissue explants to isolate individual, representative cells can be arduous and months may be spent before the actual cell culture experiments can be performed.

A major disadvantage is dedifferentiation, a loss of characteristics representative of the tissue from which the cells originated. After periods of continuous growth cell characteristics can change significantly. This is particularly a problem in long established cell lines, however it can be partially avoided by isolating cells from primary tissue, avoiding transformation (Freshney, 2004). The validity of cultured cells as a model of *in vivo* physiological functions has often been criticised (Breidahl et al., 1989; Kratz, 1998; Boyce, 2004). Cells are capable of adapting to different nutrients and stimuli, however this adaptation brings with it a change in biochemical processes within the cell. This can be problematic when cell culture is used to predict or understand processes *in vivo*. (Hence it is important to report the exact composition of media and unwise to change culture conditions during the course of experimentation.) As the cellular environment cannot always be exactly replicated *in vitro*, due to absence of cell contact or hormonal interaction with other cells, problems with characterisation may occur. Cells can be made to proliferate *in vitro* which would not survive *in vivo*, after all cell culture is an artificial system and it is necessary to understand its limitations. However as long as any *in-vitro* results are correlated to physiological events *in vivo* with caution cell culture is an extremely useful tool.

7.1.5 Senescence, 'Normal cells' and transformed cell lines

In a classic experiment by Hayflick and Moorhead it was demonstrated that human foetal lung cells could be subcultured for about 50 generations, before they started to senesce and despite being viable became incapable of dividing (Hayflick & Moorhead, 1961). The term 'cell line' is used to describe a cell population which is capable of remaining mitotic over many subcultures. All cells derived from 'normal' animal tissue possess a limited growth potential, due to senescence which is caused by telomeric attrition (Burton et al, 2005). The finite number of generations of growth is dependent on cell type, age of donor and species. It is established that embryonic normal tissue is longer lasting than those derived from adult organisms. 'Normal' cells according to Hayflick & Moorhead (1961) have a diploid chromosome number, which implied no major chromosomal damage had occurred, they are anchorage dependent- cells requiring a solid surface to attach to, they have a finite lifespan and are non-malignant.

Transformed or immortalised cells on the other hand are cells which have acquired the capacity of infinite growth at the cost of losing some of their original features. These established or continuous cell lines go through a process of transformation, which rids the cells of sensitivity to stimuli associated with growth control. Continuous cell lines have often lost their anchorage-dependence, may show chromosome fragmentation and require less growth factors for in-vitro culture (Butler, 1994). Continuous cell lines show a modified diploid state (aneuploid), have shorter doubling times are generally more robust and easier to cultivate than 'normal' cells e.g. 3T3 Swiss mouse fibroblast cell line. Cells can be immortalised by use of viruses, particularly retroviruses, oncogens or mutagens (Stöppler et al., 1997). It is important to mention that human fibroblasts remain predominantly euploid (Hayflick and Moorhead, 1961; Hayflick, 1965) and very rarely spontaneously transform, which has predisposed them to being a good model system for cellular ageing (Hubbard-Smith et al.,1992; Sandhu et al., 1994). Mouse fibroblasts and most mammalian tumour cells tend to become aneuploid in culture and can easily be transformed. Some cells, particularly of rodent origin have even been reported to transform spontaneously in culture, which is attributed to the presence of endogenous viruses (Butler, 2004).

After explantation of a tissue explant the cells divide and after the cells reach a certain confluence they are passaged by subculturing into fresh vessels. At the crisis point (transformation) a cell line may become continuous (immortalised) or senesce and subsequently die. The finite number of generations of growth is known as the 'Hayflick limit'. Even when mammalian cells are frozen they retain all characteristics, including the overall maximum number of cell divisions possible (Hayflick & Moorhead, 1961). The prolific period before onset of senescence is often termed the pre-crisis life span.

7.1.6 Continuous cell line versus primary cell line

Continuous cell lines are often unstable, due to their aneuploid state, which constitutes major problems in practice. This can lead to phenotypic and genotypic changes, which can make comparison of results between laboratories difficult. Primary cell culture or primary cell lines as opposed to continuous or immortalised cell lines are generally considered to be more physiologically similar to in vivo cells (Petricciani & Sheets, 2008). Primary cell culture can be very laborious and may require obscure and expensive specialist growth media.

7.1.7 Embryonic Swiss Mouse Fibroblast cells (3T3 line)

The Embryonic Swiss Mouse Cell Fibroblast (3T3) cell line was established from disaggregated Swiss mouse embryo tissue (*Mus musculus*) by George Todaro and Howard Green in 1962. 3T3 is a standard cell line used widely in a variety of research and industrial settings. This type of cell is easy, fast and cheap to grow and large quantities can be obtained in short time spans. Their hardiness makes them ideal candidates for preliminary experiments, as confirmation of cell death caused by a cytotoxic agent in 3T3s would imply that more sensitive, more expensive cell types would respond in an even more severe manner. Apart from being used for various purposes in their own right, 3T3 cells play an important part in allowing keratinocyte growth in-vitro. Historically in-vitro work with keratinocytes was very unsatisfactory and limited, as these cells could not be successfully cultured for longer periods of time. In 1974 Rheinwald found that keratinocytes, which grew slowly, started to grow more quickly when the growth media was supplemented with lethally irradiated 3T3 cells.

7.1.8 Rationale for using cell culture

The main reasons for employing cell culture are to investigate the biochemistry and physiology of cells, which allows the study of metabolic pathways e.g. release of interleukins. Building on this principle this method is employed for cytotoxicity testing or generally to investigate the effects of compounds on specific cell types such as the effect of bacteriophage on cell survival or interleukin release. Cell culture can be used to produce artificial tissue which may be histotypic or organotypic, mono- or multilayer cell sheets obtained by this method are routinely used as artificial skin transplants in burns treatment.

7.1.9 Cytotoxicity and viable cell quantification

In order to make any quantitative assessments of cell death or survival and to achieve reproducible results, methods to quantify cell populations are needed. The most basic method involves pipetting cell suspensions onto a haemocytometer slide and counting each individual cell, addition of dyes such as Trypan Blue allows differentiation between viable and non-viable cells (Freshney, 2004). The method estimating viability by dye exclusion is simple, fast, inexpensive, requires only a small fraction of cell suspension, however it can interfere with experimental setups, as cells growing as monolayers need to be trypsinised first to produce cell suspensions. Additionally counting large numbers of samples can be very time consuming.

Another method based on estimating viability by dye uptake uses diacetyl fluorescein, which is hydrolysed by live cells to fluorescein, to which cell membranes of viable cells are impermeable (Freshney, 2004). After staining with propidium iodide and fluorescence microscopy live and dead cells can be easily differentiated. This method has similar advantages to Trypan blue, however is invasive and time consuming.

7.1.10. Miniaturized colourimetric methods to determine cytotoxicity

Simple counting of total cells may be insufficient, in which case the most accurate procedure involves counting cells that have the ability to divide by the mitotic index method. Non-invasive methods measuring metabolic parameters determine glucose or

oxygen consumption rates, or the production of metabolites like lactic and pyruvic acid and carbon dioxide which can be correlated to actual cell numbers present if a standard curve is performed. These methods are non-destructive and permit successive sample removals, allowing the detection of dynamic changes in the cell culture.

Direct count and colony-forming efficiency and clonogenic assays may be the most reliable methods for quantitative determination of cell numbers, however they are impractical when many samples need to be analysed.

Another commonly used method is measurement of [³H]thymidine incorporation into DNA, however this requires handling radioactive isotopes, is costly and prone to artefacts, due to DNA synthesis not always correlating with viable cell numbers or mycoplasma contamination of cells or the factor tested (Jakway, 1986; Merckenschlager, 1988).

The majority of experiments where cell growth or toxicity are assessed involve comparison of a culture treated with a compound compared to an untreated control. To allow direct comparison of results, cells in both treatment arms have to be in exponential growth phase at the point of assay endpoint determination, to allow meaningful readings. Depending on the setup of the colourimetric method various requirements should be met to render the data meaningful. The minimum detectable number per well should be characterised or obtained from literature. The sensitivity of each method should be established for each cell type used in the assay. For certain assays a calibration plot establishing the linear range of absorbance versus cell number needs to be established before commencing experimentation. In addition the cell number present in all wells needs to be on the linear part of an optical density versus cell number calibration plot. It is essential to understand that cell type and assay conditions, such as volume of sample, medium used, serum concentration and serum batch can affect the outcome of a colourimetric assay. Assay duration is another relative parameter, as development of colour depends on chemical reactions, which can be affected by physicochemical parameters. It is therefore essential to appreciate that numerical values obtained through colourimetric methods cannot be considered as constant. Several positive and/or negative controls need to be included in each set of

measurements, which can complicate experimental setup significantly. It is also recommended to avoid interpretation of colourimetric data isolated from colony-formation assay or direct counts. In toxicity assays it has been found that colourimetric assays and Trypan blue exclusion correlated well with colony-formation assays after 3-4 days of drug treatment (Doyle & Griffiths, 1998; Horowitz & King, 2000); Provided that the limitations of the assays are considered and preliminary data are collected using other methods to validate the colourimetric results, miniature colourimetric assays can be of great value. It is important to combine a range of experiments to build a body of evidence for any toxicity assessment. Colourimetric assays non-invasive and therefore allow longitudinal studies of large amounts of samples. (Doyle & Griffiths, 1998).

7.1.10.1. Lactate dehydrogenase activity (LDH)

A standard method used regularly to assess cytotoxicity is to measure LDH levels, which is only released by dying or dead cells. This is a non-invasive method which requires removal of only small volumes of cell supernatant from the culturing vessel and allows observation of dynamic changes over periods of time (Freshney, 2004). This assay is a colourimetric alternative to ^{51}Cr release cytotoxicity assays. It is a quantitative measure of loss of cell viability, whereby the reduction of pyruvate to lactate can be quantitatively determined using a spectrophotometer.



LDH is a stable cytosolic enzyme released upon cell lysis. LDH assays are based on the assumption that intracellular enzymes are only released following damage to the cell membrane and that release is complete and rapid. A directly proportionate relationship between the extent of cell damage and enzyme activity is used as a mathematical assumption, on which assays are based

7.1.10.2 Assays using MTS tetrazolium compounds

Assays utilising tetrazolium compounds are popular, convenient ways to determine numbers of viable cells without employing radioactive agents. The original method using MTT to determine cell proliferation (Mosmann et al., 1983) required the

solubilisation of the formazan compound. MTT soon became replaced by the newly synthesised agent XTT (Paull, 1988), which itself had the disadvantage of requiring a freshly prepared solution prior to each assay and limited solubility and stability in solution. The most recent and most advanced assay involving tetrazolium compounds utilises [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt; MTS] also known as Owen's reagent. MTS has better solubility than XTT and consequently remains stable for several months. The principle of the assay is based on the MTS compound forming aqueous, soluble formazan compound when bio-reduced by viable cells in culture, in combination with micromolar quantities of an electron transfer reagent (Cory, 1991).

7.1.11 Colony formation assay (clonal assay)

Perhaps the most popular and widely accepted form of assaying cell survival is the colony formation in dilution cloning. The assay is based on the ability of cells to form colonies in isolation, which is achieved by using a single cell suspension which will form discrete colonies when diluted below a certain density and counting colonies that form after incubation (Kulling et al., 1998; Gordon et al., 2002; Robichova et al., 2004; Rose et al., 2004; Carmo et al., 2007; Jantova et al., 2008).

7.1.12 Cell mediators

Cytokines are mediators which facilitate communication between different cell types and are essential in wound healing (Gillitzer & Goebeler, 2001). They are proteins secreted by a cell that signals other cells in a paracrine or autocrine fashion. Cytokines, unlike hormones usually do not pass into the blood, but produce local effects on nearby cells or the cell which produced the signalling molecule itself. The concept once held 'one producer cell- one cytokine- one target cell' has been disproved. It is problematic assigning certain cytokines to specific producer cells. Most cytokines are pleiotropic, (i.e. produce many effects) while others are more restricted in their functions (Clark et al., 2007). Additionally many cytokines have overlapping functions with near identical activities (cross-talk). As a consequence one factor may functionally replace another or partially compensate for the lack of a specific cytokine. Most cytokines are not stored

ready for use, but the expression is dependent upon cell activation in response to an induction signal.

Cytokine expression can be regulated on various levels (transcription, translation, protein expression) and appears to be regulated differently dependent on cell type and developmental age (important with primary tissues). For historic reasons some cytokines have an array of different names which is confusing. It is also important to mention that the relevance of *in vitro* activities of some cytokines to *in vivo* processes is not entirely clear. In addition a single cytokine may cause a function which may under different conditions be the reverse of the original reaction. Duration, type and magnitude of cellular activities induced by a particular cytokine can be affected by factors like confluence, neighbouring cell types, and the combination of other cytokines present and temporal sequence of various cytokines acting on the same cell. Maas-Szabovski et al. (1999) even reports that gamma irradiated cultured fibroblast cells, as used in feeder layers for keratinocytes remain functionally active and continue to release cytokines. This observation is important when interpreting cytokine release where two cell types are present, particularly feeder cells, which are often referred to as 'lethally irradiated' which does not reflect Maas-Szabovski's findings. In addition cytokine expression is modulated in cocultured fibroblasts and keratinocytes, with distinct increases in KGF, IL-1 α IL-1 β , while IL-8 and TGF-1 β mRNA levels were reduced. It could be argued that up- or downregulated expression of mRNA does not equal automatic release of cytokines in an *in-vitro* situation, however these findings need to be kept in mind when designing and interpreting *in-vitro* studies of mixed cell cultures.

7.2 Aims & Objectives

The aim of this chapter was to evaluate the cytotoxicity profile of bacteriophages against relevant mammalian cell cultures.

The main objective was to develop a series of cytotoxicity assays using a variety of cells and techniques. The methodologies were initially developed using the 3T3 mouse fibroblast cell-line described above, as this was the most convenient to cultivate. Once the techniques were established they were repeated on the more relevant human dermal fibroblasts and human keratinocytes isolated from primary material.

7.3 Material and Methods

7.3.1 3T3 Swiss mouse fibroblast cell line (3T3)

3T3 Swiss albino mouse fibroblast cell line (*mus musculus*) '3T3(+3)' ECACC No. 89022402 (from stocks held at the University of Brighton) was used for all mouse fibroblast experiments, except for keratinocyte feeder layers.

7.3.1.1 Standard growth vessels for 3T3 Swiss mouse fibroblast cells

All 3T3 cell experiments were undertaken in either sterile Nunclon surface T25 flasks with filter caps or sterile Nunclon surface 24-well plates with fitting lids both Nunc, Roskilde, Denmark.

7.3.1.2 Standard growth-medium for 3T3 Swiss mouse fibroblast cells

DMEM (Dulbecco's Modified Eagle's Medium) with 1000mg glucose/L, L-glutamine and sodium bicarbonate (Sigma Aldrich, Gillingham, UK) supplemented with 10% (v/v) foetal calf serum (FCS) Biosera, Ringmer and 1% (v/v) penicillin and streptomycin (Sigma Aldrich, Gillingham, UK) was routinely used as growth-medium for 3T3 cells.

Reference to DMEM or medium for all 3T3 mouse experiments relates to the combination of reagents above.

7.3.1.3 Standard incubation of 3T3 Swiss mouse fibroblast cells

All 3T3 cells were incubated in a humidified Heraeus, Heracell, (Thermo Fisher Scientific, Waltham, MA, USA), cell incubator with 5% CO₂ saturation. 24-well plates were routinely stacked at a maximum of two plates each stack in disinfected plastic containers with loosely covered lids, to avoid accidental removal of 24-well plate lids and ensuring even moisture and temperature levels during heavy usage of the incubator. T25 flasks were routinely stacked at a maximum of three flasks each stack and flasks were checked daily to ensure filter lids were not contaminated with medium.

7.3.1.4 Trypsinisation of 3T3 mouse cells grown in T25 flasks

Cell culture work was routinely carried out under sterile conditions in a class II cell culture laminar flow cabinet (Cyttox II, Envair Ltd., Lancaster, UK). Before trypsinisation DMEM was pre-warmed to 37°C in a waterbath, and trypsin (trypsin EDTA 500mg/L Cambrex, BioWhittaker, East Rutherford, NJ, USA) was warmed to approx. 20°C. Growth flasks were removed from the incubator; the medium covering the cells was aspirated and replaced with 2ml of trypsin in T25 flasks. The vessels were incubated for between 3 and 5 minutes at 37°C, (5% CO₂ in a humidified incubator), including microscopic checks to ensure the majority of cells were detached. Persistent gentle striking of the growth vessels against a hard surface or the palm of a hand was used to aid detachment.

4ml of DMEM were added to each T25 flask, to neutralise the trypsin. The resulting suspension was then transferred to 50ml centrifuge tubes and spun at 500g for 5min. The supernatant liquid was aspirated and the cell pellet was re-suspended in 1ml DMEM to produce a homogenous cell suspension.

7.3.1.5 Trypsinisation of 3T3 mouse cells grown in 24-well plates

Trypsin was prewarmed, growth medium replaced with 200µl trypsin per well in 24-well plates, followed by incubation as described in **Section 7.3.1.4**. For 24-well plates 800µl of DMEM was used to neutralise the trypsin, and the suspensions from each well were transferred into 1.5ml Eppendorf tubes and centrifuged at 400g for 5 minutes. The

cell pellet was re-suspended in 1ml of DMEM and drawn up and down in a pipette (trituated) to obtain a homogenous cell suspension.

7.3.1.6 Standard seeding and passaging of growth vessels

24-well plates were seeded with cell suspensions (**Sections 7.3.1.4 & 7.3.1.5**). 1ml of pre-warmed medium was added immediately, the lid was applied and the plate placed back in the incubator at 37°C. In cases where volumes of cell suspensions exceeded 100µl, the quantity of pre-warmed medium was reduced accordingly to give a total volume of 1ml in each well. Empty wells were filled with sterile PBS to ensure equal humidity levels throughout the plate.

In T25 flasks 7ml of pre-warmed medium was pipetted directly to the bottom of the flask, the flasks were held upright and the cell suspension was trickled over the inside flat bottom of the flasks, to allow attachment of cells. Flask were then tilted to allow even distribution of medium and swiftly moved to the incubator.

7.3.1.7 Counting of mammalian cells with Trypan Blue stain

Immediately after trypsinisation and resuspension of the cell pellet in 1ml medium (**7.3.1.4 & 7.3.1.5**) 20µl of cell suspension were mixed with 4µl of Trypan Blue solution 0.4% (w/v) (Fluka, Buchs, Switzerland) in PBS in a clean Eppendorf tube, trituated and immediately applied to a haemocytometer. Cells were counted under an inverted light microscope (Wilover, Will, Germany) at 100 x magnification. In cases where the cell density was too high for counting 100µl of the cell suspension were diluted with 900µl of DMEM, gently trituated and 20µl of the dilution mixed with 4µl of Trypan Blue solution 0.4% (w/v) and the respective dilution factor considered in all calculations.

Unstained cells were counted as alive, cells which have taken up the Trypan Blue stain and were coloured blue were considered dead. Live/dead cell ratios were calculated using the formulae given in **Appendix 1**.

7.3.1.8 Hoechst propidium iodide staining (HPI)

Hoechst propidium iodide staining (HPI) was used to determine levels of apoptotic or necrotic cell death. Cell nuclei were stained simultaneously with DNA binding dyes by mixing 900µl of cell medium with 50 µl of a HPI solution (1mg/ml in 10ml demineralised water) and 50µl of bisBenzimide (Hoechst 33342), Sigma-Aldrich, Poole, Dorset. As the stain was light sensitive, lights in the laminar flow hood were switched off during use of the stain and the container was wrapped in aluminium foil. Cells were viewed using a Karl Zeiss, Axiovert 25 (Karl Zeiss Ltd., Welwyn Garden city, UK) inverted fluorescence microscope with 420nm filter.

Viable cells were identified by their intact nuclei with blue fluorescence (bisBenzimide stain), necrotic cells by their intact nucleus with pink fluorescence (positive for both stains) and apoptotic stains by their fragmented nuclei, fluorescence either blue (early phase) or pink (bisBenzimide and HPI positive, late phase with secondary necrosis).

7.3.1.9 Cumulative population doublings of 3T3 Swiss fibroblast cell line in 24-well plates over 72 hours

3T3 cells at Passage 20 (P20), grown in 2xT25 flasks from University of Brighton stocks were trypsinised and counted with Trypan Blue.

24-wells were seeded with 5,000 cells each, 1ml of medium was added to each well, and the plate incubated at 37°C, (5% CO₂). After 24, 48 and 72 hours of incubation, without media changes three wells at a time were harvested and counted using a haemocytometer. Doubling times were calculated using the formula given in **Appendix 1**.

The results are shown in section **7.4.1**.

7.3.1.10 Cumulative population doublings of 3T3 Swiss fibroblast cell line in T25 flasks over 96 hours

3T3 mouse cells at P39, grown in 4xT25 flasks were passaged, counted and seeded at 10,000 cells per flask (P40) into 8xT25 flasks. The T25 flasks were incubated for 96 hours without media changes. At 24, 48, 72 and 96 hours of incubation two flasks were harvested and counted using a haemocytometer. Doubling times were calculated using the formula given in **Appendix 1**.

The results are shown in section **7.4.2**.

7.3.1.11 Validation of phage activity in tissue culture conditions without mammalian cells present

The purpose of this experiment was to investigate if phage were still viable and infective after exposure to CO₂, prolonged incubation at 37°C and exposure to tissue culture medium or a combination of the above.

5 wells of a 24-well plate were filled with 0.9ml of DMEM or lambda buffer respectively. 100µl of purified and twice filtered phage suspension HER401 BS46 (**Section 6.2.10**) at count of 2.2×10^{10} pfu/ml was added to each well. The plates were incubated at 37°C, 5% CO₂. After 24 and 48 hours 100µl aliquots were withdrawn from each well and serially diluted in lambda buffer in 10-fold steps. Phage counts of the samples withdrawn at various time points were established using the soft agar overlay method (n=5);

The results are shown in section **7.4.3**.

7.3.1.12 Validation of phage activity in tissue culture conditions with mammalian cells present

The purpose of this experiment was to investigate if DMEM or FCS, the presence of 3T3 cells, their metabolites, or a combination of all these factors affected the viability of phage.

9 wells on a 24-multiwell plate were inoculated with 5,000 3T3 mouse fibroblast cells and 1ml of DMEM was added to each well. 9 empty wells were filled with 1ml of DMEM and 9 wells with 1ml of lambda buffer. Each plate was incubated at 37°C, 5% CO₂ for 24 hours.

100µl of one and two-10-fold dilutions of purified phage suspension HER401 BS46 (**Section 6.2.10**) at calculated phage counts of 2×10^9 and 2×10^8 pfu/ml (Dilutions 1 & 2) were added to the wells containing only DMEM, lambda buffer and cells in DMEM in triplicate. All plates were incubated at 37°C, 5% CO₂.

After 24, 48 and 72 hours 100µl aliquots were withdrawn from one well containing lambda buffer or DMEM and serially diluted in lambda buffer in 10-fold steps.

For the wells containing cells the volume contained in each well was removed with a sterile syringe and needle, syringe filtered and 100µl aliquots of the filtrate serially diluted in lambda buffer in 10-fold steps.

To calculate the actual number of phage particles present in each well (Baseline) 100µl of one and two-10-fold dilutions of phage suspension HER401 BS46 (Dilutions 1&2) were diluted with 1ml of lambda buffer, not incubated and further serially diluted to produce soft agar overlays.

Phage counts of all samples were established using 10-fold serial dilutions in lambda buffer with the soft agar overlay method (**Section 2.3.4**) in quintuplicate.

The results are shown in section 7.4.4

7.3.1.13 Cytotoxicity and cell proliferation assays for phage HER401 BS46 using a 3T3 mouse fibroblast cell line

As discussed above preliminary experiments were conducted to develop cytotoxicity and proliferation assays using a 3T3 mouse fibroblast cell line.

7.3.1.13.1 Cytotoxicity assay using Trypan Blue and 3T3 cells

3T3 Mouse fibroblast cells were thawed from frozen and passaged at least once before use in the cytotoxicity assay. Cells were seeded at a cell density of 200,000 in 25 cm² flasks after which DMEM medium with 10% FCS and penicillin and streptomycin was added to give a final volume of 7ml. Each flask was incubated in a humidified atmosphere at 37°C with 10% CO₂ until a confluence of 60-80% was reached. Cells were checked under a microscope for unusual colony formation or morphology.

Cells were trypsinised (**Section 7.3.1.4**) and after establishing a cell count the cell concentration was adjusted with growth medium to obtain the final concentration required.

Two 24-well plates were prepared with 1ml growth medium per well. 3T3 cells were used from passage P22 and seeded at two different densities into 24-well plates 5,000 (plate 1) and 200,000 cells per well (plate 2) the plates were gently moved to ensure even distribution of cells in the well. The multi-well plates were incubated for 24 hours at 37°C with 10% CO₂ to allow attachment of cells to the plastic. After 24 hours, 4 wells were trypsinised and counted with the Trypan Blue counting technique (**Section 7.3.1.7**), which was considered the 24-hour baseline.

After establishing the cell count baseline, 50µl aliquots of Dilutions 1, 2 and 3 of phage HER401 BS46 were added to the wells in quadruplicate. Dilutions 1, 2 and 3 were one, two and three-10-fold dilutions of a purified and twice filtered stock suspension of Phage HER401 BS46, with DMEM containing 10% FCS and penicillin/streptomycin as the diluent. Phage dilutions 1, 2 and 3 were calculated to be 2x10⁹, 2x10⁸ and 2x10⁷ pfu/ml respectively. Untreated cells were used as controls.

The multi-well plates were incubated for a further 24 hours at 37°C. After a total incubation time of 48 hours (but only 24 hours with phage) all wells were trypsinised and the number of dead/surviving cells counted using the Trypan Blue counting technique (**Section 7.3.1.7**). All counts were total counts of 5 squares in the haemocytometer. In all cases two counts were performed for each suspension from each well.

The results are shown in section 7.4.5.

7.3.1.13.2 Cytotoxicity assay using HPI staining and 3T3 cells

Cells were prepared as described in **Section 7.3.1.6**. Two 24- well plates were filled with 1ml pre-warmed growth medium per well. 3T3 cells were used from passage P28 and seeded at 5,000 cells per well into 24-well plates. The plates were gently moved to ensure even distribution of cells in the well. The multiwell plates were incubated for 24 hours at 37°C with 10% CO₂, to allow attachment of cells to the plastic.

After 24 hours incubation 6 wells were stained with HPI stain and 6 random fields of view were counted with an inverted fluorescence microscope (**Section 2.1.4**). This was considered the 24-hour baseline, which was included to ensure cells were viable and present at the expected concentrations in a countable form.

Dilutions 1, 2 and 3 were one, two and three-10-fold dilutions of a purified and twice filtered stock suspension of stock suspension Phage HER401 BS46 at concentrations 2×10^9 , 2×10^8 and 2×10^7 pfu/ml, with DMEM plus 10% FCS and penicillin/streptomycin as the diluent. After establishing the 24-hour baseline 50µl aliquots of Dilutions 1,2 and 3 of Phage HER401 BS46 were added to the wells (n=6). The multi-well plates were incubated for 72 hours at 37°C with 5% CO₂. At time points 24, 48 and 72 hours after phage addition 6 wells each containing three different concentrations of phage and controls were stained with HPI and 6 random fields of each well were counted with an inverted UV-microscope. The number of viable cells (dark blue circular cells), necrotic (pink nucleus) and apoptotic cells (dark blue cells with bright nucleus) were recorded. Wells containing cells without phage addition were used as controls.

The results are shown in section 7.4.6.

7.3.1.13.3 Cytotoxicity assay measuring LDH release of 3T3 mouse fibroblast cells

3T3 mouse fibroblast cells from passage P31, grown in T25 flasks were trypsinised with 2ml trypsin for 3 minutes and counted using a haemocytometer and Trypan Blue stain.

24-well plates were prepared with 1ml pre-warmed growth medium per well and seeded with 5,000 cells per well. 3 wells were filled with 1ml of medium, without addition of cells. Each multi-well plate was incubated for 24 hours at 37°C with 5% CO₂ after which a 24-hour baseline cell count was performed on 3 wells in duplicate, using Trypan Blue and the cell morphology checked under a microscope. 50 µl aliquots of HER401 BS46 at concentrations 2x10⁹, 2x10⁸ and 2x10⁷ pfu/ml (Dilutions 1, 2 & 3) of a purified and twice filtered stock suspension were added to the wells containing cells in triplicate. Phage was also added to 9 wells containing medium without cells. After phage addition all multi-well plates were incubated for a further 24 hours. Wells without phage addition, containing 3T3 cells were used as controls.

On the following day 1 well containing only cells without phage was chosen and 100µl of lysis solution (Promega, Madison, USA) was added to 1ml of medium. The plate was incubated at 37°C, 5% CO₂ for 45-60 minutes, which was considered the positive control (100% value). After the incubation time elapsed a 96-well plate was filled with 50µl supernatant of each well in triplicate and 50µl of substrate solution was added.

As controls 50 µl aliquots of cell supernatants without phage, lysed cells and incubated medium (without cells +/- phage) were also pipetted in triplicate into the 96 well plates and 50µl of substrate solution was added. The 96-well plate was incubated at room temperature, protected from light for 30 minutes. After incubation a stop solution consisting of acetic acid (Promega, Madison, USA) was added. The plate was read in an automatic ICN Flow Titertek Multiskan Plus MKII plate reader (ICN Flow, Herts, UK), at 492 nm.

The results are shown in **Section 7.4.7**.

7.3.1.13.4 Cell proliferation assay measuring MTS formation of 3T3 mouse fibroblast cells (I)

3T3 mouse fibroblast cells from passage P37, grown in T25 flasks were trypsinised with 2ml trypsin for 3 minutes and counted using a haemocytometer. The cell density was adjusted and 48 wells were filled with 1ml of DMEM medium and 5,000 cells each. The

multi-well plate was incubated for 24 hours at 37 °C in a humidified, 5% CO₂ atmosphere. After this period a 24-hour baseline count was performed on 3 wells and the cell morphology checked under a microscope. 50 µl aliquots of HER401 BS46 at concentrations 2x10⁹, 2x10⁸ and 2x10⁷ pfu/ml (Dilutions 1,2 &3) of a purified and thrice filtered stock suspension were added to the experimental wells, using cell medium as diluent for phage (n=3). The originally twice filtered phage stock suspension (**Section 6.2.10**) had been syringe filtered a third time to exclude contamination and spoilage during handling. 50µl of lambda buffer was added to 9 wells containing cells to serve as controls. After phage addition the cells were incubated for 24, 48 and 72 hours and the number of viable cells was determined using CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) by Promega, (Madison, USA). Immediately before use the MTS solution was thawed in a 37°C waterbath and then mixed with prewarmed DMEM in a ratio of 1 part in 20. During manipulation of the MTS solution the lights in the laminar flow hood were switched off and the container protected from light. The relevant plate was removed from the incubator, the media removed and 200µl of the MTS-DMEM mixture were added to each well prior to incubation at 37°C in a humidified, 5% CO₂ atmosphere for 2 hours. 90µl from each well were pipetted into separate wells of a flat-bottomed 96-well plate in duplicate, avoiding formation of any air bubbles. The plate was transported to the plate reader in a light protective environment and read in an automatic ICN Flow Titertek Multiskan Plus MKII plate reader (ICN Flow, Herts, UK), at 492 nm and the absorbance recorded.

The results are shown in **Section 7.4.8**.

7.3.1.13.5 Cell proliferation assay measuring MTS formation of 3T3 mouse fibroblast cells (II)

The procedure described in **Section 7.3.1.4** was repeated using cells from passage P39. After the cell density was adjusted, 48 wells were filled with 1ml of DMEM medium and 5,000, 10,000 or 15,000 cells were added per well. 50 µl aliquots of HER401 BS46 at concentrations 2x10⁹, 2x10⁸ and 2x10⁷ pfu/ml (Dilutions 1, 2&3) of a purified and thrice filtered stock suspension were added to the wells in triplicate, using lambda

buffer as diluent. 50 µl of sterile lambda buffer was added to 9 wells and 9 wells remained without additions (Controls). CellTiter 96 AQueous One Solution Cell Proliferation Assay was followed as described in Section 7.3.1.13.4.

The results are shown in Section 7.4.9.

7.3.1.13.6 Microscopy of 3T3 mouse fibroblast cells

Microscopy can be used to confirm identity and estimate growth densities. The optical appearance of 3T3 mouse fibroblast cells at P50, grown under standard growth conditions for 72 hours in T25 flasks was recorded. Seeding density was 200,000 cells/flask. For photography of cell culture samples a Nikon Eclipse, TE 200-U inverted phase contrast light microscope and Nikon D1x digital camera using 200 x magnifications were used.

The results are shown in Section 7.4.10.

7.3.2 Propagation of 3T3 Swiss mouse fibroblast cells for feeder layers

A 3T3 Swiss mouse fibroblast immortalised cell line (Section 7.3.1), (Blond McIndoe Centre cell stock) was grown antibiotic free on growth medium (Section 7.3.1.3-7.3.1.6) in Cellstar T25cm standard cell culture flasks (Greiner Bio-One, Stonehouse, Gloucestershire, UK). Incubation took place in a humidified cell incubator with 5% CO₂ saturation.

Before trypsinisation or harvesting the T25 flask growing cells was rinsed with 5ml of HBSS warmed to 37°C, which was aspirated. 1ml of trypsin warmed to 37°C was added to the T25 flask and incubated for 2-4minutes. After ensuring cells had sufficiently detached from the plastic, 4ml of medium warmed to 37°C were added to the flask and the cells gently triturated.

1.25ml of the cell suspension was used to inoculate a sterile T25 flask, 5ml of standard medium (Section 7.3.1.2) warmed to 37°C were added and the flask incubated at 37°C in a humidified cell incubator with 5% CO₂ saturation.

7.3.2.1 Standard growth-medium for 3T3 cells for feeder layers

DMEM+1000mg/l of glucose, Glutamax, pyruvate and 10% FCS (Gibco), without antibiotic or antifungal supplementation was used to grow 3T3 cells at the Blond McIndoe Centre.

7.3.2.2 Gamma irradiation of 3T3 Swiss mouse fibroblast cells for feeder layers

90% confluent T25 flasks were removed from incubation, the medium was aspirated and the cells rinsed with 5ml of HBSS (37°C), which was then aspirated. 1ml of trypsin warmed to 37°C was added to the T25 flask and incubated for 2-4 minutes at 37°C. After ensuring cells had sufficiently detached from the growth vessel by gentle tapping 4ml of medium warmed to 37°C was added, a cell suspension was produced by trituration, counted and transferred into a sterile centrifuge tube. The tube was closed with a sterile lid, and irradiated with 3mGray using a γ -irradiation source, (Gammacell 1000, Cs137 source, Isomedix Inc., Parsippany, New Jersey). Once irradiated the tube was sprayed and wiped with 70% ethanol, returned to the flow hood for further use or refrigerated for 24 hours.

7.3.2.3 Standard growth medium for isolated keratinocytes

Rheinwald Green medium (R&G) was used for the cultivation of keratinocytes (Rheinwald, 1989). One bottle of DMEM 500ml (Gibco, Invitrogen, Paisley, UK), was freshly opened and the following ingredients added to 300ml:

- F12 100ml (F-12 Nutrient Mixture Ham) (1X), liquid - with L-glutamine, Invitrogen, Paisley, UK)
- Hydrocortisone 200 μ g/500ml (Sigma, Poole, England)
- FCS 100ml/500ml (Biosera, Ringmer, UK)
- EGF 5 μ g/500ml (Recombinant Human Epidermal growth factor), Invitrogen, Paisley, UK

- Recombinant *E. coli* Cholera Toxin 5ml of a 1×10^{-10} M working solution/500ml, azide free (Gentaur Molecular products, Brussels, Belgium).

7.3.3 Isolation of epithelial cells from human biopsy-keratinocytes

The isolation method was performed at the Blond McIndoe Centre Queen Victoria Hospital, East Grinstead. A human skin flap was obtained through surgical breast reduction with informed consent of the patient. 12ml of penicillin/streptomycin (Gibco), 25ml of trypsin and four aliquots of 24.5ml HBSS Gibco were pipetted into sterile Falcon centrifuge tubes. A working solution of dispase was prepared by adding 0.5ml of dispase stock solution (Gibco) of (0.95units/mg) 100mg/ml to 4 aliquots of 24.5ml HBSS to produce a working concentration of 2mg/ml. 7.5ml of penicillin/streptomycin were added to 24.5ml of HBSS (full strength), which was mixed by gentle rotation, 7.5ml of the mixture were removed and placed in measured volumes of HBSS to produce 1/2 and 1/4 and 1/8 strength dilutions.

All liquids from stock containers were decanted and dispensed into desired aliquots before opening the skin sample container, as it was likely to introduce microbial contamination into the flow hood. The skin flap was removed from its container and placed in the centrifuge tube containing full strength penicillin/streptomycin, the tube sealed and vigorously shaken. The skin flap was consecutively transferred to 1/2, 1/4 and 1/8 strength penicillin/streptomycin in HBSS and this process repeated to aid removal of the transient and intrinsic flora of the skin sample. After de-contamination the skin flap was rinsed with 10ml HBSS. The decontaminated skin flap was transferred to a sterile Petri dish with grid. Using two sterile forceps the skin tag was rolled out flat onto the dish, the perimeter marked and the surface area of the skin tag estimated at 4cm^2 . Using sterile scalpel and forceps the skin sample was cut into 4 squares which were placed in 25ml HBSS to avoid dehydration of the sample. The skin cuts were placed in centrifuge tubes containing the working solution of Dispase in HBSS (2mg/ml), the tubes sealed and placed in an orbital shaker in horizontal position set at 37°C , (100 rev/min for 60 minutes). The skin flap was tested if it was ready for further processing and placed back in the incubator for 60 minutes.

After incubation the skin flap was placed in a sterile square Petri dish and confirmed that the tissue was ready for further processing, by prising a small area of dermis and epidermis apart. The skin flap was arranged with the stratum corneum facing downwards, rolled flat and wetted with 1ml of HBSS to avoid dehydration. Using two sterile forceps a large surface area of the epidermis and dermis was prised apart (Processing of Dermis see **Section 7.3.4**). The epidermis part of the sample was bathed in 5ml BD Difco (10x) trypsin in a sterile falcon centrifuge tube and placed in an incubator with a shaking platform set to 37°C at 100rpm. Into the remainder of the sample, which could not be separated into two skin layers, criss-cross incisions were made using a sterile scalpel, it was placed in 5ml of trypsin in a sterile centrifuge tube and refrigerated at 2-8°C for 18 hours. After storage in the refrigerator the sample was processed in the same way as described previously, by prising the epidermis and dermis apart and trypsinizing for 1hour at 37°C.

After trypsinisation the sample presented itself as a cell sheet in a viscous suspension. The viscous part was removed, attempting to keep as much of the cell sheet and dislodged cell flakes in the centrifuge tube. 10ml of Rheinwald-Green (R&G) medium was added to the centrifuge tube. The cell sheet was placed on a sterile Petri dish and pieces of epithelial skin layers were scraped off onto the dish using sterile forceps, 5ml R&G medium were trickled over the scraped surface of the skin sample and the scrapings were added to the cell flakes in the centrifuge tube. The sample was centrifuged at 400g for 4 minutes in a B4i Jouan centrifuge. The supernatant liquid was removed and the cells suspended in 5ml fresh R&G medium. The cell suspension was added to several CellStar 75cm² culture flasks, R&G medium was added to give a final volume of 10ml per flask. 1-2x10⁶ 3T3 γ -irradiated cells (**Section 7.3.2.2**) were added to each flask, marked as P0, one of the flasks was supplemented with 0.2ml of Gibco penicillin/streptomycin to give a 1:50 dilution in the final volume of medium to have at least one harvest in case of infection. Flasks were incubated in a humidified atmosphere at 37°C with 10% CO₂ for 92 hours. This sample would give rise to keratinocyte culture. After initial incubation, cell morphology was checked under a microscope, with primary cells growing in the typical 'paving-slab' style, infection was ruled out and the flask containing antibiotic supplementation discarded.

Existing medium inside the antibiotic-free flask was vigorously triturated using a 5ml pipette to remove 3T3 cells, whilst the keratinocytes remained attached to the plastic. The medium was aspirated, cells and flask were twice washed with 5ml HBSS, with the second wash appearing clear. All liquid was aspirated; 10ml of R&G medium was added together with approx. 1.6×10^6 γ -irradiated 3T3 cells (**Section 7.3.2.2**). Medium change with γ -irradiated 3T3 cell supplementation was undertaken every 48 hours on a further three occasions. Cells were incubated for another 24 hours. Harvesting was initiated by washing 3T3 cells in the flask by vigorously triturating medium over the cells; medium was aspirated and twice replaced with 5ml HBSS, which was used to wash cells and flask.

HBSS was aspirated, 2.5ml of prewarmed trypsin was added and the flask incubated for 10minutes. The flask was patted against the palm of a hand to facilitate cell detachment and 3ml of R&G medium were added. Cells in medium were transferred to 50ml centrifuge tubes and spun at 400g for 4 minutes. Cells were resuspended in 1ml of medium supplemented with 10%DMSO, counted as 4.9×10^6 cells/ml, pipetted into a cryovial, frozen at -70°C for 24 hours and transferred to liquid nitrogen storage.

7.3.4 Isolation of epithelial cells from human biopsy-fibroblasts

The dermis part of the skin sample (**Section 7.3.3**) was placed in a separate sterile Petri dish, wetted with DMEM+10%FCS and cut into 5x5mm pieces. Each piece was placed in a separate well of a sterile 6-well culture plate, and moistened with 100 μ l of medium. The plate was incubated at 37°C , 5% CO_2 for 72 hours, propped up at a 30 degree angle, with the skin sample in the middle of the well and medium collected on the inside rim. After incubation the remaining medium was aspirated and 2ml of medium added slowly to the perimeters of each well to avoid detachment of skin explants from plastic. The plate was incubated for 120 hours at 37°C 10% CO_2 , media changes were undertaken every 48 hours. After this time the explants had attached to the plastic and were each rinsed with 2ml of HBSS, which was aspirated and trypsinised by adding 1.8ml trypsin to each well. The cells were incubated at room temperature for 3 minutes, transferred to a sterile centrifuge tube and the volume made up to 5ml with DMEM+10%FCS. The

cell suspension was centrifuged at 150g, resuspended in equal volumes of medium and counted using the Trypan Blue method (**Section 7.3.1.7**).

1ml of cell suspension in medium and 5ml of DMEM+10%FCS were added to T25cm² standard growth flasks. Each flask contained a calculated cell count of 1.6×10^5 cells/ml and was incubated at 37°C, 10% CO₂. Medium was changed every 72 hours and the cells were incubated for a total of 47 days from the time of isolation. The flasks were harvested at P3, with a cumulative population doubling (CPD) of 9.269, by rinsing twice with 5ml PBS, aspirating, adding 3ml of trypsin and incubating at 37°C for 3-5 minutes. As soon as the fibroblasts rounded up 5ml of growth medium was added. The cells were centrifuged and resuspended in medium supplemented with 10%DMSO chilled to 4°C. The cell suspension were dispensed into 1.5ml cryotubes and frozen at -20°C in a polystyrene box to promote a gradual temperature drop, then moved to -80°C and finally stored in liquid nitrogen.

7.3.4.1 Human primary fibroblasts - transfer from Blond McIndoe Centre to the University of Brighton

Human primary dermal fibroblasts SKF276 previously frozen as described in **Section 7.3.3** were defrosted at the Blond McIndoe Centre and seeded into a T200 flask at 7.5×10^5 cells per flask. After 72 hours incubation at 5% CO₂, 37°C in DMEM+10% FCS without antibiotics in a humidified atmosphere the morphology of the cells was checked under a microscope. The flask was filled with approx. 150 ml of DMEM + 10% FCS, then sealed with an airtight transport cap and sterile parafilm and transported in a light-protective environment to the University of Brighton.

On arrival all excess medium was removed from the cells, centrifuged and stored at 2-8°C for future use. Cells were checked for damage under a microscope, no significant cell detachment was observed and cells appeared healthy. The medium was aspirated, 15 ml of HBSS used to rinse the flask surfaces, which was aspirated and 15ml trypsin added. The flask was incubated at 37°C for 3 minutes with 5% CO₂ in a humidified atmosphere. 30ml of DMEM+ 10% FCS was added and the content of the flask pipetted into a centrifuge tube, which was centrifuged at 500g for 5 minutes. The supernatant

liquid was aspirated and the cell pellet re-suspended in 1ml DMEM, carefully triturated and counted using a haemocytometer with the Trypan Blue method.

The cells were seeded in 10,000 cell increments in twelve T25 flasks, 7ml of DMEM+10% FCS were added per flask, incubated at 37°C with 5% CO₂ in a humidified atmosphere for 48 hours. After incubation the cells were harvested and frozen (**Section 7.3.4.2**). Flask 12 was used for further propagation, without freezing.

7.3.4.2 Harvesting and freezing of human fibroblasts SKF276

Before trypsinisation DMEM was prewarmed to 37°C in a water bath, while trypsin was warmed to approx. 20°C. Cells obtained from the Blond McIndoe Centre were trypsinised by adding 2ml trypsin-EDTA (1x) and incubating for 3 minutes at 37°C, 5%CO₂. 4ml of DMEM+10% FCS were added, the content of each flask individually centrifuged at 500g for 5 minutes.

The cell pellets of flasks 1-11 were resuspended in 1ml of cryomedium, which consisted of 70% v/v DMEM, 10%v/v DMSO and 20%v/v FCS. 20µl were removed for counting. The remaining content of each resuspended flask was transferred into Nalgene cryoware Cryovials, which were placed in Nalgene Cryo 1°C Freezing containers and immediately placed in a -80°C freezer and later transferred to liquid nitrogen storage.

Population doublings of cells in each flask were calculated and average population doublings were calculated using the formulae described in **Appendix 1**.

The cell pellet of flask 12 was immediately resuspended in DMEM+10%FCS, seeded in two T25 flasks at a concentration of 1×10^5 cells/flask and incubated at 37°C, 5%CO₂ for 48 hours (P5).

7.3.4.3 Standard incubation of human fibroblasts SKF276

SKF276 samples were routinely incubated in a humidified Heraeus, Heracell, (Thermo Fisher Scientific, Waltham, MA, USA), cell incubator with 10% CO₂ saturation.

7.3.4.4 Standard growth vessels for human fibroblasts SKF276

Cells were grown routinely in Nunclon surface T25 or T75 flasks with filter caps for sterile Nunclon surface 24-well plates with fitting lids (Nunc, Roskilde, Denmark).

Growth medium volumes were 7ml and 20ml for T25 and T75 flasks. T185 growth flasks (Nunc, Roskilde, Denmark) were used for one experiment with 50ml medium.

7.3.4.5 Standard growth-medium for Fibroblasts SKF276

Primary fibroblasts were grown on DMEM with 1000mg glucose/L, L-glutamine and sodium bicarbonate with 10% FCS, without antibiotic or antifungal supplementation for all experiments.

7.3.4.6 Seeding SKF276 cells from frozen stock

DMEM was pre-warmed to 37°C in a water bath. A cryovial was removed from liquid nitrogen and swiftly defrosted by swirling in a beaker of warm water. In T25 flasks 7ml of pre-warmed medium was pipetted to the bottom of the flask, the flasks were held upright and the cell suspension was trickled over the inside flat bottom of the flasks, to allow attachment of cells. Flasks were then tilted to allow even distribution of medium and swiftly moved to the incubator.

After incubation for 24 hours at 37°C with 10% CO₂ in a humidified atmosphere medium was changed.

7.3.4.7 Trypsinizing of human fibroblasts SKF276 in T25 flasks

Before trypsinisation DMEM was prewarmed to 37°C in a water bath and trypsin was warmed to approx. 20°C. The medium was aspirated and the cells and inside of flask were washed using 4ml HBSS, which was then aspirated. 2ml of trypsin were added to the T25 flask, incubated for 3-5 minutes at 37°C with 10% CO₂ in a humidified atmosphere and 4ml of DMEM with 10% FCS were added. Cells were transferred to a centrifuge tube, centrifuged at 400g for 5minutes, resuspended in 1ml cell medium and counted.

The routine volume of medium used to grow cells in T25 flasks was 7ml DMEM+10%FCS.

7.3.4.8 Trypsinizing of human fibroblasts SKF276 in T75 flasks

Trypsin was prewarmed, medium aspirated and growth vessels washed as described in **Section 7.3.4.7**. To each T75 flask 5ml of trypsin were added, incubated for 3-5 minutes at 37°C with 10% CO₂ in a humidified atmosphere, followed by an addition of 10ml of DMEM with 10% FCS. Cells were transferred to a centrifuge tube, centrifuged at 400g for 5minutes, resuspended in 1ml cell medium and counted.

The routine volume of medium used to grow cells in T25 flasks was 20ml DMEM+10%FCS

7.3.4.9 Trypsinizing of human fibroblasts SKF276 in 24-well plates

Before trypsinisation DMEM was prewarmed to 37°C in a water bath and trypsin was warmed to approx. 20°C. A maximum of two wells from a 24-well plate were trypsinised concurrently. Medium was aspirated from both wells, 400µl HBSS was added to each well and triturated to wash each well. The liquid was aspirated, 200µl trypsin was added to both wells and incubated for 5 minutes at 37°C with 10% CO₂ in a humidified atmosphere. 800µl DMEM with 10% FCS was added to each well, the cells resuspended in medium, transferred to a microcentrifuge tube, centrifuged at 400g for 5 minutes, resuspended in 1ml cell medium and twice counted.

7.3.4.10 Mycoplasma screening of human fibroblasts SKF276

At P74 5,000 cells were seeded per well in 12-well plate and tested using a Gibco-MycoTect kit, (Gibco, Invitrogen, Paisley, UK) with a total incubation time of 168 hours.

The results are shown in **Section 7.4.11**

7.3.4.11 Doubling times of human primary fibroblasts SKF276 in 24-well plates

A sub-culture of Flask 12 from Passage 5 (**Section 7.3.4.2**) was trypsinised, the harvested cells pooled, counted (P6) and split. One part was seeded in 24-well plates, containing 1ml cell medium at a cell density of 5,000 cells/well. The remaining part (subculture Flask 12) was used to seed two T25 flasks at a concentration of 1×10^5 cells/flask and were incubated at 37°C, 10%CO₂ for 120 hours (P6) with medium changes every 48 hours.

The cells in 24-well plates were incubated at 37°C, 5%CO₂ for 48 hours. After 24hours and 48 hours of incubation 6 wells were washed with 400µl HBSS, trypsinised and counted in duplicate.

The results are shown in **Section 7.4.13**

7.3.4.12 Cell proliferation assay of SKF276 cells (I)

A sub-culture of Flask 12 (P6) in two T25 flasks (**Section 7.3.5.10**) was trypsinised, pooled and counted in duplicate. The cell suspension (P7) was diluted, 54 wells of 24-well plates were seeded with 10,00 cells and the volume of each well was made up to 1ml using DMEM with 10% v/v FCS, 18 wells were filled with 1ml medium only (Control). Multi-well plates were incubated for 24 hours to allow attachment of cells to the plastic at 37°C with 10% CO₂ in a humidified atmosphere. After overnight incubation each well was checked for the presence of healthy mitotic cells using an inverted optical microscope.

18 wells on each plate were chosen to make additions of 50µl of two tenfold dilutions of concentrated phage HER401 BS46 and 50µl of lambda buffer. 22 wells containing cells with medium and 18 wells with medium alone were used as controls without adding further reagents. Four wells containing only cells and medium were washed with 400µl HBSS, trypsinised and counted in duplicate to perform a 24-hour baseline cell count using the Trypan Blue method, to ensure the approximate viable cell concentration per well was 10,000cells. After phage addition and baseline counting multi-well plates were

incubated for 24, 48 and 72 hours 37°C with 10% CO₂ in a humidified atmosphere and the number of viable cells was determined using an MTS assay (**Section 7.3.4.12.1**).

The results are shown in **Section 7.4.14**

7.3.4.12.1 MTS assay

After addition of reagents to experimental wells, cells were incubated for 24, 48 and 72 hours and the number of viable cells was determined using CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS). Immediately before use the MTS solution was thawed in a 37 °C water bath and mixed with prewarmed DMEM in a ratio of 1 part in 20 (100µl+1900µl). At time point 24 hours a negative control was included by aspirating medium off six wells containing cells and medium only. 20µl of 70% IMS was added to four wells and evaporated for 10 minutes under a laminar flow hood, whilst covering the other rows of cells with the plastic lid. During manipulation of the MTS solution the lights in the laminar flow hood were switched off and the container protected from light. The relevant plate was removed from the incubator and the media removed. Each well washed twice with 400µl HBSS, which was aspirated and 200µl of the MTS-DMEM mixture were added to each well and incubated at 37°C in a humidified, 10% CO₂ atmosphere for 2 hours. Wells pre-treated with IMS were not washed before addition of MTS-DMEM. 90µl from each well were pipetted into separate wells of a flat-bottomed 96-well plate in duplicate, avoiding formation of any air bubbles. The plate was transported to the plate reader in a light protective environment and read in an automatic ICN Flow Titertek Multiskan Plus MKII (ICN Flow, Herts, UK) plate reader, at 492 nm and the absorbance recorded.

7.3.4.13 Cytotoxicity assay using human fibroblasts SKF276 (I)

Human dermal fibroblasts (P5) Flask 4 from frozen stock were defrosted and propagated to CPD 17.9. Cells were harvested and used to seed 24-well plates with 100,000 cells/well, with cell medium added to reach a total volume of 1ml per well. Cells were incubated for 24 hours to allow attachment and after this period each well was checked under an inverted microscope for presence of healthy mitotic cells. 100µl

of the following samples were added to wells containing cells in (n=6): concentrated, purified and thrice filtered phage preparation at three consecutive ten-fold dilutions (phage dilution 1= 2×10^9 , dilution 2= 2×10^8 pfu/ml and dilution 3= 2×10^7 pfu/ml, lambda buffer, sonicated cells (**Section 7.3.5.1**), purified water and ethanol. Six wells were filled with DMEM without cells.

MTS (see **Section 7.3.4.12.1**) and LDH (see **Section 7.3.4.13.1**) assays were performed after incubation for 24 hours.

The results are shown in **Section 7.4.15**

7.3.4.13.1 LDH method using SKF276 cells

24-well plates were removed from incubation and 100µl of lysis solution (Promega, Madison, USA) was added to 3 wells containing SKF276 cells to obtain the maximum LDH release control. The plate was incubated at 37°C, 10% CO₂ for 45-60 minutes after which time a 96-well plate was filled with 50µl of supernatant liquid from six experimental wells in duplicate and 50µl of substrate solution was added. As controls 50 µl aliquots of six wells cell containing cells without experimental addition and incubated medium were also pipetted in duplicate into the 96 well plate and 50µl of substrate solution was added. The 96-well plate was incubated at room temperature whilst protected from light for 30 minutes. After incubation 50µl of a stop solution consisting of acetic acid (Promega, Madison, USA) was added to all wells. The plate was read in an automatic plate reader at 492 nm.

7.3.5 Preparation of samples containing endotoxin

1-2 colonies of *Acinetobacter* HER1401 and HER1151 were used to inoculate 2x10 ml TSB, which were incubated at 37°C for 16-18 hours at 120rpm. Seven to nine ml of the overnight suspension were used to inoculate one 500ml conical flask containing 300ml TSB which was incubated at 37°C, 120rpm for approx. 3-5 hours until OD₆₀₀ of either cell suspension reached 0.5. The cell suspensions were centrifuged at 2011g for 10 minutes and resuspended in equal amounts of PBS. 1ml of each of the resuspended cell suspension was withdrawn for viable counts as described in **Section 2.5**.

Results for viable counts of the resuspended cell suspension are shown in **Section 7.4.16**.

7.3.5.1 Bacterial cells disrupted using a sonicator

3x5ml of the HER1401 and HER1151 cell suspension in PBS were dispensed into Fisherbrand centrifuge tubes. The content was sonicated on ice (5x60 second bursts, setting 3-4) using a Sonicator Ultrasonic Processor XL, (Head Systems, Ultrasonic, Inc., Plainview, NY) setting 3-4, set to Pulsar Duty cycle %/sec to 95. Cell debris was removed by centrifugation at 2011g for 15 min. The supernatant liquid was syringe filtered (**Section 2.1.2**) to remove any remaining viable bacteria and stored in Eppendorf tubes at 2-8°C. The amount of endotoxin was determined using a Limulus Amoebocyte Lysate test (Cambrex, USA) (**Section 7.3.5.6**). The results are shown in **Section 7.4.18**

7.3.5.2 Bacterial cells disrupted by phage

100ml of the HER1401 cell suspension in PBS prepared as described in **Section 7.3.5** were placed in a sterile conical flask and infected with phage HER401 BS46 as described in **Section 2.7.1**. The flask containing bacteria and phage was placed in a static incubator set to 37°C for 20 minutes to facilitate phage attachment. The shaking mechanism was then activated and the flask incubated for 16-18 hours at 120rpm. The suspension was stored at ambient temperature, protected from light for 72 hours, then centrifuged at 2011g for 15 minutes and filtered using a 0.45µm syringe filter. The filtrate was stored at 2-8°C until required. The presence of viable phage in the sample was confirmed using the soft overlay method. The amount of endotoxin was determined using the LAL test (**Section 7.3.5.6**). The results are shown in **Section 7.4.18**.

7.3.5.3 Bacterial cells lysed by antibiotic

Bacterial lawns were produced by dipping a sterile cotton bud into the undiluted bacterial suspensions prepared above and spreading across overdried TSA plates. Mastring M11 Multodiscs (Mast Diagnostics, Merseyside, UK) were applied to the surface and the plates incubated for 16-18 hours at 37°C.

Results of the Multodisc experiment can be found in **Section 7.4.17**.

10ml of a 100unit/ml solution of penicillin G (Sigma Aldrich, Gillingham, UK) was prepared with sterile water and syringe filtered using a 0.45 µm syringe filter. 5ml of the resuspended cell suspension of HER1151 (5×10^8 cells/ml) were mixed with 5ml of the 100unit/ml penicillin G solution and incubated at 37°C for 20minutes. The resulting lysed cell suspension was filtered using a 0.45µm syringe filter and stored at 2-8°C.

The amount of endotoxin released from the cells was determined using the LAL test (**Section 7.3.5.6**). The results are shown in **Section 7.4.18**.

7.3.5.4 Bacterial cells disrupted using a Bead-Beater

Zirconium beads with a diameter of 0.5mm were decanted into 10ml universal glass flasks with loosely fitted metal caps and autoclaved at 121°C for 15minutes.

The small chamber of a bead-beater (Biospec Products, Bartlesville, Oklahoma, USA) was the only part of the equipment to come into contact with the cell suspension and this was rinsed with 50ml of 70% IMS and allowed to dry under UV light in a laminar flow cabinet. 25ml of cell suspension of HER1401 (prepared as described above) in PBS and 25cm³ of sterile Zirconium beads were placed in the small chamber of a bead-beater which had the following measurements: 3.5cm (height) x 2.5cm x 2.5cm. The screw-cap lid of the small chamber was firmly closed, the small chamber placed inside the outer chamber, which was filled with crushed ice to avoid overheating. A rubber gasket was secured to prevent cooling water leaking out of the system and a sealing ring was affixed the entire mechanism in place. The assembled parts were placed onto the rubber clutch and the bead-beater switched on continuously for 15minutes. After treatment the beads were allowed to sink to the bottom and the liquid was decanted through sterile Whatman filter paper grade 1 (GE Healthcare, Buckinghamshire, UK) into a 50ml centrifuge tube. The supernatant was syringe filtered, transferred to a sterile centrifuge tube and stored at 2-8°C. The amount of endotoxin was determined using the LAL test (**Section 7.3.5.6**). The results are shown in **Section 7.4.18**

7.3.5.5 Bacterial cells disrupted using an autoclave

30ml of the cell suspension of HER1401 in PBS prepared as above were placed in sterile glass bottles and autoclaved at 121°C, for 30minutes. After cooling to room temperature the bottles were stored at 2-8°C. The amount of endotoxin was determined using the LAL test (Section 7.3.5.6). The results are shown in Section 7.4.18

7.3.5.6 Endotoxin test

A Cambrex (BioWhittaker, East Rutherford, NJ, USA) Limulus Amoebocyte Lysate (LAL) QCL-1000 test was used to quantitatively determine endotoxin concentrations in various samples. The basic principle of this test kit was to mix reagents with the sample, incubate at 37°C for 10minutes, add a substrate solution and incubate for a further 6 minutes and then apply a stop reagent to halt the chemical reaction. The presence of endotoxin in the sample was indicated by the development of a yellow colour, the absorbance of which could be determined spectrophotometrically at 405-410nm. The correlation between the absorbance and the endotoxin concentration was linear in the 0.1-1.0 EU/ml range. As the absorbance was directly proportional to the endotoxin quantity in the sample, the endotoxin amount could be calculated from a calibration curve. All steps were performed in a positive pressure laminar flow hood to ensure maximal protection of the experiment, sterile individually wrapped surgical gloves were worn and sterile wrapped plasticware was used for all experiments.

pH readings of all samples (7.3.5.1-7.3.5.5) were taken and confirmed as pH=7, using pH tape (Fisherbrand, Fisher Scientific, Loughborough, UK). One vial containing Chromogenic substrate (Cambrex, BioWhittaker, East Rutherford, NJ, USA) was reconstituted with 6.5ml of LAL reagent water to yield a concentration of approx. 2mM and kept at 2-8°C in a light protective environment during storage. One vial containing 20 EU of *E. coli* endotoxin (Cambrex, BioWhittaker, East Rutherford, NJ, USA) was reconstituted with 1.0ml of LAL Reagent Water warmed to room temperature, shaken and vortexed vigorously for 20 minutes using a vortex mixer. To obtain a solution with a working concentration of 1.0EU/ml 0.1ml of Endotoxin stock solution was added to

1.9ml of LAL Reagent water. The solution was vigorously vortexed for 1minute. All solutions containing *E. coli* endotoxin were stored at 2-8°C.

In order to produce an endotoxin calibration plot, four standard concentrations (1.0, 0.5, 0.25, 0.1EU/ml) were produced using the *E.coli* endotoxin and all dilutions were vigorously vortexed for 1 minute. A 100-well Honeycomb 2 plate (Thermo Electron Corp., Basingstoke, UK) was placed in an incubator for 10 minutes to pre-equilibrate to 37°C. 50µl of samples (7.3.5.1-7.3.5.5) were pipetted into the appropriate well in duplicate taking extreme care to avoid formation of air bubbles. 2 wells were filled with endotoxin free water at room temperature. 50µl of the four standard concentrations of endotoxins were vigorously vortexed for 1 minute, then immediately pipetted into the appropriate well in duplicate. Once the control, standards and all samples were pipetted into the wells, the multi-well plate was placed in a 37°C incubator for 5minutes. The LAL lysate (Cambrex, NJ, USA) was reconstituted by piercing the lid of two vials with a needle to avoid a vacuum. 1.4ml of Endotoxin free water was then injected into each vial and gently rotated to avoid formation of foam. The content of both vials was pooled before use and the content protected from light exposure during storage. Any remaining reconstituted LAL substrate solution was frozen and stored at -20°C.

At time T=0, 50µl of LAL was added to each well, avoiding formation of air bubbles and attempting to be consistent in the order and rate of pipetting. The plate was gently tapped to facilitate mixing and placed back in the incubator at 37°C for 10 minutes.

At time T=10 minutes, 100µl of chromogenic substrate solution pre-warmed to 37°C was added to all wells, the plate gently tapped and placed back in the incubator.

At time T=16 minutes 100µl of stop reagent (acetic acid, Cambrex, NJ, USA) was added, the plate gently tapped to ensure even distribution and the absorbance was read at 405 nm in a Bioscreen C MBR (Thermo Electron Corp., Basingstoke, UK).

The results are shown in **Section 7.4.18**

7.3.5.7 Cytotoxicity assay using human fibroblasts SKF276 (II)

Human dermal fibroblasts (P5) Flask 2 from frozen stock were defrosted and propagated to CPD 21.72. 10,000 cells were seeded per well in 24-well plates and made up to 1ml of DMEM+10%FCS, the 24-well plates were stacked in doubles, placed in sterile plastic containers with loosely fitted lids, to combat drying effects and incubated for 24 hours at 37°C with 5% CO₂ in a humidified atmosphere. A 24 hour baseline count was done by trypsinizing and counting four random wells in duplicate, cells were confirmed to appear healthy, viable and attached to the wells using an inverted light microscope.

The following agents were added in 100µl aliquots to assigned wells with 10 repeats:

- HER401 BS46 purified and thrice filtered phage suspension at a concentration of 1.3×10^{14} without further dilution in lambda buffer.
- *E. coli* Endotoxin (Cambrex, BioWhittaker, East Rutherford, NJ,USA), containing 1.0EU/ml Endotoxin
- Autoclaved sample of HER1401 containing approx. 1.7EU/ml Endotoxin (**Section 7.3.5.6**)
- Sonicated sample of HER1401 containing approx. 4EU/ml Endotoxin (**Section 7.3.5.1**)
- Filtered phage lysate in PBS (**Section 7.3.5.2**) containing approx. 4.54EU/ml Endotoxin
- Endotoxin free water (Cambrex, BioWhittaker, East Rutherford, NJ,USA)
- Lambda buffer
- Ten wells contained DMEM and cells only.

All plates were incubated for a further 72 hours at 37°C with 5% CO₂ in a humidified atmosphere and analysed using LDH and MTS assays (**Sections 7.3.12.1 and 7.3.13.1**)

at time points 24, 48 and 72hours. For the MTS assay a calibration plot was completed (**Section 7.3.5.8**).

The supernatant liquids of dedicated experimental and control wells were removed in quintuplicate and frozen in 200µl aliquots for cytokine testing (**Section 7.3.5.10**) at time point 72 hours.

The results are shown in **Section 7.4.19**.

7.3.5.8 Correlation of cell numbers to MTS absorbance readings

1000, 2000, 5000, 10000, 20000, 40000, 50000, 80000 and 100000 cells with CPD 17.9 were seeded in 24-well plates, each concentration in quadruplicate. 1ml of DMEM was added and the plates were incubated for 2 hours at 37°C in a humidified, 5% CO₂ atmosphere. 4 wells each of the same concentration were used to perform an MTS assay (**7.3.4.12.1**) and the absorbance of each well was read in duplicate. The mean absorbance of eight readings could then be matched to the viable cell counts using a calibration graph **Section 7.4.20**.

7.3.5.9 Cell proliferation assay using SKF276 cells (II)

Human dermal fibroblasts (P9) Flask 2 at CPD 25.69 were counted and multiple 24-well plates were seeded with 10,000 cells/well. The volume of each well was made up to 1ml using DMEM with 10% v/v FCS and 6 wells were filled with 1ml medium only. Multi-well plates were incubated for 24 hours to allow attachment of cells to the plastic at 37°C with 5% CO₂ in a humidified atmosphere. After overnight incubation each well was checked for the presence of healthy mitotic cells using an inverted optical microscope.

18 wells on each plate were chosen to make additions of 100µl of two tenfold dilutions of concentrated phage HER401 BS46 and 100µl of lambda buffer. 22 wells containing cells with medium and 18 wells with medium alone were used as controls without adding further reagents. Four wells containing only cells and medium were washed with 400µl HBSS, trypsinised and counted in duplicate to perform a 24-hour baseline cell

count using the Trypan Blue method, to ensure the approximate viable cell concentration per well was 10,000 cells. After addition of the same agents used in **Section 7.3.5.7**, and baseline counting multi-well plates were incubated for 24, 48 and 72 hours 37°C with 5% CO₂ in a humidified atmosphere and the number of viable cells was determined using an MTS assay (**Section 7.3.4.12.1**).

The results are shown in **Section 7.4.21**.

7.3.5.10 Cytokine release of human primary fibroblasts SKN276 challenged with phage

Supernatant liquids removed in the course of cytotoxicity experiments (**Section 7.3.5.7**) were pipetted into 100-200µl aliquots and frozen at -10°C for cytokine experimentation.

7.3.5.10.1 Wash buffer for cytokine experiments

PBS with 0.05% Tween-20 was freshly prepared or used within 3 days of preparation, with 2-8°C storage.

7.3.5.10.2 Multi-well plates

For all cytokine experiments 96-well Iwaki Elisa plates (Asahi technology, Chiba, Japan) were used. Standards were diluted within the multi-well plates.

7.3.5.10.3 Preparation of IL-1β, IL-6, IL-8 and TNF-α standards

Cytokine standards (BD Bioscience, California, USA) were warmed to room temperature, reconstituted with 1ml deionised water, vortexed, pipetted in 50µl aliquots per vial and frozen at -70°C.

As required one 50µl frozen standard aliquot was allowed to thaw/equilibrate to room temperature for each 96-well plate used. For TNF-α and IL-1β standards a 500pg/ml standard was prepared from the stock standard and serially diluted within a 96-well plate, to obtain standards of 250pg/ml, 125pg/ml, 62.5pg/ml, 31.3pg/ml, 15.6pg/ml and 7.8pg/ml in a volume of 200µl. For IL-6 assays a 300 pg/ml standard was prepared from

the stock standard and serially diluted within a 96-well plate, to obtain standards of 150pg/ml, 75pg/ml, 37.5pg/ml, 18.8pg/ml, 9.4pg/ml and 4.7pg/ml in a volume of 200 μ l. For IL-8 assays a 200pg/ml standard was prepared from the stock standard and serially diluted within a 96-well plate, to obtain standards of 100pg/ml, 50pg/ml, 25pg/ml, 12.5pg/ml, 6.3pg/ml and 3.1pg/ml in a volume of 200 μ l.

For all standards respective well contents were mixed by careful trituration. Two wells were filled with only assay diluent to obtain the zero standard (0pg/ml).

7.3.5.10.4 Sample treatment and dilution of SKN276 supernatant liquids for IL-1 β assay- pilot experiment (I)

Interleukin release from HDF samples was expected to be low as Spörri & Maret (2004) reported no release of IL-1 β from unstimulated HDF. Spörri et al. (1996) reported a release of 2-3pg/ml when the same cells were directly stimulated with T-cells, while Park (2004) reported levels of IL-1 β release in HDF starved of FCS between 5(\pm 3.55) and 6.33(\pm 4.16)pg/ml, heat shock caused release of up to 7(\pm 3.82) pg/ml.

For the pilot experiments supernatants from wells containing phage dilution 1, 2 & 3; sonicated bacterial cells; cells treated with ethanol; and cells only (controls) were used undiluted and assayed in duplicate (n=6). The cytokine assay was performed as described in **Section 7.3.5.10.5**.

The results are shown in **Section 7.4.22**.

7.3.5.10.5 Cytokine assays for IL-1 β for HDF (II)

Wells of a 96-well plate were coated with Capture antibody diluted in coating buffer (BD Biosciences, San Diego, USA) after which the plate was sealed and incubated overnight at 4°C. The wells were aspirated and washed three times with over 300 μ l/well of wash buffer. After the last wash plates were inverted and blotted on absorbent paper to remove residual buffer. The plates were blocked with 200 μ l/well PBS with 10% FCS at pH7 (assay diluent) and incubated at ambient temperature for 1 hour. The wells were then aspirated and washed as before, with final blotting of residual liquid.

Standards as described in **Section 7.3.5.10.3** were prepared within each 96-well plate, to obtain a final volume of 100µl/well in duplicate for each standard.

Frozen cell supernatant liquids previously removed from experimental and control wells (**Section 7.3.5.7**) of human dermal fibroblasts SKF276 grown in 24-well plates were defrosted by bringing to ambient temperature. Samples used were supernatant liquids from cells exposed to phage dilutions, sonicated bacteria, lambda buffer and DMEM. 100µl of each freshly defrosted sample was pipetted into appropriate wells; the plates were sealed and incubated at ambient temperature for 2 hours. The wells were aspirated, washed five times with wash buffer, with blotting on absorbent paper between each wash.

Working detector (BD Bioscience) for one plate was prepared by mixing 40µl detection antibody (BD Bioscience) with 10ml assay diluent followed by vortexing. 100µl of detection antibody was added to each well after which the plates were sealed and incubated for 1 hour at ambient temperature. Wells were aspirated and washed five times with over 300µl/well of wash buffer. After the last wash plates were inverted and blotted on absorbent paper to remove residual buffer. 40µl of Enzyme Reagent (BD, Bioscience) was diluted in 10ml assay diluent and vortexed. 100µl of dilute Enzyme Reagent was added to each well after which the plates were sealed and incubated for 30minutes at ambient temperature. Wells were aspirated and washed seven times with over 300µl/well of wash buffer. After the last wash plates were inverted and blotted on absorbent paper to remove residual buffer.

100µl of Substrate solution, (BD Biosciences) (1:1 Tetramethylbenzidine and Hydrogen Peroxide) were added to each well and plates were incubated without sealing for 30minutes at ambient temperature in the dark. 50µl of Stop Solution (2N H₂SO₄) was added to each well. Absorbance was read at 450nm within 30 minutes of stopping the reaction.

The results are shown in **Section 7.4.22**.

7.3.5.10.6 Cytokine assays for IL-6, IL-8 and TNF- α for HDF

96-well plates were prepared as described in **Section 7.3.5.10.5** and cytokine standards were prepared within each 96-well plate, to obtain a final volume of 100 μ l/well in duplicate for each standard as described in **Section 7.3.5.10.3**.

The same samples as used in **Section 7.3.5.10.5** were defrosted as described there.

100 μ l of each freshly defrosted sample was diluted if necessary and pipetted into appropriate wells, the plates were sealed and incubated at ambient temperature for 2 hours. The wells were aspirated, washed five times with wash buffer, with blotting on absorbent paper between each wash. Working detector for one plate was prepared by mixing 40 μ l Detection antibody (BD Bioscience) with 40 μ l streptomycin-HRP (BD Bioscience) in 10ml Assay diluent and vortexed. 100 μ l of pre-prepared Working detector (Detection Antibody+Sav-HRP reagent) was added to each well, plates were sealed and incubated for 1hour at ambient temperature. The wells were aspirated, washed seven times with wash buffer, allowing wash buffer to soak well for 30-60 seconds for each wash step and with blotting on absorbent paper between each wash. 100 μ l of substrate solution consisting of 1:1 Tetramethylbenzidine and Hydrogen Peroxide was added to each well and plates were incubated without being sealed for 30 minutes in the dark at room temperature. 50 μ l of Stop solution (2N H₂SO₄) was added to each well. Absorbance was read at 450nm within 30 minutes of stopping the reaction. Standard curves were drawn and used to calculate interleukin levels, dilutions were accounted for in the calculations.

The results are shown in **Sections 7.4.23, 7.4.24 and 7.4.27**.

7.3.5.10.7 Sample treatment and dilution of SKN276 supernatant liquids for IL-8 assay- pilot experiment (I)

As the approximate interleukin output of the human dermal fibroblasts (HDF) was unknown, a range of dilutions were chosen for the pilot experiments. Depending on expected maximum and minimum interleukin readings from literature, samples required dilution to ensure detectable and meaningful results. IL-8 levels of unstimulated HDF

was reported to be 2ng/ml, stimulated HDF ranged nearer 200ng/ml (Spörri et al., 1996) or between 21(+4) and 65 (+20) ng/ml depending on experimental design (Müller et al., 2000). For the pilot experiments supernatant liquids from wells containing phage dilution; sonicated bacterial cells and cells only (controls) were diluted 1:10, 1:100, 1:500 and 1:1000 and assayed in duplicate. Dilutions were taken into account when calculating interleukin values.

The results are shown in **Section 7.4.25**.

7.3.5.10.8 Sample treatment and dilution of SKN276 supernatant liquids for IL-8 assay (II)

Samples were used undiluted and in 1:5 dilution, which was accounted for when calculating interleukin values.

The results are shown in **Section 7.4.26**.

7.3.5.10.9 Microscopy of SKN276 human fibroblast cells

Content of Flask 2 from frozen stock was defrosted (**Section 7.3.4.6**) and propagated to CPD 25.69, (P9) as described in **Section 7.3.4.8**. 10,000 and 20,000 cells were seeded in six wells of a 24-well plate, standard growth medium was added and the cells were incubated under standard conditions for 24 hours. 100µl phage preparation HER401 BS46 at concentrations 2×10^9 was added to one half of samples and incubated for 72h.

The microscopic appearance of the human cells was photographed using a Nikon Eclipse, TE 200-U inverted phase contrast light microscope and Nikon D1x digital camera at 200x magnification.

The results are shown in **Section 7.4.28**.

7.3.6 Cytotoxicity assay for human keratinocytes SKN0106 with 3T3 mouse fibroblast feeder layers

A T75 flask of human dermal primary keratinocytes SKN0106 (SKN) and seven 50ml Falcon centrifuge tubes containing γ -irradiated 3T3 cells (3T3) (**Section 7.3.2.2**) were

obtained from Dr. James, Blond McIndoe Centre, P2 estimated CPD 6. The growth flask had been filled completely with Rheinwald & Green (R&G) medium, sealed with a transport cap and sterile parafilm.

Upon arrival the excess R&G medium in the flask was pipetted into 50ml centrifuge tubes, centrifuged at 600g for 10minutes to remove cell debris and the supernatant liquid stored at 2-8°C in the refrigerator to be used as medium during the entire experiment. The flask containing cells was filled with 20 ml of medium, the transport cap was removed and replaced with a standard sterile filter cap. The cells were confirmed to be viable and attached to the growth vessel and the flask was incubated at 37°C with 10% CO₂ in a humidified atmosphere for 24 hours.

After overnight incubation the medium covering the cells was aspirated, the cells were washed with 2x5ml HBSS, which was aspirated and they were then trypsinised with 5ml of trypsin for 5 minutes. The trypsin was neutralised with 10ml R&G medium, the cells were centrifuged for 2-3 minutes at 400g, carefully resuspended in 10ml R&G and counted with the Trypan blue method (**Section 7.3.2.2**). The keratinocyte cell harvest was 2.9×10^6 cells.

24-well plates were prepared by adding sterile 0.9% NaCl to the outside wells to avoid excessive evaporation of medium on the edges. Keratinocytes were seeded into 24-well plates at a concentration 4×10^4 cells/well and 4×10^4 cells/well of γ -irradiated 3T3 cells were added to each well containing keratinocytes. Equal amounts of wells were seeded with mouse fibroblast cells as controls. The final volume of the wells containing cells was made up to 1ml with R&G medium. Eight wells were filled with medium without cell addition to calculate the medium background control at timepoints 24, 48 and 72 hours for the LDH experiment.

The 24-well plates were incubated overnight at 37°C with 10% CO₂ in a humidified atmosphere for 24 hours. After overnight incubation 100 μ l purified and three times filtered phage preparation HER401 BS46 in lambda buffer at a concentration of 2×10^{10} pfu/ml was added to experimental wells and 100 μ l sterile lambda buffer was added to

an equal number of wells as control. 24-well plates were incubated for a further total of 72 hours, with LDH (7.3.6.1) and MTS (7.3.6.2) assays taking place every 24 hours.

The results are shown in **Section 7.4.29**.

7.3.6.1 LDH assay for human keratinocytes SKN0106 with 3T3 mouse fibroblast feeder layers

At timepoints 24, 48 and 72 hours 24-well plates were removed from incubation. Three wells containing only SKN0106 + 3T3 cells and 3T3 cells were chosen and 100µl of lysis solution (Promega, Madison, USA) was added to 1ml of medium to determine the maximum LDH release control for SKN + 3T3 and 3T3 cells.

The lysis plate was incubated at 37°C, 10% CO₂ for 45-60 minutes. After the incubation time elapsed a 96-well plate was filled with 50µl supernatant liquid of six experimental wells in duplicate and 50µl of substrate solution was added. As controls 50 µl aliquots of six wells cell containing SKN + 3T3 and 3T3 cells with lambda buffer or phage, cells without experimental addition and incubated medium were also pipetted in duplicate into the 96 well plate and 50µl of substrate solution was added. The 96-well plate was incubated at room temperature whilst protected from light for 30 minutes. After incubation 50µl of a stop solution consisting of acetic acid (Promega, Madison, USA) was added. The plate was read in an automatic ICN Flow Titertek Multiskan Plus MKII plate reader (ICN Flow, Herts, UK), at 492 nm.

The results are shown in **Section 7.4.29**.

7.3.6.2 MTS assay for human keratinocytes SKN0106 with 3T3 mouse fibroblast feeder layers

At timepoints 24, 48 and 72 hours 24-well plates were removed from incubation and the number of viable cells was determined using CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) by Promega, Madison, USA. Immediately before use the MTS solution was thawed in a 37°C waterbath and then mixed with prewarmed R&G medium in a ratio of 1 part in 20. During manipulation of the MTS solution the lights in

the laminar flow hood were switched off and the container protected from light. The relevant plate was removed from the incubator and the media removed. 200µl of the MTS-medium mixture was then added and incubated at 37°C in a humidified, 10% CO₂ atmosphere for 2 hours. 90µl from each well were pipetted into separate wells of a flat-bottomed 96-well plate in duplicate, avoiding formation of any air bubbles. The plate was transported to the plate reader in a light protective environment and read in an automatic ICN Flow Titertek Multiskan Plus MKII plate reader (ICN Flow, Herts, UK), at 492 nm and the absorbance recorded.

The results are shown in **Section 7.4.30**.

7.3.7 Colony formation assay using V79 hamster cells

The intention behind this range of experiments was to further build on the body of evidence investigating the potential cytotoxic effect of phage using a colony formation assay (**Section 7.3.7.7**). Literature regularly cites endotoxin release during the phage lytic cycle to be a potential hindrance to phage therapy for Gram negative infections (Slopek et al., 1985; Kutter & Sulakvelidze 2005). In addition, the presence of endotoxins present in therapeutic phage preparations as an impurity has in the past been blamed for treatment failure, however, current purification protocols should prevent this from occurring now.

A number of experiments were conducted to quantify the endotoxin levels in various phage preparations used in the course of this investigation (**Section 7.3.5**). An additional focus of the investigation was to examine if lambda buffer or endotoxins - which may be contained in the phage preparation depending on the method with which it was produced and the stage of purification- may have effects on colony formation. The intention was to compare the amount of endotoxin released from *Acinetobacter* produced by different treatments such as sonication, lysis through phage, cell bursting by lytic antibiotics, shear forces and autoclaving.

7.3.7.1 V79 hamster cells

Japanese hamster lung cells, V79 originally from Japanese Cancer Research Resources Bank, Japan, from University of Brighton stocks were used.

7.3.7.2 Standard growth vessels for V79 hamster cells

All V79 cell experiments were undertaken in either sterile Nunclon surface T25 or T75 flasks with filter caps or sterile Nunclon surface 24-well plates with fitting lids both Nunc, (Roskilde, Denmark).

7.3.7.3 Standard growth medium for V79 hamster cells

V79 cells were maintained in continuous logarithmic growth and routinely propagated on DMEM with 1000mg glucose/L, L-glutamine and sodium bicarbonate (Sigma Aldrich, Gillingham, UK) supplemented with 10% FCS, (Biosera, Ringmer) 1% (v/v) penicillin and streptomycin (Sigma Aldrich, Gillingham, UK), which is hereafter referred to as 10% FCS DMEM. For all colony formation assays cells were grown in DMEM with 5% FCS added, which is referred to as 5% FCS DMEM.

7.3.7.4 Standard seeding of V79 hamster cells

All cell culture work was carried out under sterile conditions in a class II cell culture laminar flow cabinet (Cyttox II, Envair Ltd., Lancaster, UK). Cells of a known concentration were added to individual wells of a 24-well plate and then 1ml of pre-warmed medium was added to each well. The lid was applied and placed back in the incubator. All wells on the periphery were filled with sterile demineralised water to ensure equal humidity levels throughout the plate, forming a humidity ring around the wells containing V79 cells. In T25 flasks the cell suspension was pipetted over the inside back of the flasks, to allow attachment of cells, followed by 7ml of pre-warmed medium.

7.3.7.5 Standard incubation of V79 hamster cells

V79 cells were routinely incubated in a humidified Heraeus, Heracell, (Thermo Fisher Scientific, Waltham, MA, USA) cell incubator with 5% CO₂ saturation. The 24-well plates were routinely stacked at a maximum of two plates in each stack in disinfected plastic containers with loosely covered lids. This was done to avoid accidental removal of 24-well plate lids and to ensure even moisture and temperature levels during heavy usage of the incubator. T25 flasks were routinely stacked at a maximum of three flasks each stack and flasks were checked daily to ensure filter lids were not contaminated with medium.

7.3.7.6 Trypsinisation of V79 hamster cells grown in T25 flasks

Before trypsinisation DMEM was pre-warmed to 37°C in a water bath, and trypsin (trypsin EDTA 500mg/L Cambrex, BioWhittaker, East Rutherford, NJ, USA) was warmed to approx. 20°C. Growth flasks were removed from the incubator; the medium covering the cells was aspirated and replaced with 2ml of trypsin. The vessels were incubated for 3 to a maximum of 5 minutes at 37°C, 5% CO₂ in a humidified incubator, including microscopic checks to ensure the majority of cells were detached. Persistent gentle striking of the growth vessels against a hard surface or the palm of a hand was used to aid detachment.

4ml of DMEM were added to each T25 flask, to neutralise the trypsin. The cells and supernatant liquid were then transferred to 50ml centrifuge tubes and spun at 500g for 5min. The supernatant liquid was aspirated and the resulting cell pellet was re-suspended in 1ml DMEM to produce a cell suspension

7.3.7.7 Colony formation assay to assess the cytotoxicity of phage and lambda buffer on V79 cells

The colony formation assay after Rose et al. (2004) was modified to suit the needs of this investigation. V79 hamster cells were obtained from University of Brighton stock at P20 in a T75 flask, sub-cultured at confluence and maintained on 10% FCS DMEM. The confluent flasks were harvested at P22 and the cells counted and seeded at 50 cells per

well in 24 well plates. Only 16 wells were seeded per plate, the remaining 8 outside wells were filled with sterile demineralised water to combat the drying effect during the experiments. All seeded wells contained 10% FCS DMEM and the plates were incubated for 24h at 37°C, 5% CO₂ in a humidity incubator. Following incubation the medium was aspirated from each well containing cells and refilled with 0.5ml of either pure 5% FCS DMEM (Control) or medium containing phage or lambda buffer at various concentrations in triplicate.

To prepare the phage samples a concentrated, purified and thrice filtered phage suspension HER401 BS46 was diluted 10-fold in lambda buffer. The resulting phage suspension (2.2×10^9 pfu/ml) was then diluted with 5% FCS DMEM to give dilutions of 50, 25 and 12.5%. The control samples were prepared in the same fashion, using sterile lambda buffer instead of phage. Three wells containing controls were included on each 24 well plate. Each percentage dilution was plated in quadruplicate.

All plates were incubated for a further 5 days at 37°C, 5% CO₂ in a humidity incubator. After incubation the medium was aspirated and 0.5ml glutaraldehyde 25% (v/v) was added to each well and left for 30 minutes at room temperature to fix the cells. The glutaraldehyde was removed, each well was rinsed in 0.5ml PBS, which was aspirated and 0.5ml 10% (v/v) Giemsa stain (Sigma-Aldrich, Poole, Dorset, UK) added and left for 40 minutes at room temperature. Each well was rinsed with several 0.5ml increments of deionised water to remove the stain and allowed to air dry. The number of colonies in each well was counted by eye with the help of a magnification glass using a backlit colony counter. The average number of colonies counted of each extract dilution was calculated and compared to the control wells to express the effect of each dilution on colony formation.

The results are shown in **Section 7.4.31**.

7.3.7.8 Colony formation assay to assess the cytotoxicity of phage, diluents and cell extracts with varying endotoxin concentrations on V79 cells

The same setup as used in **Section 7.3.7.7** was used with a wider sample range, and cells at P23 were used for the experiment. Ten-fold dilutions of phage suspension (2.2×10^9 pfu/ml) were diluted with 5% FCS DMEM to give dilutions of 75, 50, 25 and 12.5% of phage in lambda (PL) or PBS (PPBS). The control samples were prepared in the same fashion, using sterile lambda buffer or PBS instead of phage. Three wells containing only DMEM as controls were included on each 24 well plate.

Other samples used with varying endotoxin concentrations were (**Section 7.3.5**) *E. coli* 1.0EU/ml Endotoxin (Cambrex, BioWhittaker, East Rutherford, NJ, USA), autoclaved sample of HER401 BS46, sonicated samples of HER401 BS46, syringe filtered but unpurified phage lysate of HER401 BS46 which were diluted with 5% FCS DMEM to give dilutions of 75, 50, 25 and 12.5% of extract, each of which was added to three wells.

The results are shown in **Section 7.4.32**.

7.4 Results

3T3 Swiss mouse fibroblast cell line

7.4.1 Cumulative population doublings of 3T3 Swiss fibroblast cell line in 24-well plates over 72 hours

Time since seeding	P	N _L cells seeded	N _H cells harvested (well 1)	N _H cells harvested (well 2)	N _H cells harvested (well 3)	CPD	Original cell conc.	Mean CPD
Flask 1							5.5 x10 ⁵	
0 h	21	5,000	-	-	-			
24 h	21		6.3x10 ³	8.7x10 ³	3.8 x10 ^{3*}	0.3,0.8,0		0.57
48 h	21		1.4 x10 ⁴	7.5 x10 ³	3.8 x10 ^{3*}	1.5,0.6,0		1.04
72 h	21		3.9x10 ⁴	7.5x10 ⁴	4.1x10 ⁴	2.9,3.9,3.0		3.30
Flask 2							8.5x10 ⁵	
0 h	21	5,000	-	-	-			
24 h	21		8.8 x10 ³	1.1 x10 ⁴	7.5 x10 ³	0.8,1.1,0.6		0.85
48 h	21		2.5 x10 ⁴	3.7 x10 ⁴	4.0 x10 ⁴	2.3,2.9,3.0		2.73
72 h	21		8.9x10 ⁴	8.6x10 ⁴	9.2x10 ⁴	4.2,4.1,4.2		4.15

Table 7.1 Population doublings for 3T3 cells, 24-well plates

Legend Table 7.1:

**These values were not considered in calculating the (cumulative) population doublings (CPD) for 24 hours*

P Passage, N_L cells seeded, N_H cells harvested

After 24h incubation time (**Table 7.1**) the recovered cell count in well 3 (subcultured from flask 1) dropped below the 5,000 cells seeded. The cell titre slowly recovered after prolonged incubation and gave a similar reading to wells subcultured from flask 2 after 72 hours. This demonstrates that cell titres can easily drop without intervention and addition of potentially cytotoxic agents. This effect is likely to be most prominent with low seeding densities, as employed in low surface-area 24-multiwell-plates (approx. 2cm² per well) and consequently short incubation times, as the cells are contact inhibited. This effect needs to be taken into account in conjunction with the experimental setup used. If only cell counts were relied upon to measure cytotoxicity, it would be easily possible to produce false positives.

This demonstrates the importance of using replicates, assessing cell growth over longer incubation periods and utilising more than one method to assess cytotoxicity.

The data (**Table 7.1**) demonstrated that even immortalised cell lines, grown under identical conditions have different growth rates in various wells. To account for this statistical means of multiple repeats and more than one method of cytotoxicity testing needed to be employed.

7.4.2 Determination of cumulative population doublings over 96 hours in T25 flasks

Results of cell counts of flask 1 and 2 in **Table 7.2** correlated with each other and CPD from flask two of **Table 7.1**. The cell density of cells in both flasks was typical of growth behaviour after subculture (Freshney, 2005), doubling times of 18 hours were typical for 3T3 cells according to ATCC, which aided confirmation of the identity of cells present (<http://www.lgcpromochem-atcc.com>, visited 13 May 2008). The variation in cell numbers was far less than in 24-well plates, partly because the seeding

density was double the density of 24-well plates, the volume of growth medium was higher and the cells had a larger surface area to cover without contact inhibition.

Time since seeding	Passage number	Date	N _L cells seeded	N _H cells harvested	N _H cells harvested	Mean Cumulative population doublings	Original cell conc.
				Flask 1	Flask 2	-	1.1x10 ⁸
0 h	40	23/05/05	10,000	-	-	-	
24 h	40	24/05/05		1.8x10 ⁴	2x10 ⁴	0.93	
48 h	40	25/05/05		7.4x10 ⁴	8.6x10 ⁴	3	
72 h	40	26/05/05		4.5x10 ⁵	3.8x10 ⁵	5.38	
96 h	40	27/05/05		1.1x10 ⁶	1.1x10 ⁶	6.78	

Table 7.2 Population doublings for 3T3 cells, T25 flasks

7.4.3 Validation of phage activity in tissue culture conditions without mammalian cells present

The results of this preliminary experiment demonstrated that phage remains viable for at least 48 hours, when incubated in tissue culture conditions in either lambda buffer or DMEM (**Figure 7.1**)

One-way ANOVA followed by Gabriel's multiple comparison procedure was used to test whether there was a difference between calculated phage counts (pfu/ml) in DMEM or lambda buffer over 24 and 48 hours incubation compared to controls.

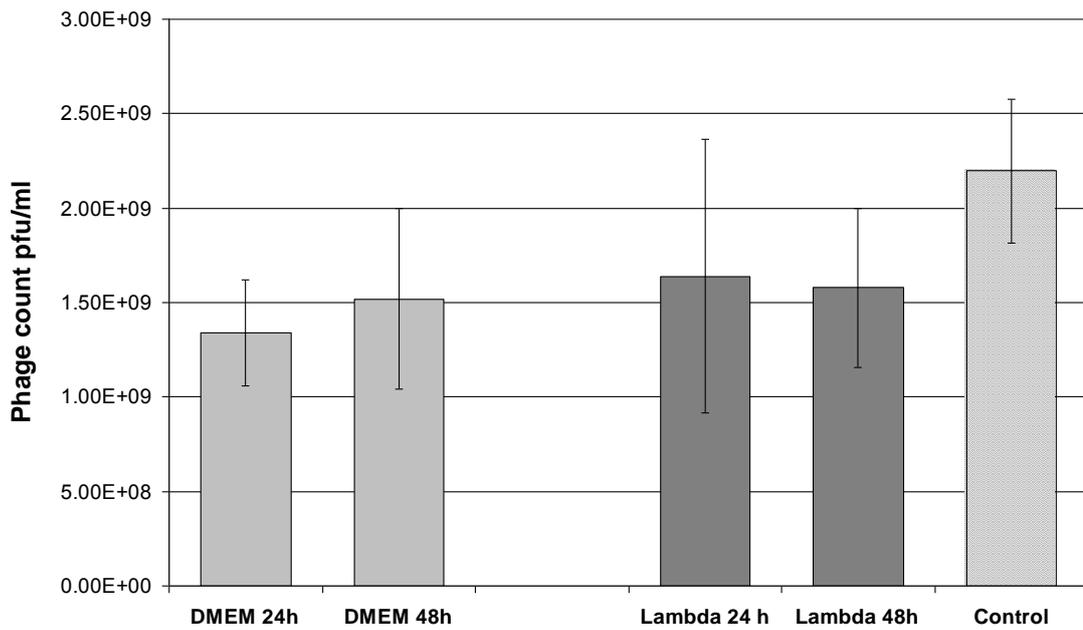


Figure 7.1 Phage particles recovered after incubation in DMEM or lambda in tissue culture conditions

Both DMEM and lambda buffer samples appeared to show less total phage recovery than controls, however there was no statistically significant difference in mean phage counts between any samples or controls ($p=0.045$, $F=2.861$). Fluctuations in recoverable phage particles may be due to adsorption onto plastic or possible inactivation of phage caused by incubation in tissue culture conditions. The average number of plaques obtained from the DMEM sample drawn after 48 hours was higher than the number of plaques after 24 hours of incubation, however these differences were not statistically significant. These insignificant fluctuations may be due to pH changes in DMEM which may be more prone to pH changes than lambda which is a buffer.

7.4.4 Validation of phage activity in tissue culture conditions with mammalian cells present

From **Figures 7.2-7.4** it appeared that the number of recoverable, viable phage was highest after a short incubation period (24h) in DMEM, regardless of the presence or absence of cells.

Statistical methods as in **Section 7.4.3** were used;

Phage Dilution 1 at timepoint 24 hours in lambda buffer gave rise to significantly lower plaque counts than any samples or baseline ($p < 0.05$; $F = 3.073$). After incubation for 48 and 72 hours the baseline phage count was significantly higher than phage titres of samples. The standard deviations of samples in DMEM (Dilution1, with or without cells) **Figure 7.2** were near 10-fold larger than standard deviations of experimental samples incubated for 48 or 72 hours (**Figures 7.3 & 7.4**). This implies that with regards to Dilution 1 there was a large drop in recoverable phage particles in the lambda buffer samples only, however after prolonged incubation the phage titres of all samples dropped significantly with respect to the baseline counts. This is evidence that at least after 48 and 72 hours incubation tissue culture conditions appeared to affect recovery of viable phage.

For phage Dilution 2 (**Figures 7.5-7.7**), there was a significant difference between the baseline phage count (not exposed to cell culture conditions) samples and cell culture control without cells at timepoints 24, 48 and 72 hours ($p < 0.001$; $F = 12.475$; 14.242; 13.484). There was no difference between any of the samples regardless of presence or absence of cells. These results imply that with regards to phage recovery the presence or absence of mammalian cells has no significant impact, however exposure to cell culture conditions statistically significantly reduced phage recovery.

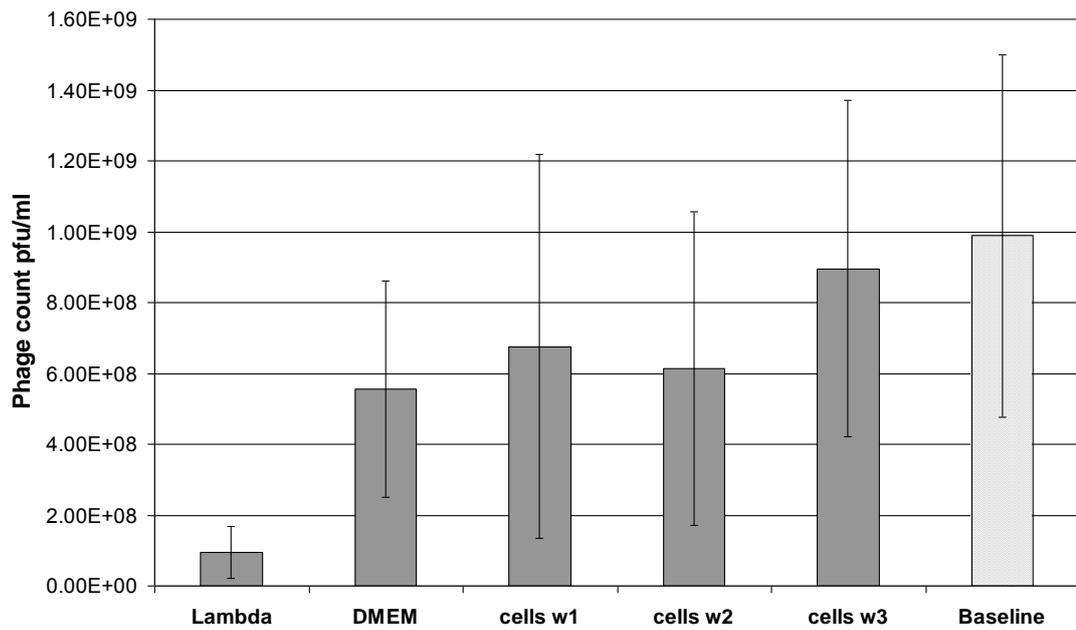


Figure 7.2 Phage count after 24h of incubation in tissue culture conditions (Dilution 1)

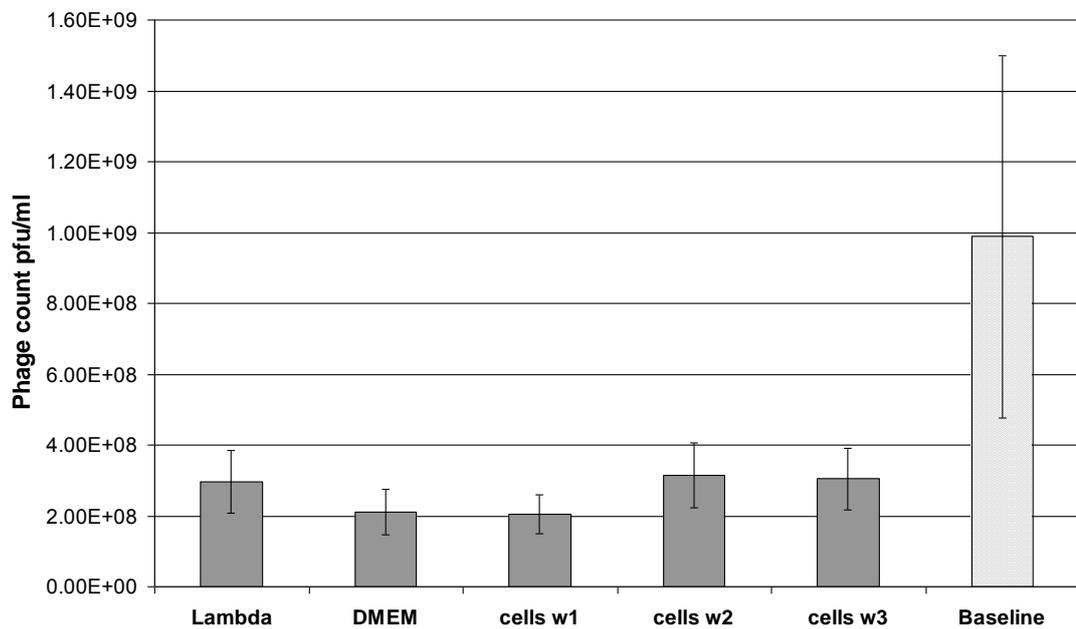


Figure 7.3 Phage count after 48h of incubation in tissue culture conditions (Dilution 1)

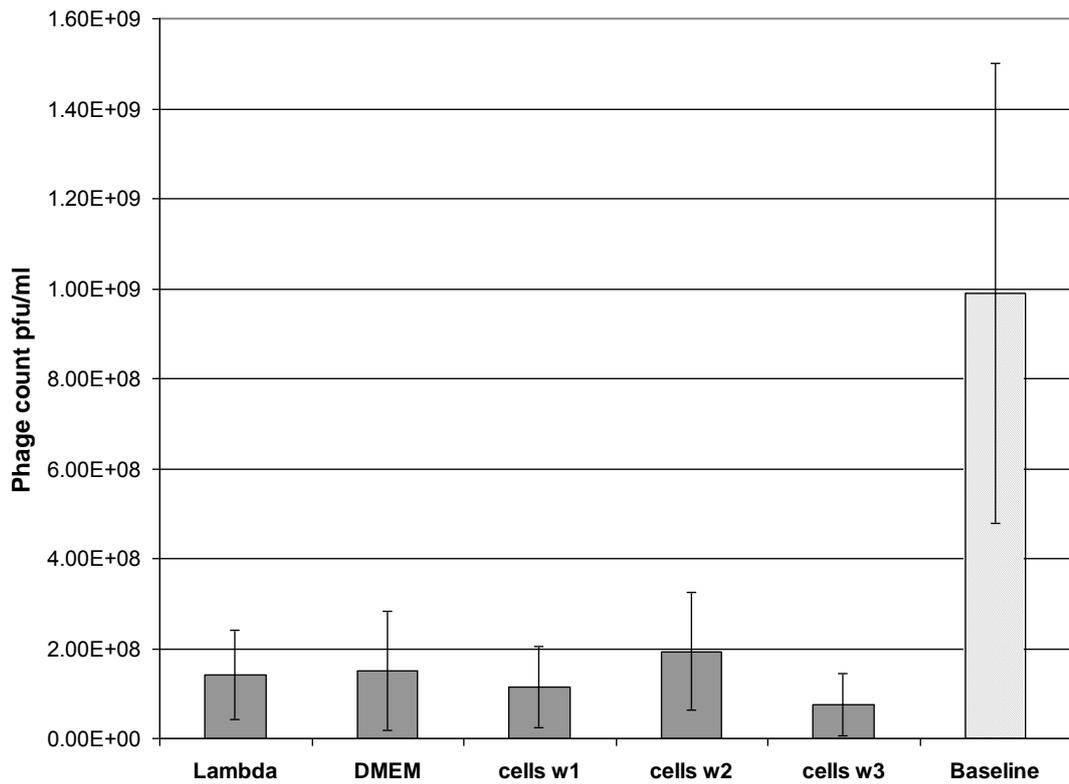


Figure 7.4 Phage count after 72h of incubation in tissue culture conditions (Dilution 1)

The changes in phage numbers may be due to variables like pH changes caused by cell metabolites or dissolved CO₂, which may in turn affect recoverability or viability of phage particles. Other contributing factors may be phage aggregation, adsorption of phage particles to plasticware or adhesion to human cells. Changes in salt concentration or pH are known to affect plaque counts (Kutter & Sulakvelidze, 2004), however it is unlikely that the changes in the original medium affected the outcome of the soft agar overlay assay, as samples underwent multiple 10-fold dilution steps in lambda buffer before plating. The detailed mechanisms would necessitate further investigation.

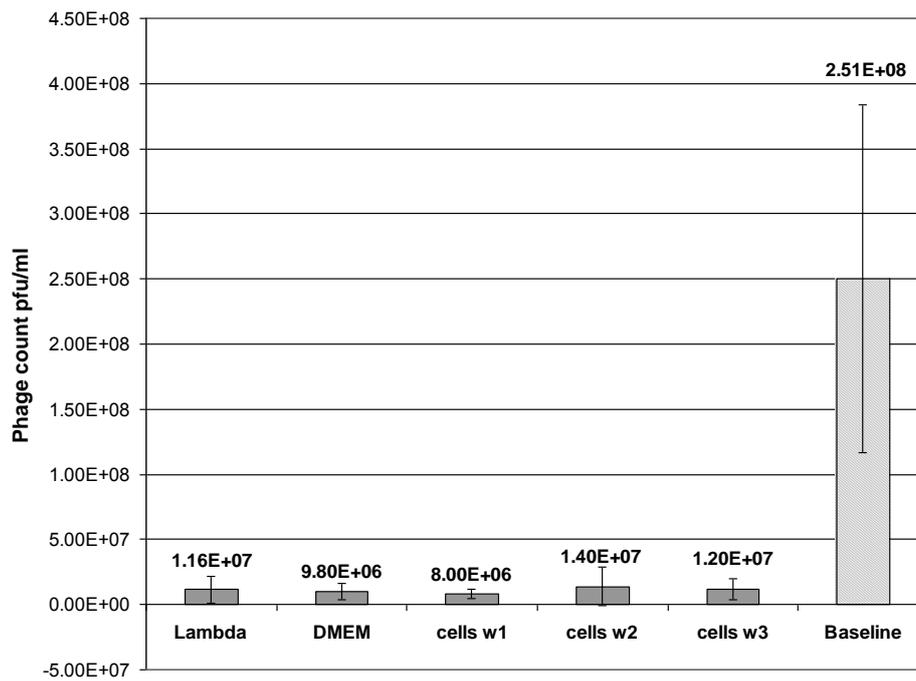


Figure 7.5 Phage count after 24h of incubation in tissue culture conditions (Dilution 2)

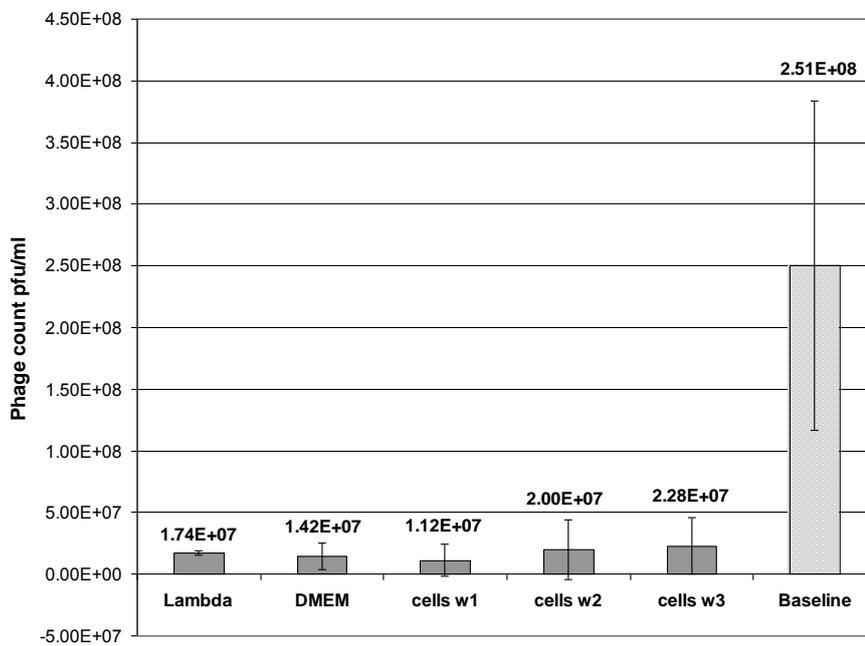


Figure 7.6 Phage count after 48h of incubation in tissue culture conditions (Dilution 2)

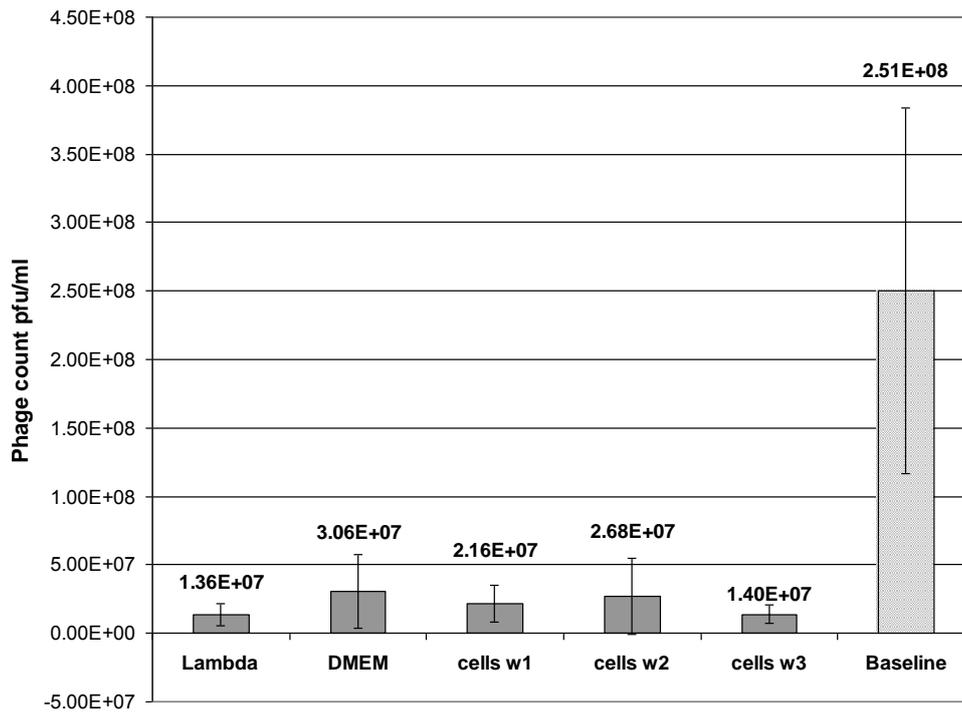


Figure 7.7 Phage count after 72h of incubation in tissue culture conditions (Dilution 2)

7.4.5 Cytotoxicity assay using Trypan Blue and 3T3 cells

The cell numbers of dead cells exposed to phage were below 3 after 24 hour incubation, regardless of the cell seeding density of 5×10^3 or 2×10^5 (**Tables 7.3-7.12**).

The Trypan blue method relies on counting small volume samples of cell suspension, which are extrapolated to calculate the overall cell number, small deviations in cell counts particularly at the lower seeding density had dramatic effects on the calculated percentage of cell death. To allow objective assessment whether the effect of phage had a significant effect on cell data was statistically treated (**Tables 7.3-7.12**).

Plate 1	live cell count	Live cells/ml	dead cell count	% live	% dead
24-hr Baseline					
Well 1 count 1	4	9.6×10^3	0	100	0
Well 1 count 2	4	9.6×10^3	0	100	0
Well 2 count 1	1	2.4×10^3	0	100	0
Well 2 count 2	3	7.2×10^3	0	100	0
Well 3 count 1	4	9.6×10^3	0	100	0
Well 3 count 2	3	7.2×10^3	0	100	0
Well 4 count 1	2	4.8×10^3	0	100	0
Well 4 count 2	5	1.2×10^4	0	100	0

Table 7.3 Cell count 24h-Baseline for Plate 1 (seeding density 5×10^3 cells per well)

Plate 1	live cell count	Live cells/ml	dead cell count	% live	% dead
Control					
Well 1 count1	7	1.7×10^4	0	100	0
Well 1 count 2	5	1.2×10^4	0	100	0
Well 2 count 1	2	4.8×10^3	0	100	0
Well 2 count 2	2	4.8×10^3	0	100	0
Well 3 count 1	3	7.2×10^3	0	100	0
Well 3 count 2	5	1.2×10^4	0	100	0
Well 4 count 1	11	2.6×10^4	0	100	0
Well 4 count 2	10	2.4×10^4	0	100	0

Table 7.4 Cell counts for Control (seeding density (sd) 5×10^3 cells/well)

Plate 1	live cell count	Live cells/ml	dead cell count	% live	% dead
Dilution 1					
Well 1 count 1	5	1.2×10^4	1	83.3	16.6
Well 1 count 2	4	1.5×10^4	1	80	20
Well 2 count 1	8	1.9×10^4	0	100	0
Well 2 count 2	7	1.7×10^4	0	100	0
Well 3 count 1	1	2.4×10^3	0	100	0
Well 3 count 2	2	4.8×10^3	0	100	0
Well 4 count 1	8	1.9×10^4	1	88.9	11.1
Well 4 count 2	7	1.7×10^4	1	87.5	12.5

Table 7.5 Cell counts for phage HER401 BS46 dilution 1 (sd 5×10^3 cells/well)

Plate 1	live cell count	Live cells/ml	dead cell count	% live	% dead
Dilution 2					
Well 1 count 1	2	4.8×10^3	0	100	0
Well 1 count 2	3	7.2×10^3	2	60	40
Well 2 count 1	7	1.7×10^4	0	100	0
Well 2 count 2	3	7.2×10^3	0	100	0
Well 3 count 1	2	4.8×10^3	0	100	0
Well 3 count 2	10	2.4×10^4	0	100	0
Well 4 count 1	2	4.8×10^3	1	66.7	33.3
Well 4 count 2	3	7.2×10^3	1	75	25

Table 7.6 Cell counts for phage HER401 BS46 dilution 2 (sd 5×10^3 cells/well)

Plate 1	live cell count	Live cells/ml	dead cell count	% live	% dead
Dilution 3					
Well 1 count 1	4	9.6×10^3	0	100	0
Well 1 count 2	5	1.2×10^4	0	100	0
Well 2 count 1	6	1.4×10^4	0	100	0
Well 2 count 2	10	2.4×10^4	0	100	0
Well 3 count 1	6	1.4×10^4	0	100	0
Well 3 count 2	7	1.7×10^4	0	100	0
Well 4 count 1	2	4.8×10^3	0	100	0
Well 4 count 2	3	7.2×10^3	1	75	25

Table 7.7 Cell counts for phage HER401 BS46 dilution 3 (sd 5×10^3 cells/well)

Plate 2	live cell count	Live cells/ml	dead cell count	% live	% dead
24hr- Baseline					
Well 1 count 1	14.8	3.6×10^4	0	100	0
Well 1 count 2	10.8	2.6×10^4	0	100	0
Well 2 count 1	13	3.1×10^4	0	100	0
Well 2 count 2	12.6	3.0×10^4	0	100	0
Well 3 count 1	12.6	3.0×10^4	0	100	0
Well 3 count 2	13.4	3.2×10^4	0	100	0
Well 4 count 1	10.6	2.5×10^4	0	100	0
Well 4 count 2	11.6	2.8×10^4	0	100	0

Table 7.8 Cell count 24hr-Baseline for Plate 2 (sd 2×10^5 cells per well)

Plate 2	live cell count	Live cells/ml	dead cell count	% live	% dead
Control					
Well 1 count 1	38	9.1×10^4	0	100	0
Well 1 count 2	51	1.2×10^5	1	98.1	1.9
Well 2 count 1	41	9.8×10^4	0	100	0
Well 2 count 2	59	1.4×10^5	0	100	0
Well 3 count 1	28	6.7×10^4	2	93.3	6.7
Well 3 count 2	25	6.0×10^4	1	96.2	3.8
Well 4 count 1	67	1.6×10^5	0	100	0
Well 4 count 2	87	2.1×10^5	3	96.7	3.4

Table 7.9 Cell counts for Control (sd 2×10^5 cells/well)

Plate 2	live cell count	Live cells/ml	dead cell count	% live	% dead
Dilution 1					
Well 1 count 1	76	1.8×10^5	0	100	0
Well 1 count 2	88	2.1×10^5	1	98.9	1.1
Well 2 count 1	67	1.6×10^5	0	100	0
Well 2 count 2	73	1.8×10^5	0	100	0
Well 3 count 1	52	1.3×10^5	0	100	0
Well 3 count 2	70	1.7×10^5	0	100	0
Well 4 count 1	62	1.5×10^5	0	100	0
Well 4 count 2	54	1.3×10^5	0	100	0

Table 7.10 Cell counts for phage HER401 BS46 dilution 1 (sd 2×10^5 cells/well)

Plate 2	live cell	Live	dead cell	% live	% dead
Dilution 2	count	cells/ml	count		
Well 1 count 1	85	2.0 x10 ⁵	0	100	0
Well 1 count 2	77	1.8 x10 ⁵	0	100	0
Well 2 count 1	43	1.0 x10 ⁵	2	95.6	4.4
Well 2 count 2	37	8.9 x10 ⁵	0	100	0
Well 3 count 1	59	1.4 x10 ⁵	0	100	0
Well 3 count 2	68	1.6 x10 ⁵	1	98.5	1.5
Well 4 count 1	38	9.1 x10 ⁵	0	100	0
Well 4 count 2	42	1.0 x10 ⁵	0	100	0

Table 7.11 Cell counts for phage HER401 BS46 dilution 2 (sd 2x10⁵ cells/well)

Plate 2	live cell	Live	dead cell	% live	% dead
Dilution 3	count	cells/ml	count		
Well 1 count 1	82	2.0 x10 ⁵	1	98.7	1.2
Well 1 count 2	93	2.2 x10 ⁵	0	100	0
Well 2 count 1	91	2.2 x10 ⁵	0	100	0
Well 2 count 2	81	1.9 x10 ⁵	0	100	0
Well 3 count 1	75	1.8 x10 ⁵	3	96.2	3.8
Well 3 count 2	122	2.9 x10 ⁵	1	99.2	0.8
Well 4 count 1	76	1.8 x10 ⁵	0	100	0
Well 4 count 2	64	1.5 x10 ⁵	0	100	0

Table 7.12 Cell counts for phage HER401 BS46 dilution 3 (sd 2x10⁵ cells/well)

Statistical data treatment of Trypan blue counting results

One-way between groups ANOVA with Tukey’s post-hoc test was used to test whether there was a difference between mean percentages of alive/dead cells treated in different ways. As percentages cannot be assumed to be normally distributed an additional nonparametric test with Kruskal-Wallis equation was included.

There was no statistically significant difference between any of the mean percentage live/dead cells at 5,000 or 20,000 seeded cells, with the same p-value for both statistical approaches $p=0.134$ and 0.173 respectively. This implies that regardless of presence or absence of phage or phage concentrations used, the Trypan blue counts remained unaffected.

7.4.6 Cytotoxicity assay using HPI staining and 3T3 cells

Data were statistically treated using Univariate General linear model and Levene’s test (Tables 7.13-7.15).

Blank	Phage 2×10^9 pfu/ml	Phage 2×10^8 pfu/ml	Phage 2×10^7 pfu/ml
96.97	99.48	100	100
98.92	99.87	100	99.5
100	100	100	99.63
98.62	99.75	100	100
94.45	99.72	100	99.01
99.47	99.58	99.76667	99.78

Table 7.13 Mean percentage viable cells (n=6x6) 24 hours after phage addition

Blank	Phage 2x10⁹pfu/ml	Phage 2x10⁸pfu/ml	Phage 2x10⁷pfu/ml
99.78	99.18	99.92	99.25
99.63	99.67	99.73	100
99.58	99.6	99.78	100
100	99.77	99.53	99.92
100	99.67	99.67	99.68
99.53	99.67	99.73	99.83

Table 7.14 Mean percentage viable cells (n=6x6) 48 hours after phage addition

Blank	Phage 2x10⁹pfu/ml	Phage 2x10⁸pfu/ml	Phage 2x10⁷pfu/ml
99.37	94.98	97.367	91
99.1	99.07	98.98	95.35
99.58	98.7	93.6	86.3
97.53	98.57	97.4	87.78
99.52	97.37	92.85	98.9
99.4	97.78	93.4	95.95

Table 7.15 Mean percentage viable cells (n=6x6) 72 hours after phage addition

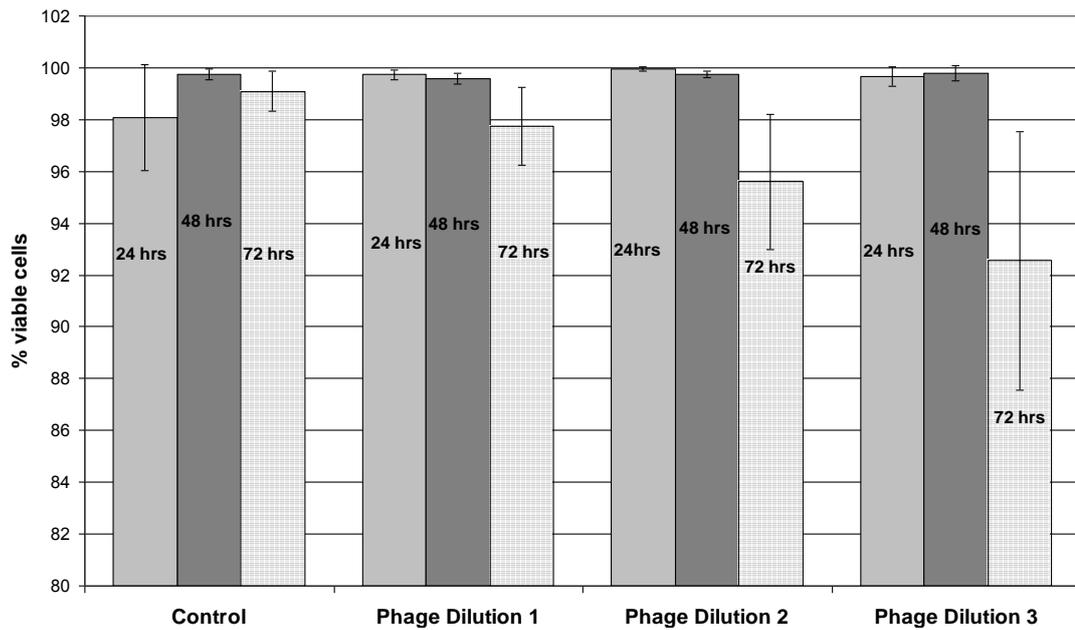


Figure 7.8 Overall mean percentages cell survivors 24, 48 and 72 hours after phage addition

Legend Figure 7.8: Bars represent standard deviations

Statistical data treatment of HPI counting results

The same statistical approach as in **Section 7.4.5** was used.

24 hours: Asymptotic significance (Kruskal Wallis test) $p=0.039$; ANOVA $p=0.039$; $F=2.873$. The mean percentage of viable cells in samples treated with Phage Dilution 2 was significantly higher than controls. There were no statistically significant differences found between the other samples or controls.

48 hours: Asymptotic significance $p=0.147$. ANOVA $p=0.418$; $F=0.952$. No difference between controls or samples.

72 hours: Asymptotic significance $p<0.001$, ANOVA $p<0.001$; $F=25.803$ Samples containing phage Dilutions 2 and 3 had significantly lower viable cell counts than controls and samples containing the highest concentration of phage (Dilution 1).

For samples containing phage, a reduction in the number of viable cells occurred after 72 hours of incubation (**Figure 7.8**). Although this effect was statistically significant compared to control it was only seen at the lowest concentrations of phage and the reduction was very small and may be within the limits of experimental error. Therefore the reduction may have been due to other influences such as contact inhibition, a drying effect and/or decreased cell survival caused by accumulation of cell metabolites.

The results of this experiment remain inconclusive and further investigation may be warranted. It would be useful to repeat this experiment with larger sample sizes and larger growth vessels (T25 flasks), which may be less affected by the accumulation of cell metabolites due to the larger volume of medium present. Alternatively 24-well plates may be used, ensuring that wells are assigned randomly to each sample for a more meaningful statistical analysis to avoid a cumulative skewing of data due to a drying-out effect on peripheral wells.

7.4.7 Cytotoxicity assay measuring LDH release of 3T3 mouse fibroblast cells

	24 h	48 h	72 h
Percentage cell death for cells + Dilution 1	7.7 %	1.3%	8.4 %
Percentage cell death for cells + Dilution 2	3.4 %	0 %	11.5 %
Percentage cell death for cells + Dilution 3	2.1 %	1.8%	7.4 %
Percentage cell death for untreated cells (Control)	0 %	3 %	7.1 %

Table 7.16 Average percentages cell death of cells exposed to phage compared with controls (N=6).

The difference in absorbance between wells containing medium only and wells containing medium and phage were negligible (**Figure 7.9 & Table 7.16**).

The percentage cell death was calculated using the following formula:

Percentage cell death= (Absorbance Experimental value (492nm) – Absorbance Medium only(492nm)) / Absorbance Lysed cells(492nm) x 100

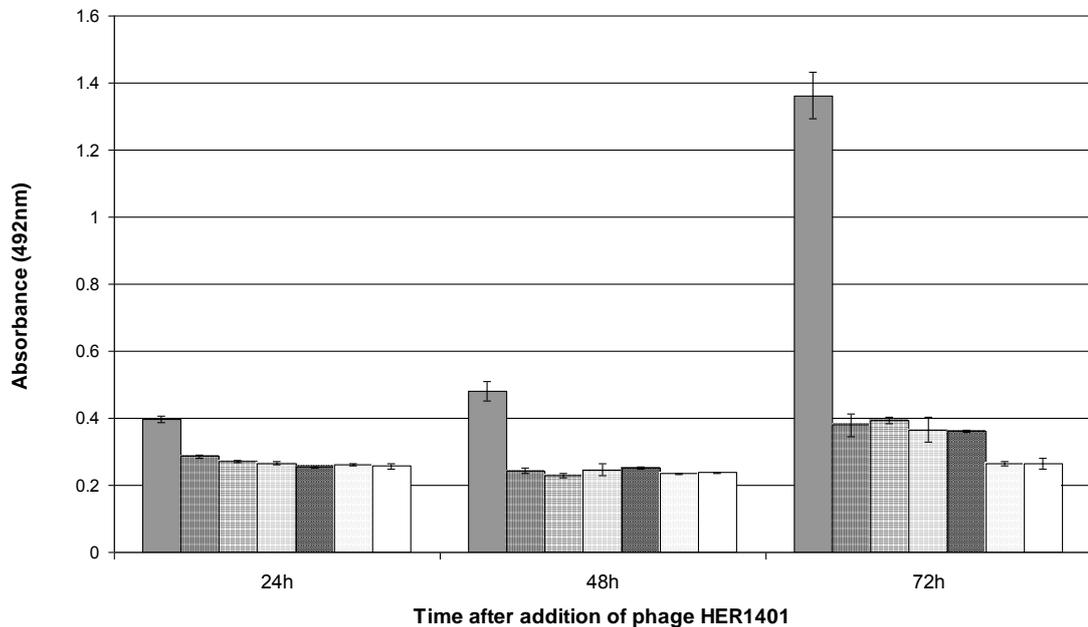


Figure 7.9 LDH readings of 3T3 cells treated with phage

Legend to Figure 7.9: From left to right: Black- lysed cells, narrow check- cells with phage dilution 1, wide check- cells with phage dilution 2, faint check-cells with phage dilution 3, large dots- cells only (Control), dotted-medium containing phage only without cells, clear-medium consisting of DMEM+10%FCS and P&S.

Statistical data treatment of LDH optical densities

One-way ANOVA followed by Gabriel’s multiple comparison procedure was used to test whether there was a difference between mean optical densities of cells exposed to phage at different concentrations and control.

Statistical tests showed there was a statistically significant difference at time point 24 hours (F=22.568, p<0.001), Gabriel’s multiple comparison showed that the mean optical density of phage at the highest concentration was significantly higher than the other means (p<0.001),

At 48 hours there was no significant difference between mean optical densities of samples ($F=3.879$, $p=0.006$).

At 72 hours there was a significant difference. Gabriel's multiple comparison showed that the mean optical densities of medium and medium containing only phage (without cells) were significantly lower than optical densities of other samples ($F=16.848$, $p<0.001$).

At timepoint 24 hours the phage dilution D1 led to higher mean optical densities than other samples, which implies that LDH release in samples treated with Dilution 1 was higher than in all other samples.

At 48 hours there was no statistically significant difference between all samples, which implies that LDH release was not increased in samples treated with phage compared with controls.

At 72 hours medium and medium containing phage only (without cells) optical densities were lower than in other samples. This is unsurprising and would be expected in samples incubated for over 72 hours. Samples treated with phage were no different with regards to LDH release than untreated cells.

Apart from dilution 1 measured at 24 hours there is no evidence to support the notion that phage exert any effect on 3T3 cells which would differ from controls. It is possible that the increased LDH levels seen at 24 hours with Dilution 1 may be due to residual chemicals present in the phage preparation, which may be compensated for by cell repair mechanisms.

7.4.8 Cell proliferation assay measuring MTS formation of 3T3 mouse fibroblast cells (I)

Data were analysed as described in **Section 7.4.7** and mean absorbance readings compared (**Figure 7.10**). Statistical tests showed there was no significant difference at any time points between absorbance readings for wells treated with lambda buffer (control) or wells exposed to phage at any concentration. ($F=0.929$; 2.887 ; 1.531 respectively, $p>0.005$).

This implies that in terms of cell proliferation the effect of phage at any of the three dilutions used was equal to the effect lambda buffer had on the 3T3 cells.

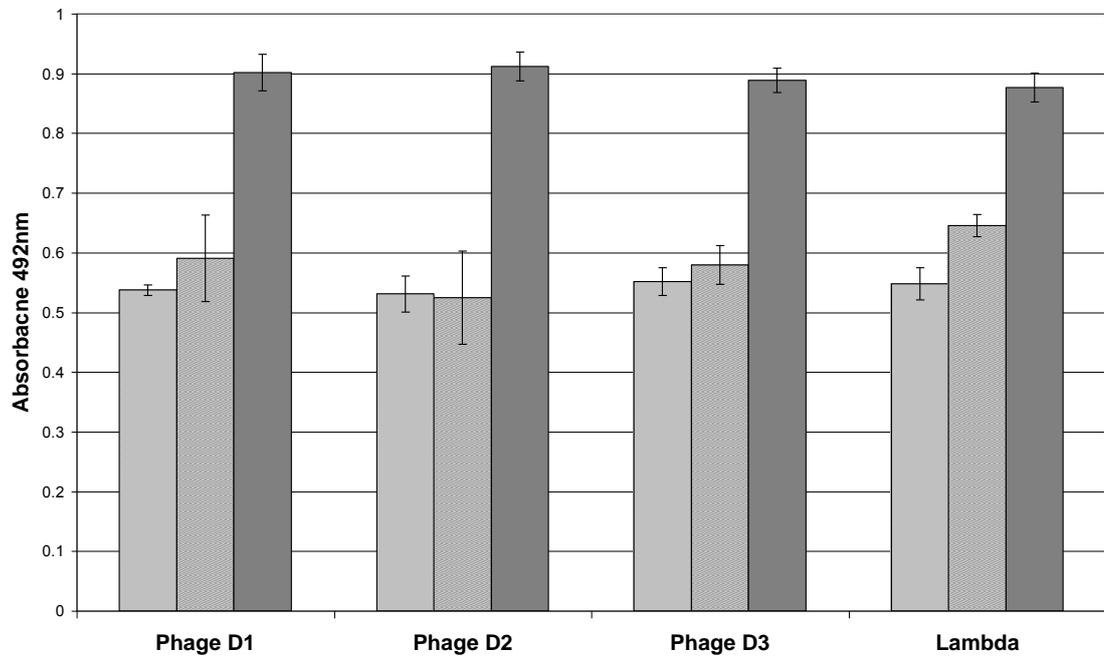


Figure 7.10 MTS formation of 3T3 cells exposed to three phage dilutions

Legend to Figures 7.10: *From left to right: Phage D1- cells with phage dilution 1, Phage D2- cells with phage dilution 2, Phage D3-cells with phage dilution 3, Lambda-addition of lambda buffer; Incubation times: faint stripes: 24h incubation, bold stripes: 48h incubation, filled: 72h.*

7.4.9 Cell proliferation assay measuring MTS formation of 3T3 mouse fibroblast cells (II)

One-way between groups ANOVA with Tukey's post-hoc test was used to test whether there was a difference between mean optical density readings (**Figures 7.11-7.13**).

At seeding densities of 5,000 cells/well at 24, 48 and 72 hours there were no significant differences ($p > 0.5$, $F = 5.888$; $p > 0.5$, $F = 0.485$; $p > 0.5$, $F = 0.660$).

At seeding densities of 10,000 cells/well at 24 hours the mean optical density of D1 was significantly lower than D3, however there was no statistically significant difference between any other samples or samples D1, D2 and D3 and controls ($p < 0.05$, $F = 7.407$). At 48 hours there was no statistically significant difference between any samples ($p > 0.5$, $F = 2.125$). At timepoint 72 hours there was a significant difference ($p < 0.05$, $F = 9.996$) between D1, D2 and samples treated with lambda buffer and controls. Dilutions 1 & 2 gave rise to higher optical density readings than lambda samples or controls.

At seeding densities of 15,000 cells/well at 24 hours Dilutions 1, 2 & 3 gave rise to significantly higher optical density readings than lambda buffer ($p < 0.05$; $F = 11.577$), there was no difference to controls. At timepoints 48 and 72 hours there was no difference between experimental wells, lambda buffer and controls ($p > 0.05$, $F = 1.428$; $p > 0.5$, $F = 0.883$). Hence there is no evidence to suggest that phage may be detrimental to cell proliferation, compared to lambda buffer or untreated cells (controls). There is however some evidence that suggests that at higher seeding densities phage may be more beneficial to cell proliferation than lambda buffer or no treatment at all.

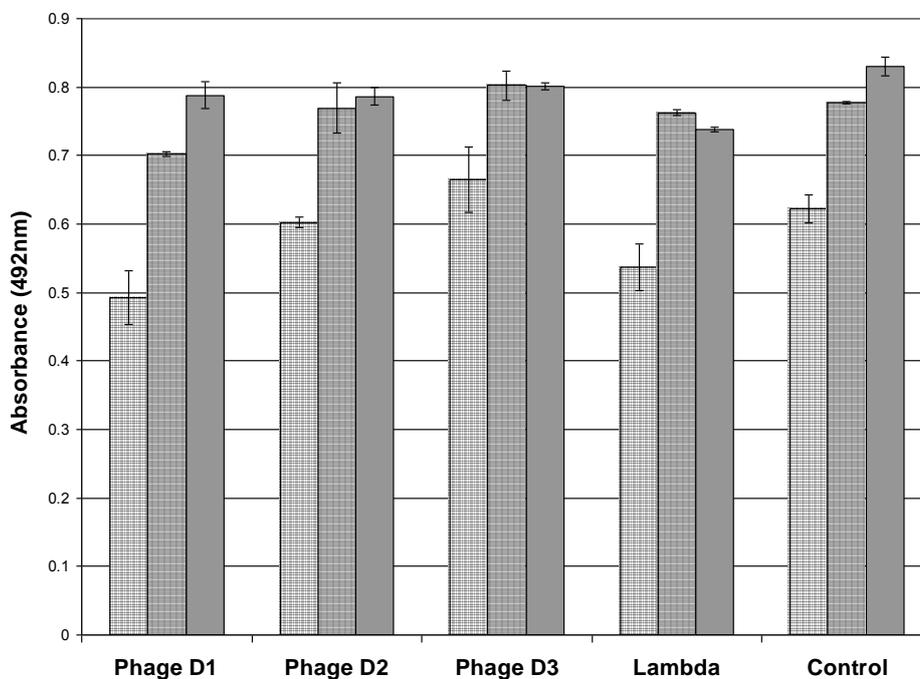


Figure 7.11 Comparison of MTS formation of 3T3 cells after 24h incubation

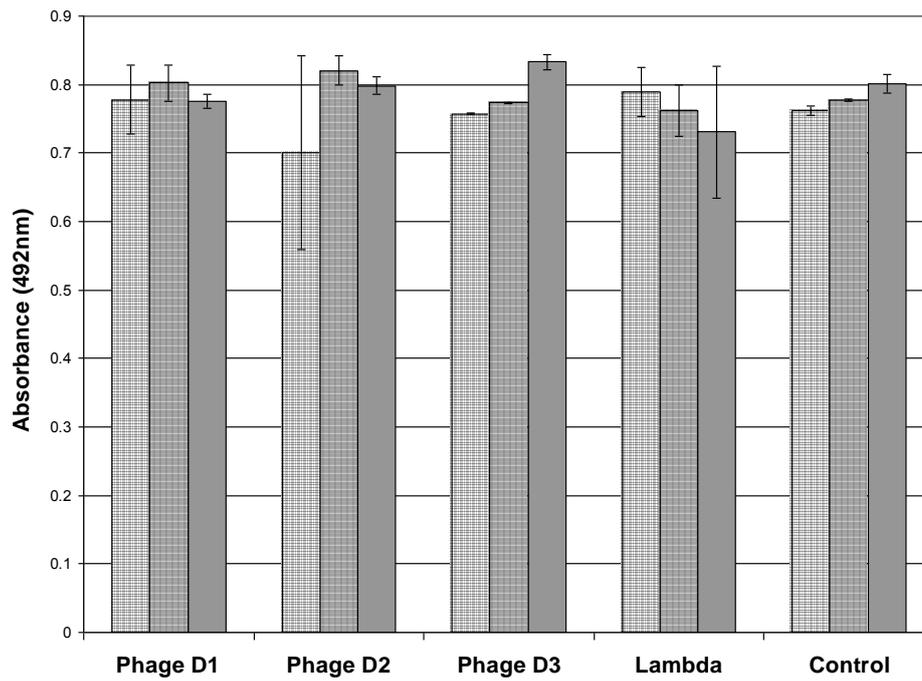


Figure 7.12 Comparison of MTS formation of 3T3 cells after 48h incubation

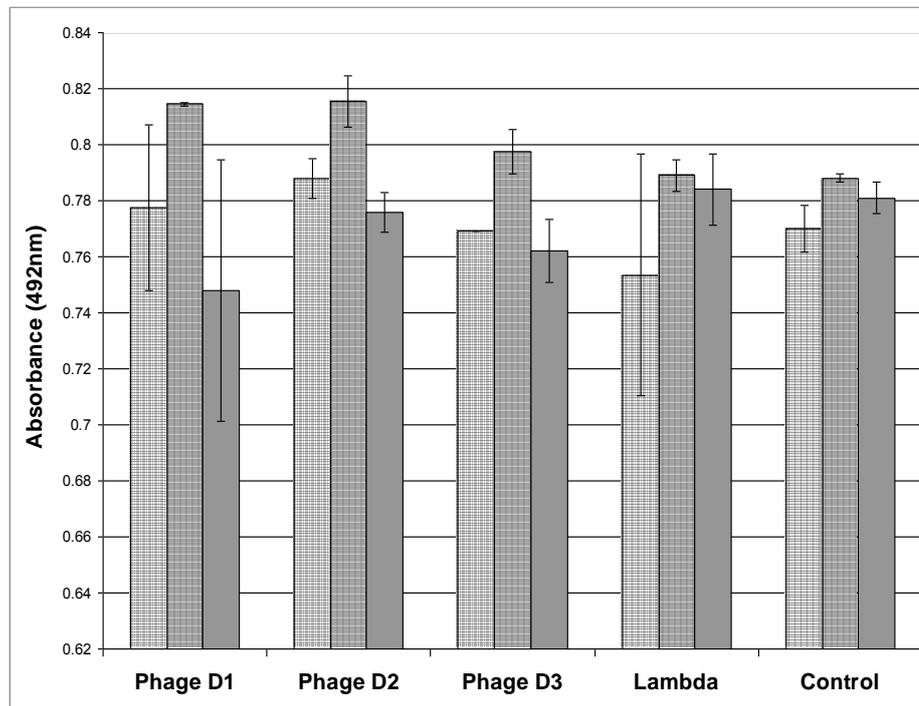


Figure 7.13 Comparison of MTS formation of 3T3 cells after 72h incubation

Legend to Figures 7.11-7.13: *From left to right: Phage D1- cells with phage dilution 1, Phage D2- cells with phage dilution 2, Phage D3-cells with phage dilution 3, Lambda-addition of lambda buffer, Control-cells only; Seeding densities: wide check: 5,000 cells, narrow checked: 10,000 cells, filled: 15,000 cells/well.*

Data shown in **Tables 7.17-7.19** are presented as post-treatment recovery (percentage live cells); Percentages of viable cells were calculated with the absorbance of the control (untreated cells) defined as 100% live cells.

Seeding density	5,000cells	10,000cells	15,000cells
Percentage live cells for Dilution 1	79.12	90.41	94.94
Percentage live cells for Dilution 2	96.79	98.97	94.76
Percentage live cells for Dilution 3	100	100	96.51
Percentage live cells for lambda	86.27	98.13	88.92

Table 7.17 Percentages of live cells after 24 hour incubation

Seeding density	5,000cells	10,000cells	15,000cells
Percentage live cells for Dilution 1	100	100	96.82
Percentage live cells for Dilution 2	91.93	100	99.69
Percentage live cells for Dilution 3	99.41	99.49	100
Percentage live cells for lambda	100	98.07	91.20

Table 7.18 Percentages of live cells after 48 hour incubation

Seeding density	5,000cells	10,000cells	15,000cells
Percentage live cells for Dilution 1	100	100	95.77
Percentage live cells for Dilution 2	100	100	99.36
Percentage live cells for Dilution 3	99.87	100	97.57
Percentage live cells for lambda	97.86	100	100

Table 7.19 Percentages of live cells after 72 hour incubation

7.4.10 Microscopy of 3T3 mouse fibroblast cells

3T3 mouse fibroblasts were confirmed as viable and the typical appearance of these cells aided in the confirmation of identity (**Figure 7.14**).

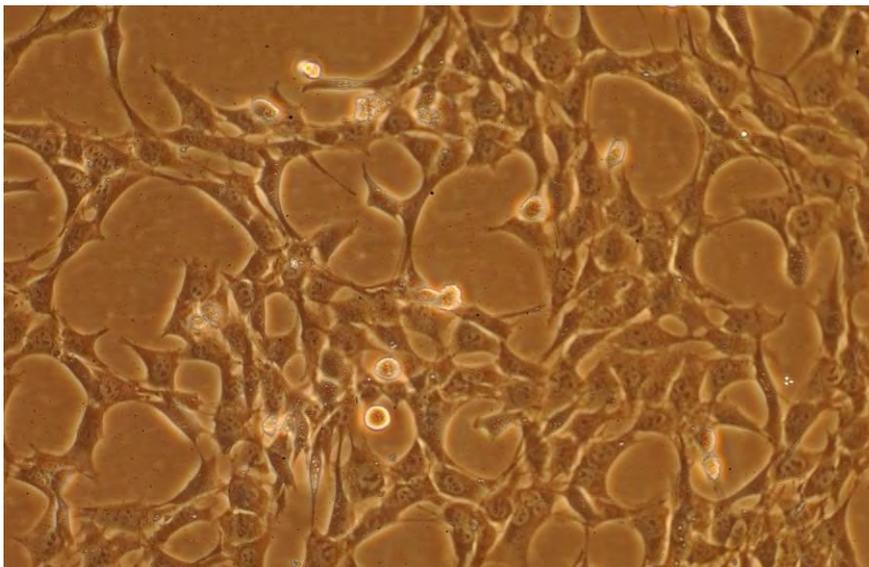


Figure 7.14: 3T3 mouse fibroblast cells, estimated 45% confluence

7.4.11 Mycoplasma screening of human fibroblasts SKF276

SKF276 human fibroblast cells were confirmed to be mycoplasma negative

7.4.12 Population history of SKF276, after receipt from the Blond McIndoe centre

Passage number	Incubation time (h)	N _L cells seeded	N _H cells harvested	Population doublings	Flask Size	CPD
3	72	7.5x10 ⁵	1.5x10 ⁶	1	T200	10.3
4	48	1x10 ⁵	2.4 x10 ⁵	1.26	T25	11.5

Table 7.20 Population history of SKF276

Cells received from Blond McIndoe Centre at CPD 10.3 (**Table 7.20**) were further propagated in twelve T25 flasks, harvested and frozen at CPDs documented in **Table 7.21**.

Numerous samples of cells were frozen at an early cumulative population doubling, to ensure cells had not entered senescence. This allowed repeatability of results with equal or purposely different CPDs at any time (**Table 7.21**).

SKF276 cells when grown under the conditions stated, doubled approximately 1.2 times within 48 hours in T25 flasks, without any media changes after seeding. The data shown in **Table 7.21** in addition to documenting the fate of propagated samples illustrate the differing growth rates in various flasks. Flask 11 only gave rise to 3x10³ cells, yet flask 3 yielded 3.0x10⁵ cells. The different numbers of cells harvested, despite being grown in the same conditions demonstrate that unlike cell lines, SKF276 cells may not always multiply at a uniform rate.

Passage number	Incubation time (h)	N_L cells seeded	N_H cells harvested	Population doublings	Flask Number	CPD
4	48	1×10^5	1.4×10^5	0.49	1	10.8
4	48	1×10^5	3.0×10^5	1.58	2	11.9
4	48	1×10^5	3.0×10^5	1.58	3	11.9
4	48	1×10^5	2.6×10^5	1.38	4	11.7
4	48	1×10^5	1.4×10^5	0.49	5	10.8
4	48	1×10^5	2.4×10^5	1.26	6	11.6
4	48	1×10^5	2.1×10^5	1.07	7	11.4
4	48	1×10^5	1.9×10^5	0.93	8	11.2
4	48	1×10^5	4.2×10^4	2.07	9	12.4
4	48	1×10^5	8×10^3	-	10	-
4	48	1×10^5	3×10^3	-	11	-
4	48	1×10^5	2.4×10^5	1.26	12	11.6

Table 7.21 Cell count before freezing and calculation of doubling times in 12x T25 flasks

7.4.13 Doubling times of human primary fibroblasts SKF276 in 24-well plates

Time since seeding	P	N _L cells seeded	N _H cells harvested (well 1)	N _H cells harvested (well 2)	N _H cells harvested (well 3)	CPD	Original cell conc.	Mean CPD
Flask 12							1.1 x10 ⁶	11.6
0 h	6	5,000	-	-	-	-		
24 h	6		4 x10 ³	1.0 x10 ³	3.6 x10 ³	-		-
48 h	6		2.5 x10 ⁴	1.0 x10 ⁴	5.9 x10 ³	1.19		12.8

Table 7.22 SKF276 cells harvested from 24-well plates

Cell recovery, despite washing with HBSS and pre-warmed trypsin from 24-well plates (**Table 7.22**) was more erratic than could be expected from an immortalised cell line like 3T3 cells (**Section 7.4.1-7.4.2**). Due to the erratic growth and/or recovery of these cells, extensive experiments involving direct counts were avoided.

7.4.14 Cell proliferation assay of SKF276 cells (I)

Time since seeding	P	NL cells seeded	NH cells harvested (well 1)	NH cells harvested (well 2)	NH cells harvested (well 3)	CPD	Original cell conc.	Mean CPD
							1.1 x10 ⁶	11.6
0 h	7	10,000	-	-	-			
24 h	7		7.2 x10 ³	8.4 x10 ³	6.0 x10 ³	-		-

Table 7.23 Baseline count for SKF276, Cell proliferation assay

The 24-hour baseline count after cell attachment was deemed satisfactory, considering SKF276 was a primary cell culture (**Table 7.23**), the cell recovery was less than expected, similarly as seen in **Table 7.22**.

One-way between groups ANOVA with Tukey’s post-hoc test was used to test whether there was a statistical difference between MTS readings (**Figures 7.15-7.17**). After 24 hour incubation phage dilution 1 gave statistically significantly higher MTS readings than any other samples-including untreated cells ($p < 0.001$, $F = 67.492$). After 48 and 72 hours ($p < 0.001$, $F = 98.896$; $p < 0.001$, $F = 444.993$) phage dilution 1 yielded significantly lower MTS readings than all other samples, except cells treated with EtOH. At 48 hours untreated cells gave rise to significantly higher MTS readings than cells exposed to lambda buffer.

As expected cells treated with ethanol gave rise to statistically significantly lower levels of MTS release than any other samples at all timepoints.

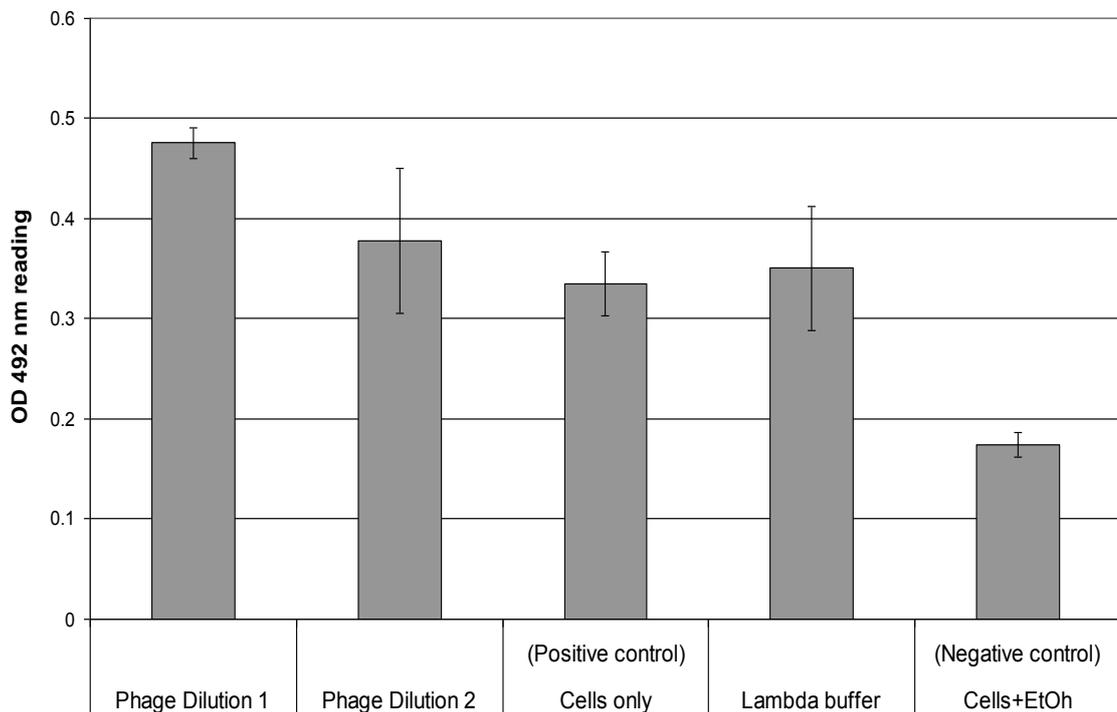


Figure 7.15 MTS readings of cells incubated with phage for 24 hours

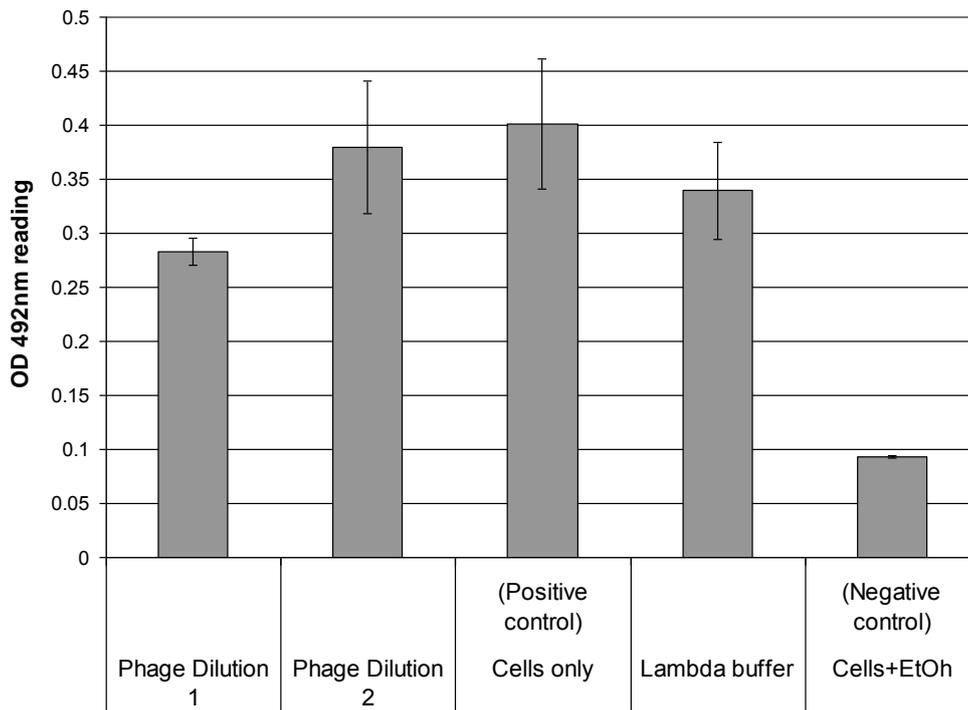


Figure 7.16 MTS readings of cells incubated with phage for 48 hours

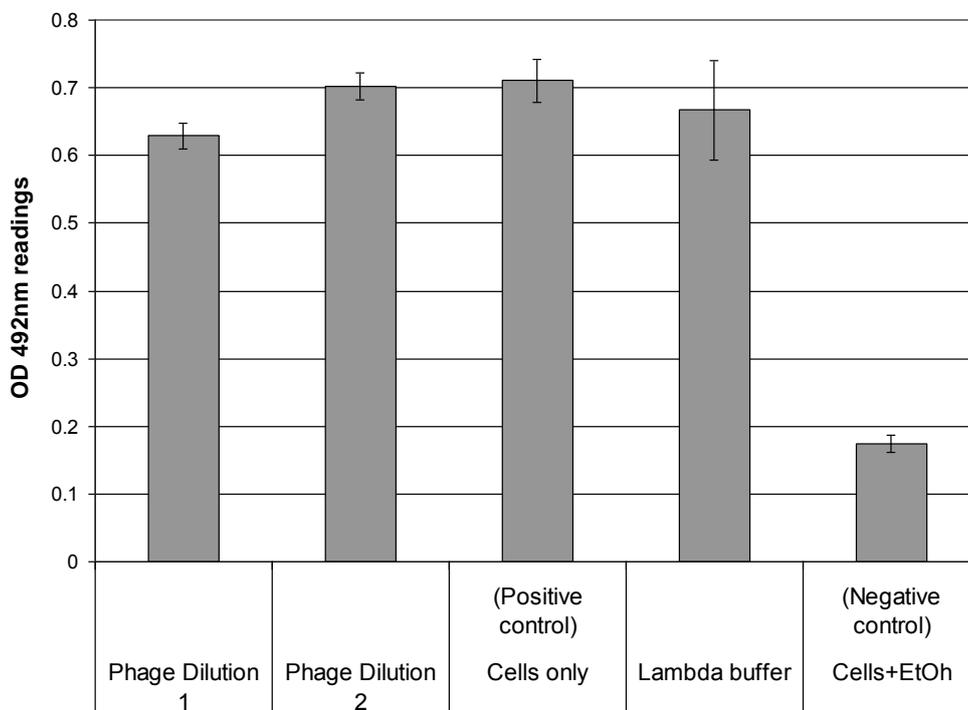


Figure 7.17 MTS readings of cells incubated with phage for 72 hours

7.4.15 Cytotoxicity assay using human fibroblasts SKF276 (I)

There was no statistical difference in MTS outputs between phage Dilution 1, Dilution 2 & 3, samples containing sonicated bacteria, lambda buffer and untreated cells (control).

Cells exposed to sonicated bacteria yielded statistically significantly lower MTS readings ($p < 0.001$, $F = 58.453$) than untreated cells and cells exposed to phage Dilution 2 (Figure 7.18).

Expectedly MTS readings of cells killed by water, ethanol and DMEM without cells were significantly lower than readings for any other samples. Phage dilution 2 also gave rise to significantly higher MTS outputs than cells exposed to sonicated bacteria.

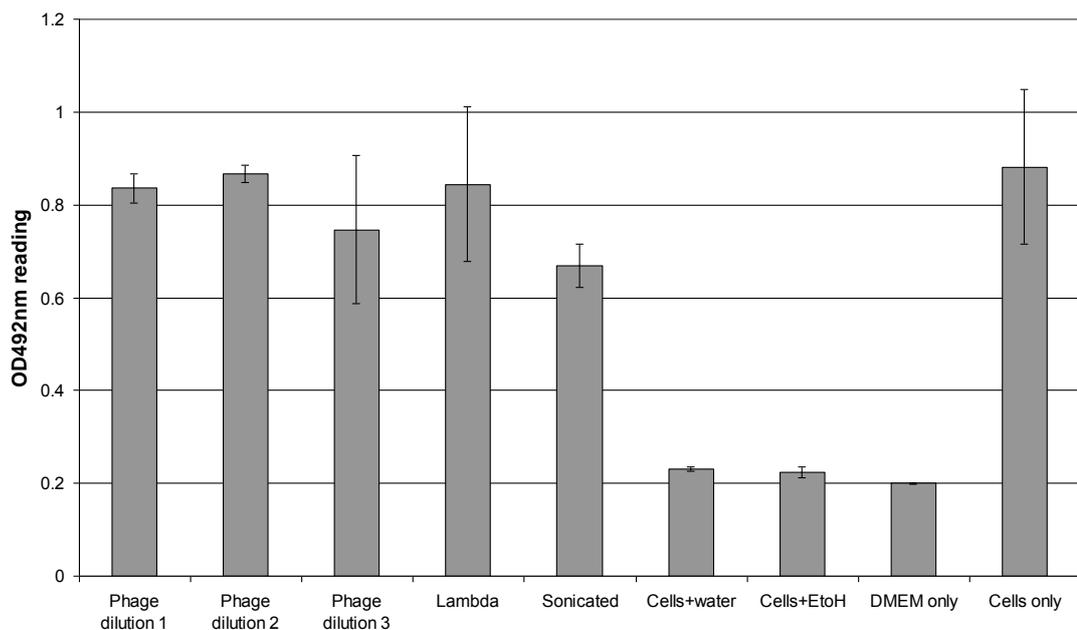


Figure 7.18 MTS readings of SKF276 cells incubated with phage

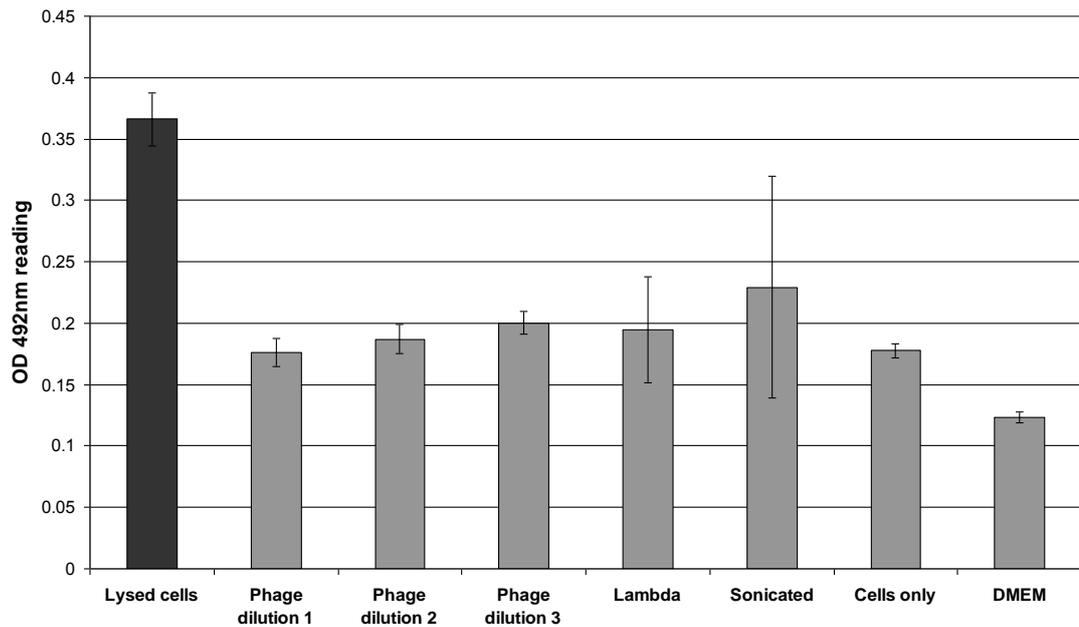


Figure 7.19 LDH reading of SKF276 cells treated with phage

Percentage cell death for cells + Phage dilution 1	14.34
Percentage cell death for cells + Phage dilution 2	17.34
Percentage cell death for cells + Phage dilution 3	20.99
Percentage cell death for lambda buffer	19.49
Percentage cell death for sonicated filtered bacteria	28.91
Percentage cell death for untreated cells (Control)	14.8

Table 7.24 Average percentages cell death of cells exposed to phage, sonicated cells or lambda buffer compared with controls (N=6).

In terms of LDH test absorbance readings, there was no statistically significant difference between phage Dilution 1&2 and all other samples, except lysed cells. The same scenario applied to phage Dilution 3, except additionally the LDH output was

significantly higher than DMEM. As expected lysed cells gave rise to significantly higher ($p < 0.001$, $F = 22.233$) LDH output than all other samples (**Figure 7.19, Table 7.24**).

7.4.16 Viable counts for samples containing endotoxins

The viable count of the resuspended bacterial suspensions, which were used in the preparation of all samples containing endotoxins were 2.1×10^8 cells/ml (HER1401) and 5×10^8 cells/ml (HER1151).

7.4.17 Multodisc clearance areas

HER1151 and HER1401 gave distinctively different areas of clearance with Mastring M11 Multodiscs (**Tables 7.25&7.26**). For the purpose of this study HER1151 exposed to penicillin G was chosen as the lytic antibiotic for the purpose of measuring endotoxin concentrations after cells had been lysed by the antimicrobial.

Antimicrobial	Concentration	Plate 1	Plate 2	Plate 3
Chloramphenicol	25 µg	2.2	2.19	2.32
Erythromycin	5 µg	1.5	1.45	1.5
Fusidic acid	10 µg	2.7	2.65	2.69
Methicillin	10 µg	0	0	0
Novobiocin	5 µg	2.3	2.37	2.37
Penicillin G	1 unit	1.1	1.09	1.1
Streptomycin	10 µg	1.28	1.35	1.22
Tetracycline	25 µg	2.39	2.29	2.23

Table 7.25 Diameters of zones of inhibitions (cm) of HER1151 using Multodiscs

Penicillin G giving areas of clearance may seem surprising, however Gradon et al.(1992) and Bergogne-Berezin et al.(1996) reported cases of successful clinical treatment of *Acinetobacter* spp. infections with this drug.

Antimicrobial	Concentration	Plate 1	Plate 2
Chloramphenicol	25µg	0.95	0.88
Erythromycin	5 µg	0	0
Fusidic acid	10 µg	0	0
Methicillin	10 µg	0	0
Novobiocin	5 µg	0	0
Penicillin G	1 unit	0	0
Streptomycin	10 µg	1.25	1.29
Tetracycline	25 µg	1.52	1.63

Table 7.26 Diameters of zones of inhibitions (cm) of HER1401 using Multodiscs

7.4.18 Endotoxin levels of various cell extracts

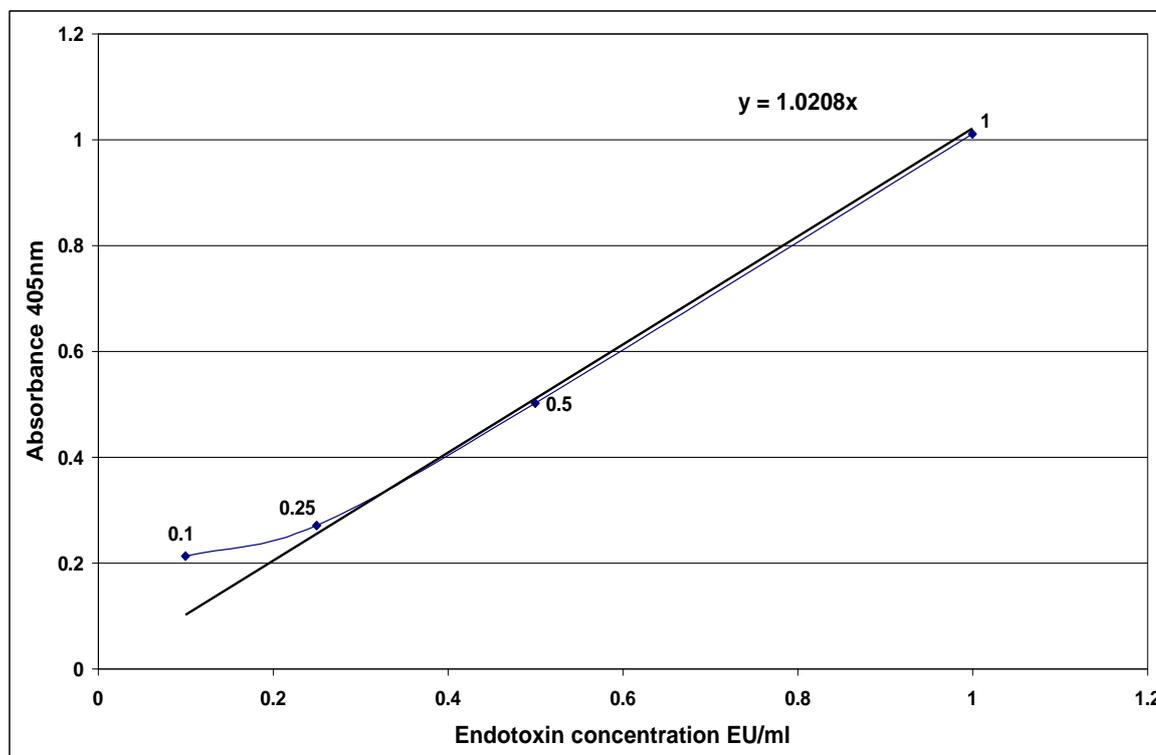


Figure 7.21 Calibration curve correlating endotoxin concentrations to absorbance

Sample	Calculated endotoxin concentration	Bacterial strain
Phage lysate	4.54 EU/ml	HER1401
Autoclaved cells	4.70 EU/ml	HER1401
Sonicated sample 1	4.12 EU/ml	HER1401
Sonicated sample 2	4.30 EU/ml	HER1401
Sonicated sample 3	4.11 EU/ml	HER1151
Beadbeater	4.32 EU/ml	HER1401
Lytic Antibiotic	3.71 EU/ml	HER1151

Table 7.27 Calculated endotoxin concentrations of various samples

A linear correlation between four standard endotoxin concentrations and absorbance could be achieved. Samples at the lower end of the scale (0.1 and 0.25) deviated from the linear trendline (**Figure 7.21**). Due to this deviation the endotoxin concentration was calculated using the Calculator method (Cambrex, BioWhittaker, East Rutherford, NJ, USA), by determining the corresponding endotoxin concentration of samples from absorbance by linear regression using a range of formulae described in **Appendix 1**.

One-way between groups ANOVA with Tukey's post-hoc test was used to test whether there was a difference between calculated endotoxins concentrations. There was no statistical difference in terms of endotoxin release, regardless of how the samples were treated ($p > 0.5$, $F = 0.44$).

Autoclaved cells gave rise to the highest endotoxin concentration, with 0.99EU/ml difference to the endotoxins released by lytic antibiotic (**Table 7.27**). Penicillin G was chosen, representative of lytic antibiotics. Only HER1151 gave clearance zones when Multodiscs 11 were used, which excluded HER1401 from this part of the experiment.

As cell suspensions of equal optical density with confirmed cell counts in a similar range (2.1×10^8 cfu/ml HER1401 and 5×10^8 cfu/ml HER1151) were used to produce the samples it may be assumed that the samples are interchangeable for the purpose of this range of experiments. Sonicated sample 3 (HER1151) gave very similar readings to sonicated samples 1 and 2 (HER1401).

7.4.19 Cytotoxicity assay using human fibroblasts SKF276 (II)

One way ANOVAs with Gabriel's post-hoc comparison were used to analyse data collected for this experiment (**Figures 7.22-7.24**). Percentages of cell death were calculated for result averages **Table 7.28**.

At 24, 48 and 72 hours lysed cells gave rise to significantly higher LDH outputs than all other samples ($p < 0.001$, $F = 131.153$; $p < 0.001$, $F = 182.357$; $p < 0.001$, $F = 210.150$). After 24 hours endotoxin free water applied to cells gave significantly higher LDH readings than untreated cells. At 48 hours DMEM (without cells) gave only rise to low background readings in the LDH test, which were significantly lower than for all other samples.

Autoclaved cells yielded significantly lower LDH outputs than lysed cells, but were higher than all other samples. *E. coli* endotoxin led to significantly lower LDH outputs than endotoxin free water applied to cells. At 72 hours DMEM yielded significantly lower LDH outputs than all other samples, except for filtered phage lysate. HER401 BS46 gave rise to LDH readings which were only significantly higher than DMEM (without cells) and lower than cells exposed to autoclaved sample. Autoclaved samples gave rise to significantly higher LDH outputs at 72 hours than HER401 BS46, filtered phage lysate and DMEM with cells. Filtered phage lysate and lambda buffer samples yielded significantly lower LDH values than endotoxin free water, autoclaved extract and sonicated sample.

Overall these results indicate that there is no statistically significant difference in LDH output between phage preparation HER401 BS 46 or filtered phage lysate and controls (DMEM+cells).

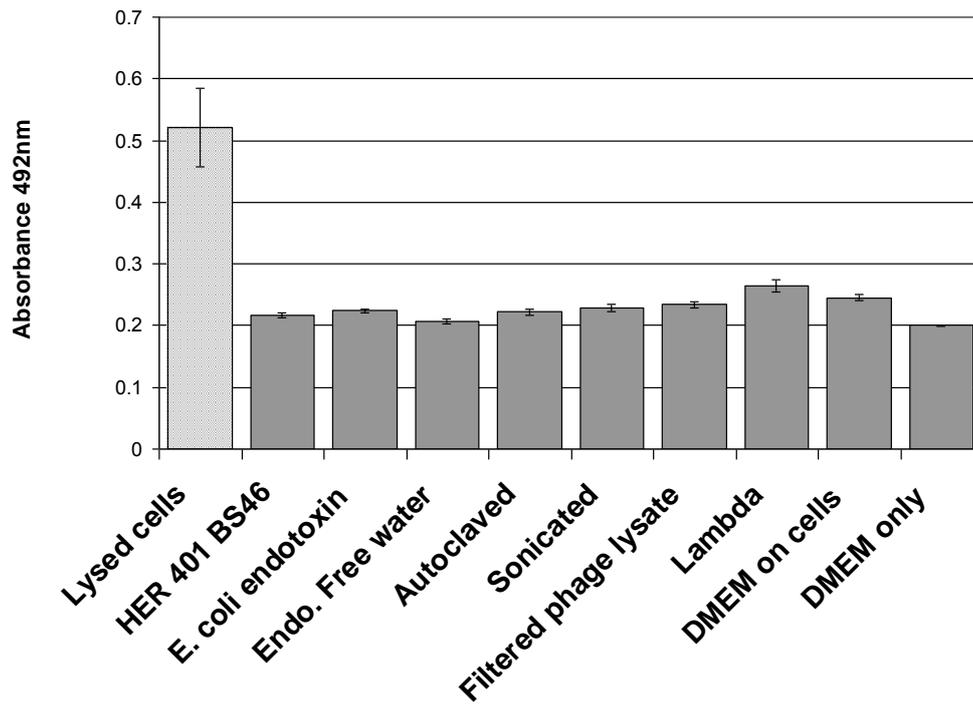


Figure 7.22 SKF276 LDH reading after 24 hours

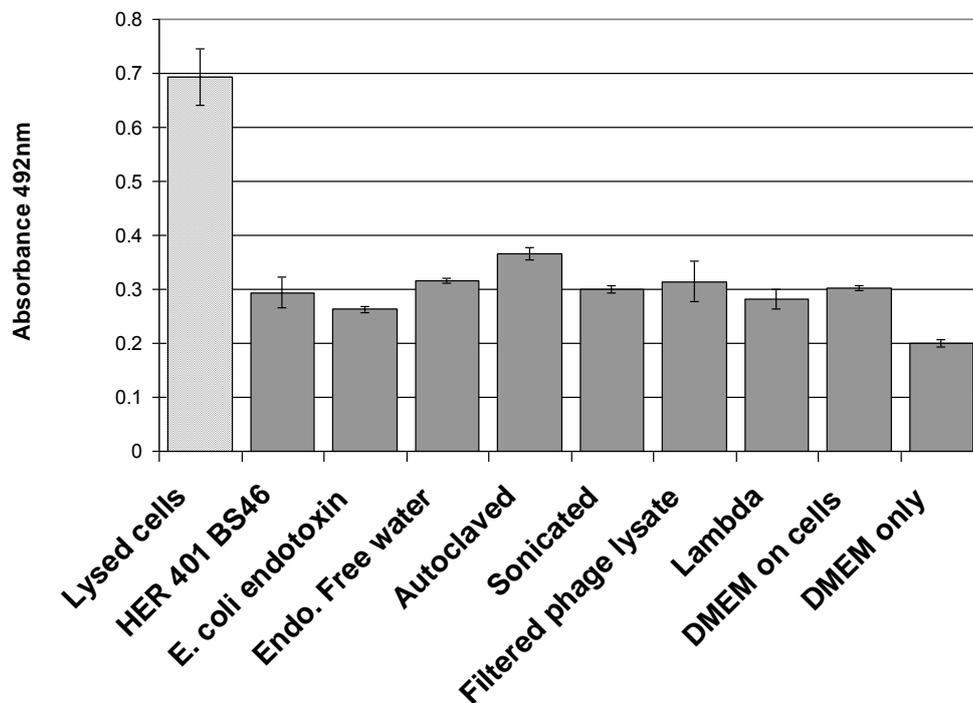


Figure 7.23 SKF276 LDH reading after 48 hours

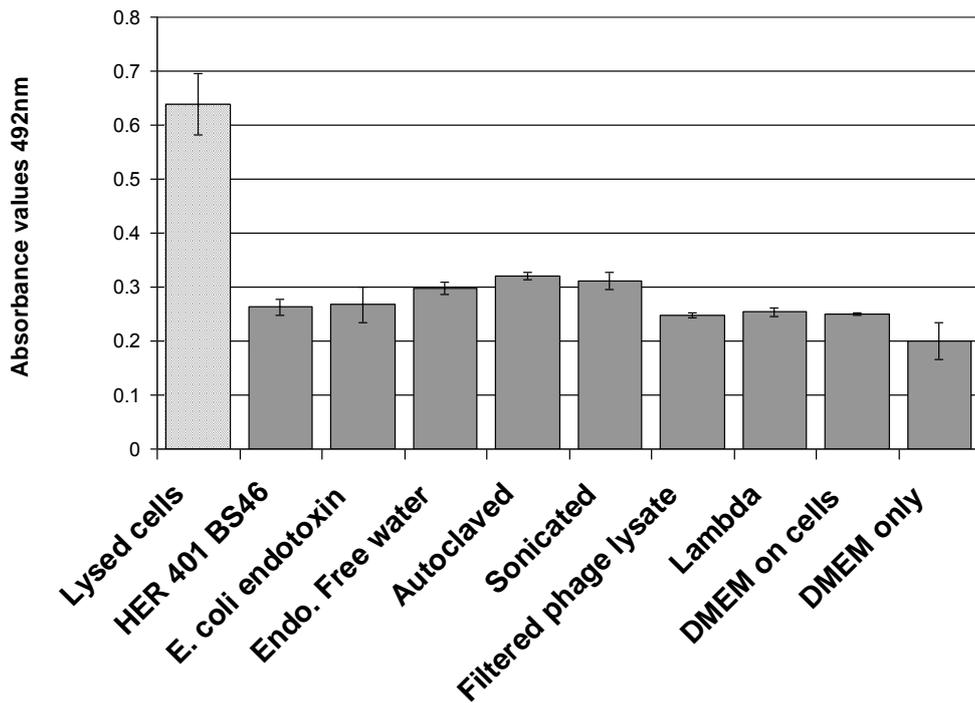


Figure 7.24 SKF276 LDH reading after 72 hours

Percentage cell death	24 h	48 h	72 h
Phage HER401 BS46	3.20	13.58	9.81
E.coli endotoxin	4.61	9.06	10.62
Endotoxin free water	1.34	16.73	15.31
Autoclaved and filtered bacteria	4.32	23.85	18.91
Sonicated and filtered bacteria	5.54	14.33	17.43
Filtered crude phage lysate	6.53	16.51	7.46
Lambda buffer	12.45	11.83	8.45
Percentage cell death for untreated cells (Control)	8.75	14.66	7.67

Table 7.28 Average percentages cell death of cells exposed to phage, or cell extracts

7.4.20 Correlation of cell numbers to MTS absorbance readings

A relationship between cell numbers/ well and absorbance values could be established (Figure 7.25). The trend line equation is: $y=4 \times 10^{-6}x+0.1863$, which allowed conversion of MTS absorbance readings to total cell numbers. The correlation coefficient of the line was $r=0.996$, indicating a linear response between the cell numbers and absorbance.

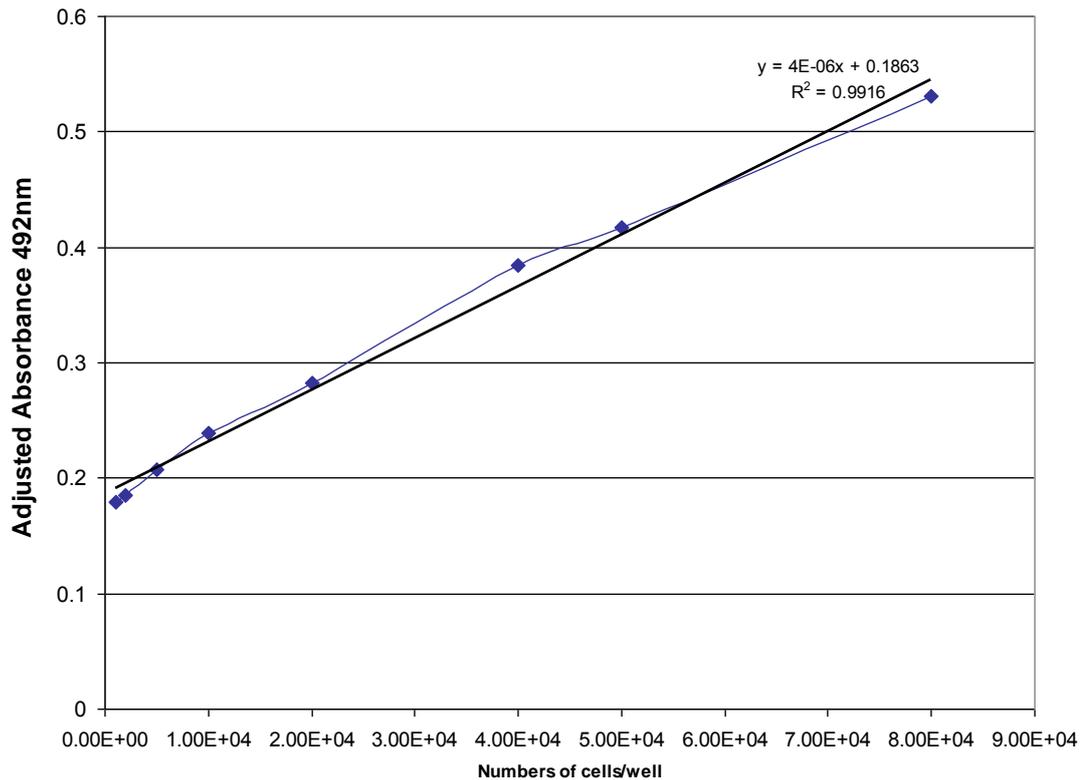


Figure 7.25 MTS Calibration plot for SKF276 cells

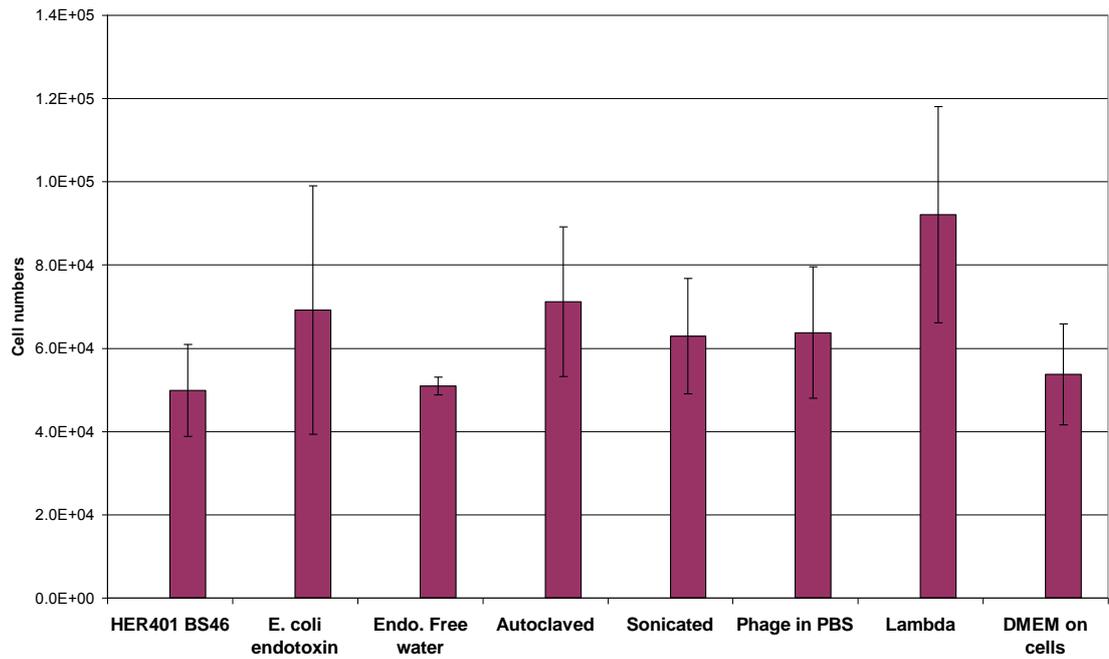


Figure 7.26 Viable cell numbers of SKF276 incubated with reagents for 24 hours

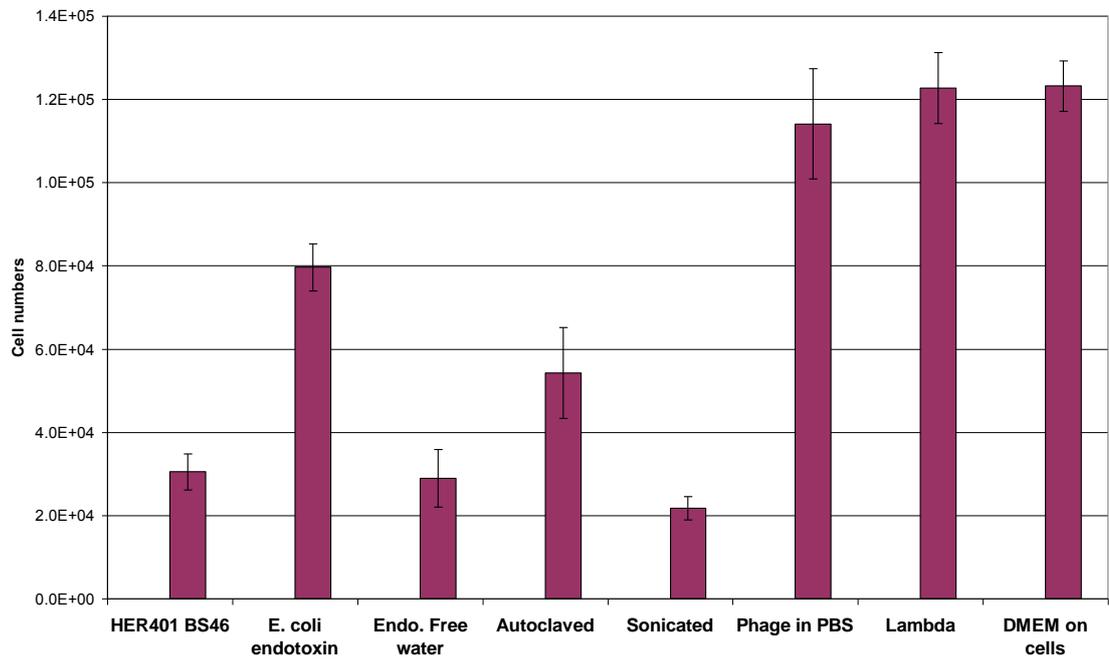


Figure 7.27 Viable cell numbers of SKF276 incubated with reagents for 48 hours

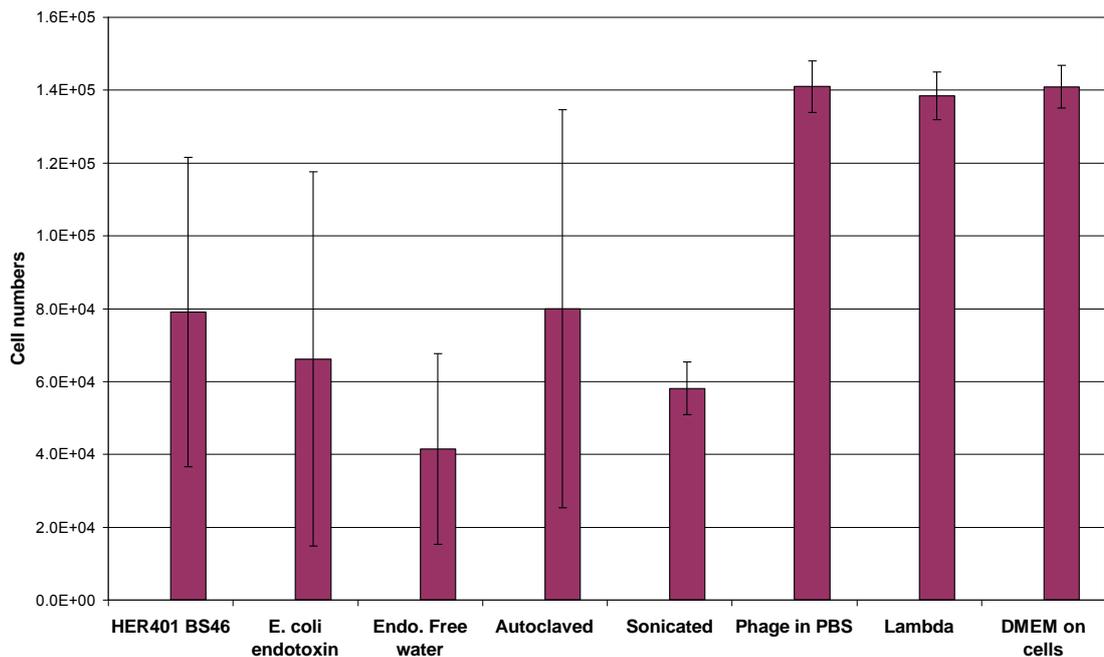


Figure 7.28 Viable cell numbers of SKF276 incubated with reagents for 72 hours

MTS readings (**Figures 7.25-7.28**): At 24 hours, cells treated with ethanol and DMEM without cells had significantly lower ($p < 0.001$, $F = 29.728$) MTS outputs than all other samples. Samples exposed to lambda buffer gave rise to significantly higher MTS outputs than cells treated with HER1401, endotoxin free water and DMEM+cells (controls).

After 48 hours MTS output for HER1401 was significantly lower ($p < 0.001$, $F = 285.575$) than all other samples except endotoxin free water and sonicated samples. There was no significant difference between DMEM+cells and filtered phage lysate or lambda buffer, however controls gave rise to significantly higher MTS outputs than all remaining samples. Lambda buffer and filtered phage lysate gave significantly higher MTS readings than HER1401, *E. coli* endotoxin, endotoxin free water, autoclaved and sonicated samples. *E. coli* endotoxin and endotoxin free water gave significantly higher MTS outputs than all other samples, except MTS outputs were significantly lower compared to filtered phage lysate, lambda buffer and controls. MTS outputs of autoclaved samples compared to other samples like endotoxin free water, except it was also significantly lower than *E. coli* endotoxin. Additionally cells plus ethanol gave rise

to significantly lower MTS readings than all samples except endotoxin free water and sonicated samples. Sonicated samples led to significantly lower MTS readings than all samples except cells treated with EtOH and DMEM. DMEM was significantly lower in MTS readings than all other samples.

After 72 hours MTS readings for HER1401, filtered phage lysate, *E.coli* endotoxin, endotoxin free water, and autoclaved sample were statistically lower than sonicated, lambda buffer and control samples ($p < 0.001$, $F = 25.599$). MTS outputs of sonicated samples and lambda buffer were significantly higher than any other samples, except control wells; There was also no significant difference between lambda buffer, sonicated samples and controls (DMEM+cells). Controls (DMEM+cells) gave rise to significantly higher MTS readings than any other samples, except sonicated and lambda buffer samples. Cells treated with ethanol and DMEM yielded significantly lower MTS outputs than any other samples, except for endotoxin free water, which was not statistically different.

The apparent large variations in cell numbers in some samples are likely to be caused by the inherent nature of the cells as they were isolated from primary material. For these cells growth rates may not be as uniform, as might be expected from immortalised cell lines. Overall results collected after 48h showed lower standard deviations, hence fewer variations in cell proliferation, than results at 24 or 72 hours. Large deviations in cell numbers after 72 hours are not unexpected, as cells may die over prolonged incubation times without media change due to accumulation of cellular metabolites. Contact inhibition was considered a less likely cause for cell death, as fibroblasts are capable of growing in cell sheets and were proliferating slowly (PD 1.2 over 48 hours).

7.4.21 Cell proliferation assay using SKF276 cells (II)

Mean baseline 24 hour cell counts were 7200cell/well. One way ANOVAs with Gabriel's post-hoc comparison were used to analyse MTS outputs collected for this experiment (**Figures 7.29-7.31**).

After 24 hours phage gave rise to statistically higher MTS readings than *E. coli* endotoxin, but was significantly lower than autoclaved, sonicated and lysate in buffer

samples ($p < 0.001$, $F = 490.538$). There was no statistical difference to other samples except DMEM and cells+IMS, which were significantly lower. DMEM and cells treated with IMS, as well as *E. coli* endotoxin yielded significantly lower MTS outputs than all other samples. Lysate in PBS gave rise to significantly higher MTS readings than phage or *E. coli* endotoxin, but was not statistically different to other samples. Endotoxin free water, lambda buffer and DMEM+cells led to significantly higher MTS readings than endotoxin samples. Autoclaved and sonicated samples were higher in MTS output than phage and endotoxin samples.

After 48 hours incubation DMEM and IMS treated cells had significantly lower MTS outputs than all other samples ($p < 0.001$, $F = 871.085$). There was no significant difference between DMEM+cells and any other samples, except DMEM and IMS+cells. Sonicated, lysate in PBS, endotoxin free water and lambda buffer samples gave rise to significantly higher MTS levels than phage. No statistical difference could be found between phage, endotoxin and autoclaved samples.

At timepoint 72 hours phage gave rise to significantly higher MTS readings than all other samples, except *E. coli* endotoxin and sonicated which showed no statistical difference ($p < 0.001$, $F = 282.589$). DMEM+cells had a statistically significantly lower MTS output than phage, there was no statistical difference to any other samples. MTS readings for IMS treated cells and DMEM were significantly lower than all other samples, except for lambda buffer, which was higher than DMEM only. MTS readings for *E. coli* endotoxin were significantly higher than lysate in PBS, endotoxin free water and lambda buffer.

Sonicated samples yielded significantly lower MTS readings than lambda buffer. Lysate in PBS, endotoxin free water and lambda buffer were all significantly lower than phage and *E. coli* endotoxin outputs.

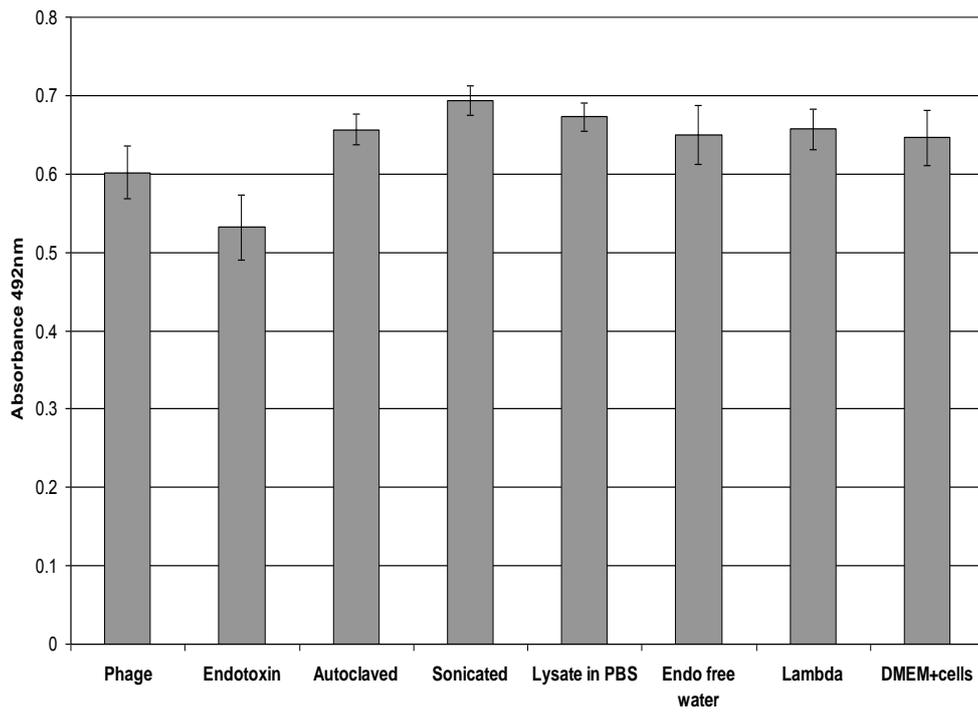


Figure 7.29 MTS assay (II) 24 hours

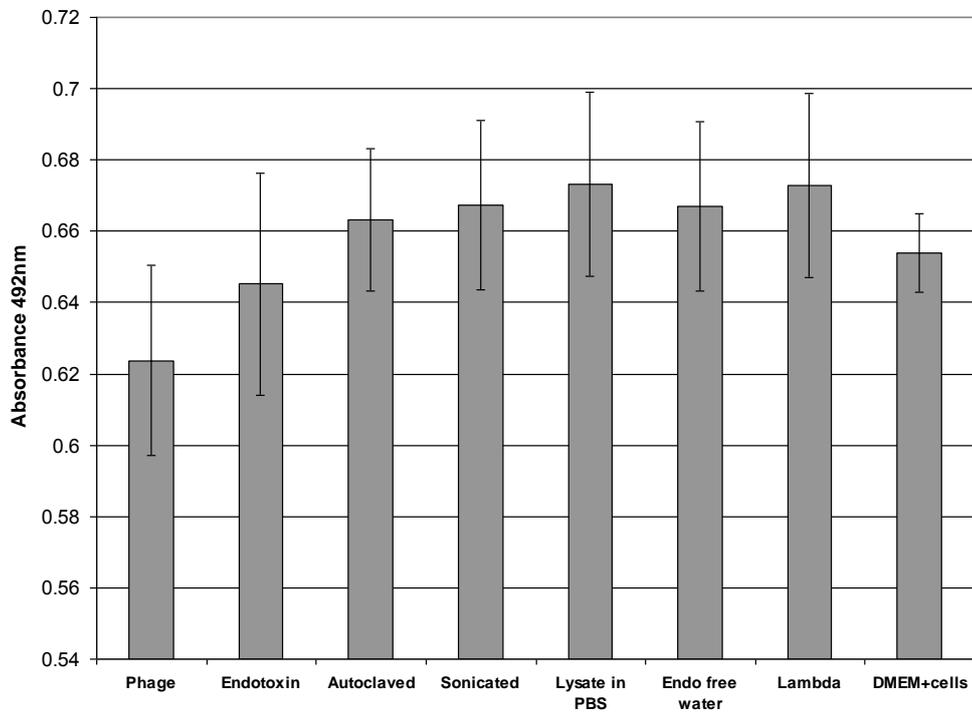


Figure 7.30 MTS assay (II) 48hours

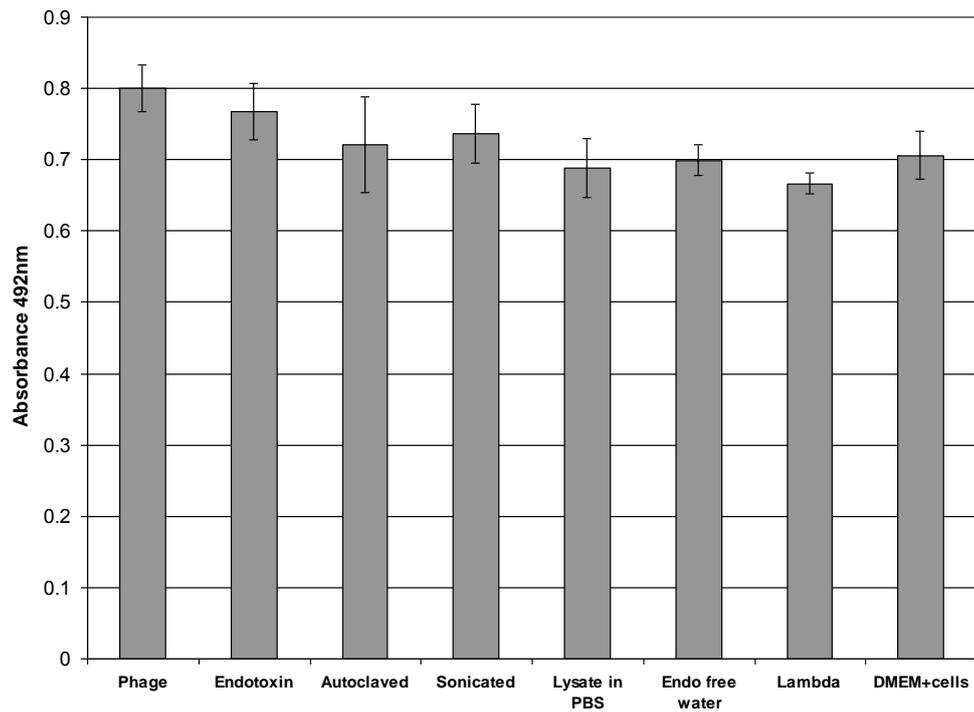


Figure 7.31 MTS assay (II) 72hours

7.4.22 Cytokine assays for IL-1 β fibroblasts SKF276 (I & II)

After normalisation of data by subtracting the mean zero standard absorbance from experimental values, the normalised absorbance value was less than or equal to zero. Despite collected cell supernatants being used undiluted, this implies that provided any IL-1 β was released at all, the output of the fibroblast cells was below the detection limit of 0.8pg/ml (BD Biosciences).

7.4.23 IL-6 release by fibroblasts SKF276-pilot experiment

In initial experiments the results for sonicated cells and controls were not conclusive (**Table 7.29**). Consequently samples were used undiluted for follow-on experiments.

Sample Name	Interleukin Reading	Standard Deviation
Phage Dilution 1	38.6	7.4
Sonicated cells	4522.5	3594.6
Controls	8338.4	2004.9

Table 7.29 IL-6 readings of SKF276 pilot experiment (pg/ml)

7.4.24 IL-6 release by fibroblasts SKF276

Cells treated with concentrated phage and crude lysate **Tables (7.30-7.31)** led to similar IL-6 release (41 & 47pg/ml). Park et al. (2004) reported baseline levels of primary foreskin HDF at passage 6-10 at 19-21pg/ml (± 15). Stimulated HDF by heat shock or starving of FCS increased IL-6 outputs to 424pg/ml and 1.3ng/ml (Park et al., 2004), while stimulation with extracts of *Sarcoptes scabiei* yielded outputs of 56ng/ml (Arlian et al., 2003).

Untreated cells released on average between 9 and 32pg/ml of IL-6 and given the thousand-fold reported differences between baseline levels of IL-6 in controls and stimulated cells (Park et al., 2004), the differences between controls and experimental samples are most likely due to experimental variation. In the context of this investigation it is however noteworthy that in the results shown in **Table 7.29** phage dilution 1 led to a sevenfold and fourfold increase in interleukin when compared to controls or other phage dilutions. IL-6 values of samples were statistically evaluated using one way ANOVAs with Gabriel's post-hoc comparison. The only statistically

significant difference between samples was between phage dilution 1 and all other samples (sig<0.001, F=107.503). IL-6 output of phage preparation 1 was significantly higher than for any other samples including sonicated extracts.

In view of thousand fold higher IL-6 outputs reported elsewhere caused by cell stimulation this may seem minor, however it would suggest the need for further investigation. It is unlikely that the slightly elevated IL-6 levels of cells exposed to phage are due to cytotoxicity caused by chemicals. It may however be possible that the cellular response to nanoparticles, such as phages, may be giving rise to some increase in IL-6. Shortkroff et al. (2002) have demonstrated that particles the size of 100nm led to IL-6 outputs of 94pg/ml, which were dependent on the surface area ratio of cells to particles and the particle size itself.

Sample Name	Interleukin reading	Standard Deviation
Phage Dilution 1	67.17	4.38
Phage Dilution 2	14.14	0.87
Phage Dilution 3	9.71	1.16
Controls	9.43	1.06
Sonicated cells	8.91	2.53
Ethanol	9.08	0.72

Table 7.30 IL-6 results of HDF (pg/ml)

Sample Name	Interleukin reading	Standard deviation
Phage HER1401	41.07	5.08
Crude lysate	47.82	11.91
Cells (Controls)	32.55	7.94
Sonicated	25.52	5.49
Endotoxin free H ₂ O	28.57	11.11
<i>E. coli</i> endotoxin	22.71	2.51
Autoclaved	26.33	2.26
Lambda	27.70	3.11

Table 7.31 IL-6 results of HDF (pg/ml)

7.4.25 IL-8 release by fibroblasts SKF276-pilot experiment (I)

Optimum sample concentrations for IL-8 readings were found to be undiluted samples and 1:5 dilutions. Cells exposed to phage, sonicated cell culture filtrates and controls released almost ten times more IL-8 than those cells subjected to sonicated bacterial culture (**Table 7.32**).

Sample Name	Interleukin reading	Standard Deviation
Phage Dilution 1	169.00	48.66
Sonicated cells	3.85	1.33
Controls	30.13	0.96

Table 7.32 IL-8 release of HDF (pilot experiment; pg/ml)

7.4.26 IL-8 release by fibroblasts SKF276

For data shown in **Table 7.33** and **Table 7.32** (pilot experiment) supernatant liquids drawn during the same experiment were used. Some differences were evident, particularly with regard to the interleukin values of the controls between the pilot experiment and data in **Table 7.32**, however there were striking correlations. It was evident that phage dilution 1 led to over 10-times more interleukin release than dilutions 2 and 3. As Dilutions 2 and 3 were 10-fold serial dilutions of dilution 1, it was surprising that dilution 3 gave a similar interleukin output as dilution 2. In comparison to control wells phage dilution 1 led to 32 times more IL-8 being released into the supernatant liquid.

IL-8 values of samples were statistically evaluated using one way ANOVAs with Gabriel's post-hoc comparison and this showed that phage dilution 1 cytokine output was significantly higher than all other samples ($p < 0.001$, $F = 164.610$). It is possible that dilution 1, which was a 10-fold dilution of the concentrated purified phage lysate, may have contained chemical remnants of the purification process which may have affected the interleukin release of cells. Another striking feature was the large variation seen only with dilution 1 of approx. 34 and 49 in **Tables 7.32** and **7.33**. The standard deviations of all other samples were < 3 . This implies that the cellular interleukin release caused by dilution 1 varied widely amongst experimental wells.

Sample Name	Interleukin reading	Standard deviation
Phage Dilution 1	191.62	34.41
Phage Dilution 2	13.78	4.77
Phage Dilution 3	11.04	1.78
Controls	5.91	0.83
Sonicated cells	5.32	2.72
Ethanol	5.21	1.02

Table 7.33 IL-8 readings experiment I (pg/ml)

Sample Name	Interleukin reading	Standard deviation
Phage HER1401	73.90	26.46
crude lysate	194.48	13.87
Cells	37.31	7.35
Sonicated	18.64	5.66
Endotoxin free H ₂ O	15.60	2.52
<i>E. coli</i> endotoxin	15.73	6.16
Autoclaved	26.85	7.74
Lambda	45.49	10.32

Table 7.34 IL-8 readings experiment II (pg/ml)

One way ANOVAs with Gabriel's post-hoc comparison were used to analyse the second set (**Table 7.34**) of IL-8 values ($p < 0.001$, $F = 116.771$). Phage and crude lysate

cytokine outputs were significantly higher than for all other samples. Endotoxin free water gave rise to significantly lower IL-8 levels than crude lysate, purified phage or untreated cells. Controls (untreated cells) gave rise to significantly higher IL-8 readings than sonicated or lambda buffer samples, but significantly lower than for phage.

This implies that crude lysate and phage elicited a more pronounced IL-8 release than any other samples. It is slightly surprising that samples containing sonicated bacterial cells elicited even lower IL-8 release than controls. Further investigation may be required.

7.4.27 TNF- α release by fibroblasts SKF276

In the pilot experiment, all calculated values were negative after normalising data by subtracting the background absorbance. In the main experiment, after data normalisation, absorbance readings converted to TNF- α values were below 1pg/ml. BD Biosciences reports the detection limit of this ELISA as 2pg/ml. This implies that under the experimental conditions the presence of phage does not induce TNF- α release by fibroblasts SKF276 sufficiently to be detected by a standard method.

7.4.28 Microscopy of SKN276 human fibroblast cells

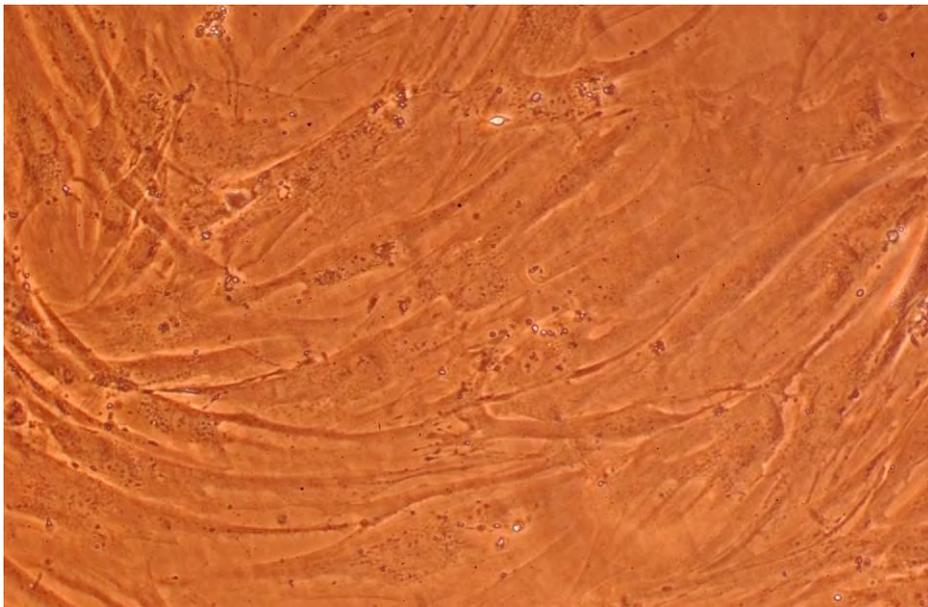


Figure 7.32 SKN276- seeding density 10,000 cells/well medium only

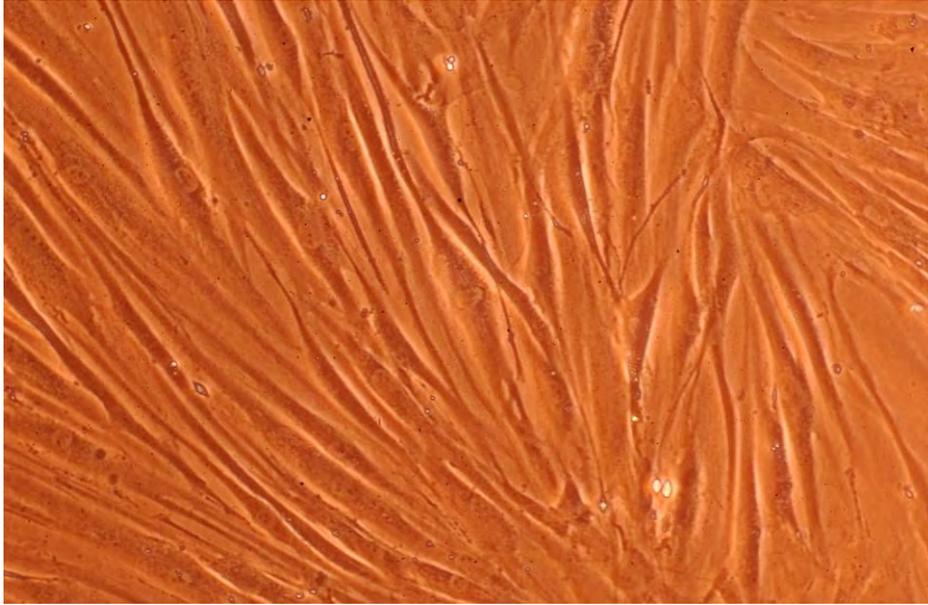


Figure 7.33 SKN276- seeding density 10,000 cells/well exposed to phage



Figure 7.34 SKN276- seeding density 20,000 cells/well exposed to phage

Fibroblasts are morphologically heterogeneous with diverse appearances depending on their location and function. SKN276 were confirmed as viable and the typical appearance of these cells (**Figures 7.32-7.35**) aided in the confirmation of identity, with typical lamellar appearance when grown on planar surfaces (Horobin et al., 2006).

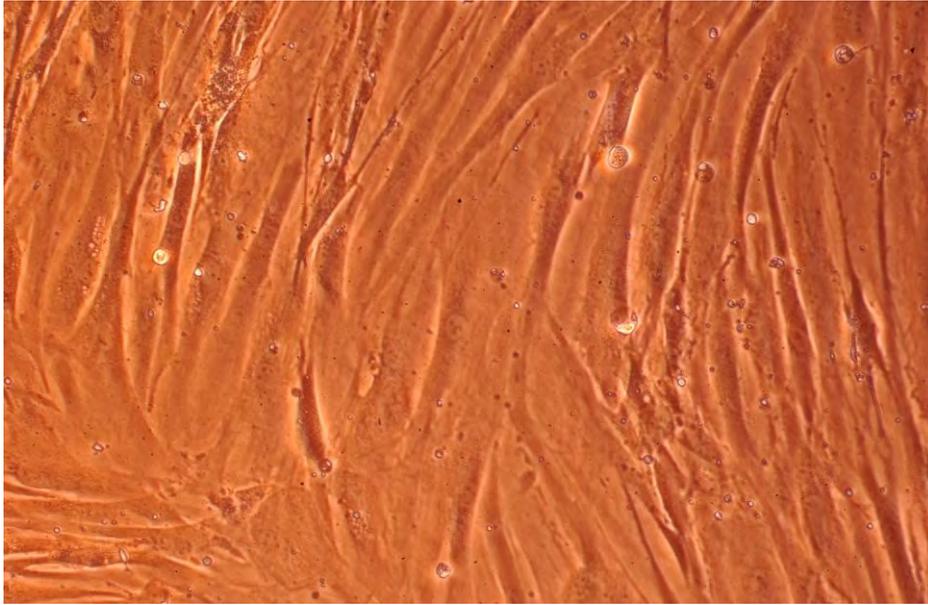


Figure 7.35 SKN276- seeding density 20,000 cells/well medium only

7.4.29 LDH assay for human keratinocytes SKN0106 with 3T3 mouse fibroblast feeder layers

Absorbance values shown in **Figure 7.36** were corrected by subtracting the absorbance of the culture medium from the experimental absorbance values as recommended by the manufacturer (Promega, Madison, USA). This corrected for any LDH activity contributed by the presence of serum and the varying amounts of phenol red in the culture medium. All wells containing keratinocytes also contained 3T3-feeder cells to enable growth of human cells. However, as a control some wells contained only 3T3 cells, which allowed calculation of the proportion of LDH released by the human effector (SKN0106) cells and the feeder cells. To allow differentiation between the feeder cell and the keratinocytes under investigation, the absorbance values of 3T3-feeder layers and keratinocyte absorbance were subtracted and the difference depicted as checked areas **Figure 7.36**. The checked areas depict the LDH release attributed to the keratinocytes, the dotted areas relates to the LDH output of the 3T3 feeder layer cells. Standard deviations relate to the overall LDH output of 3T3+SKN cells. The wells treated with lysis solution (Promega, Madison, USA) were labelled as 3T3+SKN Lysis.

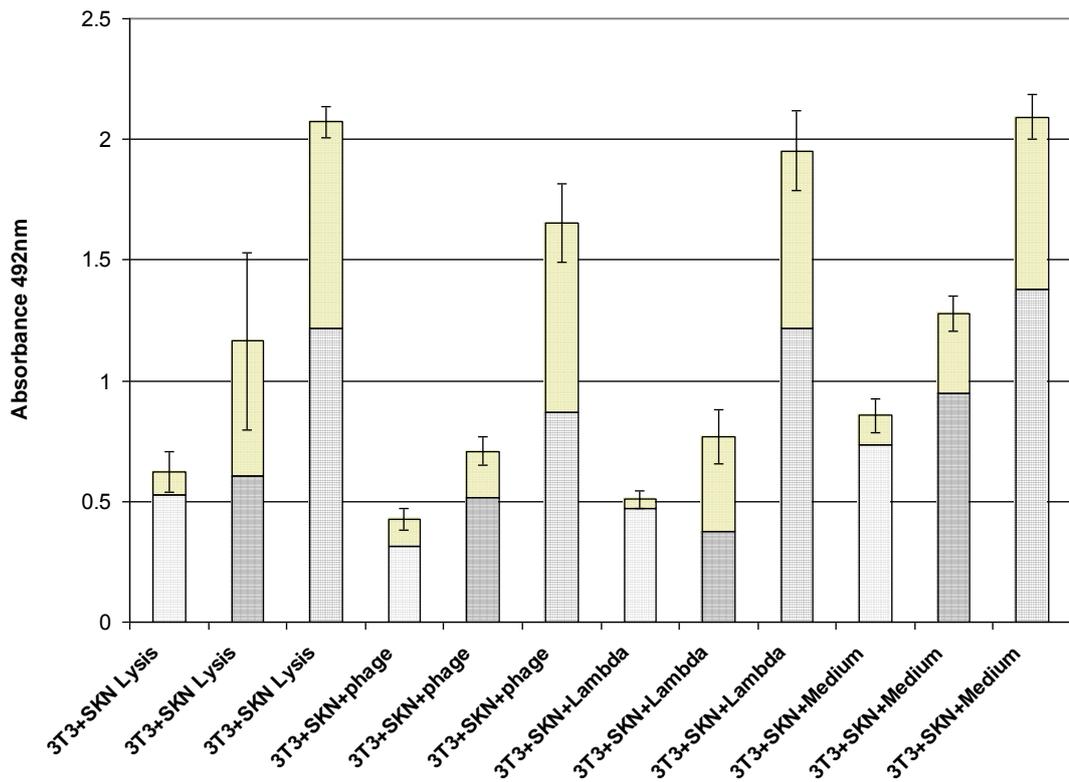


Figure 7.36 LDH assay Keratinocytes with 3T3 feeder layers 24, 48 & 72 hours

Legend Figure 7.36:

Faint check- 24h, narrow check-48h, wide check-72h LDH readings for human Keratinocytes, dotted- 3T3 LDH readings, Bars relate to the standard deviation of the overall LDH output of 3T3+SKN cells

One way ANOVAs with Gabriel's post-hoc comparison were used to analyse LDH outputs comparing mouse cells treated with phage and lambda buffer with controls and separately human cells receiving the same treatment (**Figure 7.36**). After 24 hours there was a significant statistical difference between mouse cells and human cells treated with phage and lambda buffer ($p < 0.001$, $F = 114.003$). In both cases lambda buffer gave significantly higher LDH readings than phage. Mouse and human controls (R&G medium) gave significantly higher LDH readings than all other samples. After 48 hours LDH output of controls and lambda buffer was significantly higher than cells treated with phage, for human and mouse cells ($p < 0.001$, $F = 16.483$). This suggests that phage

had a less toxic overall effect than lambda buffer on keratinocytes plus feeder cells, which is reflected in percentages of cell death (**Table 7.35**). After 72 hours the only statistically significant difference in LDH outputs was between mouse cells ($p < 0.001$, $F = 16.203$). 3T3 cells treated with lambda buffer yielded lower LDH readings than mouse controls.

Percentage cell death	24 h	48 h	72 h
Phage HER401 BS46 in lambda buffer- SKN0106	64.71	43.01	58.4
Lambda buffer- SKN0106	77.2	48.33	68.99
Phage HER401 BS46 in lambda buffer-3T3 cells	56.07	47.51	50.62
Lambda buffer- 3T3 cells	83.94	37.17	61.53
Percentage cell death for untreated SKN0106 cells	111.4	57.4	73.95
Percentage cell death for untreated 3T3 cells	108.6	56.69	50.62

Table 7.35 Percentage cell death of Keratinocytes with 3T3 feeder layers

7.4.30 MTS assay for human keratinocytes SKN0106 with 3T3 mouse fibroblast feeder layers

One way ANOVAs with Gabriel's post-hoc comparison were used to analyse MTS readings of keratinocytes plus 3T3 cells treated with either phage, lambda buffer or controls (Cells+R&G medium only). Mouse cell readings were statistically treated separately (**Figures 7.37-7.39**).

After 24 hours the only statistically significant difference between keratinocyte readings ($p < 0.001$, $F = 12.786$) was between phage and controls, with phage treated cells giving rise to significantly lower MTS readings than untreated cells. There was no statistical difference between mouse cell experimental samples. Naturally cells treated with IMS

gave significantly lower MTS readings than all other samples regardless of cell type or overall incubation time.

After 48 hour incubation keratinocytes+3T3 feeder cells treated with phage gave rise to significantly higher MTS outputs than cells exposed to DMEM or IMS ($p < 0.001$, $F = 111.535$). In comparison experimental wells with lambda buffer added yielded only significantly higher MTS readings than cells killed with IMS. Mouse cells treated with lambda buffer gave statistically higher MTS outputs than cells exposed to phage, DMEM or IMS.

After 72 hours for keratinocytes and feeder layer cells there was no statistically significant difference between phage samples and samples containing lambda buffer or medium only ($p < 0.001$, $F = 77.678$). Samples exposed to IMS gave significantly lower MTS readings than all other wells. Mouse cells containing phage yielded significantly lower MTS levels than cells with lambda buffer added or DMEM only.

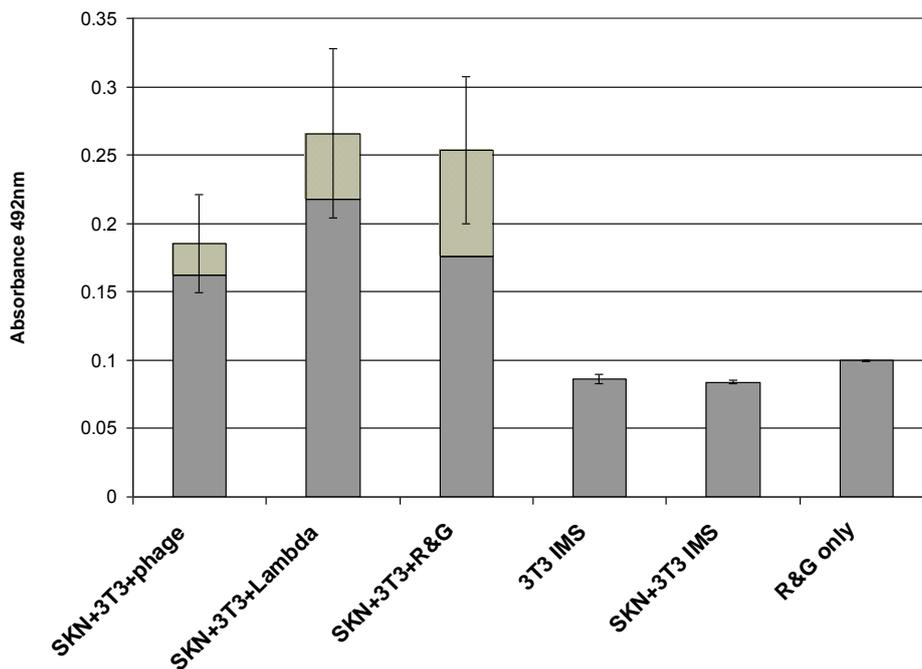


Figure 7.37 MTS assay keratinocytes with 3T3 feeder layers (24 hours)

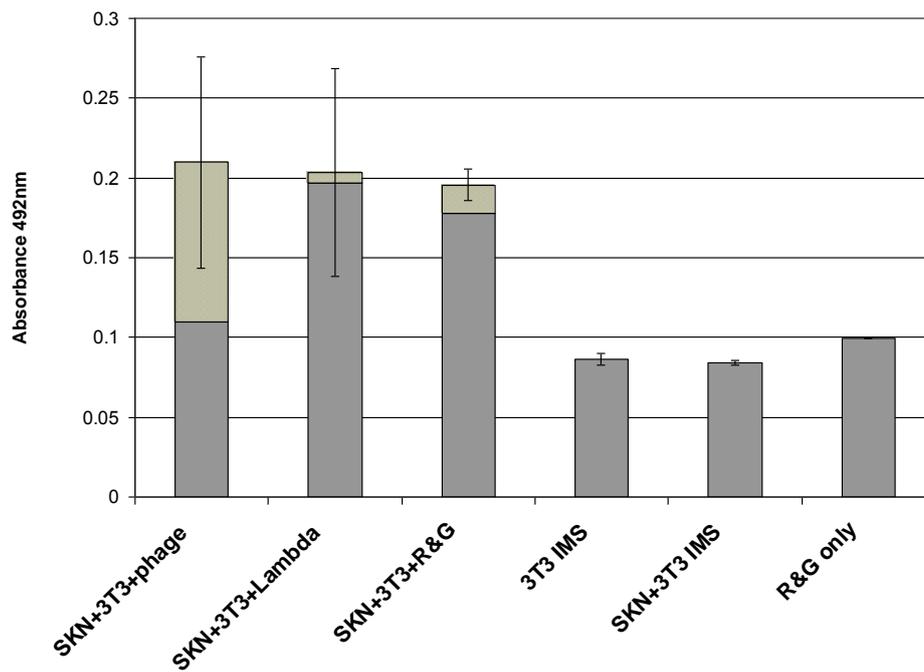


Figure 7.38 MTS assay keratinocytes with 3T3 feeder layers (48 hours)

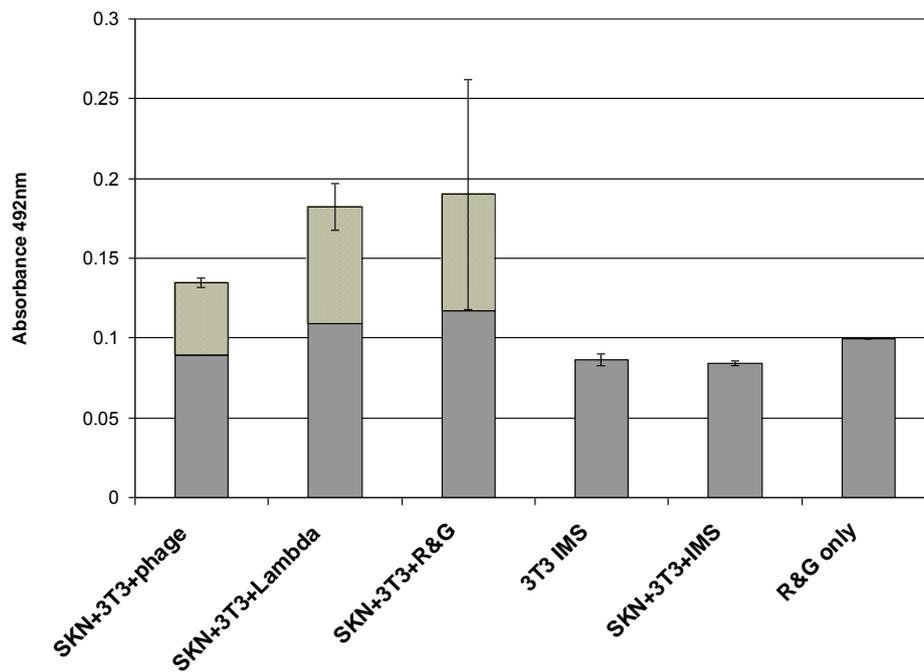


Figure 7.39 MTS assay keratinocytes with 3T3 feeder layers (72 hours)

Legend Figures 7.37-7.39:

Checked areas: MTS reading attributed to 3T3 cells alone; Bars refer to standard deviations of keratinocytes plus feeder cells

Colony numbers treated with lambda buffer and phage were compared to controls using One-way ANOVA followed by Tukey's post-hoc test ($p < 0.05$, $F = 2.469$). Samples treated with lambda buffer 50% gave rise to significantly lower numbers of colonies than samples treated with 12% and 25% lambda buffer. None of the phage or lambda buffer samples showed any significant difference to controls.

There was no statistically significant difference within phage samples or between phage samples of any concentration and lambda buffer or controls. This implies that phages do not exert a greater cytotoxic effect than lambda buffer or no cell treatment at all.

None of the preparations inhibited colony formation by or over 50%, hence an EC_{50} value could not be established.

7.4.31 Colony formation assay to assess the cytotoxicity of phage and lambda buffer on V79 cells

Samples containing 12 and 25% lambda buffer actually showed an increase in colony numbers compared to control (DMEM) (**Figure 7.40**), however this was not statistically significant. This may be due to experimental variation, as only 50 cells were seeded per well initially, the average number of colonies formed on DMEM was only 24 colonies. It is unlikely that lambda buffer would have a positive effect on the growth of the mammalian cells in lower concentrations, it is more likely however that the 15% difference (which corresponds to approx. 3 colonies), was due to experimental variation. Care was taken to pre-empt any drying effect by filling 8 wells per plate with sterile water, hence sacrificing one third of useable surface of plasticware and incubating the 24-well plates in loosely covered plastic containers, which makes a drying effect an unlikely cause for the experimental variations.

The optical appearance of stained cells cultivated with 50% phage and 50% lambda buffer was distinctively different. Dilution 50% lambda buffer gave very faint colonies, while 50% phage colonies were clearly visible and thoroughly stained (N=4).

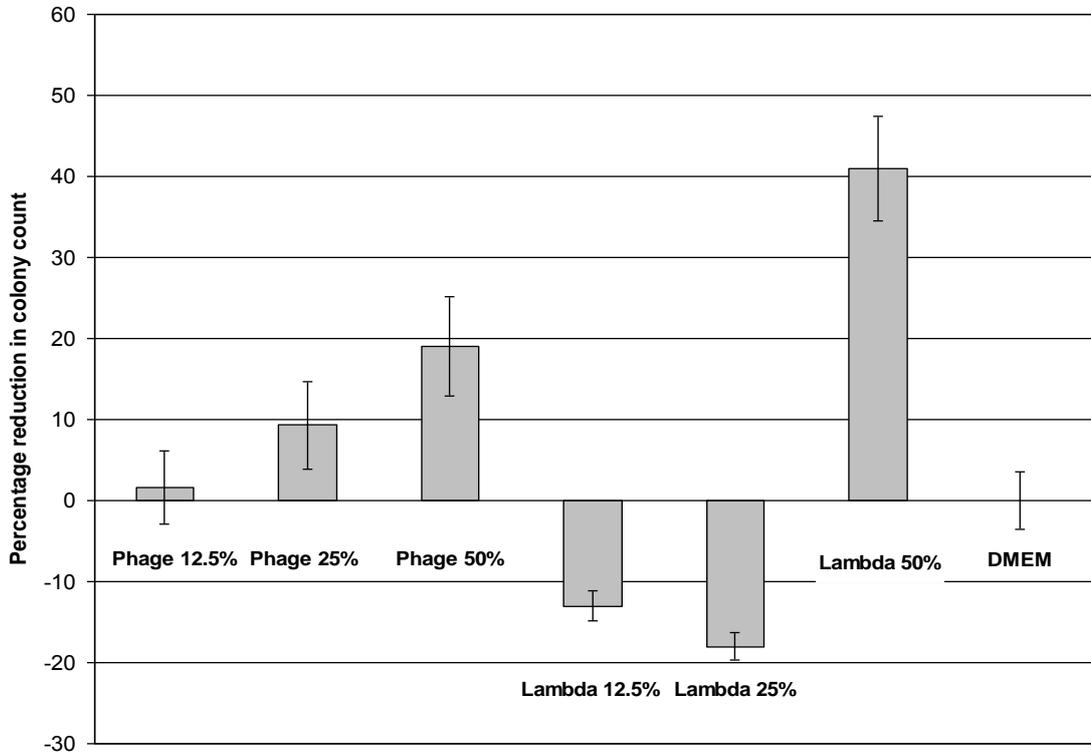


Figure 7.40 Percentage reduction of colony numbers grown with lambda buffer or phage compared to colony numbers in DMEM

Mean colony sizes of different samples, measured with callipers, were compared using one-way ANOVA followed by Gabriel's post-hoc test ($p < 0.001$, $F = 20.366$). Cells treated with 12% phage or lambda buffer gave rise to significantly larger colonies than cells exposed to lambda or phage 50%, there was no difference in terms of colony size to untreated controls (DMEM+cells). This was also the case with lambda buffer and phage 25% additionally both samples caused colonies to be significantly larger than untreated controls. Phage and lambda buffer 50% samples gave rise to significantly smaller colonies than any other phage or lambda buffer samples. Overall no statistical differences between phage or lambda buffer samples of equivalent concentrations could be found.

However statistical differences between DMEM+cells and lambda buffer and phage were significant. Lambda buffer 25% gave rise to significantly larger colony sizes than untreated controls. This was not the case with phage 25%, which showed no significant difference to controls. Lambda buffer and phage 50% caused colony sizes to be significantly smaller than controls.

7.4.32 Colony formation assay to assess the cytotoxicity of phage, diluents and cell extracts with varying endotoxin concentrations on V79 cells

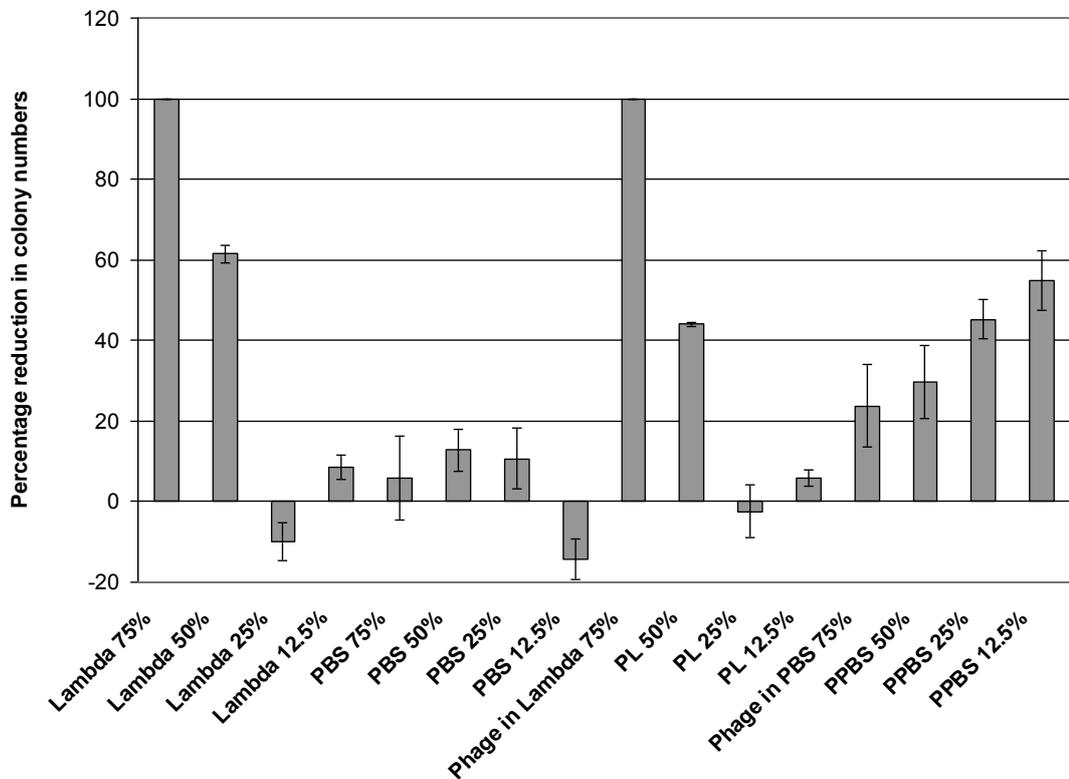


Figure 7.41: Percentage reduction in colony numbers dependent on supplementation

Legend to Figure 7.41:

PBS- Phosphate buffered saline

PL- Phage diluted in lambda

PPBS- Phage diluted in phosphate buffered saline

Numbers following code refer to percentage sample contained in 5% FCS DMEM

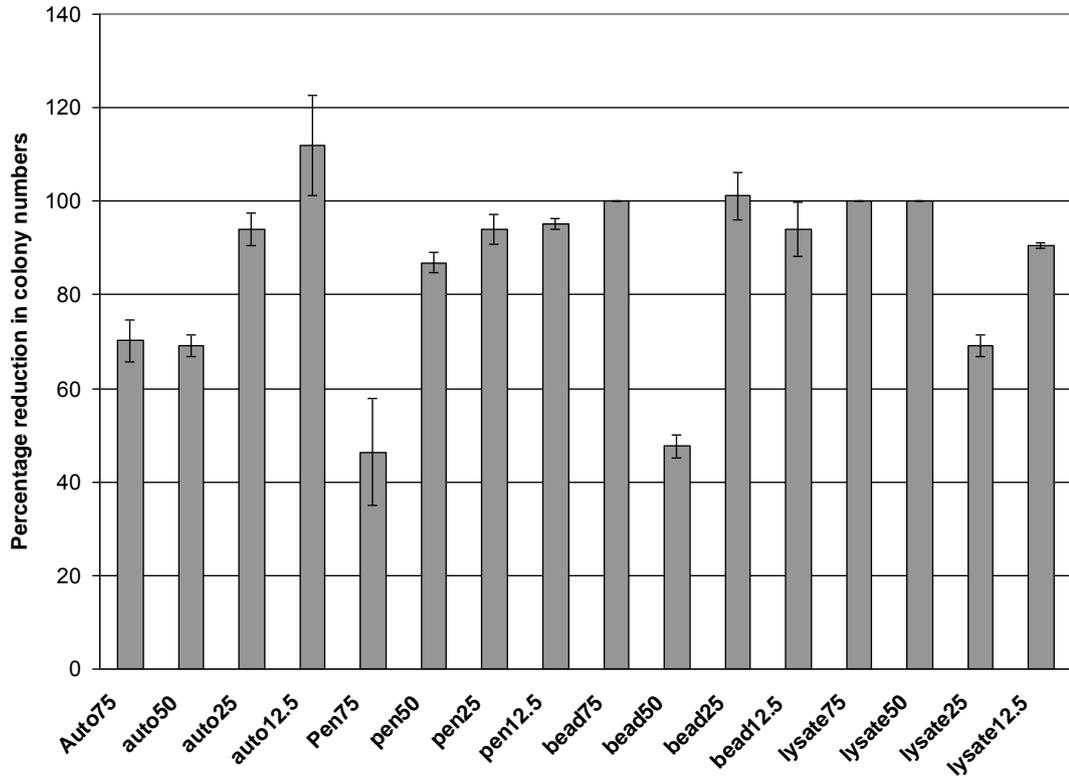


Figure 7.42 Percentage reductions in colony numbers dependent on supplementation

Legend to Figure 7.42:

Auto: Autoclaved cells

Pen: Penicillin G treated bacterial culture

Bead: Bacteria exposed to bead-beater

Lysate: Phage lysate in PBS buffer

Numbers following code refer to percentage sample contained in 5% FCS DMEM

Statistically significant differences in colony formation assays						
Significant difference						
Autoclaved 75%						
>	L75	PL75	Bd75	Lys75	Lys50	
Autoclaved 50%						
>	L75	PL75	Bd75	Lys75	Lys50	
Autoclaved 25%						
>	L75	PL75	Bd75	Lys75	Lys50	
Autoclaved 12.5%						
>	L75	L50	Phage75	Bd75	Lys75	Lys50
Beadbeater 75%						
<	Aut75	Aut50	Aut25	Aut12.5	L25	L12.5
<	PBS75	PBS50	PBS25	PBS12.5	PL25	PL12.5
<	PBS75	PBS50	Pen50	Pen25	Pen12.5	Bd25
<	Bd12.5	Lys25	Lys12.5	10%FCS	5%FCS	
Beadbeater 50%						
<	L25	PBS12.5				
Beadbeater 25%						
>	L75	L50	Bd75	Lys75	Lys50	
Beadbeater12.5%						
>	L75	PL75	Bd75	Lys75	Lys50	

Table 7.36 Statistical difference of mean colony numbers in colony formation assays

Statistically significant differences in colony formation assays						
Significant difference						
Lysate 75%						
<	L25	L12.5	PBS75	PBS50	PBS25	PBS12.5
<	PL25	PL12.5	PBS75	PBS50	Aut75	Aut50
<	Aut25	Aut12.5	Pen50	Pen25	Pen12.5	Bd25
<	Bd12.5	Lys25	Lys12.5	10%FCS	5%FCS	
Lysate 50%						
<	L25	L12.5	PBS75	PBS25	PBS12.5	PL25
<	PL12.5	PBS75	PBS50	Aut75	Aut50	Aut25
<	Aut12.5	Pen50	Pen25	Pen12.5	Bd25	Bd12.5
<	Lys25	Lys12.5	10%FCS	5%FCS		
Lysate 25%						
>	L75	PL75	Bd75	Lys50		
Lysate 12.5%						
>	L75	PL75	Bd75	Lys75	Lys50	
10% FCS						
>	L75	PL75	Lys75	Lys50		
5% FCS						
>	L75	L50	PL75	Bd75	Lys75	Lys50

Table 7.37 Statistical difference of mean colony numbers in colony formation assays

Statistically significant differences in colony formation assays						
Significant difference						
Lambda 75%						
<	L25	L12.5	PBS75	PBS50	PBS12.5	Phage25
<	PPBS75	PPBS50	Aut75	Aut50	Aut25	Aut12.5
<	Pen50	Pen25	Pen12.5	Bd25	Bd12.5	Lys25
<	Lys12.5	10%FCS	5%FCS			
Lambda 50%						
<	L25	PBS75	PBS12.5	Phage25	Aut12.5	Bd25
<	5%FCS					
Lambda 25%						
>	L75	L50	PL75	PBS12.5	Pen75	
Lambda 12.5%						
>	L75	PL75	Bd75	Lys75	Lys50	
PBS 75%						
>	L75	L50	PL75	Bd75	Lys75	Lys50
PBS 50%						
>	L75	PL75	Bd75	Lys75	Lys50	
PBS 25%						
>	L75	PL75	Bd75	Lys75	Lys50	

Table 7.38 Statistical difference of mean colony numbers in colony formation assays

Statistically significant differences in colony formation assays						
Significant difference						
PBS 12.5%						
>	L75	L50	PL75	PBS12.5	Pen75	Bd75
>	Bd50	Lys75	Lys50			
Phage in lambda 75%						
<	L25	L12.5	PBS75	PBS50	PBS12.5	Phage25
<	Phage12.5	PPBS75	PPBS50	Aut75	Aut50	Aut25
<	Aut12.5	Pen50	Pen25	Pen12.5	Bd25	Bd12.5
<	Lys25	Lys12.5	10%FCS	5%FCS		
Phage in lambda 25%						
>	L75	L50	PL75	Bd75	Lys75	Lys50
Phage in lambda 12.5%						
>	L75	PL75	Bd75	Lys75	Lys50	
Phage in PBS 75%						
>	L75	PL75	Bd75	Lys75	Lys50	
Phage in PBS 50%						
>	L75	PL75	Bd75	Lys75	Lys50	
Phage in PBS 12.5%						
<	L25	PBS12.5				

Table 7.39 Statistical difference of mean colony numbers in colony formation assays

Statistically significant differences in colony formation assays						
Significant difference						
Penicillin 75%						
<	L25	PBS12.5				
Penicillin 50%						
>	L75	PL75	Bd75	Lys75	Lys50	
Penicillin 25%						
>	L75	PL75	Bd75	Lys75	Lys50	
Penicillin 12.5%						
>	L75	PL75	Bd75	Lys75	Lys50	

Table 7.40 Statistical difference of mean colony numbers in colony formation assays

Legend to Tables 7.36-7.40:

<: indicates mean colony numbers are statistically significantly lower for the sample in the header (bold) than samples listed underneath

>: indicates mean colony numbers are statistically significantly higher for the sample in the header (bold) than samples listed underneath

L: lambda buffer, PBS: phosphate buffered saline, PL: purified phage preparation in lambda buffer, PPBS: phage in phosphate buffered saline, Aut: autoclaved, Pen: penicillin, Bd: beadbeater, Lys: unpurified lysate in PBS buffer, 10% FCS: DMEM+10% FCS, 5%FCS: DMEM+5% FCS

Numbers following sample code indicate the percentage of sample contained in DMEM+5%FCS.

A comparison of cell extracts with varying concentrations of endotoxins demonstrated the detrimental effect endotoxins and bacterial cellular waste products had on mammalian cell survival (**Figure 7.41&7.42**). Colony numbers of different samples were compared to controls using One-way ANOVA followed by Gabriel's post-hoc test ($p < 0.001$, $F = 10.642$).

Comparing PBS results with phage in PBS (PPBS) it was evident that both samples showed identical statistical differences to the remainder of samples (**Tables 7.36-7.40**). This implies that the presence or absence of phage in PBS had no significant impact on colony formation. The same scenario presented itself when lambda buffer and purified phage in lambda buffer were compared. This implies that the presence of phage does not significantly affect colony formation when compared to its control. Phage 50% and PBS 25% were not significantly different in terms of average colony numbers to any other samples.

Autoclaved samples (75%) gave rise to significantly higher colony numbers than lambda buffer 75%, phage in lambda 75%, beadbeater 75%, lysate 75% and 50%. penicillin 75% yielded lower colony numbers than lambda buffer 25% and PBS 12.5%. (**Tables 7.36-7.40**). Unpurified lysate (75%) however gave significantly lower colony numbers than a multitude of other samples, which implies that unpurified lysates may be more cytotoxic than treated bacterial cell extracts.

In terms of numbers of samples with statistically significantly different numbers of colonies (comparing 75% only) PBS, phage in PBS and autoclaved samples were comparable, having shown significantly larger colony numbers than 6, 5 and 5 other samples. Lambda buffer, phage in lambda buffer and unpurified lysate gave rise to significantly smaller colony numbers than 21, 22 and 23 other samples. Penicillin and beadbeater samples showed significantly lower colony counts than two or nine other samples respectively.

7.5 Discussion

7.5.1 Cytotoxicity tests

A number of standard assay methods are available to measure the number of viable cells in proliferation or cytotoxicity assays. The methods generally considered to be the most reliable are those involving staining cells with dyes, such as Trypan Blue or HPI and counting viable cells under a microscope. These methods however are time-consuming and invasive and consequently require large volumes of cells and materials. Trypan Blue is limited by only providing information about the number of apparently intact cells, it does not however provide further information about the proliferative stage of the cell or any minor cell damage, such as leaking membranes or cell stresses. Furthermore dye exclusion assays such as Trypan Blue overestimate viability (Freshney, 2005), hence results need to be confirmed with other experimental methods to avoid false negatives. Similarly HPI staining, which does provide information beyond the number of viable cells present, is limited to differentiation between apoptotic and necrotic cells. As there is an absence of cytotoxicity data in the literature regarding therapeutic phage preparations, in the context of this investigation it was considered important to use these two basic cell counting techniques first, followed by more discriminating experiments. Most comprehensive cytotoxicity studies combine a range of test methods and use a variety of cells, an approach employed in this investigation.

The most common technique to quantify cellular proliferation is measurement of [³H]thymidine incorporation into DNA, a method extensively reported in literature but which carries with it a range of disadvantages. The major limiting factor is that the amount of DNA synthesis measured is not necessarily directly related to the number of viable cells present.

Traditionally MTT assays were used to measure cell viability, however a more advanced standard method is the MTS assay. Only live cells are capable of reducing the tetrazolium dye to a coloured formazan product. The MTS assay is unable to distinguish between the differential responses of individual cells or the population, unlike the clonogenic assay, which was also included in this investigation. The MTS assay

measures cell proliferation based on mitochondrial activity. Small amounts of MTS are converted spontaneously in the culture medium and consequently the type of medium or serum used; pH and exposure to light are variables which may affect MTS assay absorbance values. In particular direct exposure to light and elevated pH can result in increased background absorbance values.

Lactate dehydrogenase leakage from cells is commonly used to assess toxicity of particular agents on primary cell culture (Schmidt et al. 2005), human cell lines (Vihola et al. 2005) and human fibroblasts (Issa, 2004). The LDH assay, a measure of membrane integrity, was used to assess agent induced cytotoxicity. This assay is considered to be an alternative to the ^{51}Cr release cytotoxicity assay (Decker & Lohmann-Matthes, 1988) and relies on the conversion of a tetrazolium salt (INT) into a red formazan product.

The LDH assay was used to monitor keratinocytes with feeder layers and allowed measurement of cell death in the presence of the two cell populations in particular determination of percent cytotoxicity. Factors known to interfere with the assay are spontaneous release of LDH from cells, LDH present in the medium or serum and phenol red, however controls were in place to correct for these factors. LDH quantities in animal sera vary and calf serum, which was used in this investigation, contains the highest LDH levels. Care was taken to use the same batch of medium and serum throughout each experiment. Factors affecting spontaneous LDH release from cells include vigorous pipetting, heat shock, PBS wash buffers and centrifugation of cells at speeds above 250xg.

Cytotoxicity experiments were conducted in 24-well plates since replicate determinations and experimentation relying on more than one approach was required to avoid false positive cytotoxicity results.

7.5.2 Cytotoxicity testing using 3T3 cells

The identity of 3T3(+3) cells obtained from University of Brighton stocks was confirmed by means of studying doubling times (**Sections 7.4.1 & 7.4.2**) and investigating cell morphology (**Section 7.3.10**). Initial experiments on doubling times

demonstrated that cells grown in 24-multiwell plates with lower surface area, rather than growth flasks were more susceptible to variations in cell survivors after seeding than cells grown in T25 flasks with higher seeding density and surface area. This was due to contact inhibition, smaller growth medium volume and smaller air-liquid interface.

7.5.2.1 Validation of phage activity in tissue culture conditions

In several experiments it was shown that phage HER401 BS46 retained its viability in tissue culture conditions, regardless of whether exposed to DMEM or lambda buffer. In the first experiment omitting mammalian cells, there was no statistically significant difference in phage particle recovery between samples or controls at 24 or 48 hours in the absence of mammalian cells (**Section 7.4.3**).

Experiments with 3T3 cells present (**Section 7.4.4**) showed that phage counts of some samples were significantly affected by incubation in tissue culture conditions. Higher phage concentrations (2×10^9 pfu/ml) gave rise to significant phage titre decreases after 24 hours only in lambda buffer samples-compared to controls. After prolonged incubation of 48 and 72 hours baseline phage counts were significantly higher than any samples, regardless of presence of mammalian cells. All samples of lower phage concentrations (2×10^8 pfu/ml) gave rise to significantly lower phage counts than samples not incubated in tissue culture conditions. The presence or absence of mammalian cells did not significantly affect phage recovery.

The dynamic changes in recoverable phage suggested that phage may aggregate and not be present as a homogenous suspension particularly after prolonged incubation in tissue culture conditions. It is likely that the phages, which have a surface charge may have adsorbed to the plastic ware. Some phage may have possibly adhered or adsorbed to the mammalian cells. Changes in pH due to cell metabolites and prolonged incubation with CO₂ may have affected the physical attachment of phage particles to surfaces presented, or rendered the phage non-viable. These pH and electrolyte changes are the most likely cause for the decrease in phage titre.

Future experiments may include direct phage particle counting methods like electron microscopy to investigate quantitative changes in recoverable, viable phage under prolonged tissue culture conditions.

7.5.2.2 Cytotoxicity assays using dyes

A range of experiments employing a variety of different methods were used to investigate the cytotoxicity of phage HER401 BS46 in 3T3 cells. The Trypan Blue stain method was used to count surviving cells at two seeding densities, exposed to three different concentrations of phage. No statistically significant difference in cell viability between controls and cells exposed to phage could be found. There was no significant difference in cell viability within the three phage concentrations used. This implies that in terms of total cell counts phage had no different effect on cells than no intervention at all.

Statistical analysis of the HPI staining experiment, a method allowing counts of total cells present and differentiation of apoptotic, necrotic and viable cells, remained inconclusive. After incubation with phage for 24 hours phage dilution 2 gave rise to significantly higher numbers of viable cells than controls, no statistical differences could be found between any other samples or controls. After 48 hours there was no statistically significant difference between any of the samples or controls. This implies that under the experimental conditions phage was unlikely to cause a detrimental effect on cell viability of 3T3 cells. Prolonged incubation (72 hours) may require further investigation- samples containing more dilute phage concentrations (Dilutions 2 & 3) gave rise to significantly lower cell counts than phage dilution 1 or controls. These data could be interpreted as higher concentrations of phage having a positive effect on cell survival for prolonged incubation periods.

Levene's Test pointed towards the necessity of giving consideration to the possibility of variances not being assumed equal between groups. Overall mean percentages of viable cells of experimental wells and controls were all within the range 92-95%. The phage preparation with the lowest phage titre led to the largest variations and overall lowest percentage of cell viability of 92%, after 72 hours incubation.

This suggests that any cytotoxic effect is unlikely to be caused by the presence of phage, as experimental wells with 10 and 100 times more phage gave rise to lower cell death and variation in cell numbers. As the drop in viability was less than 10% it was attributed to experimental variation such as different growth rates, drying effects or accumulation of cell metabolites rather than the potential cytotoxicity of phage

Despite 36 data points being read at 24, 48 and 72 hours, further investigation may include repeating similar experiments with even larger sample sizes. Use of larger growth vessels (T25 flasks) may give rise to more uniform growth rates and reduce the risk of accumulation of cell metabolites, due to the larger volume of medium present.

7.5.2.3 Cytotoxicity and cell proliferation assays (LDH and MTS assays)

Apart from one sample containing phage at the highest concentration (2×10^9 pfu/ml), drawn at 24 hours, there was no evidence to suggest that phage significantly affected LDH output of 3T3 cells (**Section 7.4.7**). Increased LDH levels seen in the sample may be attributed to chemicals introduced during the lysate purification process, which may have been diluted to insignificant levels at lower phage concentration or compensated for by cell repair mechanisms after prolonged incubation.

Fluctuations in LDH values and ultimately calculated percentages cell death were attributed to experimental variations. Future experiments may include attempts at reducing spontaneous LDH release by cells using media without phenol-red, containing different serum levels or attempting different lysate purification methods.

The MTS output of 3T3 cells was measured in two separate experiments (**Sections 7.4.8 & 7.4.9**). In MTS (I) experiment 5,000 cells were seeded per well and no significant differences in absorbance between samples treated with lambda buffer or phage were observed.

In the MTS (II) experiment (**Section 7.4.9**) three different seeding densities of 3T3 cells were employed. No evidence could be found that addition of bacteriophage to 3T3 cells may have a detrimental effect on cell proliferation. There was some evidence at higher

seeding densities (15,000 cells /well) that phage addition, regardless of concentration, may have been more beneficial to cell proliferation than lambda buffer after incubation for 24 hours. At lower seeding densities (10,000 cells/well) statistically significantly higher MTS outputs were attributed to cells exposed to phage (Dilutions 1 & 2) rather than lambda buffer and untreated cells (controls) after incubation for 72 hours. With regard to the 3T3 Swiss mouse cell line, it appeared that addition of phage did not exert a cytotoxic effect or affect cell proliferation negatively. There is some evidence to suggest that phage may even have a positive effect on 3T3 cell proliferation, further experimentation may be required.

7.5.3 Bacterial cell extracts with varying endotoxin concentration

A range of bacterial cell extracts were produced to provide sources of lipopolysaccharide and other bacterial cell products which may be present within a phage preparation or may result from the action of bacteriophages on infecting bacterial cells. The intention was to investigate whether any adverse reaction on the cell culture may be different if the bacterial cells were killed by bacteriophages or lytic antibiotics.

For the preparation of samples containing endotoxin *A. baumannii* HER1401 was used throughout, except for the antibiotic lysed cell sample. Preliminary experiments (**Section 6.2.7**) demonstrated that HER1401 was resistant to penicillin G, however, *A. baumannii* HER1151 was shown to be sensitive. As the bacterial suspension only served to provide endotoxins (released by antibiotic action) for use in the colony formation assay it decided to use *Acinetobacter* HER1151 for this section of the work.

Endotoxin concentrations were shown not to be statistically significantly different, regardless of the method used to process the bacterial cell suspensions.

Some authors have speculated that bacterial cells lysed by phage may give rise to excessively high concentrations of endotoxins (Clark & March, 2006) and this has been used as an argument against phage therapy. From data shown in **Section 7.4.18** there was no indication that lytic phage could give rise to statistically significantly higher levels of endotoxins than lytic antibiotics in the cell killing process.

In the course of this investigation phage stability became an issue. As reported in **Section 6.3.7**, the most concentrated phage preparation (HER1401), precipitated on storage and the phage titre dropped. This is not uncommon as reported by Ellis and Delbrück (1939), yet it caused difficulties, as regular phage enumeration was required. This occurrence highlighted several points of consideration in the context of clinical phage therapy. Phage titres may fluctuate depending on poorly understood factors. Temperature fluctuations may have been responsible for the drop in phage titre and highlights the need to investigate optimum storage conditions for phage preparations. Future experiments should include an investigation into factors such as UV-light, temperature, choice of diluents, pH, etc on phage stability in the same way as stability testing for medicines would be undertaken.

The purification process of phage may also require further investigation. Detection of residual chemicals from the chemical purification process employed in this investigation, using HPLC would elucidate the potential presence of chemical agents eliciting potential cytotoxicity. A range of different purification processes including ultracentrifugation, serial filtration with non-toxic diluents and their effect on phage yield and cytotoxicity may need to be investigated. A selection of phage diluents and their effect on phage titres and long term stability may also require further investigation. It may be possible that there could be a critical threshold concentration at which a phage preparation may be likely to precipitate in liquid form. The use of freeze drying for phage preparations may need to be considered after purification and the effect this may have on phage viability will need to be extensively investigated. Further work may also include testing of automated phage titration techniques, using absorbance readings in multiwell plates. This may make large volume, single operator phage titration and consequently published long-term phage stability data a reality.

An ideal scenario may be for phage preparations to be dispensed into airtight, sterile ampoules immediately after the purification process and include storage in a strictly temperature controlled, light protected environment.

7.5.4 Cytotoxicity testing using SKF276 human dermal fibroblasts

In this investigation part of a skin specimen was ‘transplanted’ onto a culture plate, with cells eventually migrating over the plastic surface to form explants. The enzymatic separation of dermis and epidermis ensured the absence of epidermal components in the resulting fibroblast culture and made it possible to utilise the same skin sample to establish a keratinocyte culture at the same time. The advantage of using fibroblasts obtained by this method, rather than using an immortalised cell line from a culture collection, is that cells are more likely to still possess similar characteristics to the equivalent cells found in-vivo.

Whole skin explants may have been used as an alternative, however obtaining large areas of live skin from a similar area and same donor would be extremely difficult. Human live skin samples are scarce and by propagating cells from primary origin it was possible to create conditions which allowed repeatable experimentation with statistically valid sample numbers. Cells were propagated to passage 4 and frozen at sufficient quantities, however it became apparent that despite using the same methods throughout cell recovery and growth rates varied between growth flasks. Despite improvements in methodology such as washing of cells with HBSS before trypsinizing and pre-warming the trypsin, cell recovery remained erratic, unlike the situation found with immortalised cell lines. Due to the slow growth rate of cells, the scarcity of material available and erratic recovery rates, direct counting methods for assessing cytotoxicity were not used for human dermal fibroblasts.

During cell baseline counts and doubling time experiments it also became apparent that cells were sensitive to being trypsinised or passaged (**Sections 7.4.12 & 7.4.13**). Cell recovery after 24 hours was regularly below the seeding density, which may be considered a disadvantage of using cells derived from primary material.

7.5.4.1 Cytotoxicity and cell proliferation assays (LDH and MTS assays)

In the preliminary MTS experiment comparing MTS conversion from cells exposed to lambda buffer and phage (**Section 7.4.14**), cell proliferation was more pronounced in samples containing phage at higher concentrations than samples exposed to lambda buffer alone at 24 hours. After prolonged incubation for 48 and 72 hours higher phage concentrations gave rise to significantly lower MTS readings than other samples. Samples with additions of phage containing 2×10^8 pfu/ml and 2×10^7 pfu/ml yielded MTS readings which were insignificantly different from controls. This may be evidence supporting the notion that not actual phage, but some components of the concentrated phage preparation may have some cytotoxic activity. One fold dilutions appear to reduce this effect sufficiently to not being statistically significant.

A second MTS assay (**Section 7.4.15**) combined with an LDH assay, investigated the cellular response to a wider range of samples after 24 hour incubation. No statistically significant difference could be found between samples containing phage, regardless of concentration, lambda buffer, untreated cells and samples containing sonicated bacteria.

In a third MTS assay (**Section 7.4.20**) investigating the widest range of samples, including preparations containing endotoxin released by different means, MTS outputs were correlated to absolute cell numbers with the aid of a calibration plot. There was no statistically significant difference between controls and phage samples at 24 hours. After longer incubation statistically significant differences started to emerge between phage samples and other samples. In view of previous experiments variations in cell proliferation between samples were considered to be attributable to experimental variations and another MTS assay was performed (**Section 7.4.21**).

The fourth MTS assay (**Section 7.4.21**) investigating the effect of phage and bacterial products on cell proliferation showed that after 24 and 48 hours there was no statistically significant difference between purified phage preparation and controls. Data suggested that despite differences between samples over prolonged incubation time (72 hours) and fluctuating MTS readings, phage did not have a detrimental effect on cell

proliferation compared to untreated human dermal fibroblast cells (controls). Overall there is overwhelming evidence to suggest that phages are unlikely to be a causative factor in impairment of cell proliferation.

An initial LDH experiment (**Section 7.4.15**) with short-term incubation (24 hours) showed that concentrated phage preparation regardless of final concentration in the samples showed no statistical difference to controls (untreated cells) or lambda buffer with regards to LDH output. A more extended LDH (**Section 7.4.19**) experiment with prolonged incubation and a wider selection of samples indicated that concentrated phage preparation HER401 BS 46 was not statistically significantly different than untreated cells or filtered phage lysate with regards to LDH readings.

This implies that there is no evidence to suggest that a concentrated phage preparation has a statistically significant cytotoxic effect on human primary dermal fibroblasts.

7.5.6 Cytokine assays for IL-1 released from human dermal fibroblasts SKN276

Despite being exposed to undiluted bacterial cell supernatant liquids the human dermal fibroblasts did not release any IL-1 β that could be detected with the BD Biosciences assay (**Section 7.4.22**). The lower detection limit of this assay was reported to be 7.8pg/ml (BD Bioscience). Spörri et al. (1996) could not detect any IL-1 β released from unstimulated human dermal fibroblasts (HDF), however when the same type of cells was directly stimulated using T-cells the HDF released approximately 2-3pg/ml into the supernatant liquid. Other investigators found that HDF starved of FCS released 5(\pm 3.55) pg/ml, rising to 6.33(\pm 4.16)pg/ml. HDF stimulated by heat shock released up to 7(\pm 3.82)pg/ml (Park et al., 2004). Maret (2004) reported the levels of IL-1 β and IL-1 α to be increased intracellularly, but not detectable in supernatant liquids.

This implies that with the assay available it would be impossible to determine whether the cells gave rise to IL-1 β output in response to being stimulated with phage, endotoxin or other experimental reagents compared to unstimulated cells. Cells treated with ethanol were included as positive controls, however no IL-1 output could be detected.

Extreme stimuli like heat shock may be required to release IL-1 β into the supernatant liquid in quantities detectable by this assay. Endotoxins have been reported to be the most common stimuli for IL-1 release (Dinarello 1991), alternative experimental approaches, such as lysing of cell pellets by several freeze-thawing cycles in Tris-HCl and assaying the lysate for IL-1 β may yield results of approx. 30pg/ml as found by Spörri et al. (1996). HDF have been found to express and translate the IL-1 β gene, yet IL-1 β was not secreted into cell culture supernatant liquids when stimulated with LPS, mirroring the results in this investigation (Spörri, 1996).

7.5.7 Cytokine assays for IL-6 released from human dermal fibroblasts SKN276

In the pilot experiment (**Section 7.4.22**) a large variety of dilutions were used. It was established that it would be necessary to use undiluted samples for follow-on IL-6 readings.

Phage dilution 1 (2×10^9) released 7 times more IL-6 than control cells; Overall phage dilution 1 gave statistically significantly higher IL-6 outputs than all other samples.

Reduced concentrations (2×10^8 and 2×10^7) of phage gave rise to the release of interleukin levels which were not statistically different to the unstimulated controls.

Stimulated HDF have been reported to release ng/ml quantities of IL-6 into supernatant liquids (Park et al., 2004; Arlian et al., 2003), however, the samples in this investigation remained in the pg/ml range. This thousand fold difference between maximum reported levels and experimental results implies that differences between concentrated phage, crude phage samples and controls were potentially due to experimental variation, however further investigation may be warranted.

Shortkroff et al. (2002) discovered that cells exposed to nano-sized particles elicited similar IL-6 levels to unstimulated control cells, while particles of 100nm increased IL-6 to 94pg/ml. HER401 BS46 phage, is a member of the Leviviridae group and estimated to be approximately 23nm in diameter. Phage may be capable of inducing IL-6 (or release of other cytokines or other cell responses) not in the traditional context of

chemical cytotoxicity, but as a response to phage as a nanoparticle. Some nanoparticles have been known to enter cells and cause specific changes to cell surfaces (Panessa-Warren et al., 2006). It should be excluded by future experimentation that phage particles do not behave in the same way and do not cause damage on a microscopic or genetic level.

Erdag & Morgan (2002) found that cultured composite keratinocyte grafts supplemented with IL-6 improved the immune response of the cells and enhanced antibacterial properties. If phage could stimulate local autocrine IL-6 release in wounds and grafted wounds this may provide additional antibacterial advantages and improve overall graft survival.

7.5.8 Cytokine assays for IL-8 release from human dermal fibroblasts SKN276

It was evident from the pilot experiment that phage dilution 1 gave over five times the IL-8 output of untreated cells (**Section 7.4.25**). This was confirmed in the main IL-8 experiment (**Section 7.4.26**), where purified and crude phage lysate gave rise to significantly higher IL-8 outputs than all other samples. Interestingly untreated cells (controls) yielded significantly higher IL-8 levels than sonicated bacterial cells or lambda buffer, however IL-8 levels remained significantly lower than for phage.

7.5.9 Cytokine assays for TNF- α release from human dermal fibroblasts SKN276

TNF- α levels in this investigation were below measureable detection limits. Perfetto et al. (2003) stimulated HDF with lipopolysaccharides and porins, yet TNF gene expression remained undetectable. Park et al. (2004) reported average TNF- α levels of 20 ± 15 pg/ml in human dermal primary fibroblasts (human foreskin, P6-10) during a 48 hour incubation in controls and samples subjected to heat shock. Fahey et al. (2002) described how normal subcutaneous mouse fibroblasts did not release any measurable amounts of TNF- α in response to lipopolysaccharides, however fibroblasts cultured from wound sites (polyvinyl alcohol sponges subcutaneously implanted for 2 weeks in

Balb/c mice) released significant TNF- α levels, associated with upregulation of TNF- α mRNA in response to the same stimuli. This implies that wound-derived fibroblasts as used by Fahey (2002) or HDF from a different source may indeed release measurable TNF- α levels. Future experiments may involve investigating TNF- α release in myofibroblasts and HDF from various donor sites in response to phage preparations. Additionally TNF- α mRNA production may be measured in the HDF used in this investigation.

Evidence has suggested that cytokines have antagonistic activities against tissue growth factor- β 1 an important cytokine in fibrosis and wound healing (Roberts et al., 2003). TNF- α specifically antagonised the TGF- β 1-induced upregulation of type I and III collagens in fibroblasts in mouse models. Goldberg et al. (2007) suggested that the antagonism between TGF- β 1 and TNF- α may be important in extracellular matrix formation, maintaining tissue homeostasis and ultimately wound healing. In a contraction wound model Goldberg et al. (2007) demonstrated that TNF- α negated the myofibroblast contractility induced by TGF- β 1 in fibroblast-populated collagen lattices. This may have clinical implications in terms of wound contraction and aesthetics. This issue is particularly important in the context of burns wounds, as contraction of large areas of tissue may lead to loss of function and if occurring in the thoracic region suffocation.

In a wound fibroblasts may transform into myofibroblasts, which may be present in fibrotic and normal wound healing. These activated fibroblasts (myofibroblasts) express α -smooth muscle actin (Darby et al. 1990) and produce stress fibres under the influence of mechanical stress- and more importantly in the context of this investigation- the presence of cytokines (Gabbiani, 2003; Goldberg et al., 2007). Stressful stimuli such as heat shock and lipopolysaccharides induce cytokine expression including TNF - α , IL-1 and IL-6, which may in turn affect collagen metabolism. The effects of phage on collagen metabolism markers may be investigated and provide further information on whether phage may affect wound healing and in-vivo conversion of fibroblasts to myofibroblasts. Future experiments may include further studies of interleukins in human dermal fibroblasts, cell surface area measurements of attached cells exposed to

phage preparations, ultrastructural cell modifications, formation of stress fibres in HDF and myofibroblasts exposed to LPS, bacterial cell extracts and various phage preparations (Moulin et al. 1998).

7.5.10 Cytotoxicity testing using SKF276 human dermal keratinocytes

Rheinwald and Green had successfully cultured and subcultured keratinocytes in clonal cell densities on a feeder-layer of lethally irradiated mouse fibroblasts (Rheinwald & Green, 1975). Green subsequently suggested that these cells were suitable for therapeutic use (Green 1979, 1991). Keratinocytes grown at the Blond McIndoe Centre were grown on antibiotic free medium with 3T3 feeder layers, for clinical reasons. Keratinocytes could be grown successfully without 3T3 feeder layers, utilizing 'complete' media, however early senescence and cell differentiation, as well as high costs of media have been discouraging factors (Fu et al., 2003). The reason why the Rheinwald & Green method was chosen was that keratinocytes processed using the same protocols were used for patient burn wound grafting at the Blond McIndoe Centre. As the keratinocytes were cultured in the 'gold standard' method on 3T3 feeder layers it posed an additional technical problem with regards to the MTS and LDH assays used. The overall total LDH and MTS absorbance comprised the portion released by keratinocytes plus 3T3 cells and could be easily read for each sample. As the keratinocytes would not survive without another cell type present, only 3T3 cells could be grown on their own. The LDH and MTS absorbance readings of 3T3 cells alone were then subtracted from SKN+3T3 cells. This was an indirect method to calculate the absorbance attributable to the keratinocyte portion. The LDH release from 3T3 cells was expected to be prone to fluctuations, as irradiation of 3T3 cells damaged cell membranes. Despite Rheinwald & Green (1975) originally describing irradiated 3T3 cells as 'lethally irradiated', these cells retain some of their viable functions which may interfere with LDH and MTS tests. Promega asserted that CytoTox96, the LDH assay used specifically allowed the measurement of cell death in the presence of two cell populations.

Keratinocytes and 3T3 feeder layers exposed to lambda buffer gave rise to significantly higher LDH outputs than phage after 24 hours incubation. After 48 hours controls and

lambda buffer treated cells released significantly more LDH than cells exposed to phage, regardless of cell type. After prolonged incubation (72 hours) the only significant differences were those seen between lambda buffer and controls in mouse cells. This implies that phage had no significant effect compared to lambda buffer or controls after long incubation.

Overall there was no evidence to suggest that phage had a cytotoxic effect on keratinocytes grown on mouse fibroblast feeder layers. There may be even some evidence that phage have some beneficial effect on cell survival compared to controls and lambda buffer, this would require further investigation.

In the cell proliferation assay phage gave rise to significantly higher MTS outputs than controls for keratinocytes grown on feeder cells after 24 hours. There was no significant difference between any mouse samples. After 48 hours phage treated keratinocytes + 3T3 cells yielded significantly higher MTS outputs than controls. Mouse cells exposed to lambda buffer showed significantly higher MTS readings than phage treated wells or controls. After 72 hours there was no statistical difference between phage, lambda buffer samples and controls with regard to keratinocytes. Mouse cells exposed to phage gave rise to significantly lower MTS outputs than lambda buffer.

Overall in the MTS assay phage did not appear to exert a detrimental effect on keratinocytes grown on feeder layers. Results read at 24 and 48 hours implied that phage treated keratinocytes may have a more beneficial effect on human cells than no treatment. It needs to be borne in mind that the 3T3 mouse cells had been irradiated and were expected to have erratic MTS outputs.

7.5.11 Colony formation assay using V79 hamster cells

The colony formation (or clonogenic) assay was designed as a long-term test, demonstrating survival of cells rather than short-term toxicity, which may be reversible. Survival of cells indicated that the regenerative capacity of cells was intact (Freshney, 2005). The colony formation assay is a standard in-vitro method of toxicity testing, using primary cells or, as employed in this experiment, cell lines. It works on the assumption that healthy cells attach to the plastic and form colonies which can be

stained and seen with the naked eye. In the presence of cytotoxic material the cells do not form colonies on the bottom of the wells.

An initial colony formation assay was designed to compare only phage and lambda buffer at three concentrations with controls (DMEM only) in **Section 7.4.31**. Colony numbers and colony sizes were statistically compared using ANOVAs and Gabriel's post-hoc tests.

In terms of colony sizes no statistical differences could be found between samples containing equivalent concentrations of phage or lambda buffer. There was one difference compared to controls (DMEM+cells), where lambda buffer 25% gave rise to significantly larger colony sizes, phage 25% showed no statistical difference to controls.

This implies that in terms of colony sizes phages have no statistically significantly different effects than lambda buffer of equivalent volume.

With regard to colony numbers, (the measure of cell survival), there was no statistically significant difference within phage samples of different concentrations or between phage samples of any concentration and lambda buffer or controls. Subsequent experiments (**Section 7.4.32**) differed from the preliminary colony formation assay in that a wider sample variety and size was used. The rationale behind using the various extracts was to shed light on which components of a phage preparation may be responsible for cytotoxicity or treatment failure, particularly potential damage to skin-grafts. Endotoxins have been implicated in the literature for treatment failure of phage preparations. Apart from the various endotoxin-containing preparations an additional sample tier was introduced whereby phages were diluted in PBS rather than lambda buffer and compared to PBS alone, to investigate if lambda buffer itself may have some inhibitory effect on colony formation.

With regard to statistical significance, the presence or absence of phage in PBS had no significant impact on colony formation. This was equally the case when lambda buffer and purified phage in lambda buffer were compared. This implies that the presence of phage does not significantly affect colony formation when compared to its control. However when phage in lambda buffer or lambda buffer alone were compared to phage

in PBS or PBS alone it became evident that lambda buffer itself may have some properties which may inhibit colony formation.

The percentage reductions in colony numbers (**Figure 7.42**) were not proportional to endotoxin concentration as the autoclaved cell extract (75%) with the highest measured endotoxin concentration of 4.70EU/ml gave rise to significantly more colonies than a range of other samples with less endotoxin. This may be an indicator that other factors, possibly bacterial cell metabolites, cell remnants or choice of diluents (lambda buffer) affected colony formation. These factors which are likely to be present in crude and partly purified phage preparation may be responsible for cytotoxicity rather than phage itself. The effect of phage diluents and contaminants present in phage preparation due to the nature of their production or possibly chemical purification on cell viability requires further investigation.

Rose et al. (2004) used Eagle's minimal essential medium instead of DMEM, however DMEM has been successfully used in a range of investigations using V79 cells in a similar context. Rose et al. (2004) also used an initial FCS concentration of 5% for standard growth of V79 cells which was reduced to 2.5% during the colony formation assay. In this investigation the initial FCS concentration was 10%, which was dropped to 5% for the assay. It could be argued that the higher serum concentration may have masked potential cytotoxic effects. A higher initial FCS concentration was chosen as the overall cytotoxicity of phage or the diluent lambda buffer was unknown and it was important to produce meaningful results with a wider range than would have been obtained with the lower concentrations of FCS. Some experimental setups included only exposure to the putative cytotoxic agent during pre-incubation, in this investigation phage were added throughout clonal cell growth, which made this experimental setup a more stringent assay (Freshney, 2005). Various serum concentrations were acceptable and desirable in clonogenic assays (Freshney, 2005). The principle remained the same, that cells were exposed to lower concentrations of FCS during the assay, which made them sensitive to cytotoxic influences. Future experiments could include different negative standards, repetition of all experiments using different concentrations of FCS and use of diluents other than lambda buffer. A wider range of concentrations and phage

preparations may be used with various serum concentrations, particularly 5% halved to 2.5% during experiments. Longer exposure to phage may provide further useful data, as cells may be more sensitive in longitudinal studies due to cell cycle effects and cumulative damage (Freshney, 2005). In view of all other results it is unlikely that phage were cytostatic, however to exclude this possibility colony sizes may be compared between controls and experimental wells. Significant differences in colony sizes, without reducing the number of colonies present may point towards an agent being cytostatic.

7.6 Conclusion

Bacteriophages, when introduced to the tested cell culture, reduced in titre, yet were recoverable sufficiently to allow meaningful experimentation. It was postulated that phages which possess surface charges may aggregate or adsorb to plastic ware and possibly even mammalian cells. Changes in pH, due to cell metabolites and incubation with CO₂, and also changes in electrolytes were considered to be the most likely causes for the reduction in recoverable phage titres.

A range of experimental approaches were used to investigate the putative cytotoxicity of phage when added to 3T3 cells in culture. With the Trypan blue method no statistically significant difference could be detected between controls and phage.

HPI stain experiments remained inconclusive, while there was some evidence that phage may have had a positive effect on cell survival it was concluded that they were unlikely to be a contributing factor to cell death.

The differences in LDH release from 3T3 cells between controls and experimental wells were considered negligible. Fluctuations in LDH values and ultimately calculated percentages cell death were attributed to experimental variations.

In two MTS experiments with 3T3 cells utilising different seeding densities, differences between experimental samples and controls were considered to be due to experimental variations. Overall there was no evidence to suggest that phage preparation HER401 BS46 at Dilutions 1, 2 or 3 exerted any significant cytotoxic effect on 3T3 cells. There

was however, some evidence to suggest that phage may have had some beneficial effect on cell proliferation.

There was no evidence to suggest that bacteria lysed by phage liberated significantly more endotoxins than other methods of cell disruption such as lytic antibiotics or sonication.

A range of MTS experiments involving human dermal primary fibroblasts led to the overall conclusion that phage were unlikely to have shown any cytotoxic effect on these cells. There was an indication that components of the concentrated purified phage preparation HER401 BS46 had some effect on cell proliferation, and this may require further investigation.

Two LDH experiments with human dermal primary fibroblasts suggested that there was no evidence to suggest that concentrated phage preparation had a statistically significant cytotoxic effect on human primary dermal fibroblasts.

Interleukin release of human dermal primary fibroblasts exposed to phage at three dilutions was measured. There was no detection of IL-1 β or TNF- α release in response to phage or other stimuli. The most concentrated phage preparation (dilution 1 with 2×10^9 pfu/ml) gave rise to significantly higher IL-6 outputs than any other samples. 1:10 dilutions of the same phage preparation were not statistically different to any other samples, including controls. This may be another pointer that components of the concentrated phage preparation HER401 BS46, rather than phage itself may have had a negative effect on phage proliferation. With regard to IL-8 release purified and crude phage lysate gave rise to significantly higher IL-8 outputs than all other samples.

In LDH experiments with wells containing keratinocytes with 3T3 feeder layers, there was no evidence to suggest that phage had a cytotoxic effect. There may be even some evidence that phage may have some beneficial effect on cell survival compared to controls and lambda buffer, but this would require further investigation.

The MTS assays implied that phage did not exert a detrimental effect on keratinocytes grown on feeder layers. Results read at 24 and 48 hours suggested that phage treatment may have a more beneficial effect on human cells than no treatment.

The first colony formation assay suggested that in terms of colony size there was no statistical difference between phage and lambda buffer at the same concentration. With regard to colony numbers there was also no statistical difference between phage samples of any concentration, lambda buffer or untreated cells.

In the second colony formation assay the presence or absence of phage did not appear to have any statistically significant impact on colony numbers whether diluted in PBS or lambda buffer when compared to its respective diluents.

Overall no conclusive evidence could be found that phages were cytotoxic in any of the cell culture systems used. Some evidence pointed towards phage potentially having a beneficial effect on cell proliferation or survival. Lambda buffer, the diluent most frequently used for phage preparations in the literature, may itself have some cytotoxic properties.

Chapter EIGHT

General Discussion

8.1 Antibiotic resistance of *Acinetobacter* spp. strains

In this investigation all local Sussex isolates (Chapter 3) including at least one representative of an outbreak strain and some isolated from burns patients were multiply antibiotic resistant. One strain was resistant to all antibiotics tested in this investigation, according to BSAC breakpoints. Similarly strain HER1424 was resistant to seven antibiotics out of 12 with given breakpoints (Chapter 5).

Canadian isolates displayed resistance against a multitude of antibiotics, information with regards to their origin and clinical significance was not available (Chapter 6). Particularly with regards to the Sussex isolates, where at least one clinical isolate had become virtually resistant to all tested antibiotics, the need for alternative treatment options, become apparent.

The problematic nature of *Acinetobacter* spp. was recognised in the 1980s (Bergogne-Berezin & Joly-Guillou, 1985). More recent sources refer to a multi-drug resistant *Acinetobacter* epidemic (Morgan et al., 2009) and a public health problem (Karageorgopoulos & Falagas, 2008), as clinical treatment requires antibiotic combinations (Walsh, 2008). Reflecting this decrease of therapeutic choice, the BSAC Disc Diffusion method Version 7 (2008) only lists MICs and zone diameter breakpoints for six antimicrobial preparations, including tigecycline, yet colistin remains absent. *Acinetobacter* spp. has even shown resistance to polymyxins, considered the last resort drug group (Fernandez-Reyes et al., 2009).

With the emergence of pan-drug resistant bacterial strains of *Acinetobacter* (Giamarellou et al., 2008; Souli et al., 2008) and pharmaceutical companies focussing

research efforts on the development of agents for the treatment of chronic conditions rather than antimicrobials, it seems appropriate to look elsewhere for alternatives (Pachon & Vila, 2009). Phage therapy has shown promise as a viable therapeutic option in the past, even in pan-drug resistant strains, and should be considered as an alternative or complement to orthodox treatment (Weber-Dabrowska, 2000; Debattista, 2004; Hanlon, 2005, 2007; Sulakvelidze, 2005; Kropinski, 2006). For phage therapy to become an acceptable complementary or alternative treatment option to antibiotics, inhibiting factors which may limit acceptability or hamper success, and previous reasons for phage therapy failure, as well as potential applications need to be investigated.

8.2 Identification, taxonomic status of *Acinetobacter* spp.

Early on in this investigation it became clear that reliance on phenotypic properties for strain identification was problematic. This is clearly recognised in the literature; API20NE kit is universally considered the most useful out of the choices of phenotypic identification kits for *Acinetobacter* spp. (Gerner-Smidt & Tjernberg, 1991; Bosshard et al., 2006). Due to the unclear official taxonomic position of most *Acinetobacter* isolates it was difficult to clearly assign isolates to a particular subspecies, a phenomenon encountered by other investigators (Bosshard et al., 2006). This shortcoming could affect phage therapy in a clinical context; clear identification even to species level would require involvement of costly or time consuming techniques. Being unsure about the taxonomic status of the infecting agent could mean delays in identifying clinically suitable phages, unnecessarily wasting of large quantities of phage material in the host-screening process and delays in sharing of therapeutic bacteriophage preparations in medical emergencies. This problem does not affect antibiotic therapy to the same extent, as it is unimportant in a clinical emergency which subspecies causes the infection, as long as it is sensitive to antimicrobial therapy. Since phages are considered to have a narrower host spectrum than antibiotics, then phage preparations would need to be screened before use. With the wide diversity of *Acinetobacter* spp, statistically the numbers of phage preparations to be tested to find a few suitable preparations for clinical use becomes very large. Overall the taxonomic status for most *Acinetobacter* genospecies remains chaotic and complicated, due to historic and continuing changes (Gerischer, 2007; Giamarellou et al., 2008; Nemeč et al., 2009). Having clear official

taxonomic guidelines combined with a fast, reliable phenotypic identification system would allow narrowing down of the numbers of potentially clinically suitable phage preparations for testing.

8.3 Selective media for the isolation of *Acinetobacter* spp.

In this investigation Leeds Acinetobacter Medium (LAM) was used to serve different purposes. In agar form it was utilised to support the identification of *Acinetobacter* spp. Provided the bacteria grew, it gave rise to a reliable indicator change in the agar, which translated to the agar changing to mauve and colonies appearing as faint pink.

Differentiation of *Acinetobacter* spp. growth from other organisms was simple and reliable. In this respect LAM agar proved useful.

It was also attempted to use LAM as a selective medium to isolate *Acinetobacter* spp. in the presence of other contaminating bacteria, this was unsuccessful. Jawad et al. (1994) had made the observation that existing selective media were too inhibitory for some *Acinetobacter* strains, yet did not exclude other species sufficiently and consequently developed a new medium (LAM) which allegedly had superior properties over Herellea and Holton's agars when tested on 134 *Acinetobacter* strains. In the course of this investigation three out of nine Sussex isolates, the Leiden isolate and five out of ten Canadian isolates failed to grow on LAM as described by Jawad et al. (1994). Overall 45% of isolates tested on LAM failed to grow on this medium, which emphasises the need for improvement of this agar. Attempts were made during this investigation to modify the antibiotic supplementation, by changing cephalosporins to permit growth of more *Acinetobacter* isolates, while making the medium more restrictive with respect to other species, but these did not have any beneficial outcome.

The concept of using the presence of a host bacterium also as an indicator for phage in a sample, at the point of isolation, has been previously explored (Mandilara et al., 2006). LAM liquid was used in this investigation for host isolation and enrichment with the rationale that the simple means of a colour change may indicate the presence of host bacterium and ultimately the potential presence of *Acinetobacter* phage. LAM was also intended to suppress proliferation of background contamination, which was prone to overgrow *Acinetobacter* spp. at the same time as breeding *Acinetobacter* phage with the indicator host present in waste water samples or sewage. LAM liquid was unsuitable for

such a purpose. For enrichment and phage isolation a selective liquid medium which sufficiently suppresses contaminants, yet supports growth of *Acinetobacter* spp. and consequently phage, would be very useful.

8.4 Availability of *Acinetobacter* phage

In this investigation *Acinetobacter* spp. with varying antimicrobial susceptibility profiles and some isolates responsible for severe infections in the local QVH burns unit, were readily available. Finding lytic *Acinetobacter* phage matching the bacterial isolate collection proved to be much more difficult. Various experimental approaches, including standard enrichment techniques did not lead to isolation of lytic phage. Reference sources like Kutter & Sulakvelidze (2005) or Adams (1959) provide generalised phage isolation protocols. Other authors such as Ackermann & Turcotte (1970), Coffi (1995), Herman & Juni (1974), Twarog & Blouse (1968), Soothill (1992) provided some details of the successful isolation of *Acinetobacter* spp. phage. Such instructions, such as inclusion of enrichment steps or two different approaches of direct plate selection methods, were employed with a multitude of samples (Chapter 4). Despite using appropriate isolation methods phage material could not be obtained from the samples, which indicates that more specific approaches with regards to ambient temperature, sampling location or other know-how may be needed. Sampling of hospital waste water during the presence of a hospitalised patient infected with *Acinetobacter* spp. was generally a useful principle, as the digestive tract is a reported reservoir for *Acinetobacter* spp. in hospitalised patients (Bergogne-Berezin et al., 1996). Presence of detergents in the waste water samples and seasonal variations may have contributed to the lack of successful phage isolation. Kloos & Musselwhite (1975) reported that *Acinetobacter* spp. are isolated more commonly in the summer months. This notion may be supported by more frequent reports of community-acquired pneumonia caused by *Acinetobacter* spp. in developed tropical countries (Barnes et al., 1988; Anstey et al., 1992). A seasonal pattern of infection by *Acinetobacter anitratus* was also reported by Retailiau et al., (1979). Following the notion that abundant presence of the indicator bacteria is a predictor for phage presence, optimum samples may be drawn on a day preceded by several consecutive hot days.

The concept that phage exists in abundance in the environment and can be readily isolated is perpetuated in the literature (Holt, 1984; Kutter & Sulakvelidze, 2005). Bergey's Manual of Systematic Bacteriology (Holt, 1984) holds this assertion in the context of *Acinetobacter* citing two references (Twarog & Blouse, 1968; Herman & Juni, 1974). It could be speculated that at least one author of each paper was a well-respected expert on *Acinetobacter* spp. at the time and consequently was likely to be experienced in at least host and possibly phage isolation. In the 1960s and until the 1980s phage typing was considered a routine method and it is likely that for this particular reason phage isolation expertise was profuse in Western Europe at that time. It could be also speculated that wastewater and sewage treatment systems may have been different in the last century and household chemicals, which may reduce phage counts in samples, less readily available.

Specific *Acinetobacter* phage isolation protocols remain unpublished; closely guarded know-how on optimum sampling conditions and increasing phage yields are yet to be made available in the public domain. Pure methodologies have become unfashionable matters of publication and *Acinetobacter* spp., despite becoming increasingly clinically significant, remains in relative obscurity.

Local or international collections investigated did not hold *Acinetobacter* phage (Chapter 4). Interestingly ATCC had held three phages for *Acinetobacter calcoaceticus* in the past, which could have been very useful in this investigation. Such material had been deaccessioned and is now irreplaceably lost to the research community. It is speculative if the advent of more modern bacterial identification techniques than phage typing rendered some phages less financially viable for a culture collection. One research group readily shared one phage and host pair; however, this proved unsuitable for therapeutic use, due to a complex temperature-dependent lytic behaviour. An attempt to obtain phages from a well-respected source in Canada proved problematic with lengthy delays and bureaucracy, partly due to anti-terrorist legislation. Other researchers who had previously worked with *Acinetobacter* phage did not respond to communications, were no longer in post or were unwilling to share material due to Intellectual Property issues. For some phages, hosts (Chapter 4) could not be found and the few lytic phages which matched to *Acinetobacter* strains appeared to be specific for

one host (Chapter 6). The availability of potential therapeutic phage was therefore severely limited which in turn may hamper further attempts at building an extensive phage library, suitable for treating bacterial infections in (burns) patients.

As a consequence, before phage therapy for *Acinetobacter* infections can be considered as a viable option it will be necessary to source a wider range of lytic phages from the environment. This process has been conducted successfully in the past for other phages, but was outside the scope of this thesis.

8.5 Narrow host range

One major difference between antibiotics and phages is that the bacterial host range of phage is generally narrower than that found in antibiotics. None of the nine Sussex isolates could be lysed by phage HER424, yet were sensitive *in vitro* to a variety of antimicrobials. Similarly there was evidence that each Canadian strain could be lysed by only one phage, if at all, whilst all strains were sensitive to a variety of antibiotics *in vitro* (Chapters 4, 5 & 6). The Pasteur material (Chapter 4) contained a large selection of viable isolates and phages, yet were not matched and none of the available phage preparations exhibited evidence of lysis against the Pasteur isolates. This highlights the issue of keeping appropriate records of matched hosts with the phage material, to ensure phage can be propagated.

Most phages lyse only a particular species of bacteria and many are only capable of infecting a small number of strains within a species (Merril et al., 2003; Kutter & Sulakvelidze, 2005). Herman & Juni (1974) and Bergogne-Berezin et al. (1996) recognised that most *Acinetobacter* phages are specific to only one host strain, on which they were isolated, reflecting the findings of this investigation.

The narrow host range may be looked at as an advantage in terms of the decreased harm exerted on the natural flora, compared to broad-spectrum antibiotics, which can give rise to diarrhoea or secondary infections like *C. difficile*. It was unfortunately also a determining reason in the decline of phage therapy in the West (Pirisi, 2000).

Due to the narrow host range of phage, it is essential to undertake phage susceptibility testing of clinical isolates before initiating therapy, to exclude automatic treatment failure. This may require the development and sharing of standardised testing

methodologies, similar to antibiotic sensitivity testing and may necessitate the involvement of UK agencies like BSAC.

In this investigation two different direct plate selection methods were used to ascertain suitable hosts for phage material. A multipoint inoculator was deemed unsuitable for phage screening, due to heavy bacterial growth at the point of inoculation, which inhibited plaque detection (Chapter 4); using multipoint pipettors however proved labour saving. An optimised surface spotting method meant swabbing washed cell suspension with a known cell concentration over agar plates, instead of spreading. This enhanced method gave rise to very even lawns and easier plaque detection, compared to established methods (Chapter 6).

The combination of two direct plate screening methods (surface spotting, soft agar spotting) was instrumental to predicting whether phage would lyse a prospective host-seven host and phage pairs gave rise to clearance zones, yet only four resulted in plaques (Chapter 6).

Luciferase reporter phages have been successfully used for drug susceptibility testing in *Mycobacterium tuberculosis* isolates (Riska et al., 1999; Hazbon et al., 2003). The principle of this method depends on phage harbouring the *fflux* reporter gene and being able to infect and express its genome in viable bacteria, with luciferase activity being detected only in the presence of cellular ATP, by means of a luminometer. This principle has proved suitable for semi-automatic assays (Banaiee et al., 2008).

Similarly phage typing could be conducted in an automated and labour saving fashion. Using phages coupled with luciferase, local laboratories could test phage susceptibility using 100-well plates with sterile pre-filled lytic phage preparations, with results being generated within minutes, rather than days, which is the usual timeframe for antibiotic susceptibility testing.

Mass spectrometry and DNA microarray technologies may potentially be used for phage typing (Merril et al., 2003).

The diversity of *Acinetobacter* strains in this investigation may compound the issue of narrow host range. Local isolates, which have caused significant clinical complications, did not just include *Acinetobacter baumannii/calcoaceticus* complex, but additionally

Acinetobacter lwoffii and *Acinetobacter calcoaceticus* var. *anitratus*. Isolates from other locations identified to at least species level also included *Acinetobacter haemolyticus* and *Acinetobacter junii/johnsonii*, which highlights the phenotypic and genetic diversity of strains on hand, which combined with a narrow phage host range may not be a promising outlook for phage therapy in *Acinetobacter spp.* infections. Human trials with phage therapy for cholera, organised by the World Health Organisation highlighted that one of the problems encountered was the diversity of serotypes of *Vibrios* (Summers, 2001). Changes in pH, additions of lysozymes to therapeutic preparation, attempts to isolate more virulent and clinically relevant phages during each cholera season (Pollitzer, 1955), use of phage cocktails and high titre preparations above 10^{12} pfu/ml (Monsur et al., 1970) were attempts to overcome this issue.

Narrow *Acinetobacter* host ranges and genetic diversity of clinical *Acinetobacter* isolates may be compensated for by developing a large collection of well-characterised, possibly promiscuous phages. Pooling of efforts between laboratories and national support with involvement of the NHS may be useful. Molecular techniques may be used to widen host ranges of specific phages (Merril, 2003), however it may be resource intensive. *E. coli* bacteriophages have been engineered to express two tail fibre proteins, allowing infection of two strains (Thiel, 2004). There is a possibility that this principle may be applicable to *Acinetobacter* phages. Traditionally narrow phage host ranges have been compensated for by combining several phages in one medicinal product (phage cocktails).

Suggestions to use phage as prophylaxis to decolonise (immunocompromised) patients or patients waiting for surgery (Pirisi, 2000), in place of a disinfectant or broad-spectrum antibiotic need to be critically evaluated. This idea is perpetuated in literature and may contribute to false expectations, which would ultimately lead to perceived treatment failure. *Acinetobacter* with varying serotypes and the narrow host spectrum of material currently available does not lend itself to empirical decolonisation, instead phage typing would need to be used to ensure suitably lytic phage material is used in a clinical scenario.

The peculiarities of phage will require a paradigm shift for clinicians for phage therapy to be successful. Phage typing must become an integral part in phage therapy protocols, a pre-condition to prescribing and administration. In clinical practice phage preparations may be treated like formulary restricted antibiotics and only supplied on Consultant Microbiologist recommendation.

8.6 Resistance to bacteriophage

Bacteria are capable of developing resistance to bacteriophages in a similar manner to antibiotics. This was observed soon after the discovery of the therapeutic potential of phages by d'Hérelle himself, and identified as a potential shortcoming of phage therapy (d'Hérelle, 1926; Summers, 1999). Bacteria employ different mechanisms of resistance to bacteriophage (Alisky et al., 1998).

There are few data on the frequency of spontaneous mutations of various phage types which may lead to phage-resistance, such as changes in surface receptor structures. It is estimated that mutation rates in bacteriophage Q β occur at 6.5 mutations per genome per replication (Drake et al., 1998). Kutter & Sulakvelidze (2005) suggested that frequencies of spontaneous bacterial mutations which may confer resistance to phage are thought to be “comparable to those seen for antibiotics”. The same authors postulated that resistance rates could be reduced by using phage cocktails and that bacterial resistance to phage has not been reported to be a clinical problem in human studies, however this may be due to use of phage cocktails. The issue of potential resistance of bacteria to phage highlights another problem namely that licensing authorities need to be flexible enough to allow for constant updating of phage preparations (Kutter & Sulakvelidze, 2004). Chanishvili et al. (2001), postulated that resistance was least likely to develop when the chances of spreading of host and phage to other hosts was avoided. They recommended keeping patients treated with phage in isolation and possibly even isolating burnt limbs on a specific patient.

One positive aspect about resistance mechanisms of bacteria to phage is that mutations that enable the bacteria to become resistant to antibiotics, do not lead to bacterial resistance to phage and vice versa (Carlton, cited by Pirisi, 2000). This confirms that bacterial strains resistant to antibiotics are ideal candidates for phage therapy.

D'Hérelle investigated whether bacteria develop resistance following exposure to phage and therefore if phages ultimately induce resistance (Summers, 2001). He concluded that bacteria adapted to the presence of phage (Summers, 2001) and hence resistance was a consequence of exposure to phage. Later experiments by Luria and Delbrück (1943) provided evidence that the presence of phage was not required for bacterial mutations leading to resistance to occur.

Two different types of basic phage resistance mechanism have been reported - one where spontaneous mutations occur, leading to modified phage receptors on the bacterium, so that phage tail fibres cannot attach to the host (Adams, 1959); Therefore- a given population of cells will contain some cells intrinsically resistant to a given phage (Pisman & Pechurkin, 2008). The second mechanism is resistance related to lysogeny, whereby the host utilises restriction endonucleases (Summers, 2001), this type of resistance to phage is of concern. Temperate phages may integrate their genome into the host chromosome and transfer genetic material encoding for toxins or help enhance bacterial pathogenesis (Merril et al., 2003). To avoid this occurrence phage genomes can be checked against GenBank online to ensure absence of unwanted genes in clinical phage preparations (Merril et al., 2003).

8.7 Phage cocktails

A combination of therapeutic phage strains in one medicinal product, referred to as phage cocktails (Sulakvelidze & Kutter, 2004) or polyvalency (Summers, 2001), may be a viable option to increase the chances of treatment success. This could cover for the eventuality of resistant mutants spontaneously arising during the course of phage therapy and may be of use in combating a narrow host range. Traditionally Georgian researchers always use phage cocktails (Personal communication, Dr. Nina Chanishvili, 18 February 2005; Thiel, 2004), containing up to 30 different phage strains, with hospital specific modifications in the mixture to allow for local resistance patterns. In 2004 (Thiel, 2004), the FDA were advocating use of single strain over polyvalency, but this view appears to have changed with the recent licensing of the use of phage cocktails against *Listeria* in food (U.S. Food and Drug Administration, 2006; Docket No. 2002F-0316).

8.8 Restriction and modification

Preparing phage stocks on laboratory strains and then administering them to a patient infected with different restriction-modification specificity could lead to phage DNA being degraded on entry into the pathogen. This phenomenon, described as host induced modification, is almost as if the host most recently used for phage replication affected its ability to infect other bacterial strains (Kutter & Sulakvelidze, 2005). This may lead to the false assumption that a specific phage may be unsuitable in a therapeutic scenario. To combat this problem, phages could be engineered to contain genomes without restriction sites recognized by a non-permissive host or incorporating genes into the phage genome which facilitate inhibition of bacterial modification or restriction enzymes (Merril, 2003). To avoid complex phage engineering it may be more practical to breed phage with the target strain before therapeutic use, which would modify phage DNA by methylation, thereby rendering it resistant to host restriction enzymes. D'Hérelle recommended therapeutic phage stocks to be grown using patient specific isolates, as this was a way for the phage to acquire the modification pattern of the isolate to be treated (Summers, 2001). There is a realistic risk that licensing authorities may be reluctant to support this tailor made approach; it may be for the sake of standardisation and total reproducibility that phage may have to be produced on a large scale using one strain only (Sulakvelidze, 2005). This lack of flexibility in updating therapeutic phage preparations by breeding with current clinical isolates may make some phage preparations less useful. Summers (2001) commented that historically investigators who prepared their own phage stocks appeared to be more [clinically] successful than investigators evaluating commercial products. This emphasises how very different phages are to antibiotics and that it requires a paradigm shift in thinking about treatment of bacterial infections if phage therapy is to be seriously undertaken in the UK. Researchers and health professionals would need to dedicate considerable resources on continuous education for stakeholders in phage therapy, particularly clinicians and licensing authorities, to ensure the limitations of phage are understood.

Producing large quantities of phage preparations and distributing them to hospitals in a „one-size-fits-all“ approach, may be convenient and cost effective, however it may be insufficient to ensure phage therapy is used for maximum patient benefit. It may be

necessary to prepare at least some phage stocks on an ad-hoc basis, by breeding patient specific phage preparations, as is done in Tbilisi. This may be labour and cost intensive (Monsur et al., 1970), yet may be the only viable method to ensure the success of phage therapy.

8.9 Phage behaviour *in-vitro* compared to *in-vivo*

Bacteria can alter gene expression in response to changing environments and in turn phage may interact with such changed bacterial clones differently than expected. Bacterial densities have an impact on phage kinetics (Payne & Jansen, 2001) and the presence of body fluids may also affect infectivity of phage (Merril, 2003).

8.10 Lytic nature of phages and transduction

Therapeutic phage must be lytic as the presence of temperate phages in therapeutic preparations may facilitate the transfer of harmful genes bacteria (Kutter & Sulakvelidze, 2005). It remains unclear whether a distinct separation exists in nature between lytic and temperate phages. Currently there are no specific methods available to differentiate temperate from virulent phages, however plaque morphology (Adams, 1959) may be an indicator. PCR-based protocols (Sander & Schmieger, 2001) which detect host cell DNA, have been used as indicators in predicting phage transduction in *Salmonella* phage. Kutter & Sulakvelidze (2005) suggested full sequencing of every phage used for agricultural or therapeutic purposes. Data deposition and sharing of phage sequencing in available databases could help to avoid therapeutic use of phage carrying genes associated with antibiotic resistance or bacterial virulence.

In this investigation one particular phage only formed plaques when very specific conditions had been met (Chapter 5). Phage AP205 (HER424) appeared to give rise to plaques only at or below 30°C. For successful plaque formation the infection temperature of host with phage was more important than the incubation temperature at which the cell suspension was originally grown. It was established that regardless of incubation temperature, phage attached to its host, but did not complete the lytic cycle at higher temperatures. Temperature dependent lytic behaviour with parallels was reported in *Listeria monocytogenes* epidemic clone II phages (Kim & Kathariou, 2009). Phages with a broad host range produced plaques with clone II at 37°C, but not at 30°C or

below. Regardless of bacterial incubation temperatures, similar to HER424, the *Listeria* phages attached to host bacteria.

The temperature dependency phenomenon made HER424 potentially unsuitable for therapeutic use, yet it may give more insight into the mechanisms involved in switching from a lytic to a lysogenic cycle.

One peculiarity of HER424 was a non-homogenous plaque size distribution; plaques propagated from small or large plaques gave in turn rise to plaques of heterogeneous size. This phenomenon has been previously described by Hava & Camilli (2001) in the temperature-sensitive generalised transducing phage of *Vibrio cholera* CP-T1ts. Similarly to HER424, plaque formation in CP-T1ts only occurred at 30°C and not at higher temperatures.

8.11 Lack of interest of the pharmaceutical industry in phage

The huge financial burden of bringing new chemical entities to the market has led the pharmaceutical industry to reappraise its position with regard to drug development. The tendency in recent years has been to focus their research effort on improving well-established structures (Taylor et al., 2002), rather than attempting to discover structurally novel compounds and this is particularly true in terms of antibacterial agents. Taylor et al. (2002) put forward the idea that it may be more in the interest of patients to develop selective antibacterial agents, specific for a small group of pathogens, rather than more broad-spectrum antibiotics. The authors claim that development of such specific agents (like phage) does not present attractive commercial returns and is unlikely to be of interest to the pharmaceutical industry.

A range of companies specialising in bacteriophage have appeared or become established in recent years, which demonstrates that there is growing commercial interest behind phage therapy. Companies currently undertaking research and development into the use of bacteriophages are all small to medium sized enterprises. **Tables 8.1 & 8.2** list publicly known companies commercially dealing with phage products.

Company Name and URL	Company Focus	Company Location
Intralytix, Inc. http://www.intralytix.com/	Food safety, environmental hygiene, veterinary and human phage therapy	Maryland, USA
Phage Therapeutics International (Phage Tx)	Supplier of Eliava phage for phage therapy centre in Mexico; research on MRSA, <i>P. aeruginosa</i>	Washington, USA
Exponential Biotherapies, Inc. http://www.expobio.com/	Vancomycin-resistant <i>Enterococcus faecium</i> , MRSA and <i>P. aeruginosa</i> ; phase II clinical trials	New York, USA
Biochimpharm http://www.biochimpharm.ge/indexeng.html	Phage cocktails: Pyobacteriophage („purulent“ infections), Intestibacteriophage (intestinal infections)	Tbilisi, Georgia
GangaGen, Inc. http://www.gangagen.com/home.asp	<i>S. aureus</i> & MRSA, <i>E.coli</i> O157:H7, <i>Salmonella</i> phage for veterinary use, <i>P.aeruginosa</i>	Bangalore, India; Ontario, Canada
Phage Biotech Ltd. http://www.phage-biotech.com/	<i>P.aeruginosa</i> , phage pharmaceuticals, „Immunopreparat Ufa“, keratitis, otitis media	Rehovot, Israel
Hexal gentech http://www.hexal-gentech.com/	Packaging of lethal molecules into bacteriophage heads, „Lethal agent delivery system“	Holzkirchen, Germany

Table 8.1 List of companies commercially dealing with phage products

Company Name and URL	Company Focus	Company Location
Biophage http://www.biophagepharma.net/index.html	<i>E.coli</i> , <i>Salmonella</i> ; „Listex“ control of <i>Listeria monocytogenes</i> in cheese, FDA approved.	Montreal, Canada
DD Pharma	Use of Eliava phage, phage typing	Melbourne, Australia
Biopharm Pharmaceuticals	Phage cocktails	Tbilisi, Georgia
EBI Food Safety http://www.foodprocessing-technology.com	<i>Listeria monocytogenes</i> - Listex P100 for food use	Wageningen, Netherlands
Novolytics	Wound care, disinfection, crop pathogens	Coventry, UK
Phage Biocontrol LLC http://www.phagebiocontrol.com/about.php	Prevention of pipeline corrosion and reservoir souring	Texas, USA
Biocontrol Ltd. http://www.biocontrol.ltd.uk/the-rapy.html	Ear infections, cystic fibrosis- <i>P. aeruginosa</i>	Southampton, UK
Omnilytics http://www.omnilytics.com/	Food & water safety, crop pathogens	Salt Lake City, USA

Table 8.2 List of companies commercially dealing with phage products

It is evident that there are major difficulties with the patenting of phage which are simply isolated from the environment. Investing in a product without full patent protection may not prove to be financially viable. For these reasons some companies are exploring other avenues, such as patenting specific phage sequences, pursuing novel concepts like using phage deficient in their lytic system (Matsuda et al., 2005), using genetically engineered phage as a vector for lethal genes (Westwater et al., 2003) or

molecules (Yacoby et al., 2006) or employing phage products such as cell hydrolases also known as lysins (Fischetti et al., 2006). As successful phage isolation depends on expertise and know-how, it is in the interest of investigators who have developed successful protocols for phage isolation and improving phage virulence for this expertise to remain in their own laboratories.

8.12 Regulatory agency approval

Phage-based products intended for clinical use are still not covered by existing regulatory guidelines; however a product for spraying onto ready-to eat meat and poultry products has been successfully given GRAS status by the US FDA in 2006. There has been much debate over whether regulatory agencies would regulate phage like a biotechnology product (Thiel, 2004). The question arose of whether phage should be considered a product of which proteins and polypeptides are components or a DNA product (Withington, 2001). This is not purely an academic debate within the phage community, as the outcome of this argument may make the difference between phage therapy becoming a successful mainstream treatment option or not. The crux lies with whether the emphasis should be placed on process controls or on characterising each single phage strain used for therapeutic purposes after the production process. For biological products more emphasis is placed on process controls than conventional medicines, as they are multi-component mixtures and nearly impossible to characterise solely by using analytical techniques.

With *Listeria monocytogenes* GRAS approval by the US FDA for use in food, it appears that phage was classified similarly to DNA products. Each one of the host strains used for phage production had to be classified and deposited in the ATCC, structural protein profiles of each phage, DNA sequences of complete genomes and phage titres had to be submitted. It is very likely that the same approach would be used for phage products intended for therapeutic administration in humans.

One particular issue is that phage therapy may be considered inherently financially unattractive. Phages have an even narrower spectrum than antibiotics and infections are generally acute illnesses which do not guarantee a regular sale of a specific pharmaceutical product, profit margins may be low. Future research efforts may be

focussed on finding phage products aimed at providing financial returns, rather than combating infectious disease.

The approach taken by the US FDA does not allow for regular updating of host strains, with phages having a narrower spectrum than antibiotics, therapeutic phage products may be rendered unsuitable faster than any antibiotic. Kutter & Sulakvelidze (2005) pointed out that updating with new strains is not unprecedented in the West and suggested therapeutic phage could be treated similarly to influenza-vaccines in terms of regulation. The authors report that in the former Soviet Union only the „principal“ phage preparation and manufacturing and quality control protocols needed official approval, a similarly flexible approach in the West would foster the success of phage therapy. The advantage of allowing licensing of therapeutic phage products as a biotechnology product would be that it would allow continuous changes of phage strains in response to evolution of dominant antibiotic-resistant bacterial strains and allow phage preparations to have maximum clinical effectiveness.

8.13 Intellectual property

Bacteriophages have been used as therapeutic agents since the beginning of the last century, which renders the concept of phage therapy unpatentable (Thiel, 2004). Marginal protection could be secured if an individual strain was characterised, however if another strain was isolated which was equally or more effective all efforts would have been in vain (Thiel, 2004). With phage being freely available in abundance in the environment, this scenario combined with the risk of the target bacteria developing resistance could easily discourage investors. Some researchers have been circumventing the issue of IP by securing patents on modified phage products (Hanlon, 2005; 2007). Formulation of delivery systems may offer another avenue for IP protection, like dressings, skin graft material or sutures impregnated with phage.

8.14 Safety of phage- safety of phage preparations

Several authors emphasise that phages are inherently safe, using the argument that phages are abundant in the environment (Kutter & Sulakvelidze, 2005).

Barrow & Soothill (citing Ochs, 1971) pointed out that phages have been used to test antibody function in immunodeficient patients, thus implying that phages are non-toxic.

However an absence of any published cytotoxicity data regarding bacteriophages is surprising in this context and any assumptions as to whether phage may be safe or not should be challenged and verified by scientific experiments. Du Bow summed this up concisely: “What we think we know about phages has to be verified and then deemed reproducible, safe and effective” (DuBow cited by Pirisi, 2000). Some vaccines were found to have been contaminated with phage in the 1970s and after the issue of an Executive Order the continued use of contaminated vaccines was permitted (Merril, 1972, 1975). The US FDA (U.S. Food and Drug Administration, 2006; Docket No. 2002F-031) has designated a mixture of *Listeria monocytogenes* phages for use in foods as GRAS (Generally Recognized as Safe), another argument to support the safety of phage (Garcia et al., 2009); however phage therapy may entail single or repeated use of concentrated phage preparations with high titres in a patient, administered by a variety of routes, which is quite different to ingesting minute quantities contained in food products.

Broudy and Fischetti (2003) described phage-associated conversion of Tox^- *Streptococcus pyogenes* into Tox^+ bacteria in vivo. Other phage-associated toxins like botulinum, shiga and diphtheria toxins may be inducible by phage, Skurnik & Strauch (2006) see this as a potential warning of the possible side effects of clinical phage use.

It is important to differentiate between the safety of a specific phage and the safety of a particular phage preparation. Several authors report cases of insufficiently purified phage preparations and attribute treatment failures to this problem (Pirisi, 2000; Merrill, 2003; Kutter & Sulakvelidze, 2005). In a particular case treatment of infections with *Y. pestis* in 1932 with phage lead to a significantly higher mortality rate than in control groups, both in mice and 33 human patients, all of whom died (Merril, 2003). Treatment failure was attributed to failure to remove endotoxins from phage preparations (Pirisi, 2000).

8.15 Cytotoxicity- Nanotoxicity

It is surprising that there appear to be no previously conducted cytotoxicity experiments for phage. The reasons may be that phages are assumed to be „clinically safe“, due to a lack of reports of adverse effects during human and animal experimentation and possibly decades of reported human phage therapy mainly in Georgia, Russia and Poland. A lack of *in vitro* cytotoxicity studies also includes lack of data on phage cytotoxicity in wound models. It needs to be borne in mind that therapeutic phage would be locally applied in very high concentrations, to cells which are involved in a very complex immune system involving cytokines and other biochemical messenger systems; any minor interference by phage preparations in the wound healing and graft-take processes could affect clinical outcomes dramatically.

A range of cytotoxicity assays, utilising immortalised cell lines and human dermal fibroblasts and keratinocytes isolated from skin samples, showed no statistically significant cytotoxic effect of phage (Chapter 7).

Cell proliferation and cytotoxicity assays (3T3 cells) indicated there was some evidence that lambda buffer and remnants of the phage breeding and purification process were more detrimental to cell survival than phage itself. This highlights an information vacuum in literature, regarding optimum phage carriers and purification protocols for clinical use. The issue of phage preparations containing bacterial products is not new (Merril, 2003), has had clinical implications and may have contributed to phage therapy failure in the past. This thesis has shown that lambda buffer, which is frequently used as a vehicle for phages, may itself exert adverse effects on mammalian cells. A change of phage vehicle raises the issue of phage titre stability in any other system. An absence of phage preparation stability data in the literature is evident and pharmaceutical formulation experience of therapeutic phage preparations is not currently in the public domain.

Phages are structures within the 1-100nm size range and could be seen as nanoparticles. It may be useful to study phages in a similar way as nanoparticles, investigating surface charge, aggregation (Panessa-Warren et al., 2006) and nanotoxicity. The possibility of migration and incorporation of phages into mammalian cells, as has been seen with similarly sized nanoparticles (Jiang et al., 2008) needs to be considered and studied.

8.16 Cytokine release

Little has been published so far on the effect phage may have on cytokine release in graft- or wound models. Supplementation of graft material with certain interleukins has led to enhanced antibacterial properties, if phage was to have an effect on interleukin release, this may supplement its own antibacterial properties.

The only published studies concerned with cytokine release in connection with phage *in vitro* are by Weber-Dabrowska et al., (2002). The human model investigated spontaneous and lipopolysaccharide induced TNF- α and IL-6 production in whole blood cell cultures of patients treated with phage for suppurative infections. Lipopolysaccharides did not appear to have a significant effect on IL-6 release. Spontaneous IL-6 release in blood cell cultures from patients treated by phage progressively normalised, initial low IL-6 production was increased significantly and initially high production decreased over a period of 21 days. TNF- α readings in the same experimental setup also normalised (high initial responses decreased and low levels increased), regardless of LPS exposure.

In vitro results (Chapter 7) in human dermal fibroblasts exposed to purified and crude phage lysates and endotoxin containing samples showed that IL-6 and IL-8 release of purified phage dilution 1 (2×10^9 pfu/ml) was significantly higher than all other preparations (67pg/ml). The IL-6 output of HDF treated with phage dilutions 2 & 3 at lower phage titres (2×10^8 & 2×10^7 pfu/ml) were statistically not different from controls. IL-1 β and TNF- α levels were found to be below detectable limits (Chapter 7).

8.17 Immunogenicity

It is claimed in the literature that intravenously administered phages may evoke a substantial immune response (Merril et al., 2003; Taylor et al., 2003). Merrill et al., (2003) stated that repeated exposure to the same phage strain would activate the adaptive immune system and result in antibody production. It is unknown whether purified phage can elicit allergic reactions, though immune responses eliminate phage (Barrow & Soothill, 1997).

8.18 Ethics

To date no debate about the ethics of phage therapy has been published. Attention has been focussed on presenting positive evidence of human and animal case studies and possible reasons why phage therapy has failed and may do so in the future. With court cases and public pressure forcing licensing authorities and governments to enable fast-track licensing schemes for drugs treating HIV, MS and cancer, it is interesting why a similar scenario has not been seen regarding phage therapy. A major reason is the absence of good, demonstrable evidence in the form of clinical trials.

There are no UK statistics reflecting the mortality rate of bacterial infections in communal establishments other than MRSA and *C. difficile* (UK Office for National Statistics Online; 2.June, 2008). 26,099 and 11,481 people had *C. difficile* or MRSA respectively listed as cause of death on their death certificates in the years 2001-2006 in England and Wales. There is a lack of national statistics available representing morbidity and mortality associated with bacterial strains untreatable with antibiotics. Such data would highlight the reality of casualties due to an absence of alternatives to the current antibiotics available. Governments may be understandably reluctant to collate and publish such figures; however pressure groups of patients may force decision makers to invest in alternatives.

The Declaration of Helsinki states: “In the treatment of a patient, where proven prophylactic, diagnostic and therapeutic methods do not exist or have been ineffective, the physician, with informed consent from the patient, must be free to use unproven or new prophylactic, diagnostic and therapeutic measures, if in the physician's judgement it offers hope of saving life, re-establishing health or alleviating suffering. Where possible, these measures should be made the object of research, designed to evaluate their safety and efficacy. In all cases, new information should be recorded and, where appropriate, published. The other relevant guidelines of this Declaration should be followed. “ (<http://www.wma.net/e/policy/b3.htm>; 2.July 2008).

With reported treatment success when phage therapy is used as a last resort, in infections non-responsive to antibiotics (Weber-Dabrowska, 2000), the question arises

whether it can actually be considered ethical to continue not pursuing phage therapy in the UK.

As the NHS is publicly funded, one way for phage therapy to gain a foothold in the UK would be to secure NHS funding and work within the structure of the NHS, possibly using unlicensed phage preparations on a named-patient basis under the emergency clause. In future the potentially strongest driving force behind phage therapy may become the public, provided phage therapy is appropriately lobbied. This may be difficult as individual researchers may not have the resources to start PR campaigns and patient groups may be unaware of the potential of phage therapy.

For chronic infections a form of health tourism may emerge, where patients with „untreatable“ infections travel to Phage Therapy centres in Georgia or Mexico (<http://www.phageinternational.com/>; visited 2 July 2008).

Self-referral to phage therapy clinics can be conveniently done on the internet, with choice of clinic on a sophisticated website, complete with wire-transfer options (http://www.phagetherapycenter.com/pii/PatientServlet?command=static_home, visited 2 July 2008).

8.19 Paradigm shifts

Phages are biologically active particles, genetically changeable, with very narrow activity spectra and display entirely different pharmacokinetics to antibiotics. As with any paradigm shift, the „old“ antibiotic paradigm, the concept of using chemicals to treat bacterial infections, is needed to explore the new paradigm- that of phage therapy. Yet, although the outcome –the killing of bacteria- is similar, the means by which this is achieved is entirely different. The kinetics of phage therapy are very different from antibiotic pharmacokinetics (Payne & Jansen, 2003) and these differences have a dramatic impact on the use of phage, compared to antimicrobials (Skurnik & Strauch, 2006).

There is a serious risk that if these differences are not reiterated to healthcare professionals, peers and particularly licensing authorities, and accounted for when attempting phage therapy, the nature and potential of phage may be misunderstood and

phage therapy may fail a second time in the West. In practice this may be overcome by treating therapeutic phage preparations in the same way as a restricted antibiotic, by dispensing to individual patients only on specialist Consultant Microbiologist advice, after rigorous testing of suitability.

8.20 Absence of rigorous proof of efficacy

Despite the overwhelming number of reports that phage has been used successfully for a multitude of infections, there remains an urgent need for double blind, placebo-controlled studies. Factors discussed previously, particularly narrow host ranges, hurdles involving licensing issues and lack of patentability of most phages are most likely to discourage investors. Clinical trials are cost intensive, require years of resources and qualified support but without them phage therapy has little chance of success.

8.21 Conclusion

Phage therapy has undoubtedly huge potential, whether it will be fulfilled will depend on a variety of factors. The multi-national pharmaceutical companies are unlikely to invest in phages which cannot be patented, have a very narrow spectrum and promise little financial return. It is most likely that the pioneers in phage therapy will remain in the ranks of academia and small spin-off companies, which may mean lengthy delays for phage therapy to ever reach mainstream therapeutics.

Acinetobacter phage therapy may be seriously hampered by the absence of available material, which may be remedied by international collaborations, the building of national phage libraries and using phage cocktails. Licensing authorities would need to be convinced of the benefits of regularly updating phage mixtures or alternatively phage may be used in unlicensed form on a named-patient basis in collaboration with physicians.

Successful phage therapy is likely to require a certain minimum infrastructure, which private companies or academia alone may not easily provide. Bacterial isolates would need to be isolated from the patient, and rapidly identified and screened against available phage strains, to ensure maximum clinical efficacy. On the basis of these

results the phage cocktail may then be mixed and formulated into a medicinal product. Within a large hospital environment, particularly with close ties to academia, this infrastructure, which may require effective cooperation between clinicians, biomedical scientists, microbiologists, pharmacists and other healthcare professionals, already exists.

Phage preparations may need to be treated like a restricted antibiotic, with Consultant Microbiologist involvement. Adequate phage prescribing and administration may need to be monitored by microbiologists and pharmacists, and excellent communication within the healthcare team would be essential.

Not pursuing the avenue of phage therapy may become ethically unacceptable. It appears that pharmaceutical companies are unlikely to invest in phage therapy for various reasons. It will need to become unacceptable in the public eye for patients to die of infectious diseases which may have been curable with phage.

8.22 Suggestions for future work

The development of selective agar based medium which permits growth of 90% of *Acinetobacter* strains and successfully inhibits background contamination may prove a useful tool in phage isolation and in increasing the *Acinetobacter* spp. collection. Additionally the development of a selective liquid medium for clinical *Acinetobacter* strains, which suppresses background contamination and at the same time supports growth and indicates metabolic activity of *Acinetobacter*, which is suitable for phage enrichment is called for.

Clarification on the taxonomic status of *Acinetobacter* spp. in general, and officially published, detailed correlation between taxonomic identification on species level and phenotypic properties would aid comparison and sharing of material.

Future work may involve the development of an *Acinetobacter* phage isolation protocol, which can be easily followed without requiring specialist know-how, followed by isolation of a multitude of lytic phages. The collection of a library of lytic, characterised purified *Acinetobacter* phage lysates would be a prerequisite to further attempts to consider *Acinetobacter* phages as future therapeutic agents.

Future experiments may include breeding and diluting phage in cell-neutral diluents, which do not affect phage viability or change phage properties. Investigation of different purification and concentration methods using high speed centrifugation could foster the production of safer, endotoxin-free products. Stability studies on liquid purified phage preparations may prove useful for future licensing of phage preparations. Experimentation involving freeze drying of phage as a means of long term storage to avoid fungal or bacterial spoilage of liquid phage preparations and to aid removal of unwanted chemical components by evaporation may be valuable.

Future cytotoxicity experiments may include primary dermal fibroblasts being compared to a dermal fibroblast immortalised cell line. Emphasis should be placed on using samples from similar donor sites, use donors of various ages, and trialling cells with a range of cumulative population doubling times. Longitudinal experiments over many population doublings, exploring the effect of a variety of phage preparations in various (cell-neutral) diluents on fibroblasts grown on larger surfaces areas, may require large resources, yet may give useful insights.

Foetal as well as adult human dermal fibroblasts from donors of varying ages and a variety of donor sites would be useful in a range of similar experiments. Different biochemical parameters such as IL-10 or IL-1 α may be used to shed further light on the interaction between phage and mammalian cells. Exploring changes in cellular appearance and biochemical parameters in the presence of phage over several population doublings with a variety of cell seeding densities and phage concentrations may be of interest.

Additional experiments may include the trialling of non-feeder layer keratinocyte systems, whilst ensuring non-interference of experimental conditions with cytotoxicity assays. Longitudinal studies with keratinocyte sheets in growth flasks exposed to phage may be useful. Studies of potential cell morphology and cell differentiation in response to phage and bacterial compounds may be warranted. Exposure of keratinocytes and composite grafts to bacteria and phage cocktails characterizing interactions between mammalian, bacterial cells and lytic phage. Mammalian cell survival, bacterial and viral proliferation, histological changes and interleukin release could be investigated in this system or skin explants may be used as alternatives.

Interleukin releases of keratinocytes exposed to phage preparations may be studied. Future experiments may aid investigating the effects of phage and bacterial products on keratinocyte cytokine release. The effects of phage on a more complex creation - a bilayered organotypic assay where an epidermis layer, separated by a filter may trigger the autocrine release of cytokine(s) from a dermis component-may be studied.

In-vivo experiments may include incorporation of purified, endotoxin-free phage preparations in wound cover or closure material including cellular graft material in various forms. Local and systemic interleukin release, graft take, overall healing rates and differences in cosmetic appearance may be studied.

Appendix 1

Equations

Counting of mammalian cells with Trypan Blue stain

Calculation of Live cells/ml

$$\text{Live cells/ml} = (\text{Count live cells}/5) \times 10^4 \times 1.2$$

$$1.2 \text{ dilution factor} = 20\mu\text{l haemocytometer volume} / (20\mu\text{l of cell volume} + 4\mu\text{l dye})$$

Calculation of percentage dead cells

$$\text{Dead cells (\%)} = 100 / \text{Total number of cells} \times \text{count dead cells}$$

Total cell numbers

$$\text{Total number of cells} = \text{sum of count live} + \text{count dead cells}$$

Mean cell to phage ratio for each plate

$$(\text{Mean live cells/ml}) / (\text{Mean phage pfu/well})$$

Calculation of doubling times

Definition of Population doubling: The interval required by a cell population to double in density

Population doubling calculations:

$$N_H/N_L = 2^x$$

$$\text{Hence } \log(N_H) - \log(N_L) = X \log 2$$

X= number of populations

N_L cells seeded – Total number of cells per flask or well

N_H cells harvested- Total number of cells/ml

Calculation of endotoxin levels of various cell extracts

$$y\text{-intercept} = \Sigma y/N - (\Sigma x/N \times \text{slope})$$

$$\text{Endotoxin concentration} = [\Delta \text{Absorbance} - (y\text{-intercept})] / \text{slope}$$

x = Endotoxin concentration in EU/ml

y = Mean Δ Absorbance Value

N = Number of standards used

Appendix 1

Σx = Summation of concentration of standards used in EU/ml

Σy = Summation of Mean Δ Absorbance values

Σxy = Summation of the standard concentration times Mean Δ Absorbance values

S_x = Standard deviation of x

S_y = Standard deviation of y

$$r = \frac{N\Sigma xy - (\Sigma x)(\Sigma y)}{N(N - 1)S_x S_y}$$

$$\text{slope} = \left(\frac{S_y}{S_x}\right)r$$

$$S_x = \sqrt{\frac{N\Sigma x^2 - (\Sigma x)^2}{N(N - 1)}}$$

$$S_y = \sqrt{\frac{N\Sigma y^2 - (\Sigma y)^2}{N(N - 1)}}$$

Colony formation assay using V79 cells

The effect of the extract applied to the cells was calculated using the following formula:

Effect of extract dilution= (Average colony count at a given dilution/Average colony count Control wells) x100

Appendix 2

Personal Communications

Personal communication Van den Worm, co-author of Klovins et al. (2002)-9 January 2003.

Jan is sending you some lysate and *Acinetobacter* sp186 today.

Acinetobacter grows on rich medium at 28 deg C. For infection with AP205, a culture should be started in rich medium with 100 mM CaCl₂. This is necessary for phage attachment later on. When the culture has reached OD₆₅₀ ~0.3, stop shaking and add phage at a multiplicity of 5-10. I don't know the titer of the lysate I sent you (especially not after mailing), but I advise you to add 50 ul lysate to 20 ml of culture. Incubate (still without shaking) for 15 minutes to allow attachment and continue growing at 28 deg C. Stop incubating after about 3 hours. Usually, the culture does not lyse, but still a lot of phage is present.

It might help if you grow the cells first on minimal medium (M9 from Maniatis supplied with trace elements) to stimulate pili formation. It helps with *E. coli*. The medium we use is called LC and contains per liter 10 g Bactotrypton (Difco), 8 g NaCl, 5 g yeast extract, 1 g MgSO₄*7 H₂O, 20 mg thymine and 1 ml 1 M Tris*Cl pH 7.9. For a titer determination I never add CaCl₂, only for the actual infection.

Personal communication Van den Worm, co-author of Klovins et al. (2002) –14 February 2003

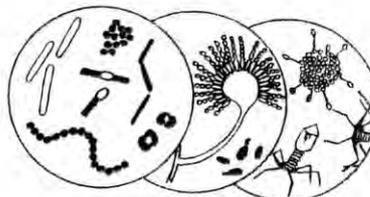
We tried to determine the function of the first ORF and assume it codes for a lysis protein. As you might have read, the protein stops growth in *E. coli* but actual lysis was never shown. I also tried to determine pfu/ml when I was purifying the phage, but never got plaques. I didn't bother too much since it was more important for me to have phage (for EM) than how much it was.

**Dr. J. A. Child, Consultant Microbiologist at the Surrey and Sussex
NHS Trust, December 2000**

Sensitivity data and isolation history for 'Sussex' strains

SURREY & SUSSEX NHS TRUST
Crawley Hospital
West Green Drive
Crawley
West Sussex
RH10 7DH

☎ 01293 600300 ext 3093 (secretary3090)
Fax 01293 600404
e-mail: ja.child@doctors.org.uk



Department of Microbiology
Dr J.A.Child, MBBS MRCPATH
Consultant Microbiologist

Dr Geoff Hanlon,
Reader in Pharmaceutical Microbiology
School of Pharmacy & Biomolecular Sciences
University of Brighton
Moulscumb
Brighton BN2 4JG

6th December 2000

Dear Geoff,

Here is a selection from our collection of clinical isolates of *Acinetobacters*. I have picked a few multiply-resistant isolates, of which three represent the outbreak strain at the Queen Victoria Hospital. Two other multiply-resistant isolates are definitely of another strain altogether. One of these represents an outbreak strain at another Sussex Hospital.

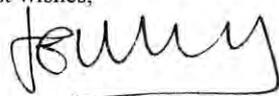
They can be almost an incidental finding, even in blood-cultures, where they can be contaminants... most of the non-outbreak strains I've sent you (the more sensitive ones) fell into this category. The outbreak strains are more virulent, certainly survive longer and are the cause of bacteraemia and ventilator-associated pneumonia in ITU patients. At both the QVH and Crawley, we've had some pretty severe soft-tissue infections with them too, but this is less common.

If they are not viable or are not as I have said they should be, please let me know!

None have been tested for susceptibility to antiseptics or disinfectants. Of the former, Triclosan would be interesting to look at. Not only is this used in the "Bacteroban" range of everything from washing-up liquid to chopping boards, but is used extensively in hospitals as part of the MRSA eradication protocol. There is a lot splashed around at the QVH, home of our delightful outbreak strain, for this reason. The other commonly used disinfectants used are hypochlorite and ethanol or isopropanol. Chlorhexidine and betadine are used as hand-scubs and pre-operative skin-preps.

With best wishes,

Jenny



Additional Sensitivity data

		MIC (mg/L)															
		amik	amp	cip	ctx	caz	gent	imi	mero	pip	Pip/taz	min	Amp/sul	net	azith	co-trim	col
W5211	8		>256	2	>256	>256	32	4	4	>256	>256	2	8	4	4	2	1
R45502	>128																1

amik-amikacin; gent-gentamicin, am-ampicillin; amp/sul-ampicillin/sulbactam, pip/ piperacillin pip/taz-piperacillin-tazobactam; ctx-cefotaxime
 caz-ceftazidime; imi-imipenem; mero-meropenem; co-trim-co-trimoxazole; azith-azithromycin, net-netilmicin. Min-minocycline

- ampicillin/sulbactam : NCCLS breakpoint for this combination is 8 mg/L. sulbactam is known to have anti-acinetobacter activity in its own right, although the mechanism is not clear. This is *not* β -lactamase inhibition -see results for pip & pip/taz ; tazobactam and sulbactam are both inhibitors of the same β -lactamase class.

R45502 is one of the QVH outbreak strains, but differs from the others in that it has high-level resistance to amikacin. The amikacin MIC for the others was typically 8-12 mg/L.

Acinetobacter strains from Dr Jenny Child

- W6492 *A. baumannii*, blood-culture isolate, medical patient
- R45502 multiply-resistant isolate from a patient with a severe burn. Became amikacin resistant. QVH outbreak strain. *A. baumannii*- complex
- R1973 multiply-resistant isolate from a patient with a severe burn. Became amikacin resistant. QVH outbreak strain. *A. baumannii*- complex
- R46383 isolate from burns patient. NOT an endemic strain.
- R3824 multiply-resistant isolate from surgical wound infection of a patient in a general ITU. *Not* QVH outbreak strain
- R3417 multiply-resistant isolate representative of an outbreak strain at another Sussex Hospital. *Not* QVH outbreak strain
- R2751 multiply-resistant isolate, QVH outbreak strain. From wound of patient on surgical ward. Treated with IV colistin.
- W5211 multiply-resistant isolate, QVH outbreak strain. From blood-culture of patient with 80% burns. Later died.
- W6108 blood-culture isolate from patient on a general medical ward
- R4474 QVH outbreak strain. Blood-culture isolate from patient with severe burn, who later died, acinetobacter infection was a contributory factor.

These should all grow very easily at 37°C in either air or 5% CO₂ on blood agar, CLED or MacConkey.

Antibiotic-sensitivity testing should be carried out on a recognised medium such as IsoSensitest agar or Mueller-Hinton agar, in air at 37°C.

Personal communication Jenny Andrews, BSAC-5 March 2003

McFarland communication

To: Jenny Andrews at BSAC, Jenny.andrews@swbh.nhs.uk

Putting in McFarland in the search window does not seem to produce results. Could you please let me know where I can find the optical densities of McFarland standards (table form). Need to know 0.5 McFarland especially.

Reply from: Jenny Andrews at BSAC

Your email has been passed to me from BSAC HQ.

The only current recommendations are from NCCLS, but they are incorrect. We are currently doing some work to establish absorbance ranges for the 0.5 McFarland standard.

REFERENCES

ABEDON, S. T., HYMAN, P. & THOMAS, C. (2003) Experimental examination of bacteriophage latent-period evolution as a response to bacterial availability. *Applied and Environmental Microbiology*, 69, 7499-7506.

ABEDON S. T. (ED.) (2008) Bacteriophage Ecology: Population Growth, Evolution, and Impact of Bacterial Viruses (Advances in Molecular and Cellular Microbiology), Cambridge University Press.

ABUL-HASSAN H.S., EL-TAHAN K MASSOUD B. & GOMAA R (1990) Bacteriophage therapy of *Pseudomonas* burn wound sepsis. *Annals of the Mediterranean Burns Club*, 3, 262-265.

ACKERMAN.H-W. & TURCOTTE, J. (1970) Properties of a phage of *Bacterium-anitratum* (b5w). *Revue Canadienne De Biologie*, 29, 317-&.

ACKERMANN, H. W., BROCHU, G. & KONJIN, H. P. E. (1994) Classification of *Acinetobacter* phages. *Archives of Virology*, 135, 345-354.

ADAMS, M. H. (1959) Bacteriophages, Interscience Publisher.

AHMAD, S. I. (2002) Treatment of post-burns bacterial infections by bacteriophages, specifically ubiquitous *Pseudomonas* spp. notoriously resistant to antibiotics. *Medical Hypotheses*, 58, 327-331.

ALI, S. N., SOUEID, A., RAO, K. & MOIEMEN, N. (2006) Self-inflicted burns, outcome and cost. *Burns*, 32, 463-466.

- ALISKY, J., ICZKOWSKI, K., RAPOPORT, A. & TROITSKY, N. (1998) Bacteriophages show promise as antimicrobial agents. *Journal of Infection*, 36, 5-15.
- ALLGOWER, M. (1995) Burning the largest immune organ - Prologue. *Burns*, 21, S5-S6.
- AMERICAN TYPE CULTURE COLLECTION LGC Standards, visited 13 May 2008, <http://www.lgcstandards-atcc.org/>.
- ANDERSON, E. S., WARD, L. R., DESAXE, M. J. & DESA, J. D. H. (1977) Bacteriophage-typing designations of *Salmonella typhimurium*. *Journal of Hygiene*, 78, 297-300.
- ANDREWS, J. M. (2001) The development of the BSAC standardized method of disc diffusion testing. *Journal of Antimicrobial Chemotherapy*, 48, 29-42.
- ANDREWS, J. M. (2004) Disc Diffusion Method for Antimicrobial Susceptibility Testing, The British Society for Antimicrobial Chemotherapy, Version 3.1.
- ANDREWS, J. M. (2005) Disc Diffusion Method for Antimicrobial Susceptibility Testing, The British Society for Antimicrobial Chemotherapy, Version 4.
- ANDREWS, J. M. (2006) Disc Diffusion Method for Antimicrobial Susceptibility Testing, The British Society for Antimicrobial Chemotherapy, Version 5.
- ANDREWS, J. M. (2008) Disc Diffusion Method for Antimicrobial Susceptibility Testing, The British Society for Antimicrobial Chemotherapy, Version 7.
- ANON (1997) API 20NE identification manual, bioMérieux, Marcy l'Etoile, France, 6th Ed.
- ANON (2006) Medicines Compendium, Datapharm Publications Ltd.
- ANSTEY, N. M., CURRIE, B. J. & WITHNALL, K. M. (1992) Community-acquired *Acinetobacter* pneumonia in the northern territory of Australia. *Clinical Infectious Diseases*, 14, 83-91.

- ARLIAN, L. G., MORGAN, M. S. & NEAL, J. S. (2003) Modulation of cytokine expression in human keratinocytes and fibroblasts by extracts of scabies mites. *American Journal of Tropical Medicine and Hygiene*, 69, 652-656.
- ASHWORTH, H. L., CUBISON, T. C. S., GILBERT, P. M. & SIM, K. M. (2001) Treatment before transfer: the patient with burns. *Emergency Medicine Journal*, 18, 349-351.
- ATIYEH, B. S., HAYEK, S. N. & GUNN, S. W. (2005) New technologies for burn wound closure and healing - Review of the literature. *Burns*, 31, 944-956.
- AYERS, D. E. & KAY, A. R. (2005) Management of burns in the wilderness. *Travel Med Infect Dis*, 3, 239-48.
- BALDWIN, A. S. (2001) The transcription factor NF-kappa B and human disease. *Journal of Clinical Investigation*, 107, 3-6.
- BALIN, A. K., FISHER, A. J., ANZELONE, M., LEONG, I. & ALLEN, R. G. (2002) Effects of establishing cell cultures and cell culture conditions on the proliferative life span of human fibroblasts isolated from different tissues and donors of different ages. *Experimental Cell Research*, 274, 275-287.
- BANAIEE, N., JANUARY, V., BARTHUS, C., LAMBRICK, M., RODITI, D., BEHR, M. A., JACOBS, W. R. & STEYN, L. M. (2008) Evaluation of a semi-automated reporter phage assay for susceptibility testing *Mycobacterium tuberculosis* isolates in South Africa. *Tuberculosis*, 88, 64-68.
- BARNES, D. J., NARAQI, S. & IGO, J. D. (1988) Community-acquired *Acinetobacter* pneumonia in adults in papua-new-guinea. *Reviews of Infectious Diseases*, 10, 636-639.

References

- BARON, C., DOMKE, N., BEINHOFER, M. & HAPFELMEIER, S. (2001) Elevated temperature differentially affects virulence, VirB protein accumulation, and T-pilus formation in different *Agrobacterium tumefaciens* and *Agrobacterium vitis* strains. *Journal of Bacteriology*, 183, 6852-6861.
- BARROW, P., LOVELL, M. & BERCHIERI, A. (1998) Use of lytic bacteriophage for control of experimental *Escherichia coli* septicemia and meningitis in chickens and calves. *Clinical and Diagnostic Laboratory Immunology*, 5, 294-298.
- BARROW, P. A. (2001) The use of bacteriophages for treatment and prevention of bacterial disease in animals and animal models of human infection. *Journal of Chemical Technology and Biotechnology*, 76, 677-682.
- BARROW, P. A. & SOOTHILL, J. S. (1997) Bacteriophage therapy and prophylaxis: Rediscovery and renewed assessment of potential. *Trends in Microbiology*, 5, 268-271.
- BAUMANN, P., DOUDOROF, M. & STANIER, R. Y. (1968) Study of *Moraxella* group 1, genus *Moraxella* and *Neisseria catarrhalis* group. *Journal of Bacteriology*, 95, 58-&.
- BAUMANN, P., DOUDOROF, M. & STANIER, R. Y. (1968) A study of *Moraxella* group .2. oxidative-negative species (genus *Acinetobacter*). *Journal of Bacteriology*, 95, 1520-&.
- BENHAR, I. (2001) Biotechnological applications of phage and cell display. *Biotechnology Advances*, 19, 1-33.
- BERCHIERI, A., LOVELL, M. A. & BARROW, P. A. (1991) The activity in the chicken alimentary-tract of bacteriophages lytic for *Salmonella typhimurium*. *Research in Microbiology*, 142, 541-549.
- BERGERON, M. G. & OUELLETTE, M. (1998) Preventing antibiotic resistance using rapid DNA-based diagnostic tests. *Infection Control and Hospital Epidemiology*, 19, 560-564.

- BERGH, O., BORSHEIM, K. Y., BRATBAK, G. & HELDAL, M. (1989) High abundance of viruses found in aquatic environments. *Nature*, 340, 467-468.
- BERGHMAN, L. R., ABI-GHANEM, D., WAGHELA, S. D. & RICKE, S. C. (2005) Antibodies: An alternative for antibiotics? *Poultry Science*, 84, 660-666.
- BERGOGNE-BEREZIN, E. & JOLY-GUILLOU, M. L. (1985) An underestimated nosocomial pathogen, *Acinetobacter calcoaceticus*. *Journal of Antimicrobial Chemotherapy*, 16, 535-537.
- BERGOGNE-BEREZIN, E., JOLY-GUILLOU, M.-L. & TOWNER, K. J. (1995) *Acinetobacter*: Microbiology, Epidemiology, Infections, Management, CRC Press.
- BERGOGNE-BEREZIN, E. & TOWNER, K. J. (1996) *Acinetobacter* spp, as nosocomial pathogens: Microbiological, clinical, and epidemiological features. *Clinical Microbiology Reviews*, 9, 148-&.
- BERLAU, J., AUCKEN, H. M., HOUANG, E. & PITT, T. L. (1999) Isolation of *Acinetobacter* spp including *A. baumannii* from vegetables: implications for hospital-acquired infections. *Journal of Hospital Infection*, 42, 201-204.
- BIOCHIMPHARM visited 20 February 2009, <http://www.biochimpharm.ge/indexeng.html>.
- BIOCONTROL LTD. visited 11 February 2009, <http://www.biocontrol-ltd.com/>.
- BIOPHAGE visited 20 February 2009, <http://www.biophagepharma.net/index.html>.
- BISWAS, B., ADHYA, S., WASHART, P., PAUL, B., TROSTEL, A. N., POWELL, B., CARLTON, R. & MERRIL, C. R. (2002) Bacteriophage therapy rescues mice bacteremic from a clinical isolate of vancomycin-resistant *Enterococcus faecium*. *Infection and Immunity*, 70, 204-210.

References

- BORREGO, J. J., CORNAX, R., PRESTON, D. R., FARRAH, S. R., MCELHANEY, B. & BITTON, G. (1991) Development and application of new positively charged filters for recovery of bacteriophages from water. *Applied and Environmental Microbiology*, 57, 1218-1222.
- BOSSHARD, P. P., ZBINDEN, R., ABELS, S., BODDINGHAUS, B., ALTWEGG, M. & BOTTGER, E. C. (2006) 16S rRNA gene sequencing versus the API 20 NE system and the VITEK 2 ID-GNB card for identification of nonfermenting gram-negative bacteria in the clinical laboratory. *Journal of Clinical Microbiology*, 44, 1359-1366.
- BOU, G., CERVERO, G., DOMINGUEZ, M. A., QUEREDA, C. & MARTINEZ-BELTRAN, J. (2000) Characterization of a nosocomial outbreak caused by a multiresistant *Acinetobacter baumannii* strain with a carbapenem-hydrolyzing enzyme: High-level carbapenem resistance in *A. baumannii* is not due solely to the presence of beta-lactamases. *Journal of Clinical Microbiology*, 38, 3299-3305.
- BOUVET, P. J. M. & GRIMONT, P. A. D. (1986) Taxonomy of the genus *Acinetobacter* with the recognition of *Acinetobacter baumannii* sp nov, *Acinetobacter haemolyticus* sp nov, *Acinetobacter johnsonii* sp nov, and *Acinetobacter junii* sp nov and emended descriptions of *Acinetobacter calcoaceticus* and *Acinetobacter lwoffii*. *International Journal of Systematic Bacteriology*, 36, 228-240.
- BOUVET, P. J. M. & GRIMONT, P. A. D. (1987) Identification and biotyping of clinical isolates of *Acinetobacter*. *Annales De L Institut Pasteur-Microbiologie*, 138, 569-578.
- BOUVET, P. J. M. & JEANJEAN, S. (1989) Delineation of new proteolytic genomic species in the genus *Acinetobacter*. *Research in Microbiology*, 140, 291-299.
- BOWLER, P. G., DUERDEN, B. I. & ARMSTRONG, D. G. (2001) Wound microbiology and associated approaches to wound management. *Clinical Microbiology Reviews*, 14, 244-269.

- BOYCE, S. T. (2004) Fabrication, quality assurance, and assessment of cultured skin substitutes for treatment of skin wounds. *Biochemical Engineering Journal*, 20, 107-112.
- BRADLEY, D. E. (1972) Study of pili on *Pseudomonas aeruginosa*. *Genetical Research*, 19, 39-&.
- BRADLEY, D. E. (1972) Evidence for retraction of *Pseudomonas aeruginosa* RNA phage pili. *Biochemical and Biophysical Research Communications*, 47, 142-&.
- BRADLEY, D. E. (1972) Shortening of *Pseudomonas aeruginosa* pili after RNA-phage adsorption. *Journal of General Microbiology*, 72, 303-&.
- BRADLEY, D. E. (1972) Stimulation of pilus formation in *Pseudomonas aeruginosa* by RNA bacteriophage adsorption. *Biochemical and Biophysical Research Communications*, 47, 1080-&.
- BRADLEY, D. E. (1973) Basic characterization of a *Pseudomonas aeruginosa* pilus-dependent bacteriophage with a long noncontractile tail. *Journal of Virology*, 12, 1139-1148.
- BRADLEY, D. E. (1974) Adsorption of *Pseudomonas aeruginosa* pilus-dependent bacteriophages to a host mutant with nonretractile pili. *Virology*, 58, 149-163.
- BRADLEY, D. E. (1980) Function of *Pseudomonas aeruginosa* PAO polar pili - twitching motility. *Canadian Journal of Microbiology*, 26, 146-154.
- BRAVO, D., RIGLEY, T. H., GIBRAN, N., STRONG, D. M. & NEWMAN-GAGE, H. (2000) Effect of storage and preservation methods on viability in transplantable human skin allografts. *Burns*, 26, 367-378.
- BREIDAHN, A. F., JUDSON, R. T. & CLUNIE, G. J. A. (1989) Review of keratinocyte culture techniques - problems of growing skin. *Australian and New Zealand Journal of Surgery*, 59, 485-497.

- BROUDY, T. B. & FISCHETTI, V. A. (2003) In vivo lysogenic conversion of Tox(-) *Streptococcus pyogenes* to Tox(+) with lysogenic streptococci or free phage. *Infection and Immunity*, 71, 3782-3786.
- BROWN, S. & AMYES, S. (2006) OXA beta-lactamases in *Acinetobacter*: the story so far. *Journal of Antimicrobial Chemotherapy*, 57, 1-3.
- BRUSSOW, H. & HENDRIX, R. W. (2002) Phage genomics: Small is beautiful. *Cell*, 108, 13-16.
- BRUTTIN, A. & BRUSSOW, H. (2005) Human volunteers receiving *Escherichia coli* phage T4 orally: a safety test of phage therapy. *Antimicrobial Agents and Chemotherapy*, 49, 2874-2878.
- BRUYNOGHE, R. A. J. M. (1921) Essais de therapeutique au moyen du bacteriophage du staphylocoque. *COMPTE RENDUS DES SEANCES DE LA SOCIETE DE BIOLOGIE ET DE SES FILIALES*, 85, 1120-1121
- BUCHWALD, U. K. & PIROFSKI, L. (2003) Immune therapy for infectious diseases at the dawn of the 21(st) century: the past, present and future role of antibody therapy, therapeutic vaccination and biological response modifiers. *Current Pharmaceutical Design*, 9, 945-968.
- BURCH, R. M. & MCMILLAN, B. A. (1991) Sucralfate induces proliferation of dermal fibroblasts and keratinocytes in culture and granulation-tissue formation in full-thickness skin wounds, *Agents and Actions*, 34, 229-231.
- BURTON, D. G. A., ALLEN, M. C., BIRD, J. L. E. & FARAGHER, R. G. A. (2005) Bridging the gap: ageing pharmacokinetics and pharmacodynamics. *Journal of Pharmacy and Pharmacology*, 57, 671-679.
- BUTLER M. (ED.) (2004) Cell Culture and Upstream Processing, Taylor & Francis, Taylor & Francis.

- CARMO, H., BRULPORT, M., HERMES, M., OESCH, F., DE BOER, D., REMIAO, F., CARVALHO, F., SCHON, M. R., KREBSFAENGER, N., DOEHMER, J., BASTOS, M. D. & HENGSTLER, J. G. (2007) CYP2D6 increases toxicity of the designer drug 4-methylthioamphetamine (4-MTA). *Toxicology*, 229, 236-244.
- CARR, E. L., KAMPFER, P., PATEL, B. K. C., GURTLER, V. & SEVIOUR, R. J. (2003) Seven novel species of *Acinetobacter* isolated from activated sludge. *International Journal of Systematic and Evolutionary Microbiology*, 53, 953-963.
- CARRIERE, C., RISKKA, P. F., ZIMHONY, O., KRIAKOV, J., BARDAROV, S., BURNS, J., CHAN, J. & JACOBS, W. R. (1997) Conditionally replicating luciferase reporter phages: Improved sensitivity for rapid detection and assessment of drug susceptibility of *Mycobacterium tuberculosis*. *Journal of Clinical Microbiology*, 35, 3232-3239.
- CARSON, C. F., HAMMER, K. A. & RILEY, T. V. (1995) Broth micro-dilution method for determining the susceptibility of *Escherichia coli* and *Staphylococcus aureus* to the essential oil of *Melaleuca alternifolia* (tea tree oil). *Microbios*, 82, 181-185.
- CARSON, C. F. & RILEY, T. V. (2003) Non-antibiotic therapies for infectious diseases. *Commun Dis Intell*, 27 Suppl, S143-6.
- CASADEVALL, A. (1996) Antibody-based therapies for emerging infectious diseases. *Emerging Infectious Diseases*, 2, 200-208.
- CASADEVALL, A. & SCHARFF, M. D. (1994) Serum therapy revisited - animal-models of infection and development of passive antibody therapy. *Antimicrobial Agents and Chemotherapy*, 38, 1695-1702.
- CERVENY, K. E., DEPAOLA, A., DUCKWORTH, D. H. & GULIG, P. A. (2002) Phage therapy of local and systemic disease caused by *Vibrio vulnificus* in iron-dextran-treated mice. *Infection and Immunity*, 70, 6251-6262.

- CHANISHVILI, N., CHANISHVILI, T., TEDIASHVILI, M. & BARROW, P. A. (2001) Phages and their application against drug-resistant bacteria. *Journal of Chemical Technology and Biotechnology*, 76, 689-699.
- CHILLER, K., SELKIN, B. A. & MURAKAWA, G. J. (2001) Skin microflora and bacterial infections of the skin. *Journal of Investigative Dermatology Symposium Proceedings*, 6, 170-174.
- CISLO, M., DABROWSKI, M., WEBERDABROWSKA, B. & WOYTON, A. (1987) Bacteriophage treatment of suppurative skin infections. *Archivum Immunologiae Et Therapiae Experimentalis*, 35, 175-183.
- CLARK, J. R. & MARCH, J. B. (2006) Bacteriophages and biotechnology: vaccines, gene therapy and antibacterials. *Trends in Biotechnology*, 24, 212-218.
- CLARK, R. A. F. (1996) *The Molecular and Cellular Biology of Wound Repair*, Springer, 2nd Ed.
- CLARK, R. A. F., GHOSH, K. & TONNESEN, M. G. (2007) Tissue engineering for cutaneous wounds. *Journal of Investigative Dermatology*, 127, 1018-1029.
- CLOKIE, M. & KROPINSKI, A. (2009) *Bacteriophages: 2 (Methods in Molecular Biology)*, Humana Press.
- COELHO, J., WOODFORD, N., TURTON, J. & LIVERMORE, D. M. (2004) Multiresistant acinetobacter in the UK: how big a threat? *Journal of Hospital Infection*, 58, 167-169.
- COFFI, H. (1995) *Lysotypie des Acinetobacter*, Université Laval, Thèse (M. Sc.).
- COLLINS, C. & LYNE, P. *Collins and Lyne's Microbiological Methods*, Butterworth-Heinemann Ltd, 7th Ed.

COMPTON, C. C., GILL, J. M., BRADFORD, D. A., REGAUER, S., GALLICO, G. G. & OCONNOR, N. E. (1989) Skin regenerated from cultured epithelial autografts on full-thickness burn wounds from 6 days to 5 years after grafting - a light, electron-microscopic and immunohistochemical study. *Laboratory Investigation*, 60, 600-612.

CORBELLA, X., MONTERO, A., PUJOL, M., DOMINGUEZ, M. A., AYATS, J., ARGERICH, M. J., GARRIGOSA, F., ARIZA, J. & GUDIOL, F. (2000) Emergence and rapid spread of carbapenem resistance during a large and sustained hospital outbreak of multiresistant *Acinetobacter baumannii*. *Journal of Clinical Microbiology*, 38, 4086-4095.

CORBELLA, X., PUJOL, M., AYATS, J., SENDRA, M., ARDANUY, C., DOMINGUEZ, M. A., LINARES, J., ARIZA, J. & GUDIOL, F. (1996) Relevance of digestive tract colonization in the epidemiology of nosocomial infections due to multiresistant *Acinetobacter baumannii*. *Clinical Infectious Diseases*, 23, 329-334.

CORY, A. H., OWEN, T. C., BARLTROP, J. A. & CORY, J. G. (1991) Use of an aqueous soluble tetrazolium formazan assay for cell-growth assays in culture. *Cancer Communications*, 3, 207-212.

DA SILVA, G. J., LEITAO, R. & PEIXE, L. (1999) Emergence of carbapenem-hydrolyzing enzymes in *Acinetobacter baumannii* clinical isolates. *Journal of Clinical Microbiology*, 37, 2109-2110.

DAEHNEL, K., HARRIS, R., MADDERA, L. & SILVERMAN, P. (2005) Fluorescence assays for F-pill and their application. *Microbiology-Sgm*, 151, 3541-3548.

DALLAS, S. & KINGSBERY L. (1997) Bacteriophage plaques, on primary isolation media of urine culture growing *Escherichia coli*. *Clinical Microbiology Newsletter*, 19, 53-56.

DALY, M., BUCKLEY, J., POWER, E., O'HARE, C., CORMICAN, M., CRYAN, B., WALL, P. G. & FANNING, S. (2000) Molecular characterization of Irish *Salmonella enterica* serotype *typhimurium*: Detection of class I integrons and assessment of genetic relationships by DNA amplification fingerprinting. *Applied and Environmental Microbiology*, 66, 614-619.

DAMASKO, C., KONIETZNY, A., KASPAR, H., APPEL, B., DERSCH, P. & STRAUCH, E. (2005) Studies of the efficacy of enterocolitacin, a phage-tail like bacteriocin, as antimicrobial agent against *Yersinia enterocolitica* serotype O3 in a cell culture system and in mice. *Journal of Veterinary Medicine Series B-Infectious Diseases and Veterinary Public Health*, 52, 171-179.

DARBY, I., SKALLI, O. & GABBIANI, G. (1990) Alpha-smooth muscle actin is transiently expressed by myofibroblasts during experimental wound-healing. *Laboratory Investigation*, 63, 21-29.

DEBATTISTA, J. (2004) Phage therapy: where East meets West. *Expert Rev Anti Infect Ther*, 2, 815-9.

DECKER, T. & LOHMANNMATTES, M. L. (1988) A quick and simple method for the quantitation of lactate-dehydrogenase release in measurements of cellular cyto-toxicity and tumor necrosis factor (TNF) activity. *Journal of Immunological Methods*, 115, 61-69.

DELBRÜCK, M. (1940) The growth of bacteriophage and lysis of the host. *Journal of General Physiology*, 23, 643-660.

DEVAUD, M., KAYSER, F. H. & BACHI, B. (1982) Transposon-mediated multiple antibiotic-resistance in *Acinetobacter* strains. *Antimicrobial Agents and Chemotherapy*, 22, 323-329.

D'HERELLE, F. (1926) *The Bacteriophage and its behaviour*, Tindall & Cox, London/Williams and Wilkins Company, Baltimore, translated by Smith, G. H.

- D'HERELLE, F. (2007) On an invisible microbe antagonistic dysenteric bacilli: brief note by Mr. F. D'Herelle presented by Mr. Roux. *Research in Microbiology*, 158, 553-554.
- DIMASI, J. A., HANSEN, R. W. & GRABOWSKI, H. G. (2003) The price of innovation: new estimates of drug development costs. *Journal of Health Economics*, 22, 151-185.
- DINARELLO, C. A. (1991) Interleukin-1 and cytokine antagonism. *Atherosclerosis : Its Pathogenesis and the Role of Cholesterol*, 23, 161-168.
- DIXON, B. (2001) Progeny of the phage school. *Asm News*, 67, 432-433.
- DRAKE, J. W., CHARLESWORTH, B., CHARLESWORTH, D. & CROW, J. F. (1998) Rates of spontaneous mutation. *Genetics*, 148, 1667-1686.
- DROST, A. C., BURLESON, D. G., CIOFFI, W. G., JORDAN, B. S., MASON, A. D. & PRUITT, B. A. (1993) Plasma cytokines following thermal-injury and their relationship with patient mortality, burn size, and time postburn. *Journal of Trauma-Injury Infection and Critical Care*, 35, 335-339.
- DUCKWORTH, D. H. (1976) Who discovered bacteriophage. *Bacteriological Reviews*, 40, 793-802.
- DUNCAN, C. O., SHELTON, R. M., NAVSARIA, H., BALDERSON, D. S., PAPINI, R. P. G. & BARRALET, J. E. (2005) In vitro transfer of keratinocytes: Comparison of transfer fibrin membrane and delivery by aerosol spray. *Journal of Biomedical Materials Research Part B-Applied Biomaterials*, 73B, 221-228.
- DVORANKOVA, B., SMETANA, K., KONIGOVA, R., SINGEROVA, H., VACIK, J., JELINKOVA, M., KAPOUNKOVA, Z. & ZAHRADNIK, M. (1998) Cultivation and grafting of human keratinocytes on a poly(hydroxyethyl methacrylate) support to the wound bed: a clinical study. *Biomaterials*, 19, 141-146.

- EATON, M. & BAYNE-JONES, S. (1934) Bacteriophage therapy. Review of the principles and results of the use of bacteriophage in the treatment of infections. *J Am Med Assoc*, 23, 1769-1939.
- EBI FOOD SAFETY visited 20 February 2009, <http://www.foodprocessing-technology.com>
- EDWARDS-JONES, V. & GREENWOOD, J. E. (2003) What's new in burn microbiology? James Laing Memorial Prize Essay 2000. *Burns*, 29, 15-24.
- EHRlich, H. P. (2004) Understanding experimental biology of skin equivalent: from laboratory to clinical use in patients with burns and chronic wounds. *American Journal of Surgery*, 187, 29S-33S.
- ELLIS E. & DELBRÜCK, M. (1939) The growth of bacteriophage. *J.Gen.Physiol.*, 22.
- ERDAG, G. & MORGAN, J. R. (2002) Interleukin-1 alpha and interleukin-6 enhance the antibacterial properties of cultured composite keratinocyte grafts. *Annals of Surgery*, 235, 113-124.
- ERICSSON, H. M. & SHERRIS, J. C. (1971) Antibiotic sensitivity testing - report of an international collaborative study. *Acta Pathologica Et Microbiologica Scandinavica Section B-Microbiology and Immunology*, 1-&.
- EXPONENTIAL BIOTHERAPIES, I. visited 20 February 2009, <http://www.expobio.com/>.
- FAHEY, T., TURBEVILLE, T. & MCINTYRE, K. (1995) Differential TNF secretion by wound fibroblasts compared tonormal fibroblasts in response to LPS. *J. Surg. Res*, 58, 759-764.
- FALAGAS, M. E., BLIZIOTIS, I. A. & SIEMPOS, II (2006) Attributable mortality of *Acinetobacter baumannii* infections in critically ill patients: a systematic review of matched cohort and case-control studies. *Critical Care*, 10.

- FERNANDEZ-GACIO, A., UGUEN, M. & FASTREZ, J. (2003) Phage display as a tool for the directed evolution of enzymes. *Trends in Biotechnology*, 21, 408-414.
- FERNÁNDEZ-REYES M, RODRÍGUEZ-FALCÓN M, CHIVA C, P. J., ANDREU D & L., R. (2009) The cost of resistance to colistin in *Acinetobacter baumannii*: a proteomic perspective. *Proteomics*, 9, 1632-1645.
- FIEROBE, L., LUCET, J. C., DECRE, D., MULLER-SERIEYS, C., DELEUZE, A., JOLY-GUILLOU, M. L., MANTZ, J. & DESMONTS, J. M. (2001) An outbreak of imipenem-resistant *Acinetobacter baumannii* in critically ill surgical patients. *Infection Control and Hospital Epidemiology*, 22, 35-40.
- FISCHETTI, V. A. (2006) Using phage lytic enzymes to control pathogenic bacteria. *BMC Oral Health*, 6 Suppl 1, S16.
- FORSTER, D. H. & DASCHNER, F. D. (1998) *Acinetobacter* species as nosocomial pathogens. *European Journal of Clinical Microbiology & Infectious Diseases*, 17, 73-77.
- FRANTZ, S., VINCENT, K. A., FERON, O. & KELLY, R. A. (2005) Innate immunity and angiogenesis. *Circulation Research*, 96, 15-26.
- FRESHNEY, I. (2005) Culture of Animal Cells: A Manual of Basic Technique, WileyBlackwell, 5th Ed.
- FRIEDLAND, I., STINSON, L., IKAIDDI, M., HARM, S. & WOODS, G. L. (2003) Phenotypic antimicrobial resistance patterns in *Pseudomonas aeruginosa* and *Acinetobacter*: results of a Multicenter Intensive Care Unit Surveillance Study, 1995-2000. *Diagnostic Microbiology and Infectious Disease*, 45, PII S0732-8893(02)00503-5.
- FU, B. J., QUINTERO, J. & BAKER, C. C. (2003) Keratinocyte growth conditions modulate telomerase expression, senescence, and immortalization by human papillomavirus type 16 E6 and E7 Oncogenes. *Cancer Research*, 63, 7815-7824.

- GABBIANI, G. (2003) The myofibroblast in wound healing and fibrocontractive diseases. *Journal of Pathology*, 200, 500-503.
- GAJIWALA, K. & GAJIWALA, A. L. (2004) Evaluation of lyophilised, gamma-irradiated amnion as a biological dressing. *Cell and Tissue Banking*, 5, 73-80.
- GANGAGEN, I. visited 20 February 2009, <http://www.gangagen.com/home.asp>.
- GARAU, J. (1998) The growing problem of nosocomial bacterial resistance - An epidemiological perspective with emphasis on the fluoroquinolones. *Clinical Drug Investigation*, 15, 1-8.
- GARCÍA P., MARTÍNEZ B, OBESO J. & RODRÍGUEZ A. (2009) Bacteriophages and their application in food safety. *Letters in Applied Microbiology*, 47, 479-485.
- GARRISON, R. G. (1963) Occurrence of *Bacterium anitratum* in secretions of pulmonary origin. *American Journal of Clinical Pathology*, 40, 260-&.
- GEIER, M. R., ATTALLAH, A. F. M. & MERRIL, C. R. (1975) Characterization of *Escherichia coli* bacterial viruses in commercial sera. *In Vitro-Journal of the Tissue Culture Association*, 11, 55-58.
- GERISCHER U. (ED.) (2007) *Acinetobacter* Molecular Biology, Caister Academic Press.
- GERNER-SMIDT, P. (1994) *Acinetobacter*: epidemiological and taxonomic aspects. *APMIS Suppl*, 47, 1-41.
- GERNER-SMIDT, P., TJERNBERG, I. & URSING, J. (1991) Reliability of phenotypic tests for identification of *Acinetobacter* species. *Journal of Clinical Microbiology*, 29, 277-282.
- GIAMARELLOU, H., ANTONIADOU, A. & KANELLAKOPOULOU, K. (2008) *Acinetobacter baumannii*: a universal threat to public health? *International Journal of Antimicrobial Agents*, 32, 106-119.

- GIAMMANCO, A., VIEU, J. F., BOUVET, P. J. M., SARZANA, A. & SINATRA, A. (1989) A comparative assay of epidemiologic markers for *Acinetobacter* strains isolated in a hospital. *Zentralblatt Fur Bakteriologie-International Journal of Medical Microbiology Virology Parasitology and Infectious Diseases*, 272, 231-241.
- GILARDI G. (ED.) Glucose Nonfermenting Gram Negative Bacteria in Clinical Microbiology, CRC Press 1978.
- GILL, J. M. & ABEDON, S. T. (2003) Bacteriophage Ecology and Plants. APSnet Feature, November 2003, American Phytopathological Society, visited 30 July 2008, <http://www.apsnet.org/online/feature/phages/>.
- GILLITZER, R. & GOEBELER, M. (2001) Chemokines in cutaneous wound healing. *Journal of Leukocyte Biology*, 69, 513-521.
- GOLDBERG, M. T., HAN, Y. P., YAN, C., SHAW, M. C. & GARNER, W. L. (2007) TNF-alpha suppresses alpha-smooth muscle actin expression in human dermal fibroblasts: An implication for abnormal wound healing. *Journal of Investigative Dermatology*, 127, 2645-2655.
- GOODRIDGE, L., CHEN, J. R. & GRIFFITHS, M. (1999) The use of a fluorescent bacteriophage assay for detection of *Escherichia coli* O157 : H7 in inoculated ground beef and raw milk. *International Journal of Food Microbiology*, 47, 43-50.
- GORDON, T., SPANIER, J., BUTALA, J. H., LI, P. & ROSSMAN, T. G. (2002) In vitro bioavailability of heavy metals in pressure-treated wood dust. *Toxicological Sciences*, 67, 32-37.
- GRADON, J. D., CHAPNICK, E. K. & LUTWICK, L. I. (1992) Infective endocarditis of a native valve due to *Acinetobacter* - case-report and review. *Clinical Infectious Diseases*, 14, 1145-1148.

- GRANT, I., WARWICK, K., MARSHALL, J., GREEN, C. & MARTIN, R. (2002) The co-application of sprayed cultured autologous keratinocytes and autologous fibrin sealant in a porcine wound model. *British Journal of Plastic Surgery*, 55, 219-227.
- GRAY H. (1995) Gray's Anatomy. Descriptive and Surgical, Parragon.
- GREEN, H. (1979) The keratinocyte as a differentiated cell type. *Harvey Lect.*, 74.
- GREEN, H. (1991) Cultured cells for the treatment of disease. *Scientific American*, 265, 96-102.
- GREENBERG, S., MARGULIS, A. & GARLICK, J. A. (2005) In vivo transplantation of engineered human skin. *Methods Mol Biol*, 289, 425-30.
- GREHN, M. & VONGRAEVENTITZ, A. (1978) Search for *Acinetobacter calcoaceticus* subsp *anitratus* - enrichment of fecal samples. *Journal of Clinical Microbiology*, 8, 342-343.
- GUARDABASSI, L., PETERSEN, A., OLSEN, J. E. & DALSGAARD, A. (1998) Antibiotic resistance in *Acinetobacter* spp. isolated from sewers receiving waste effluent from a hospital and a pharmaceutical plant. *Applied and Environmental Microbiology*, 64, 3499-3502.
- HAGENS, S., HABEL, A., VON AHSEN, U., VON GABAIN, A. & BLASI, U. (2004) Therapy of experimental *Pseudomonas* infections with a nonreplicating genetically modified phage. *Antimicrobial Agents and Chemotherapy*, 48, 3817-3822.
- HALCON, L. & MILKUS, K. (2004) *Staphylococcus aureus* and wounds: A review of tea tree oil as a promising antimicrobial. *American Journal of Infection Control*, 32, 402-408.
- HAMMER, K. A., CARSON, C. F. & RILEY, T. V. (1999) Antimicrobial activity of essential oils and other plant extracts. *Journal of Applied Microbiology*, 86, 985-990.
- HANCOCK, R. E. W. (1997) Peptide antibiotics. *Lancet*, 349, 418-422.

- HANLON, G. W. (2005) The emergence of multidrug resistant *Acinetobacter* species: a major concern in the hospital setting. *Letters in Applied Microbiology*, 41, 375-378.
- HANLON, G. W. (2007) Bacteriophages: An appraisal of their role in the treatment of bacterial infections. *International Journal of Antimicrobial Agents*, 30, 118-128.
- HATFULL, G. F. (2002) Microbiology - A tail of two specificities. *Science*, 295, 2031-2032.
- HATT (1976) The American Type Culture Collection Catalogue of Strains, 12th Ed.
- HÄUSLER, T. (2003) *Gesund durch Viren*, Piper Verlag.
- HÄUSLER, T. (2006) Bug killers. *Nature Medicine*, 12, 600-601.
- HAVA, D. L. & CAMILLI, A. (2001) Isolation and characterization of a temperature-sensitive generalized transducing bacteriophage for *Vibrio cholerae*. *Journal of Microbiological Methods*, 46, 217-225.
- HAYFLICK, L. (1965) Limited *in vitro* lifetime of human diploid cell strains. *Experimental Cell Research*, 37, 614-&.
- HAYFLICK, L. & MOORHEAD, P. S. (1961) Serial cultivation of human diploid cell strains. *Experimental Cell Research*, 25, 585-&.
- HAZBON, M. H., GUARIN, N., FERRO, B. E., RODRIGUEZ, A. L., LABRADA, L. A., TOVAR, R., RISK, P. F. & JACOBS, W. R. (2003) Photographic and luminometric detection of luciferase reporter phages for drug susceptibility testing of clinical *Mycobacterium tuberculosis* isolates. *Journal of Clinical Microbiology*, 41, 4865-4869.
- HEALTH PROTECTION AGENCY (2008) *Acinetobacter* spp bacteraemia, England, Wales, and Northern Ireland: 2003 to 2007, Voluntary surveillance data 2003 - 2007, visited 18 November 2008, <http://www.hpa.org.uk>.

HEALTH PROTECTION AGENCY (2008) National Standard Methods, Oxidase Test, BSOP TP 26, Standards Unit, Evaluations and Standards Laboratory Centre for Infections, visited 18 November 2008, <http://www.hpa-standardmethods.org.uk>.

HEALTH PROTECTION AGENCY (2008) National Standard Methods, Motility Test, BSOP TP 21, Standards Unit, Evaluations and Standards Laboratory Centre for Infections, visited 18 November 2008, <http://www.hpa-standardmethods.org.uk>.

HEALTH PROTECTION AGENCY (2008) National Standard Methods, Catalase Test, BSOP TP 8, Standards Unit, Evaluations and Standards Laboratory Centre for Infections, visited 18 November 2008, <http://www.hpa-standardmethods.org.uk>.

HEINEMANN, B., WISPLINGHOFF, H., EDMOND, M. & SEIFERT, H. (2000) Comparative activities of ciprofloxacin, clinafloxacin, gatifloxacin, gemifloxacin, levofloxacin, moxifloxacin, and trovafloxacin against epidemiologically defined *Acinetobacter baumannii* strains. *Antimicrobial Agents and Chemotherapy*, 44, 2211-2213.

HENRICHSEN, J. (1983) Twitching motility. *Annual Review of Microbiology*, 37, 81-93.

HERMAN, N. J. & JUNI, E. (1974) Isolation and characterization of a generalized transducing bacteriophage for *Acinetobacter*. *Journal of Virology*, 13, 46-52.

HERMOSO, J. A., GARCIA, J. L. & GARCIA, P. (2007) Taking aim on bacterial pathogens: from phage therapy to enzybiotics. *Current Opinion in Microbiology*, 10, 461-472.

HERNDON, D. E. (1996) Total Burn Care, Saunders Company Ltd

HERRUZO-CABRERA, R., VIZCAINOALCAIDE, M. J., MAYER, R. F. & REYCALERO, J. (1992) A new *in vitro* model to test the effectiveness of topical antimicrobial agents - use of an artificial eschar. *Burns*, 18, 35-38.

HETTIARATCHY, S. & DZIEWULSKI, P. (2004) ABC of burns: pathophysiology and types of burns (vol 328, pg 1427, 2004). *British Medical Journal*, 329, 148-148.

HETTIARATCHY, S. & DZIEWULSKI, P. (2004) ABC of burns - Introduction. *British Medical Journal*, 328, 1366-1368.

HETTIARATCHY, S. & PAPINI, R. (2004) ABC of burns - Initial management of a major burn: II - assessment and resuscitation. *British Medical Journal*, 329, 101-103.

HEXAL GENTECH visited 20 February 2009, .

HOLT, J. & KRIEG N. (ED.) (1984) *Bergey's Manual of Systematic Bacteriology*, Lippincott Williams and Wilkins, 4th Ed.

HOLTON, J. (1983) A note on the preparation and use of a selective differential medium for the isolation of *Acinetobacter* spp from clinical sources. *Journal of Applied Bacteriology*, 54, 141-142.

HORCH, R. E., KOPP, J., KNESER, U., BEIER, J. & BACH, A. D. (2005) Tissue engineering of cultured skin substitutes. *Journal of Cellular and Molecular Medicine*, 9, 592-608.

HOROBIN, A. J., SHAKESHEFF, K. M. & PRITCHARD, D. I. (2006) Promotion of human dermal fibroblast migration, matrix remodelling and modification of fibroblast morphology within a novel 3D model by *Lucilia sericata* larval secretions. *Journal of Investigative Dermatology*, 126, 1410-1418.

HOROWITZ, D. & KING, A. G. (2000) Colorimetric determination of inhibition of hematopoietic progenitor cells in soft agar. *Journal of Immunological Methods*, 244, 49-58.

HSUEH, P. R., TENG, L. J., CHEN, C. Y., CHEN, W. H., YU, C. J., HO, S. W. & LUH, K. T. (2002) Pandrug-resistant *Acinetobacter baumannii* causing nosocomial infections in a university hospital, Taiwan. *Emerging Infectious Diseases*, 8, 827-832.

HUBBARD-SMITH, K., PATSALIS, P., PARDINAS, J. R., JHA, K. K., HENDERSON, A. S. & OZER, H. L. (1992) altered chromosome-6 in immortal human fibroblasts. *Molecular and Cellular Biology*, 12, 2273-2281.

- HUBNER, G., BRAUCHLE, M., SMOLA, H., MADLENER, M., FASSLER, R. & WERNER, S. (1996) Differential regulation of pro-inflammatory cytokines during wound healing in normal and glucocorticoid-treated mice. *Cytokine*, 8, 548-556.
- HUGO, W. & RUSSELL A. (ED.) (1992) *Pharmaceutical Microbiology*, Blackwell Scientific Publications, 5th Ed.
- ICH HARMONISED TRIPARTITE GUIDELINE (1999) Q6B: Specifications : Test Procedures and Acceptance Criteria for Biotechnological/Biological Products, International Conference on Harmonisation Expert Working Group.
- INTRALYTIX INC. visited 20 February 2009, <http://www.intralytix.com/>.
- ISSA, Y., WATTS, D. C., BRUNTON, P. A., WATERS, C. M. & DUXBURY, A. J. (2004) Resin composite monomers alter MTT and LDH activity of human gingival fibroblasts in vitro. *Dental Materials*, 20, 12-20.
- JACOBS, M. R. & HORNFELDT, C. S. (1994) *Melaleuca* oil poisoning. *Journal of Toxicology-Clinical Toxicology*, 32, 461-464.
- JAKWAY, J. P. (1986) The hazards of mycoplasma contamination in the screening of hybridoma supernatants for effects on [H-3] thymidine incorporation. *Methods in Enzymology*, 121, 481-484.
- JANSSEN, A. M., LUIJENDIJK, T. J. C., SCHEFFER, J. J. C. & SVENDSEN, A. B. (1987) Caraway oil - composition and antimicrobial properties. *Pharmaceutisch Weekblad-Scientific Edition*, 9, 156-156.
- JANTOVA, S., THEISZOVA, M., LETASIOVA, S., BIROSOVA, L. & PALOU, T. M. (2008) In vitro effects of fluor-hydroxyapatite, fluorapatite and hydroxyapatite on colony formation, DNA damage and mutagenicity. *Mutation Research-Genetic Toxicology and Environmental Mutagenesis*, 652, 139-144.

- JANZEKOVIC, Z. (1970) A new concept in the early excision and immediate grafting of burns. *J Trauma*, 10, 1103-8.
- JAWAD, A., HAWKEY, P. M., HERITAGE, J. & SNELLING, A. M. (1994) Description of Leeds-Acinetobacter-medium, a new selective and differential medium for isolation of clinically important *Acinetobacter* spp, and comparison with herellea agar and holtons agar. *Journal of Clinical Microbiology*, 32, 2353-2358.
- JEEJEEBHOY, K. N. (2001) Enteral and parenteral nutrition: evidence-based approach. *Proceedings of the Nutrition Society*, 60, 399-402.
- JEPSON, C. D. & MARCH, J. B. (2004) Bacteriophage lambda is a highly stable DNA vaccine delivery vehicle. *Vaccine*, 22, 2413-2419.
- JIANG, W., KIM, B. Y. S., RUTKA, J. T. & CHAN, W. C. W. (2008) Nanoparticle-mediated cellular response is size-dependent. *Nature Nanotechnology*, 3, 145-150.
- JOHNSON, J. L., ANDERSON, R. S. & ORDAL, E. J. (1970) Nucleic acid homologies among oxidase-negative *Moraxella* species. *Journal of Bacteriology*, 101, 568-&.
- JOLY-GUILLOU, M. L., BERGOGNE-BEREZIN, E. & VIEU, J. F. (1990) A study of the relationships between antibiotic-resistance phenotypes, phage-typing and biotyping of 117 clinical isolates of *Acinetobacter* spp. *Journal of Hospital Infection*, 16, 49-58.
- JONES, I., CURRIE, L. & MARTIN, R. (2002) A guide to biological skin substitutes. *British Journal of Plastic Surgery*, 55, 185-193.
- JORI, G., FABRIS, C., SONCIN, M., FERRO, S., COPPELLOTTI, O., DEI, D., FANTETTI, L., CHITI, G. & RONEUCCI, G. (2006) Photodynamic therapy in the treatment of microbial infections: Basic principles and perspective applications. *Lasers in Surgery and Medicine*, 38, 468-481.
- JOSHI, A., SIDDIQI, J. Z., RAO, G. R. K. & CHAKRAVORTY, M. (1982) Mb78, a virulent bacteriophage of *Salmonella typhimurium*. *Journal of Virology*, 41, 1038-1043.

- JOSHI, A., SIDDIQUI, J. Z., VERMA, M. & CHAKRAVORTY, M. (1982) Participation of the host protein(s) in the morphogenesis of bacteriophage-p22. *Molecular & General Genetics*, 186, 44-49.
- JUDKINS, K. (2000) Burns resuscitation: what place albumin? *Hospital Medicine*, 61, 116-119.
- KAMOLZ, L. P., LUEGMAIR, M., WICK, N., EISENBOCK, B., BURJAK, S., KOLLER, R., MEISSL, G. & FREY, M. (2005) The Viennese culture method: cultured human epithelium obtained on a dermal matrix based on fibroblast containing fibrin glue gels. *Burns*, 31, 25-29.
- KÄMPFER, P. (1993) Grouping of *Acinetobacter* genomic species by cellular fatty-acid composition. *Medical Microbiology Letters*, 2, 394-400.
- KÄMPFER, P., TJERNBERG, I. & URSING, J. (1993) Numerical classification and identification of *Acinetobacter* genomic species. *Journal of Applied Bacteriology*, 75, 259-268.
- KANCHANAPOOM T, KHARDORI N. & (2002) Management of infections in patients with severe burns: Impact of multiresistant pathogens. *J Burns.*, 1, 1–10.
- KARAGEORGOPOULOS, D. E. & FALAGAS, M. E. (2008) Current control and treatment of multidrug-resistant *Acinetobacter baumannii* infections. *Lancet Infectious Diseases*, 8, 751-762.
- KAUTZKY F., HARTINGER A., KÖHLER L. & VOGT H. (1996) In vitro cytotoxicity of antimicrobial agents to human keratinocytes. *Journal of the European Academy of Dermatology and Venereology*, 6, 159 - 166.
- KEARNEY, J. N. (2001) Clinical evaluation of skin substitutes. *Burns*, 27, 545-551.
- KIM, J. & KATHARIOU, S. (2009) Temperature-Dependent Phage Resistance of *Listeria monocytogenes* Epidemic Clone II. e-pub, ahead of print.

KIS, K., BODAI, L., POLYANKA, H., EDER, K., PIVARCSI, A., DUDA, E., SOOS, G., BATA-CSORGO, Z. & KEMENY, L. (2006) Budesonide, but not tacrolimus, affects the immune functions of normal human keratinocytes. *International Immunopharmacology*, 6, 358-368.

KLAENHAMMER, T. R. (1988) Bacteriocins of lactic-acid bacteria. *Biochimie*, 70, 337-349.

KLOOS, W. E. & MUSSELWHITE, M. S. (1975) Distribution and persistence of *Staphylococcus* and *Micrococcus* species and other aerobic bacteria on human skin. *Applied Microbiology*, 30, 381-395.

KLOVINS, J., OVERBEEK, G. P., VAN DEN WORM, S. H. E., ACKERMANN, H. W. & VAN DUIN, J. (2002) Nucleotide sequence of a ssRNA phage from *Acinetobacter*: kinship to coliphages. *Journal of General Virology*, 83, 1523-1533.

KNEZEVIC, I., STACEY, G., PETRIECIANI, J. & SUBSTRATES, W. H. O. S. G. (2008) WHO Study Group on cell substrates for production of biologicals, Geneva, Switzerland, 11-12 June 2007. *Biologicals*, 36, 203-211.

KONEMAN, E., ALLEN, S., JANDA, W. & SCHRECKENBERGER, P. (1994) Color Atlas and Textbook of Diagnostic Microbiology, Lippincott Williams and Wilkins, 5th Ed.

KRATZ, G. (1998) Modelling of wound healing processes in human skin using tissue culture. *Microscopy Research and Technique*, 42, 345-350.

KROPINSKI, A. (2006) Phage Therapy-Everything Old is New again. *Can J Infect Dis Med Microbiol*, 17, 297-306.

KROPINSKI A. (2006) Phage Therapy - Everything Old is New Again. *Can. J. Infect. Dis. Med. Microbiol.* , 17, 297-306.

- KUCHAREWICZ-KRUKOWSKA, A. & SLOPEK, S. (1987) Immunogenic effect of bacteriophage in patients subjected to phage therapy. *Archivum Immunologiae Et Therapiae Experimentalis*, 35, 553-561.
- KULLING, S. E., JACOBS, E., PFEIFFER, E. & METZLER, M. (1998) Studies on the genotoxicity of the mammalian lignans enterolactone and enterodiol and their metabolic precursors at various endpoints in vitro. *Mutation Research-Genetic Toxicology and Environmental Mutagenesis*, 416, 115-124.
- KUTTER, E. (2004) Phage therapy: bacteriophages as natural, self-replicating and self-limiting antibiotic, a historical perspective. *Journal of Pharmacy and Pharmacology*, 56, 292.
- KUTTER, E. & SULAKVELIDZE A. (ED.) (2004) Bacteriophages: Biology and Applications: Molecular Biology and Applications, CRC Press.
- LA HEI, E. R., HOLLAND, A. J. A. & MARTIN, H. C. O. (2006) Laser Doppler Imaging of paediatric burns: Burn wound outcome can be predicted independent of clinical examination. *Burns*, 32, 550-553.
- LAMBRECHTS, S. A. G., AALDERS, M. C. G., VERBRAAK, F. D., LAGERBERG, J. W. M., DANKERT, J. B. & SCHUITMAKER, J. J. (2005) Effect of albumin on the photodynamic inactivation of microorganisms by a cationic porphyrin. *Journal of Photochemistry and Photobiology B-Biology*, 79, 51-57.
- LANGLEBEN, D. (2001) Antibiotic resistance: are we destined to return to the pre-penicillin days? . *The Pharmaceutical Journal*, 267 470-481.
- LAROCCA, D., KASSNER, P. D., WITTE, A., LADNER, R. C., PIERCE, G. F. & BAIRD, A. (1999) Gene transfer to mammalian cells using genetically targeted filamentous bacteriophage. *Faseb Journal*, 13, 727-734.

- LEE, S. S., LIN, T. M., CHEN, Y. H., LIN, S. D. & LAI, C. S. (2005) "Flypaper technique" a modified expansion method for preparation of postage stamp autografts. *Burns*, 31, 753-757.
- LEIBOVICH, S. J. & ROSS, R. (1975) Role of macrophage in wound repair - study with hydrocortisone and antimacrophage serum. *American Journal of Pathology*, 78, 71-99.
- LEWIN, B. (2008) *Genes*, Jones and Bartlett Publishers Inc., 9th Ed.
- LIN, Z. Q., KONDO, T., ISHIDA, Y., TAKAYASU, T. & MUKAIDA, N. (2003) Essential involvement of IL-6 in the skin wound-healing process as evidenced by delayed wound healing in IL-6-deficient mice. *Journal of Leukocyte Biology*, 73, 713-721.
- LIVERMORE, D. M. (2005) Tigecycline: what is it, and where should it be used? *Journal of Antimicrobial Chemotherapy*, 56, 611-614.
- LOESSNER, M. J., REES, C. E. D., STEWART, G. & SCHERER, S. (1996) Construction of luciferase reporter bacteriophage A511::luxAB for rapid and sensitive detection of viable *Listeria* cells. *Applied and Environmental Microbiology*, 62, 1133-1140.
- LOHR, J. E., CHEN, F. & HILL, R. T. (2005) Genomic analysis of bacteriophage Phi JL001: Insights into its interaction with a sponge-associated alpha-proteobacterium. *Applied and Environmental Microbiology*, 71, 1598-1609.
- LORTHOLARY, O., FAGON, J. Y., HOI, A. B., MAHIEU, G. & GUTMANN, L. (1998) Colonization by *Acinetobacter baumannii* in intensive-care-unit patients. *Infection Control and Hospital Epidemiology*, 19, 188-190.
- LORTHOLARY, O., FAGON, J. Y., HOI, A. B., SLAMA, M. A., PIERRE, J., GIRAL, P., ROSENZWEIG, R., GUTMANN, L., SAFAR, M. & ACAR, J. (1995) Nosocomial acquisition of multiresistant *Acinetobacter baumannii* - risk-factors and prognosis. *Clinical Infectious Diseases*, 20, 790-796.

- LU, Z., BREIDT, F., FLEMING, H. P., ALTERMANN, E. & KLAENHAMMER, T. R. (2003) Isolation and characterization of a *Lactobacillus plantarum* bacteriophage, Phi JL-1, from a cucumber fermentation. *International Journal of Food Microbiology*, 84, 225-235.
- LURIA, S. E. & DELBRÜCK, M. (1943) Mutations of bacteria from virus sensitivity to virus resistance. *Genetics* 28, 491-511.
- MAAS-SZABOWSKI, N., SHIMOTOYODOME, A. & FUSENIG, N. E. (1999) Keratinocyte growth regulation in fibroblast co-cultures via a double paracrine mechanism. *Journal of Cell Science*, 112, 1843-1853.
- MACFADDIN, J. (1985) Maintenance of Medical Bacteria, Lippincott Williams & Wilkins.
- MAHONY, D. E., LIM-MORRISON, S., BRYDEN, L., FAULKNER, G., HOFFMAN, P. S., AGOCS, L., BRIAND, G. G., BURFORD, N. & MAGUIRE, H. (1999) Antimicrobial activities of synthetic bismuth compounds against *Clostridium difficile*. *Antimicrobial Agents and Chemotherapy*, 43, 582-588.
- MANDEL, A. D., WRIGHT, K. & MCKINNON, J. M. (1964) Selective medium for isolation of *mima* + *herellea* organisms. *Journal of Bacteriology*, 88, 1524-&.
- MANDILARA, G. D., SMETI, E. M., MAVRIDOU, A. T., LAMBIRI, M. P., VATOPOULOS, A. C. & RIGAS, F. P. (2006) Correlation between bacterial indicators and bacteriophages in sewage and sludge. *Fems Microbiology Letters*, 263, 119-126.
- MANN, C. M. & MARKHAM, J. L. (1998) A new method for determining the minimum inhibitory concentration of essential oils. *Journal of Applied Microbiology*, 84, 538-544.
- MARCH, J. B., CLARK, J. R. & JEPSON, C. D. (2004) Genetic immunisation against hepatitis B using whole bacteriophage lambda particles. *Vaccine*, 22, 1666-1671.

- MARCUK, L. M., NIKIFORO.VN, SCERBAK, J. F., LEVITOV, T. A., KOTLJARO.RI, NAUMSINA, M. S., DAVYDOV, S. U., MONSUR, K. A., RAHMAN, M. A., LATIF, M. A., NORTHRUP, R. S., CASH, R. A., HUQ, I., DEY, C. R. & PHILLIPS, R. A. (1971) Clinical studies of use of bacteriophage in treatment of cholera. *Bulletin of the World Health Organization*, 45, 77-&.
- MARET, M., CHICHEPORTICHE, R., DAYER, J. M. & GABAY, C. (2004) Production of intracellular IL-1 alpha, IL-1 beta, and IL-1Ra isoforms by activated human dermal and synovial fibroblasts - Phenotypic differences between human dermal and synovial fibroblasts. *Cytokine*, 25, 193-203.
- MARTIN-LOZANO, D., CISNEROS, J. M., BECERRIL, B., CUBEROS, L., PRADOS, T., ORTIZ-LEYBA, C., CANAS, E. & PACHON, J. (2002) Comparison of a repetitive extragenic palindromic sequence-based PCR method and clinical and microbiological methods for determining strain sources in cases of nosocomial *Acinetobacter baumannii* bacteremia. *Journal of Clinical Microbiology*, 40, 4571-4575.
- MATSUDA, T. (2005) Lysis-deficient bacteriophage therapy decreases endotoxin and inflammatory mediator release and improves survival in a murine peritonitis model. *Surgery* 137, 639-46.
- MATTEY, M. & SPENCER, J. (2008) Bacteriophage therapy - cooked goose or Phoenix rising? *Current Opinion in Biotechnology*, 19, 608-612.
- MCGOWAN, J. E. (2006) Resistance in nonfermenting gram-negative bacteria: Multidrug resistance to the maximum. *American Journal of Infection Control*, 34, S29-S37.
- MCKAY, L. D., CHERRY, J. A., BALES, R. C., YAHYA, M. T. & GERBA, C. P. (1993) A field example of bacteriophage as tracers of fracture flow. *Environmental Science & Technology*, 27, 1075-1079.

- MCVAY, C. S., VELASQUEZ, M. & FRALICK, J. A. (2007) Phage therapy of *Pseudomonas aeruginosa* infection in a mouse burn wound model. *Antimicrobial Agents and Chemotherapy*, 51, 1934-1938.
- MEIKLE, M. C. (2006) The evolution of plastic and maxillofacial surgery in the twentieth century: The Dunedin connection. *Surgeon-Journal of the Royal Colleges of Surgeons of Edinburgh and Ireland*, 4, 325-334.
- MERKENSCHLAGER, M., KARDAMAKIS, D., RAWLE, F. C., SPURR, N. & BEVERLEY, P. C. L. (1988) Rate of incorporation of radiolabeled nucleosides does not necessarily reflect the metabolic state of cells in culture - effects of latent mycoplasma contamination. *Immunology*, 63, 125-131.
- MERRIL, C., SCHOLL, D. & ADHYA, S. L. (2003) The prospect for bacteriophage therapy in Western medicine. *Nature Reviews Drug Discovery*, 2, 489-497.
- MERRIL, C. R. (1975) Phage in human vaccines. *Science*, 188, 8-8.
- MERRIL, C. R., BISWAS, B., CARLTON, R., JENSEN, N. C., CREED, G. J., ZULLO, S. & ADHYA, S. (1996) Long-circulating bacteriophage as antibacterial agents. *Proceedings of the National Academy of Sciences of the United States of America*, 93, 3188-3192.
- MERRIL, C. R., YARKIN, R., KRELL, K., ATTALLAH, A. F., FRIEDMAN, T. B. & GEIER, M. R. (1972) Isolation of bacteriophages from commercial sera. *In Vitro-Journal of the Tissue Culture Association*, 8, 91-&.
- MILCH, H. & FORNOSI, F. (1975) Bacteriophage contamination in live poliovirus vaccine. *Journal of Biological Standardization*, 3, 307-310.
- MILES, A. M., S. (1938) The estimation of the bactericidal power of the blood. *J. Hygiene*, 38, 732-49.

- MONSUR, K. A., RAHMAN, M. A., HUQ, F., ISLAM, M. N., NORTHRUP, R. S. & HIRSCHHON (1970) Effect of massive doses of bacteriophage on excretion of *Vibrios*, duration of diarrhoea and output of stools in acute cases of cholera. *Bulletin of the World Health Organization*, 42, 723-&.
- MOODY, E. E. M., TROUSDALE, M. D., JORGENSEN, J. H. & SHELOKOV, A. (1975) Bacteriophages and endotoxin in licensed live-virus vaccines. *Journal of Infectious Diseases*, 131, 588-591.
- MORGAN, D. J., WEISENBERG, S. A., AUGENBRAUN, M. H., CALFEE, D. P., CURRIE, B. P., FURUYA, E. Y., HOLZMAN, R., MONTECALVO, M. C., PHILLIPS, M., POLSKY, B. & SEPKOWITZ, K. A. (2009) Multidrug-Resistant *Acinetobacter baumannii* in New York City-10 Years Into the Epidemic. *Infection Control and Hospital Epidemiology*, 30, 196-197.
- MORGAN, E. D., BLEDSOE, S. C. & BARKER, J. (2000) Ambulatory management of burns. *American Family Physician*, 62, 2015-2026.
- MORRISON, J. L., BREITLING, R., HIGHAM, D. J. & GILBERT, D. R. (2006) A lock-and-key model for protein-protein interactions. *Bioinformatics*, 22, 2012-2019.
- MOSMANN, T. (1983) Rapid colorimetric assay for cellular growth and survival - application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, 65, 55-63.
- MUHD FAKHRUDDIN, B., AMINUDDIN, B., MAZLYZAM, A., RUSZYMAH, B. (2004) The effects of age on monolayer culture of human keratinocytes for future use in skin engineering. *The Medical Journal of Malaysia*, 59 Suppl, B182-183.
- MOULIN, V., CASTILLOUX, G., AUGER, F. A., GARREL, D., O'CONNOR-MCCOURT, M. D. & GERMAIN, L. (1998) Modulated response to cytokines of human wound healing myofibroblasts compared to dermal fibroblasts. *Experimental Cell Research*, 238, 283-293.

- MUGDAL P., BREIDT, F., LUBKIN S. & SANDEEP K. (2006) Quantifying the significance of phage attack on starter cultures: a mechanistic model for population dynamics of phage and their hosts isolated from fermenting sauerkraut. *Applied and Environmental Microbiology*, 72, 3908-3915.
- MUHD FAKHRUDDIN, B. H., AMINUDDIN, B. S., MAZLYZAM, A. L. & RUSZYMAH, B. H. I. (2004) The effects of age on monolayer culture of human keratinocytes for future use in skin engineering. *Med J Malaysia*, 59 Suppl B, 182-3.
- MULCZYK, M. & SLOPEK, S. (1974) Use of a new phage preparation in prophylaxis and treatment of shigellosis. *Acta Microbiologica Academiae Scientiarum Hungaricae*, 21, 115-119.
- MULLER, K. M., BICKEL, M., WIESMANN, U. N. & SPORRI, B. (2000) Natural killer cells activate human dermal fibroblasts. *Cytokine*, 12, 1755-1762.
- MULLER M., PEGG S. & RULE M. (2001) Determinants of death following burn injury. *Br J Surg*, 88, 583-7.
- NAIDU, B. & AVARI, C. (1932) Bacteriophage in the treatment of plague. *Ind Jour Med Res* 19, 737-748.
- NAKAI, T. & PARK, S. C. (2002) Bacteriophage therapy of infectious diseases in aquaculture. *Research in Microbiology*, 153, 13-18.
- NAKAI, T., SUGIMOTO, R., PARK, K. H., MATSUOKA, S., MORI, K., NISHIOKA, T. & MARUYAMA, K. (1999) Protective effects of bacteriophage on experimental *Lactococcus garvieae* infection in yellowtail. *Diseases of Aquatic Organisms*, 37, 33-41.
- NATIONAL BURN CARE REVIEW COMMITTEE REPORT (2002) Standards and strategy for burn care: a review of burn care in the British Isles, visited 14 July 2008, <http://www.baps.co.uk>.

NATIONAL BURN CARE REVIEW COMMITTEE REPORT (2006) Standards and strategy for burn care: a review of burn care in the British Isles, visited 14 July 2008, <http://www.baps.co.uk>.

NEMEC A, MUSÍLEK M, MAIXNEROVÁ M, DE BAERE T, VAN DER REIJDEN TJ, VANECHOUTTE M & L., D. (2009) *Acinetobacter beijerinckii* sp. nov. and *Acinetobacter gyllenbergii* sp. nov., haemolytic organisms isolated from humans. *Int J Syst Evol Microbiol.*,59(Pt 1), 118-24.

NEMEC, A., DE BAERE, T., TJERNBERG, I., VANECHOUTTE, M., VAN DER REIJDEN, T. J. K. & DIJKSHOORN, L. (2001) *Acinetobacter ursingii* sp nov and *Acinetobacter schindleri* sp nov., isolated from human clinical specimens. *International Journal of Systematic and Evolutionary Microbiology*, 51, 1891-1899.

NEMEC, A., DIJKSHOORN, L., CLEENWERCK, I., DE BAERE, T., JANSSENS, D., VAN DER REIJDEN, T. J. K., JEZEK, P. & VANECHOUTTE, M. (2003) *Acinetobacter parvus* sp nov., a small-colony-forming species isolated from human clinical specimens. *International Journal of Systematic and Evolutionary Microbiology*, 53, 1563-1567.

NEWELL, D., DOYLE, A. & GRIFFITHS, J. (1993) *Cell and Tissue Culture: Laboratory Procedures in Biotechnology*, John Wiley & Sons.

NGUYEN, T. T., GILPIN, D. A., MEYER, N. A. & HERNDON, D. N. (1996) Current treatment of severely burned patients. *Annals of Surgery*, 223, 14-25.

NILSSON, N., MALMBORG, A. C. & BORREBAECK, C. A. K. (2000) The phage infection process: A functional role for the distal linker region of bacteriophage protein 3. *Journal of Virology*, 74, 4229-4235.

NISHIMURA, Y., INO, T. & IIZUKA, H. (1988) *Acinetobacter radioresistens* sp. nov isolated from cotton and soil. *International Journal of Systematic Bacteriology*, 38, 209-211.

- NIYONSABA, F. & OGAWA, H. (2005) Protective roles of the skin against infection: Implication of naturally occurring human antimicrobial agents beta-defensins, cathelicidin LL-37 and lysozyme. *Journal of Dermatological Science*, 40, 157-168.
- OCHS, H. D., DAVIS, S. D., CRAIN, J. D., ROSEN, F. S. & WEDGWOOD, R. J. (1971) Lack of immunologic response to bacteriophage OX 174 in X-linked agammaglobulinemia (X-A). *Journal of Clinical Investigation*, 50, A71-&.
- O'CONNOR, N. E., MULLIKEN, J. B., BANKSSCHLEGEL, S., KEHINDE, O. & GREEN, H. (1981) Grafting of burns with cultured epithelium prepared from autologous epidermal cells. *Lancet*, 1, 75-78.
- OKABE N. & GOTO, M. (1963) Bacteriophages of plant pathogens. *Annu Rev Phytopathology* 1, 397-418.
- OMNILYTICS visited 20 February 2009, <http://www.omnilytics.com/>.
- ONG, Y. S., SAMUEL, M. & SONG, C. (2006) Meta-analysis of early excision of burns. *Burns*, 32, 145-150.
- ONO, I., GUNJI, H., ZHANG, J. Z., MARUYAMA, K. & KANEKO, F. (1995) A study of cytokines in burn blister fluid related to wound-healing. *Burns*, 21, 352-355.
- ORAM, M. & FISHER, L. M. (1991) 4-Quinolone resistance mutations in the DNA gyrase of *Escherichia coli* clinical isolates identified by using the polymerase chain-reaction. *Antimicrobial Agents and Chemotherapy*, 35, 387-389.
- ORTEGA, M. R., GANZ, T. & MILNER, S. M. (2000) Human beta defensin is absent in burn blister fluid. *Burns*, 26, 724-726.
- PACHON, J. & VILA, J. (2009) Treatment of multiresistant *Acinetobacter baumannii* infections. *Current Opinion in Investigational Drugs*, 10, 150-156.

- PANESSA-WARREN, B. J., WARREN, J. B., WONG, S. S. & MISEWICH, J. A. (2006) Biological cellular response to carbon nanoparticle toxicity. *Journal of Physics-Condensed Matter*, 18, S2185-S2201.
- PAPINI, R. (2004) ABC of burns - Management of burn injuries of various depths. *British Medical Journal*, 329, 158-160.
- PARISIEN, A., ALLAIN, B., ZHANG, J., MANDEVILLE, R. & LAN, C. (2008) Novel alternatives to antibiotics: bacteriophages, bacterial cell wall hydrolases, and antimicrobial peptides *Journal of Applied Microbiology*, 104, 1-13.
- PARK, C. H., LEE, M. J., AHN, J., KIM, S., KIM, H. H., KIM, K. H., EUN, H. C. & CHUNG, J. H. (2004) Heat shock-induced matrix metal loproteinase (MMP)-1 and MMP-3 are mediated through ERK and JNK activation and via an autocrine interleukin-6 loop. *Journal of Investigative Dermatology*, 123, 1012-1019.
- PARK, S. C. & NAKAI, T. (2003) Bacteriophage control of *Pseudomonas plecoglossicida* infection in ayu *Plecoglossus altivelis*. *Diseases of Aquatic Organisms*, 53, 33-39.
- PARK, S. C., SHIMAMURA, I., FUKUNAGA, M., MORI, K. I. & NAKAI, T. (2000) Isolation of bacteriophages specific to a fish pathogen, *Pseudomonas plecoglossicida*, as a candidate for disease control. *Applied and Environmental Microbiology*, 66, 1416-1422.
- PAULL, K. D., SHOEMAKER, R. H., BOYD, M. R., PARSONS, J. L., RISBOOD, P. A., BARBERA, W. A., SHARMA, M. N., BAKER, D. C., HAND, E., SCUDIERO, D. A., MONKS, A., ALLEY, M. C. & GROTE, M. (1988) The synthesis of XTT - a new tetrazolium reagent that is bioreducible to a water-soluble formazan. *Journal of Heterocyclic Chemistry*, 25, 911-914.

- PAYAN, A., EBDON, J., TAYLOR, H., GANTZER, C., OTTOSON, J., PAPAGEORGIOU, G. T., BLANCH, A. R., LUCENA, F., JOFRE, J. & MUNIESA, M. (2005) Method for isolation of Bacteroides bacteriophage host strains suitable for tracking sources of fecal pollution in water. *Applied and Environmental Microbiology*, 71, 5659-5662.
- PAYNE, R. J. H. & JANSEN, V. A. A. (2001) Understanding bacteriophage therapy as a density-dependent kinetic process. *Journal of Theoretical Biology*, 208, 37-48.
- PAYNE, R. J. H. & JANSEN, V. A. A. (2003) Pharmacokinetic principles of bacteriophage therapy. *Clinical Pharmacokinetics*, 42, 315-325.
- PEREIRA, C., MURPHY, K., JESCHKE, M. & HERNDON, D. N. (2005) Post burn muscle wasting and the effects of treatments. *International Journal of Biochemistry & Cell Biology*, 37, 1948-1961.
- PERFETTO, B., DONNARUMMA, G., CRISCUOLO, D., PAOLETTI, I., GRIMALDI, E., TUFANO, M. A. & BARONI, A. (2003) Bacterial components induce cytokine and intercellular adhesion molecules-1 and activate transcription factors in dermal fibroblasts. *Research in Microbiology*, 154, 337-344.
- PETRENKO, V. A. & VODYANOY, V. J. (2003) Phage display for detection of biological threat agents. *Journal of Microbiological Methods*, 53, 253-262.
- PETRICCIANI, J. & SHEETS, R. (2008) An overview of animal cell substrates for biological products. *Biologicals*, 36, 359-362.
- PHAGE BIOCONTROL LLC visited 20 February 2009, <http://www.phagebiocontrol.com/about.php>.
- PHAGE BIOTECH LTD. visited 20 February 2009, <http://www.phage-biotech.com/>.

PHAGE INTERNATIONAL visited 2 July 2008,
<http://www.phageinternational.com/press/185318.html>;
<http://www.phageinternational.com/ptc.htm>.

PHAGE THERAPY CENTER visited 2 July 2008,
http://www.phagetherapycenter.com/pii/PatientServlet?command=static_home.

PIDDOCK, L. J. (1995) Mechanisms of resistance to fluoroquinolones: state-of-the-art 1992-1994. *Drugs*, 49 Suppl 2, 29-35.

PIRISI, A. (2000) Phage therapy - advantages over antibiotics? *Lancet*, 356, 1418-1418.

PISMAN, T. I. & PECHURKIN, N. S. (2008) Host-parasite interactions in closed and open microbial cultivation system. *Advances in Space Research*, 41, 773-776.

POLK, H. C. (1979) Supportive therapy in burn care. Consensus summary on infection. *J Trauma*, 19, 894-6.

POLLITZER, R., SWAROOP, S. & BURROWS, W. (1959) Cholera. *Monogr Ser World Health Organ*, 58, 1001-19.

PRESCOTT, L. (2002) *Microbiology*, McGraw-Hill Publishing, 5th Ed.

PROJAN, S. (2004) Phage-inspired antibiotics? *Nature Biotechnology*, 22, 167-168.

PROJAN, S. J. (2003) Why is big Pharma getting out of antibacterial drug discovery? *Current Opinion in Microbiology*, 6, 427-430.

PRUITT, B. A. (1980) Improvements in burn care. *Jama-Journal of the American Medical Association*, 244, 2090-2090.

PRUITT, B. A. (1984) The diagnosis and treatment of infection in the burn patient. *Burns*, 11, 79-91.

- PRUITT, B. A. & LEVINE, N. S. (1984) Characteristics and uses of biologic dressings and skin substitutes. *Archives of Surgery*, 119, 312-322.
- PRUITT, B. A., MCMANUS, A. T., KIM, S. H. & GOODWIN, C. W. (1998) Burn wound infections: Current status. *World Journal of Surgery*, 22, 135-145.
- QUALE, J., BRATU, S., LANDMAN, D. & HEDDURSHETTI, R. (2003) Molecular epidemiology and mechanisms of carbapenem resistance in *Acinetobacter baumannii* endemic in New York City. *Clinical Infectious Diseases*, 37, 214-220.
- RAB, M., KOLLER, R., RUZICKA, M., BURDA, G., KAMOLZ, L. P., BIEROCHS, B., MEISSL, G. & FREY, M. (2005) Should dermal scald burns in children be covered with autologous skin grafts or with allogeneic cultivated keratinocytes? "The Viennese concept". *Burns*, 31, 578-586.
- RAMESH, V., FRALICK, J. A. & ROLFE, R. D. (1999) Prevention of *Clostridium difficile* induced ileocectitis with bacteriophage. *Anaerobe*, 5, 69-78.
- RANDERSON, J. (2003) Phage therapy - Virus beats food bug. *New Scientist*, 178, 22-22.
- REID, G. E., GRIM, S. A., ALDEZA, C. A., JANDA, W. M. & CLARK, N. M. (2007) Rapid development of *Acinetobacter baumannii* resistance to tigecycline. *Pharmacotherapy*, 27, 1198-1201.
- REJZEK A. , WEYER F., EICHBERGER R. & W. GEBHART W. (2001) Physical changes of amniotic membranes through glycerolization for the use as an epidermal substitute. light and electron microscopic studies *Cell and Tissue Banking*, 2, 95-102.
- RELLO, J. (1999) *Acinetobacter baumannii* infections in the ICU - Customization is the key. *Chest*, 115, 1226-1229.
- RENNEKAMPF, H., HANSBROUGH, J., KIESSIG, V., DORE, C., STICHERLING, M. & SCHRÖDER, J. (2000) Bioactive interleukin-8 is expressed in wound and enhances wound healing. *J. Surg. Res.*, 93, 41-54.

RENNEKAMPFF, H., HANSBROUGH, J., WOODS, V., DORE, C., KIESSIG, V. & SCHRÖDER, J. (1997) Role of melanoma growth stimulatory activity (MGSA/gro) on keratinocyte function in wound healing. *Arch. Dermatol. Res.*, 289, 204–212.

RETAILLIAU, H. F., HIGHTOWER, A. W., DIXON, R. E. & ALLEN, J. R. (1979) *Acinetobacter calcoaceticus* - nosocomial pathogen with an unusual seasonal pattern. *Journal of Infectious Diseases*, 139, 371-375.

REVATHI, G., PURI, J. & JAIN, B. K. (1998) Bacteriology of burns. *Burns*, 24, 347-349.

RHEINWALD, J. G. & GREEN, H. (1975) Serial cultivation of strains of human epidermal keratinocytes - formation of keratinizing colonies from single cells. *Cell*, 6, 331-344.

RISKA, P. F., SU, Y., BARDAROV, S., FREUNDLICH, L., SARKIS, G., HATFULL, G., CARRIERE, C., KUMAR, V., CHAN, J. & JACOBS, W. R. (1999) Rapid film-based determination of antibiotic susceptibilities of *Mycobacterium tuberculosis* strains by using a luciferase reporter phage and the Bronx box. *Journal of Clinical Microbiology*, 37, 1144-1149.

RIVARA, F. P. (2000) Burns: the importance of prevention. *Inj Prev*, 6, 243-4.

ROBERTS, A. B., RUSSO, A., FELICI, A. & FLANDERS, K. C. (2003) Smad3: A key player in pathogenetic mechanisms dependent on TGF-beta. *Tissue Remodeling*, 995, 1-10.

ROBICHOVA, S., SLAMENOVA, D., GABELOVA, A., SEDLAK, J. & JAKUBILOVA, J. (2004) An investigation of the genotoxic effects of N-nitrosomorpholine in mammalian cells. *Chemico-Biological Interactions*, 148, 163-171.

ROBSON, M. C., BARNETT, R. A., LEITCH, I. O. W. & HAYWARD, P. G. (1992) Prevention and treatment of postburn scars and contracture. *World Journal of Surgery*, 16, 87-96.

- ROSE, S. F., LEWIS, A. L., HANLON, G. W. & LLOYD, A. W. (2004) Biological responses to cationically materials charged phosphorylcholine-based in vitro. *Biomaterials*, 25, 5125-5135.
- ROSENTHAL, S. & TAGER, I. B. (1975) Prevalence of Gram-negative rods in normal pharyngeal flora. *Annals of Internal Medicine*, 83, 355-357.
- RUBIRES, X., MERINO, S., AGUILAR, A., NOGUERAS, M. M. & TOMAS, J. M. (1998) Isolation of three different bacteriophage from mesophilic *Aeromonas* sp. that use different types of monopolar flagella as their primary receptor. *Fems Microbiology Letters*, 161, 53-57.
- RUSSELL W. & HERWALD H. (EDS) (2005) Concepts in Bacterial Virulence, Karger.
- RYAN, C. M., SCHOENFELD, D. A., THORPE, W. P., SHERIDAN, R. L., CASSEM, E. H. & TOMPKINS, R. G. (1998) Objective estimates of the probability of death from burn injuries (vol 338, pg 362, 1998). *New England Journal of Medicine*, 338, 1849-1850.
- RYAN, C. M., SCHOENFELD, D. A., THORPE, W. P., SHERIDAN, R. L., CASSEM, E. H. & TOMPKINS, R. G. (1998) Objective estimates of the probability of death from burn injuries. *New England Journal of Medicine*, 338, 362-366.
- RYBNIKER, J., KRAMME, S. & SMALL, P. L. (2006) Host range of 14 mycobacteriophages in *Mycobacterium ulcerans* and seven other mycobacteria including *Mycobacterium tuberculosis* - application for identification and susceptibility testing. *Journal of Medical Microbiology*, 55, 37-42.
- SAFFLE, J. R. (1998) Predicting outcomes of burns. *New England Journal of Medicine*, 338, 387-388.
- SANDER, M. & SCHMIEGER, H. (2001) Method for host-independent detection of generalized transducing bacteriophages in natural habitats. *Applied and Environmental Microbiology*, 67, 1490-1493.

- SANDHU, A. K., HUBBARD, K., KAUR, G. P., JHA, K. K., OZER, H. L. & ATHWAL, R. S. (1994) Senescence of immortal human fibroblasts by the introduction of normal human chromosome 6. *Proceedings of the National Academy of Sciences of the United States of America*, 91, 5498-5502.
- SANTOS FERREIRA, M. O., VIEU, J. F. & KLEIN, B. (1984) Phage-types and susceptibility to 26 antibiotics of nosocomial strains of *Acinetobacter* isolated in Portugal. *J Int Med Res*, 12, 364-8.
- SCHMIDT, M., SCHMITZ, H., BAUMGART, A., GUEDON, D., NETSCH, M. I., KREUTER, M. H., SCHMIDLIN, C. B. & SCHRENK, D. (2005) Toxicity of green tea extracts and their constituents in rat hepatocytes in primary culture. *Food and Chemical Toxicology*, 43, 307-314.
- SCHOLL, D., ROGERS, S., ADHYA, S. & MERRIL, C. R. (2001) Bacteriophage K1-5 encodes two different tail fiber proteins, allowing it to infect and replicate on both K1 and K5 strains of *Escherichia coli*. *Journal of Virology*, 75, 2509-2515.
- SEIFERT, H., DIJKSHOORN, L., GERNERSMIDT, P., PELZER, N., TJERNBERG, I. & VANEECHOUTTE, M. (1997) Distribution of *Acinetobacter* species on human skin: Comparison of phenotypic and genotypic identification methods. *Journal of Clinical Microbiology*, 35, 2819-2825.
- SELVAAG, E., HOLM, J. O. & THUNE, P. (1995) Allergic contact-dermatitis in an aroma therapist with multiple sensitizations to essential oils. *Contact Dermatitis*, 33, 354-355.
- SESSLER, D. I. (2006) Non-pharmacologic prevention of surgical wound infection. *Anesthesiol Clin*, 24, 279-97.
- SHAKESPEARE, P. G. (2005) The role of skin substitutes in the treatment of burn injuries. *Clinics in Dermatology*, 23, 413-418.

- SHERETZ R. & SULLIVAN M. (1985) An outbreak of infections with *Acinetobacter calcoaceticus* in burn patient: contamination of patient's mattress. *J. Infect. Dis.* 2 (1985), 151, 252–258.
- SHERIDAN, R. L., REMENSNYDER, J. P., SCHNITZER, J. J., SCHULZ, J. T., RYAN, C. M. & TOMPKINS, R. G. (2000) Current expectations for survival in pediatric burns. *Archives of Pediatrics & Adolescent Medicine*, 154, 245-249.
- SHKILNYJ, P. & KOUDELKA, G. B. (2007) Effect of salt shock on stability of lambda(imm434) lysogens. *Journal of Bacteriology*, 189, 3115-3123.
- SHORTKROFF, S., TURELL, M., RICE, K. & THORNHILL, T. S. (2002) Cellular response to nanoparticles. *Nanoparticulate Materials*, 704, 375-380.
- SICARD, M., TABART, J., BOEMARE, N. E., THALER, O. & MOULIA, C. (2005) Effect of phenotypic variation in *Xenorhabdus nematophila* on its mutualistic relationship with the entomopathogenic nematode *Steinernema carpocapsae*. *Parasitology*, 131, 687-694.
- SIMBOLI, N., TAKIFF, H., MCNERNEY, R., LOPEZ, B., MARTIN, A., PALOMINO, J. C., BARRERA, L. & RITACCO, V. (2005) In-house phage amplification assay is a sound alternative for detecting rifampin-resistant *Mycobacterium tuberculosis* in low-resource settings. *Antimicrobial Agents and Chemotherapy*, 49, 425-427.
- SIMHON, A., RAHAV, G., SHAZBERG, G., BLOCK, C., BERCOVIER, H. & SHAPIRO, M. (2001) *Acinetobacter baumannii* at a tertiary-care teaching hospital in Jerusalem, Israel. *Journal of Clinical Microbiology*, 39, 389-391.
- SIVROPOULOU, A., KOKKINI, S., LANARAS, T. & ARSENAKIS, M. (1995) Antimicrobial activity of mint essential oils. *Journal of Agricultural and Food Chemistry*, 43, 2384-2388.

SKERMAN, V., MCGOWAN, V., SNEATH, P., MOORE, W. & MOORE, L. (1989) Approved Lists of Bacterial Names and Index of the Bacterial and Yeast Nomenclatural Changes, American Society for Microbiology,. 1.

SKURNIK, M. & STRAUCH, E. (2006) Phage therapy: Facts and fiction. *International Journal of Medical Microbiology*, 296, 5-14.

SLOPEK, S., DURLAKOWA, I., WEBERDABROWSKA, B., DABROWSKI, M. & KUCHARWICZKUKOWSKA, A. (1984) Results of bacteriophage treatment of suppurative bacterial-infections, Detailed evaluation of the results obtained in further 150 cases. *Archivum Immunologiae Et Therapiae Experimentalis*, 32, 317-335.

SLOPEK, S., DURLAKOWA, I., WEBERDABROWSKA, B., KUCHARWICZKUKOWSKA, A., DABROWSKI, M. & BISIKIEWICZ, R. (1983) Results of bacteriophage treatment of suppurative bacterial-infections, General evaluation of the results. *Archivum Immunologiae Et Therapiae Experimentalis*, 31, 267-291.

SLOPEK, S., KUCHARWICZKUKOWSKA, A., WEBERDABROWSKA, B. & DABROWSKI, M. (1985) Results of bacteriophage treatment of suppurative bacterial-infections, Evaluation of the results obtained in 370 cases. *Archivum Immunologiae Et Therapiae Experimentalis*, 33, 219-240.

SLOPEK, S., KUCHARWICZKUKOWSKA, A., WEBERDABROWSKA, B. & DABROWSKI, M. (1985) Results of bacteriophage treatment of suppurative bacterial-infections, Evaluation of the results obtained in children. *Archivum Immunologiae Et Therapiae Experimentalis*, 33, 241-259.

SLOPEK, S., KUCHARWICZKUKOWSKA, A., WEBERDABROWSKA, B. & DABROWSKI, M. (1985) Results of bacteriophage treatment of suppurative bacterial-infections, Analysis of treatment of suppurative staphylococcal infections. *Archivum Immunologiae Et Therapiae Experimentalis*, 33, 261-273.

- SLOPEK, S., WEBERDABROWSKA, B., DABROWSKI, M. & KUCHARAWICZKRUKOWSKA, A. (1987) Results of bacteriophage treatment of suppurative bacterial-infections in the years 1981-1986. *Archivum Immunologiae Et Therapiae Experimentalis*, 35, 569-583.
- SMITH, G. P. (1985) Filamentous fusion phage - novel expression vectors that display cloned antigens on the virion surface. *Science*, 228, 1315-1317.
- SMITH, H. W. & HUGGINS, M. B. (1982) Successful treatment of experimental *Escherichia coli* infections in mice using phage - its general superiority over antibiotics. *Journal of General Microbiology*, 128, 307-318.
- SMITH, H. W. & HUGGINS, M. B. (1983) Effectiveness of phages in treating experimental *Escherichia coli* diarrhea in calves, piglets and lambs. *Journal of General Microbiology*, 129, 2659-2675.
- SMITH, H. W., HUGGINS, M. B. & SHAW, K. M. (1987) The control of experimental *Escherichia coli* diarrhea in calves by means of bacteriophages. *Journal of General Microbiology*, 133, 1111-1126.
- SMITH, H. W., HUGGINS, M. B. & SHAW, K. M. (1987) Factors influencing the survival and multiplication of bacteriophages in calves and in their environment. *Journal of General Microbiology*, 133, 1127-1135.
- SOMERVILLE, D. A. & NOBLE, W. C. (1970) A note on the gram negative bacilli of human skin. *Revue Europeenne d'Etudes Cliniques et Biologiques*, 15, 669-671.
- SOOTHILL, J. S. (1992) Treatment of experimental infections of mice with bacteriophages. *Journal of Medical Microbiology*, 37, 258-261.
- SOOTHILL, J. S. (1994) Bacteriophage prevents destruction of skin-grafts by *Pseudomonas aeruginosa*. *Burns*, 20, 209-211.

- SOULI, M., GALANI, I. & GIAMARELLOU, H. (2008) Emergence of extensively drug-resistant and pandrug-resistant Gram-negative bacilli in Europe. *Euro Surveill*, 13.
- SPELLBERG, B., MILLER, L. G., KUO, M. N., BRADLEY, J., SCHELD, W. M. & EDWARDS, J. E. (2007) Societal costs versus savings from wild-card patent extension legislation to spur critically needed antibiotic development. *Infection*, 35, 167-174.
- SPELLBERG, B., POWERS, J. H., BRASS, E. P., MILLER, L. G. & EDWARDS, J. E. (2004) Trends in antimicrobial drug development: Implications for the future. *Clinical Infectious Diseases*, 38, 1279-1286.
- SPÖRRI, B., BICKEL, M., LIMAT, A., WAELTI, E. R., HUNZIKER, T. & WIESMANN, U. N. (1996) Juxtacrine stimulation of cytokine production in cocultures of human dermal fibroblasts and T cells. *Cytokine*, 8, 631-635.
- STICHERLING M., HETZEL F., SCHRÖDER J. & CHRISTOPHERS E. (1993) Time- and stimulus-dependent secretion of NAP-1/IL-8 by human fibroblasts and endothelial cells. *Journal of Investigative Dermatology* 101.
- STÖPPLER, H., HARTMANN, D. P., SHERMAN, L. & SCHLEGEL, R. (1997) The human papillomavirus type 16 E6 and E7 oncoproteins dissociate cellular telomerase activity from the maintenance of telomere length. *Journal of Biological Chemistry*, 272, 13332-13337.
- SULAKVELIDZE, A. (2005) Phage therapy: On attractive option for dealing with antibiotic-resistant bacterial infections (vol 10, pg 808, 2005). *Drug Discovery Today*, 10, PII S1359-6446(05)03510-5.
- SULAKVELIDZE, A., ALAVIDZE, Z. & MORRIS, J. G. (2001) Bacteriophage therapy. *Antimicrobial Agents and Chemotherapy*, 45, 649-659.
- SUMMERS, W. C. (1999) Félix d'Herelle and the Origins of Molecular Biology, Yale University Press

SUMMERS, W. C. (2001) Bacteriophage therapy. *Annual Review of Microbiology*, 55, 437-451.

SWEETMAN S. (ED.) (2006) Martindale: The Complete Drug Reference, Pharmaceutical Press, 35th.

TAPLIN, D., ZAIAS, N. & REBELL, G. (1963) Human skin as a source of *Mima Herellea* infections. *Jama-Journal of the American Medical Association*, 186, 952-&.

TAYLOR, D., WHATLING, C., KEARNEY, J. N., MATTHEWS, B. & HOLLAND, K. T. (1990) The effect of bacterial products on human fibroblast and keratinocyte detachment and viability. *British Journal of Dermatology*, 122, 23-28.

TAYLOR, P. W., STAPLETON, P. D. & LUZIO, J. P. (2002) New ways to treat bacterial infections. *Drug Discovery Today*, 7, PII S1359-6446(02)02498-4.

TEBBLE N, ADAMS R, THOMAS D & PRICE P (2005) Anxiety and self-consciousness in patients with facial lacerations one week and six months later. . *British Journal of Oral & Maxillofacial Surgery* 44, 520-525.

THE WORLD MEDICAL ASSOCIATION visited 2 July 2008,
<http://www.wma.net/e/policy/b3.htm>.

THIEL, K. (2004) Old dogma, new tricks - 21st century phage therapy. *Nature Biotechnology*, 22, 31-36.

TILLEY, P. A. G. & ROBERTS, F. J. (1994) Bacteremia with *Acinetobacter* species - risk-factors and prognosis in different clinical settings. *Clinical Infectious Diseases*, 18, 896-900.

TOWNER, K. J. & CHOPADEV, B. A. (1987) Biotyping of *Acinetobacter calcoaceticus* using the Api 20NE system. *Journal of Hospital Infection*, 10, 145-151.

TRANTINO (2007) Agency Response Letter GRAS Notice No. GRN 000218, U.S. Food and Drug Administration, U.S. Food and Drug Administration, CFSAN/Office of Food Additive Safety.

TWAROG, R. & BLOUSE, L. E. (1968) Isolation and characterization of transducing bacteriophage bp1 for *Bacterium anitratum* (*Achromobacter* sp.). *Journal of Virology*, 2, 716-&.

TWORT F. (1915) *Lancet*. *An investigation on the nature of ultra-microscopic viruses*, 186, 1241-1243.

TWORT F. (1920) Researches on dysentery. *Brit J Exp Path* 1, 237-43.

UK OFFICE FOR NATIONAL STATISTICS ONLINE (2008) Deaths involving MRSA, visited 2 June 2008, <http://www.statistics.gov.uk/>.

UK OFFICE FOR NATIONAL STATISTICS ONLINE (2008) Deaths involving *Clostridium difficile*, visited 2 June 2008, <http://www.statistics.gov.uk>.

UNITED STATES HEALTH PROTECTION AGENCY (2001) Method 1601: Male-specific (F+) and Somatic Coliphage in Water by Two-step Enrichment Procedure, Office of Water, Washington D.C.

US FOOD AND DRUG ADMINISTRATION (2006) Human Services, Food Additives Permitted for Direct Addition to Food for Human Consumption; Bacteriophage Preparation

Federal Register /Vol. 71, No. 160 / Friday, August 18, 2006 /Rules and Regulations 47729, 21 CFR Part 172 [Docket No. 2002F-0316 (formerly 02F-0316)]

VAN NIEL, C. B. (1955) Natural selection in the microbial world - the 2nd Marjory Stephenson memorial lecture. *Journal of General Microbiology*, 13, 201-217.

- VANSCHIE, B. J., ROUWENHORST, R. J., VANDIJKEN, J. P. & KUENEN, J. G. (1989) Selection of glucose-assimilating variants of *Acinetobacter-calcoaceticus* lmd-7941 in chemostat culture. *Antonie Van Leeuwenhoek Journal of Microbiology*, 55, 39-52.
- VIEU, J. F., MINCK, R. & BERGOGNEBEREZIN, E. (1979) *Acinetobacter* bacteriophages and phage-typing. *Annales De Microbiologie*, A130, 405-406.
- VIHOLA, H., LAUKKANEN, A., VALTOLA, L., TENHU, H. & HIRVONEN, J. (2005) Cytotoxicity of thermosensitive polymers poly(N-isopropylacrylamide), poly(N-vinylcaprolactam) and amphiphilically modified poly(N-vinylcaprolactam). *Biomaterials*, 26, 3055-3064.
- VILA, J., RUIZ, J., GONI, P., MARCOS, A. & DEANTA, T. J. (1995) Mutation in the *gyra* gene of quinolone-resistant clinical isolates of *Acinetobacter-baumannii*. *Antimicrobial Agents and Chemotherapy*, 39, 1201-1203.
- VILLERS, D., ESPAZE, E., COSTE-BUREL, M., GIAUFFRET, F., NININ, E., NICOLAS, F. & RICHEL, H. (1998) Nosocomial *Acinetobacter baumannii* infections: Microbiological and clinical epidemiology. *Annals of Internal Medicine*, 129, 182-+.
- VINDENES, H. & BJERKNES, R. (1995) Microbial colonization of large wounds. *Burns*, 21, 575-579.
- VISAVADIA, B. G., HONEYSETT, J. & DANFORD, M. H. (2008) Manuka honey dressing: An effective treatment for chronic wound infections. *British Journal of Oral & Maxillofacial Surgery*, 46, 55-56.
- VISCONTI, N. (1953) Resistance to lysis from without in bacteria infected with bacteriophage-T2. *Journal of Bacteriology*, 66, 247-253.
- WAINWRIGHT, M. (1998) Photodynamic antimicrobial chemotherapy (PACT). *Journal of Antimicrobial Chemotherapy*, 42, 13-28.

- WALL, D. & KAISER, D. (1999) Type IV pili and cell motility. *Molecular Microbiology*, 32, 1-10.
- WALSH, T. R. (2008) Clinically significant carbapenemases: an update. *Current Opinion in Infectious Diseases*, 21, 367-371.
- WAYNE, P. (2002) M100-S12: Performance Standards for Antimicrobial Susceptibility Testing: Twelfth Informational Supplement, National Committee for Clinical Laboratory Standards.
- WEBER-DABROWSKA B, MULCZYK M & GORSKI A. (2001) Bacteriophage therapy for infections in cancer patients. *Clin Appl Immunol Rev*, 1, 131-134.
- WEBER-DABROWSKA, B., DABROWSKI, M. & SLOPEK, S. (1987) Studies on bacteriophage penetration in patients subjected to phage therapy. *Archivum Immunologiae Et Therapiae Experimentalis*, 35, 563-568.
- WEBER-DABROWSKA, B., MULCZYK, M. & GORSKI, A. (2000) Bacteriophage therapy of bacterial infections: An update of our institute's experience. *Archivum Immunologiae et Therapiae Experimentalis*, 48, 547-551.
- WEBER-DABROWSKA, B., ZIMECKI, M. & MULCZYK, M. (2000) Effective phage therapy is associated with normalization of cytokine production by blood cell cultures. *Archivum Immunologiae Et Therapiae Experimentalis*, 48, 31-37.
- WEBER-DABROWSKA, B., ZIMECKI, M., MULCZYK, M. & GORSKI, A. (2002) Effect of phage therapy on the turnover and function of peripheral neutrophils. *Fems Immunology and Medical Microbiology*, 34, PII S0928-8244(02)00375-9.
- WEINBREN, M. J. (2001) Pharmacokinetics of antibiotics in burns patients - J Antimicrob Chemother 2001; 47: 720. *Journal of Antimicrobial Chemotherapy*, 47, 720-720.
- WERNER, S. & GROSE, R. (2003) Regulation of wound healing by growth factors and cytokines. *Physiological Reviews*, 83, 835-870.

- WESTWATER, C., KASMAN, L. M., SCHOFIELD, D. A., WERNER, P. A., DOLAN, J. W., SCHMIDT, M. G. & NORRIS, J. S. (2003) Use of genetically engineered phage to deliver antimicrobial agents to bacteria: an alternative therapy for treatment of bacterial infections. *Antimicrobial Agents and Chemotherapy*, 47, 1301-1307.
- WETZLER, C., KAMPFER, H., STALLMEYER, B., PFEILSCHIFTER, J. & FRANK, S. (2000) Large and sustained induction of chemokines during impaired wound healing in the genetically diabetic mouse: Prolonged persistence of neutrophils and macrophages during the late phase of repair. *Journal of Investigative Dermatology*, 115, 245-253.
- WILLIAMS, P., CAMARA, M., HARDMAN, A., SWIFT, S., MILTON, D., HOPE, V. J., WINZER, K., MIDDLETON, B., PRITCHARD, D. I. & BYCROFT, B. W. (2000) Quorum sensing and the population-dependent control of virulence. *Philosophical Transactions of the Royal Society B-Biological Sciences*, 355, 667-680.
- WILLS, Q. F., KERRIGAN, C. & SOOTHILL, J. S. (2005) Experimental bacteriophage protection against *Staphylococcus aureus* abscesses in a rabbit model. *Antimicrobial Agents and Chemotherapy*, 49, 1220-1221.
- WILSON, S. J., KNIPE, C. J., ZIEGER, M. J., GABEHART, K. M., GOODMAN, J. E., VOLK, H. M. & SOOD, R. (2004) Direct costs of multidrug-resistant *Acinetobacter baumannii* in the burn unit of a public teaching hospital. *American Journal of Infection Control*, 32, 342-344.
- WISPLINGHOFF, H., PERBIX, W. & SEIFERT, H. (1999) Risk factors for nosocomial bloodstream infections due to *Acinetobacter baumannii*: A case-control study of adult burn patients. *Clinical Infectious Diseases*, 28, 59-66.
- WITHINGTON, R. (2001) Regulatory issues for phage-based clinical products. *Journal of Chemical Technology and Biotechnology*, 76, 673-676.

WOMMACK, K. E., HILL, R. T., MULLER, T. A. & COLWELL, R. R. (1996) Effects of sunlight on bacteriophage viability and structure. *Applied and Environmental Microbiology*, 62, 1336-1341.

WONG, T. H., TAN, B. H., LING, M. L. & SONG, C. (2002) Multi-resistant *Acinetobacter baumannii* on a burns unit - clinical risk factors and prognosis. *Burns*, 28, PII S0305-4179(02)00012-8.

WOOD, F. M., KOLYBABA, M. L. & ALLEN, P. (2006) The use of cultured epithelial autograft in the treatment of major burn injuries: A critical review of the literature. *Burns*, 32, 395-401.

YACOBY, I., SHAMIS, M., BAR, H., SHABAT, D. & BENHAR, I. (2006) Targeting antibacterial agents by using drug-carrying filamentous bacteriophages. *Antimicrobial Agents and Chemotherapy*, 50, 2087-2097.

ZHANG, J. Z., MARUYAMA, K., IWATSUKI, K., ONO, I. & KANEKO, F. (1994) Effects of prostaglandin E1 on human keratinocytes and dermal fibroblasts: a possible mechanism for the healing of skin ulcers. *Exp Dermatol*, 3, 164-70.

Abstracts & conference proceedings

Henein, A.E., Hanlon, G.W., Maillard, J.-Y. & Denyer, S.P. 2004. Temperature dependent plaque formation in phage AP205 (HER424) and *Acinetobacter haemolyticus*. (04-GM-A-4923) ASM abstract, New Orleans (USA).

Henein, A.E., Hanlon, G.W., Maillard, J.-Y. & Denyer, S.P. (2004). Temperature dependent plaque formation in Phage AP205 (HER424) and *Acinetobacter haemolyticus*. *Journal of Pharmacy and Pharmacology*, 56, 239.

Henein, A.E., Hanlon, G.W., Maillard, J.-Y. & Denyer, S.P. 2004. BPC, Manchester, September 2004