Abundance, distribution and functional characterisation of gut-associated Type II toxin-antitoxin systems

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Abstract

Prokaryotic toxin-antitoxin (TA) systems (also known as addiction modules), are ubiquitous genetic modules first discovered due to their role in stabilising vertical transmission of plasmids. Generally they are two-gene systems encoding a stable toxin (Tx) and an unstable antitoxin (ATx). Loss of the TA module leads to rapid ATx degradation and depletion, leaving the Tx free to interact with cellular targets and inhibit growth. For plasmid encoded TA systems, this leads to the death of plasmid free daughter cells, ensuring plasmid maintenance in a population, and gives rise to the term "addiction module". More recently, the expansion in microbial genome data has highlighted the prevalence and diversity of TA systems, and demonstrated that they are common features of many bacterial chromosomes. In addition, metagenomic surveys have pointed to the enrichment of some TA families in particular microbial ecosystems; a prime example from surveys of the human gut microbiome and RelBE TA family. Collectively, these observations indicate a wider role for TA modules in bacterial function, with numerous roles for TA systems now hypothesised. These include: i) Stabilisation of TA associated chromosomal DNA during vertical transmission; ii) Formation of "persister" cells resistant to environmental stresses, and; iii) Population level resistance to bacteriophage attack. Additionally, some Tx components have shown activity in eukaryotic cells, raising the potential for a role in prokaryote-eukaryote interaction. Here we undertook a systematic study of Type II TA systems, to provide a comprehensive assessment of their distribution and relative abundance, to confirm activity of prevalent TA systems, and to understand putative roles these may play in gut associated bacteria and the gut microbiome.

A comparative genomic and metagenomic analysis of 3919 bacterial chromosomes, 4580 plasmids, 711 bacteriophage genomes, and 781 metagenomes encompassing 16 distinct habitats was conducted using all known Type II TA systems present in the Toxin Antitoxin Database (~10,100 TA genes ~1:1 Tx:ATx). Of the 817 Type II TA system homologues found in human gut datasets, 686 were observed to have significantly higher relative abundance in the human gut microbiome over other microbial ecosystems. In parallel to these in silico findings, PCR and qPCR surveys of microbiomes from 65 stool samples obtained from healthy volunteers, as well as those with polyps or colorectal cancer, were undertaken. This demonstrated a higher ATx presence than Tx or complete module, however no differences in Tx copy number between health groups was seen.

To confirm the activity of the most abundant TA system homologues identified in sequence surveys, ORFs were amplified from gut metagenomic DNA, and individual Tx or ATx cloned under the control of inducible promoters. Induction of Tx expression under normal growth conditions resulted in bacterial growth inhibition, while live dead staining showed entry into a viable but non-cultivable state, commensurate with TA function. Experiments simulating environmental stresses encountered during colonisation of the GI tract (starvation, low pH, bile), indicated that expression of these TA systems could increase cell survival when carbon or nitrogen availability was limited (starvation). Since antibiotics are also commonly encountered by gut associated-bacteria (both as residents of the GI tract and during colonisation of other body sties) a role for gut associated TA systems in facilitating survival during antibiotic exposure was also explored. This revealed an increased number of cells surviving two hours post-treatment with β-lactams when Tx genes were expressed, and in keeping with an impact on cell growth.

To test the hypothesis that TA systems may stabilize associated regions of DNA, the composition of gene neighbourhoods surrounding TA systems were also explored. ORFs surrounding TA system homologues identified in metagenomic and genomic datasets were identified using the Metagene annotator, and ORF functions predicted based on searches of the Clusters of Orthlogous Groups (COG) database. This revealed significant increases in ORFs with functions related to replication/recombination/repair and those with unknown functions. It also identified a decrease in the proportion of ORFs encoding functions such as carbohydrate and lipid transport and metabolism in regions surrounding TA systems, suggesting involvement with stabilization of mobile elements.

Finally, we explored the potential for gut associated TA systems to modulate phage-microbe, and host-microbe interactions. In the case of phage-host interactions, TA systems have previously been shown to function as mediators of phage resistance at the population level, by directing cells towards a dormant state which prevents phage replication, and permits a sub-set of cells to survive phage attack. Our findings indicated the potential for gut associated TA systems to provide some degree of protection during particular host-phage interactions, but specific modules did not provide universal protection against phage. In the case of host-microbe interaction, some Type II TA system Tx components have been shown to be functional in cultured eukaryotic cells, promoting apoptosis when introduced and expressed in these cell types. However, no studies to date have examined the potential for bacterially expressed TA systems to influence eukaryotic cell health in co-culture models. To investigate this, we assessed the impact of bacterial TA system expression on the health of the intestinal epithelial cell line Caco-2 in co-culture systems specifically focusing on cell apoptosis and necrosis whilst in the presence of *Escherichia coli* expressing p22-RelBE.

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Authors 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 yet been previously submitted to this or any other university for a degree, and does not incorporate any material already submitted for a degree.

Signed:

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Abbreviations and definitions

.CSV	Comma separated file
(p)ppGpp	Guanosine pentaphosphate
aa	Amino acid
Abi	Abortive infection
ACLAME	A classification of mobile genetic elements database
AGJ	Artificial gastric juice
Am	Ampicillin
ANOVA	Analysis of variance
AP	Adenomatous polyposis
ASL	Average sequence length
AT	Annealing temperature
ATP	Adenosine Triphosphate
ATx	Antitoxin
BI	Broad Institute
BSA	Bovine serum albumin
BSH	Bile salt hydrolases
c-di-GMP	cyclic diguanylate
CAMERA	Community cyber-infrastructure for advanced Microbial research and analysis
CD	Crohn's disease
CD	Conserved domains
CDD	Conserved domains database
CDMEM	Complete DMEM
cDNA	Complimentary DNA
CFU	Colony forming units
COG	Clusters of orthologous groups
CRC	Colorectal cancer
CRISPR	Clustered regularly interspaced short palindromic repeats
DDBJ	DNA Databank of Japan

DDBs	Double stranded breaks
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Dinucleoside triphosphate
E.coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
EMBL	European molecular biology database
EtBr	Ethidium Bromide
ExPEC	Extra-intestinal pathogenic strain E.coli
FBS	Fetal bovine serum
FEG	Field emission gun
FOV	Field of view
Gastro-intestinal	GI
gDNA	Genomic DNA
GFP	Green fluorescent protein
GNAT	GCN5-Related N-Acetyltransferases
HMCJ	Human metagenome consortium Japan
HMP	Human Microbiome Project
Ho	Null hypothesis
HPI	Hoechst 33342-PI staining
HTH	Helix-turn-helix
INT	Tetrazolium salt
IPTG	Isopropyl β-d-thiogalactopyranoside
Km	Kanamycin
LB	Luria Bertani
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
M.tuberculosis	Mycobacterium tuberculosis
MGE	Mobile genetic elements
MIC	minimum inhibitory concentration

MOI	Multiplicity of infection
mRNA	Messenger Ribonucleic acid
NAG	N-acetylglucosamine
NAM	N-acetylmuramic
NCBI	National Center for Biotechnology Information
NEAA	Non-essential amino acids
NFW	Nuclease free water
nPFU	Normalised PFU (by CFU)
O/N	Overnight
OD	Optical density
ORF	Open reading frame
p22	pTRACA22
PBP	Penicillin binding protein
PBS	Phosphate buffer saline
PCD	Programmed cell death
PCR	polymerase chain reaction
PEG	Polyethylene glycol
PERL	Practical extraction and report language
PFU	Plaque forming units
PGSR	Phage genome signature-based recovery
PI	Propidium iodide
PSK	Post segregational Killing
qPCR	Quantitative polymerase chain reaction
RBS	Ribosomal binding site
REDDB	Resistance determinants database
RFLP	Restriction fragment length polymorphisms
RHH	Ribbon-helix-helix
RLU	Relative light units
RNA	Ribonucleic acid
ROI	Region of interest
RPM	revolutions per minute

rRNA	ribosomal Ribonucleic acid
RT	Room temperature
RTPCR	Reverse transcription PCR
S.Typhimurium	Salmonella Typhimurium
SEM	Standard error of mean
SGFM	Serum and glucose-free DMEM
SOC	Super optimal broth with catabolite repression
sRNA	Small Ribonucleic acid
STEM	Scanning transmission electron microscope
TADB	Toxin-antitoxin Database
TAE	Tris acetate
TB	Tris buffer
TE	Tris-Cl EDTA
TA	Toxin-antitoxin
TRACA	Transposon aided capture analysis
tRNA	Transfer Ribonucleic acid
TSA	Tryptone soya agar
TSB	Tryptone soya broth
Tx	Toxin
UDP	Uridine diphosphate
UPEC	Uropathogenic E.coli
UT	Urinary tract
UTI	Urinary tract infection
UV	Ultraviolet
VF	Virulence factors
VFDB	Virulence factor database
VLPS	Virus like particles
WT	Wild Type
X-Gal	5-bromo-4-chloro-3-indolyl β-d-galactopyranoside
σs	Sigma factor

Chapter 1: General introduction

1.1 The human gut microbiome

Humans are considered to be germ-free until the point of birth where they encounter and acquire their first microbes as they pass through the birth canal and subsequent exposure to the outside environment (Dominguez-Bello *et al.*, 2011). After initial colonisation of the body, continued development of the ecosystem and microbial community occurs, leading to complex communities establishing on many body sites including the oral cavity, skin and the gastro-intestinal (GI) tract. These communities can be extremely large with human microbiome collectively believed to have approximately 100 times the number of genes we have in our genome (Ley *et al.*, 2008, Qin *et al.*, 2010). The mammalian microbiota is thought to have co-evolved over millennia with the host, leading to a symbiotic relationship with mutual benefits and playing a critical role in the maintenance of mammalian, and subsequently, human health (Dethlefsen *et al.*, 2007, Ley 2008, Wallace *et al.*, 2011).

Of the numerous body sites colonised by our microbiota the GI tract is home to the largest with the average adult distal colon harbouring an estimates 10^{13}-10^{14} prokaryotic cells belonging to between 150-800 species (Qin *et al.*, 2010, Tap *et al.*, 2009, Gill et al., 2006). Often referred to as an organ of the human "supra-organism", the gut microbiome undertakes a number of beneficial functions and helps shape the development of host physiology. These functions include the salvage of energy from the diet through fermentation of inaccessible dietary waste, protection against pathogens through competitive exclusion or active antagonism, maturation of the immune system, maintenance of epithelial homeostasis, brain development, protection from enteropathogenic infections and maintenance of gut

motility (Wilks 2007, Martin *et al.*, 2009, Turner 2009, Samual *et al.*, 2008, Quigley 2011, Tlaskalova-Hogenova *et al.*, 2011). These bacterial residents benefit in turn through a near constant supply of nutrients as well as a relatively stable environment (Ley *et al.*, 2008). With so many vital processes undertaken by the human gut microbiome, there has understandably been a push for a greater understanding of: phylogenetic distribution of species, interactions within the community and also interactions between the host and microbial community. The more recent arrival of gene-sequence based approaches have allowed genomic and metagenomic analysis and characterisation of the gut microbial community, leading to a more comprehensive understanding of this ecosystem including relationships which significantly affect many aspects of normal mammalian physiology (Dethlefsen *et al.*, 2007).

1.2 Toxin-antitoxin systems

Investigating the mobile metagenome of the human gut microbiome, Jones *et al.*, (2010) utilised the culture independent transposon aided capture analysis (TRACA, Jones & Marchesi 2007) to isolate novel plasmids from the human gut microbiota and analysed relative abundance of encoded ORFs and putative functions in a range of metagenomes from distinct environments. Of the functions analysed, one of the most prevalent was a putative RelBE toxin-antitoxin module located on the plasmid pTRACA22, which was most closely related to the *Firmicutes*.

Toxin-antitoxin (TA) systems are widespread throughout the prokaryotic kingdom, demonstrating huge diversity on both a structural and functional level (Pandey & Gerdes 2005). Generally these systems encode two components; an

unstable antitoxin which neutralises a relatively stable toxin. The first TA system was described 33 years ago due to its ability to stabilise the F plasmid during vertical transmission through the mechanism known as post segregational killing (PSK) (Ogura & Hiraga 1983, Gerdes *et al.*, 1986). PSK is achieved by eliminating daughter cells, which do not inherit a copy of the plasmid containing the TA module during division. This mechanism relies heavily on the differing stability of the toxin and antitoxin components of the module. For daughter cells, which have not inherited a copy of the plasmid and therefore the TA module, the individual toxin and antitoxin are degraded, with a more rapid depletion of the antitoxin due to the lesser stability of the module. Figure 1.1A depicts the PSK system. This eventually leads to freeing of the stable toxin within the cytoplasm and death of the plasmid-free cell. Plasmid-encoded TA modules have also regularly been referred to as "addiction" modules because this process renders the cell population addicted to antitoxin production and thus the TA genes (Yarmolinsky 1995).

It has recently been suggested that to qualify as a TA system, specifically for Type II systems (discussed in detail below), that several common traits are required to qualify as a TA module (Gerdes 2013). For the purposes of studies undertaken here, these traits will be the working definition used:

1) Two genes adjacent or overlapping with each other, encoding a labile antitoxin and a relatively stable toxin.

2) The antitoxin auto-represses TA system transcription with co-repression of transcription by the toxin.

3) TA operon transcription is regulated by conditional cooperativity, which is inhibited by excess toxin.

4) Present on both prokaryotic chromosomes and mobile genetic elements with extremely broad phylogeny based on toxin sequence similarity.
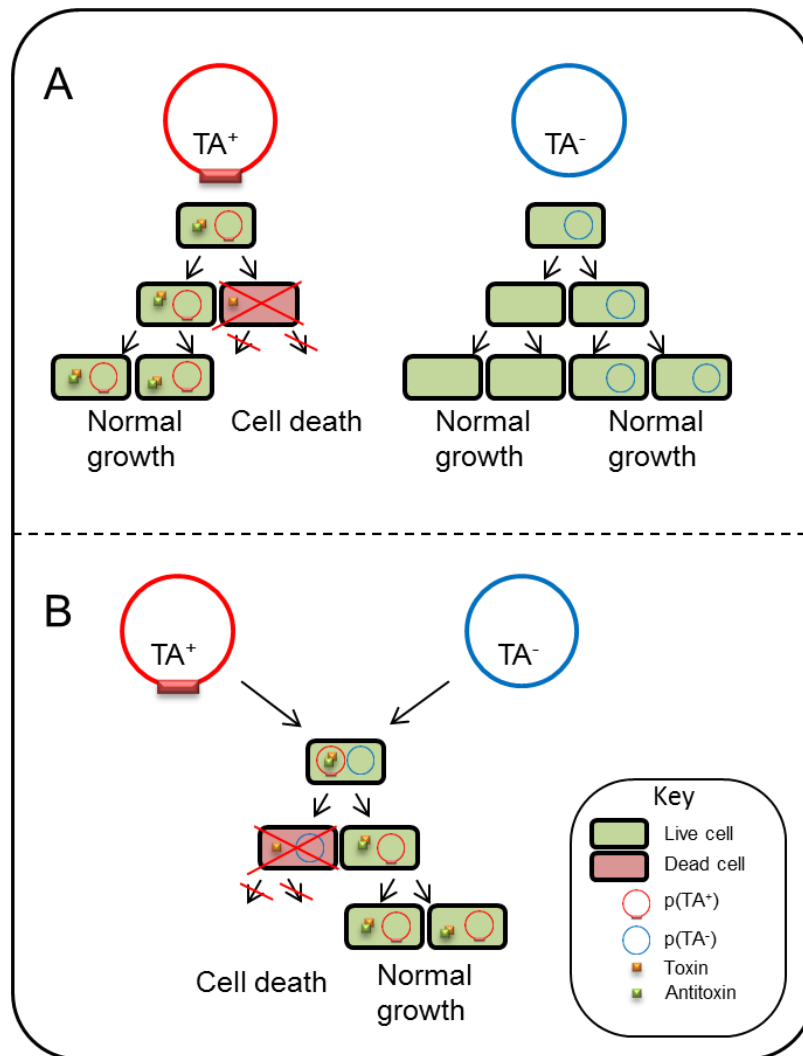
Figure 1.1: The plasmid-encoded TA module addition effect and inter-plasmid competition. Adapted from Van Melderen & De Bast 2009.

(A) Plasmid encoded TA modules increase the prevalence of plasmids in bacterial populations by PSK. Those daughter cells which inherit the plasmid grow normally after cell division whilst degradation of the antitoxin by ATP-dependent proteases (usually Lon) enable free toxin to interact with its intended cellular target leading to selective killing of plasmid free bacteria.

(B) When plasmids belong to the same incompatibility group, those harbouring TA modules will outcompete those without through PSK. Conjugants containing both plasmids from the same group cannot be maintained and the loss of one subsequently occurs. If the plasmid carrying a TA module is lost the cell is killed and only those that do not lose the TA-carrying plasmid will survive and persist in a population.

Of course, exceptions to the norm exist with some TA families not fulfilling all criteria. An example of this being the inclusion of a third toxin component within a TA system with regulatory functions such as the MazF homologue in *Myxococcus xanthus* described by Nariya & Inouye (2008), where a general transcription factor, MrpC, involved in developmental regulation behaves as a transcriptional activator. Three gene systems do not contradict the above criteria and in fact appear to be more widespread than previously thought based on recent studies (Smith & Rawlings 1997, Hallez *et al.*, 2010, Bordes *et al.*, 2011). Another exception is the ccdA/B family having a comparatively narrow host phylogeny when compared to other systems (Wilbaux *et al.*, 2007).

1.3 TA families, characteristics and classification

Since the initial identification of TA systems in prokaryotes; 11 Type I, 44 Type II, 3 Type III, 3 Type IV and 1 Type V confirmed TA pairs have been identified with many more individual putative TA genes identified (Gerdes 2013, Tan *et al.*, 2011, Wang *et al.*, 2013). In 2003 Hayes suggested a method for classification of TA systems based on the nature and mechanism of action of a given antitoxin. At the time this allowed TA modules to be divided into two distinct families (Types I & II) based on the nature of the antitoxin component and the mechanism of toxin neutralisation. More recent discoveries in the field have allowed identification of a further three Types (III, IV and V).

1.3.1 Type I
For Type I TA systems, gene expression of the toxin is downregulated by base pairing of antisense sRNAs that inhibit translation of a toxin encoding mRNA (Brand 2012). These sRNA antitoxins are often co-transcribed with the toxin gene, with large overlaps occurring at the 5' or 3' ends of the transcript and in reverse

orientation (Fozo *et al.*, 2008). In some cases they have been shown to be transcribed divergently but both genes still remain within the same intergenic regions with approximately 20 nucleotides of contiguous complimentary sequence (Fozo *et al.*, 2007). The antisense RNA antitoxin then anneals to the mRNA of the toxin, firstly inhibiting translation by preventing complex formation with the ribosome and secondly by forcing conformational changes which destabilise the toxin mRNA allowing degradation by RNAse activity (Fozo *et al.*, 2007). All toxins of Type I TA systems consist of very small hydrophobic polypeptides, usually 60 amino acids (aa) or less and so far all characterised in detail have a highly similar α-helical secondary structure (Altuvia 2007). These appear to be little more than a transmembrane domain with either a short N-terminus or C terminus tail and are predicted to reside and form pores in the inner membrane inhibiting ATP synthesis by destroying membrane potential (Livny & Waldor 2007, Henriques *et al.*, 2006).

The first Type I TA system to be discovered was the hok/sok module of the *E.coli* plasmid R1, identified due to its ability to stabilise heterologous replicons within a population (Gerdes *et al.*, 1986a). It was found that this module increased plasmid stability more than 100-fold through the mechanism of PSK described above and work done on hok/sok elucidated many of the key principles which guided further study of specifically, Type I TA systems, but also TA loci in general (Gerdes *et al.*, 1986a).

The principle components of the hok/sok system are (i) the hok mRNA, which encodes two genes; the toxin 'host killing – hok', 'modulation of killing – mok' which overlaps extensively with the former and is required for its translation and (ii) 'suppression of killing – sok' a cis-encoded antitsense RNA that blocks translation of mok (Gerdes *et al.*, 1986b). Sok-RNA is transcribed from the opposite strand to the two other genes involved and is complimentary to the mok

initiation region enabling the sRNA antitoxin to indirectly inhibit toxin translation through the regulator. Sok RNA and hok mRNA form a duplex, which is rapidly cleaved by RNase III (Gerdes *et al.*, 1992). As with most TA systems the antitoxin, in this case sok, is extremely unstable with a half-life of approximately 20 seconds. However, transcription of sok is driven by a strong promoter, whilst transcription of the toxin (hok) is driven by a weak promoter but has a half-life of 20 minutes (Gerdes & Wanger 2007). Previous to this, it was found that the sok RNA is in considerable molar excess to the hok mRNA with plasmid free cells showing the TA system-characteristic rapid depletion of sok, leaving an unregulated pool of hok mRNA which can then be translated (Gerdes 1990).

1.3.2 Type II and the pTRACA 22-RelBE module

The Type II TA systems are the most extensively studied class of TA modules and examples of these include MazEF, RelBE and YefM-Yeo. All modules within this class have a protein antitoxin which forms a protein-protein complex with the toxin resulting in neutralisation of its cellular effects (Gerdes 2013). As with all known TA modules the toxin is relatively stable whilst the antitoxin is highly vulnerable to degradation by Clp and Lon proteases (Lehnherr & Yarmolinsky 1995, Cherny & Gazit 2004, Diago-Navarro *et al.*, 2013, Smith & Rawlings 1998, Roberts *et al.*, 1994). The genetic arrangement of these operons is most often found to have the antitoxin open reading frame (ORF) upstream and slightly overlapping by between 6-20bp the toxin ORF (Yamaguchi & Inouye 2009). An exception to this rule is higBA module where the toxin, higB, is located upstream of the higA antitoxin (Tian *et al.*, 2001).

Regulation of Type II systems tends to be done at a transcriptional level by both free antitoxin and the toxin-antitoxin complex, with different ratios of both components within the complex altering the affinity for the promoter and effectivity

of regulation (Bukowski *et al.*, 2011, Overgaard *et al.*, 2009). As with Type I, Type II TA systems were initially discovered because of their ability to promote plasmid maintenance in growing populations by selectively killing plasmid free daughter cells through the mechanism of PSK (Ogura & Hiraga 1983). The first Type II loci identified through a role in PSK was Ccd_F. The CcdB toxin component of Ccd_F targets and inhibits DNA gyrase, one of the four topoisomerases found in bacteria and an essential enzyme for the regulation of DNA topology (Collin *et al.*, 2011).

Within this family of TA modules, three-component systems have also been reported such as ω-ε-ζ family encoded by *Streptococcus pyogenes* plasmid pSM19035 (Camacho *et al.*, 2002). In this module the regulator, ω, regulates the system expression through binding to the promoter (Meinhart *et al.*, 2003, Zielenkiewicz *et al.*, 2009). In addition, the PaaR-PaaA-ParE TS system in *E.coli* is also a 3-gene system however in contrast to above the toxin-antitoxin complex as well as the regulator is involved in transcriptional regulation (Smith & Rawlings 1997, Smith & Rawlings 1998). Initially Type II TA systems were placed into 14 superfamilies based on sequence similarity and gene structure (Pandey & Gardes 2005, Park *et al.*, 2013) however many hybrid systems (containing cross-family toxins and antitoxins together) have since had their functionality investigated and subsequently confirmed. More recently it has now been suggested that within Type II modules both the antitoxin and toxin should be classified separately. So far there have been 20 antitoxin and 13 toxin superfamilies proposed (Leplae *et al.*, 2011, Gugielmini & Van Melderen 2011).

As the field of toxin-antitoxin module study has grown and with the expansion of sequenced prokaryotic genomes available, an even greater expansion of putative TA genes has taken place. It has become apparent that these modules are ubiquitous throughout prokaryotic genomics. TA modules (often

in multiple copies) exist in genomes of free living-slow growing species, obligatory intracellular species, pathogens, commensals and even extremophile archaeal species (Pandey & Gerdes 2005). There is also increasing evidence that TA modules move between genomes through horizontal gene transfer with the distribution varying greatly even between isolates of the same species, implying that TA modules are highly mobile. With modern advances in sequencing technology and the introduction of new genomic and metagenomic analysis, there is now access to the uncultured fraction of environmental communities revealing genes, novel association between these and functions previously hidden by the necessity for culture.

Previously, TA modules have been identified through extensive experimental validation however Leplae *et al.*, (2011) undertook a set of comprehensive searches of 2181 prokaryotic genomes using 24 toxin and counterpart antitoxin sequences. This revealed (i) almost all toxin and antitoxin super-families identified to date originate from distinct ancestors and (ii) that TA modules found on plasmids appear to be both promiscuous and widespread suggesting movement through horizontal gene transfer supporting previous experimental work (Leplae *et al.*, 2011).

As mentioned above, investigations by Jones *et al.*, (2010) revealed a putative RelBE toxin-antitoxin module to be prevalent through another culture independent technique. Furthermore it was also revealed in this study through phylogenetic analysis that the distribution of the RelE toxin was found throughout all major bacterial phyla however absent from gut-associated archaeal species. In this same study RelBE relative abundance was compared to that of the Type II MazEF and ParDE and higAB modules across the human gut, murine gut, soil and marine environments. Firstly RelBE homologues were shown to be much more

abundant in the human gut in comparison to other environments and secondly that it was also significantly more abundant that the other three modules in the human gut environment.

The *relE* gene and the subsequent full module of *Escherichia coli* K-12 was first recognised as the toxin component of a TA locus in 1998 (Gotfredson & Gerdes 1998) with homologues of this module identified a year later (Gronlund & Gerdes 1999), with at least seven now identified within the genome of K-12 (Christensen-Dalsgaard *et al.*, 2010). The relE gene is located immediately downstream of the relB, as with many Type II TA modules (Gerdes 2013). Originally identified through single point mutations in RelB (now known to be the antitoxin) affecting the 'delayed-relaxed response', where synthesis of stable RNA stops for about 10 hours after the onset of the starvation activated stringent response, and then continues after this period (Bech *et al.*, 1985). *E.coli* responds to amino acid (aa) starvation by the synthesis of (p)ppGpp, which initiates the stringent response helping with the nutritional stress. One major function of this is the prevention of general rRNA transcription leading to a reduction in use of charged tRNAs and stimulation of the biosynthetic pathways instead (Magnusson *et al.*, 2005). Additionally relB mutants had and significantly extended growth lag when introduced into rich media again suggesting activation of a translational inhibitor (the toxin relE, Christensen & Gerdes 2004).

A protein like all other TA module toxins, RelE toxin is a ribosome-dependent mRNAse cleaving mRNA positioned at the ribosomal A-site, between the second and third nucleotide (Pedersen *et al.*, 2003). It has been shown to cleave all codons with preference to those with Guanine at the 3rd position and has no activity independent of ribosomes. (Christensen & Gerdes 2003). Whilst structurally similar to other bacterial RNAses, relE lacks a conserved aa which

gives RNase activity (Li *et al.*, 2009). However when bound to the ribosomal A-site, a slight conformational change occurs which when combined with the induced conformational change of mRNA, it promotes cleavage (Neubauer *et al.*, 2009). RelE cleavage leads to stalled ribosomes, unable to finish processing the mRNA and trapping it. This also leads to ribosomes upstream also translating the strand of mRNA to stall (Ivanoa et al., 2005, Keiler 2008).

The *relBE* promoter (P*relBE*), a single promoter which leads to transcription of both genes, is regulated through negative feedback with free RelB antitoxin repressing transcription (Gotfredsen & Gerdes 1998). On its own RelB is a dimer which interacts with operator DNA through its N-terminal ribbon-helix-helix (RHH) domain (Overgaard *et al.*, 2009) however when in the presence of the RelE toxin, is able to form a complex with this further increasing the ability of the complex to bind (Li *et al.*, 2009). There is some debate as to whether the repressor complex stoichiometry is $RelB_2RelE_2$ (Li *et al.*, 2008) or whether it is $RelB_2RelE$ (Overgaard *et al.*, 2008) however regardless of this, all agree that the antitoxin-toxin complex greatly increases binding and the subsequent level repression. Regulation of RelB antitoxin levels is achieved through Lon Protease degradation similar to 10 other Type II modules in the genome of *E.coli* K-12 hinting at the possibility of a common cellular mechanism controlling at least Type II TA loci (Christensen *et al.*, 2001, Wang *et al.*, 2011, Jorgensen *et al.*, 2009). The RelE toxin when in complex with the antitoxin can be released and become active again (Christensen *et al.*, 2001). It has been suggested this is down to Lon protease able to gain access, releasing RelE through degradation of RelB however no direct evidence exists for this yet (Gerdes 2013). This apparent global regulation of many Type II antitoxins may suggest functions all undertake, such as escape from amino acid starvation.

The abundance of the putative pTRACA22-RelBE module within the human gut microbiome may be related to addictiveness of TA systems however it has also been hypothesised that it may be due to the module having a function (or several) within this environment (Jones 2010), as in section 1.5.

1.3.3 Type III

As with Type I systems, the antitoxins of Type III modules are sRNAs, however unlike Type I systems, the mode of toxin neutralisation is different. The discovery of Type III TA modules is relatively recent. They were first identified following studies of the *toxIN* locus that was identified on a cryptic plasmid of *Pectobacterium atrosepticum*; strain SCRI1039 (Fineran *et al.*, 2009). So far three distinct families have been found in the genomes of distantly related bacterial species, *toxIN*, *cptIN* and *tempIN* and all have had their functionality confirmed within *E.coli* (Blower *et al.*, 2012). Furthermore, they have been identified on chromosomes, plasmids and even a bacteriophage genome suggesting widespread distribution (much like Types I and II) and evidence suggestions a role in providing bacteriophage resistance (Blower *et al.*, 2012).

Unlike Type I modules where the antitoxin and toxin RNAs interact together, what defines Type III is the interaction directly between the RNA antitoxin and proteinaceous toxin (Blower *et al.*, 2011a) The *toxN* gene is preceded by a short inverted repeat, which behaves as a terminator for regulating the amount of antitoxin sRNA and toxin mRNA, and several direct repeats which are the target within the *toxIN* transcript for RNAse activity of the ToxN protein. This leads to the release of the active RNA antitoxin (Blower *et al.*, 2011b, Short et al., 2013). Specifically it was later revealed by structural studies that the macromolecular complex formed pseudoknots of antitoxin RNA that coils around the endoribonuclease toxin inhibiting it (Blower *et al.*, 2011b).

The ToxIN system was found to confer bacteriophage resistance by carriage of this module, specifically because it behaved as an abortive infection system (Abi, Blower *et al.*, 2012). An Abi system is any process that enables a cell to undergo cellular suicide to protect the larger population from phage infection (Chopin *et al.*, 2005). Previously other types of TA modules, such as the *hok/sok* and *mazEF* systems (Pecota & Wood 1996, Hazan & Engelberg-Kulka 2004), have been shown to confer a degree of phage resistance however that provided by ToxIN appears to be much greater and can affect a wide range of phage across a number of different bacterial backgrounds (Blower *et al.*, 2012). Notably the Abi phenotype has also been identified TenpIN systems suggesting this function may be a general to Type III TA modules (Blower *et al.*, 2012).

1.3.4 Type IV

The yeeU/yeeV Type IV TA system was initially discovered in *E.coli* and was identified as the first toxin to interfere with cell morphology and division (Tan *et al.*, 2011). More specifically, interrupting the polymerisation of cytoskeleton proteins MreB and FtsZ, the prokaryotic homologues of actin and tubulin (Van den Ent *et al.*, 2001), both of which are essential for cellular morphology. MreB is responsible for the maintenance of cell shape and polarity in rod-shaped bacteria, chromosomal segregation and coordination of cell biosynthesis and division (Wachi *et al.*, 1987, Kruse *et al.*, 2005 Garner *et al.*, 2011). FtsZ forms ring structures within the cell, which act as scaffolds during the assembly of multi-protein complexes required for cell division.

The yeeU antitoxin, is a protein and supresses the activity of yeeV by stabilising the target MreB/FtsZ proteins. Interestingly the presence of yeeV actively enhances the bundling of both (Masuda *et al.*, 2012). In addition it appears that yeeU also protects from other inhibitors of MreB/FtsZ proteins such as S-(3, 4-

dichlorobenzyl)isothiourea (Iwai *et al.*, 2002) and MinCD (Pichoff & Lutkenhaus 2001), suggesting global stabilisation of these proteins. This is the first (and to date only) report of an antitoxin of a TA module having a role outside of neutralising TA toxicity, providing evidence of further TA functionality than has been previously been hypothesised.

1.3.5 Type V

Most recently, Type V TA systems were proposed by Wang *et al.*, (2013) demonstrating a proteinacious antitoxin which behaves as an endoribonuclease with high specificity, targeting the counterpart toxin mRNA. Designated GhoT/GhoS or GhoST because the toxin behaves as a membrane lytic peptide which causes ghost cell formation (lysed cells with severely damaged membranes) this is the only module within this type. With the exception of verifying the individual components function (within the confines of a TA-specific functions), tertiary structural analysis of the antitoxin and antitoxin activity; very little is known of this modules effect on a cellular level (Wang *et al.*, 2013).

In addition to all Types mentioned above toxin-antitoxin shuffling from different families and even Types probably occurs with evidence for high 3D structural similarities between ToxN of the Type III ToxIN system and MazF toxin of the Type II MazEF system recently being found (Blower *et al.*, 2011b).

1.4 Cellular targets of TA module toxins

The toxin components of TA modules have been shown to have a very wide range of cellular targets including metabolic processes as well as structures. Whilst not encompassing all known toxins and their targets, Table 1.1 lists many of those from well-characterised TA modules. Most well characterised TA toxins, especially

those classified as Type II, act as translational inhibitors effecting protein biosynthesis with examples of these including MazF (Zhang *et al.*, 2003) and MsqR (Yamaguchi *et al.*, 2009), which cleave free mRNA with preference for specific codons. In addition other Type II systems whilst also translational inhibitors interrupt this process through binding to ribosomal subunits and forcing conformational change (Liu *et al.*, 2008), blocking one or more sites within the ribosome and cleaving mRNA or tRNA when in complex with the ribosome (Christensen & Gerdes 2003, Winther & Gerdes 2011).

Interestingly MazF has also been shown to function in a secondary manner, targeting the 16s rRNA within the 30S ribosomal subunit, removing 43 nucleotides which contain the complimentary sequence for the Shine-Dallgarno, required for initiation of translocation. Furthermore, a mycobacterial homologue of this toxin has been shown to target a region of the 23S rRNA in a similar fashion destabilising the 30S/50S subunits and inhibiting translation (Vesper *et al.*, 2011). The structural similarities between MazF and ToxI suggest this Type III module toxin functions in a comparable way (Blower *et al.*, 2011).

Replication can also be a target for TA toxins with both the CcdB and ParE Type II toxins targeting this process, specifically by inhibiting DNA gyrase, essential for regulating DNA topology (Collin *et al.*, 2011). For CcdB this is achieved through binding to DNA gyrase whilst in its open conformational state (prior to the binding of DNA and subsequent passage) inhibiting regulation and forming a CcdB-DNA-DNA gyrase complex eventually leading to cell death (Maki *et al.*, 1992, Dao-Thi *et al.*, 2005). Whilst achieving the same effect overall, cell death, the ParE toxin appears to function entirely differently to CcdB which is supported by both proteins have completely different tertiary folding and belonging to two different super families (Leplae *et al.*, 2011). ParE is toxic due to the inhibition of DNA-gyrase-

mediated supercoiling and the introduction of double stranded breaks (DSBs) in the DNA (Jiang *et al.*, 2002).

Less commonly the cell wall and inner membrane have also been shown to be targeted by TA toxins. The ζ toxin of ω-ε-ζ phosphorylates UDP-N-acetylglocosamine, a precursor of peptidoglycan inhibiting the MurA enzyme and its activity catalysing the initial step of peptidoglycan synthesis, impairing cell wall formation (Mutschler *et al.*, 2011). Many Type I modules encode small hydrophobic proteins which introduce pores into the inner membrane which subsequently interferes with ATP synthesis due to the loss of membrane potential (Gerdes et al., 1986, Unoson & Wagner 2008, Brantl 2012). Hok and TisB are both Type I modules, listed in table 1.1, which behave in this manner. Similarly, whilst the target for the Type V GhoT toxin has not been investigated in depth, it is a small hydrophobic protein and has two transmembrane proteins predicted with expression of the toxin causing cell populations to lyse with damaged membranes (Wang *et al.*, 2012). It is highly likely that this toxin functions in a similar way to both Type I toxins mentioned above.

1.5 Biological roles of TA systems and relevance to the human gut environment

The roles of TA systems appear to be highly diverse and dependent on several factors such as host species, genomic location and external environments. While several roles are unsupported as of yet, other functions now appear, with the strong body of evidence supporting them, to be accepted fact.

Table 1.1: A range of TA module toxins, the counterpart antitoxins and the cellular targets of each.

Toxin	Antitoxin	Type	Toxin activity	Cellular process	Ref
Hok	Sok	I	Integrates into the inner cell membrane	ATP synthesis	Gerdes *et al.*, 1986
TisB	IstR-1	I	Integrates into the inner cell membrane	ATP synthesis	Unoson & Wagner 2008
SymE	SymR	I	mRNA cleavage	Translation	Kawano *et al.*, 2007
CcdB	CcdA	II	Inhibition of DNA gyrase	Replication	Maki *et al.*, 1992
ParE	ParD	II	Inhibition of DNA gyrase	Replication	Jiang *et al.*, 2002
MazF	MazE	II	Ribosome-independent mRNA cleavage, Cleavage of 16s rRNA	Translation	Zhang *et al.*, 2003, Vesper *et al.*, 2011
MazxF-mt6	MazE-mt6	II	Ribosome-independent mRNA cleavage, Cleavage of 23s rRNA	Translation	Schifano *et al.*, 2013
Kid	Kis	II	Ribosome-independent mRNA	Translation	Munoz-Gomez *et al.*, 2005
HicA	HicB	II	Ribosome-independent mRNA	Translation	Jorgensen *et al.*, 2009
RelE	RelB	II	Cleavage of ribosome-bound mRNA	Translation	Christensen & Gerdes 2003
VapC	VapB	II	Cleavage of tRNA	Translation	Winther & Gerdes 2011
Doc	Phd	II	Binds to 30S ribosomal subunit	Translation	Liu *et al.*, 2008
RatA	RatB	II	Binds to 50S ribosomal subunit	Translation	Zhang & Inouye 2011
HipA	HipB	II	Phosphorylation of EF-Tu	Translation	Schumacher *et al.*, 2009
ζ	ε	II	Phosphorylation of UDP-N-acetylglucosamine	Peptidoglycan synthesis	Mutschler *et al.*, 2011
ToxN	ToxI	III	RNA cleavage	Translation	Short *et al.*, 2009
YeeV	YeeU	IV	Inhibition of FtsZ and MreB polymerisation	Cytoskeleton	Masuda *et al.*, 2012
CptA	CptB	IV	Inhibition of FtsZ and MreB polymerisation	Cytoskeleton	Masuda *et al.*, 2012
GhoT	GhoS	V	Integration into inner cell membrane	ATP synthesis	Wang *et al.*, 2012

Modified from Unterholzner *et al.,* 2013.

1.5.1 DNA stabilisation

Plasmid stabilisation through PSK is well documented as a primary function of TA systems and would allow retention of plasmids (and other MGE) within the gut microbiome in the absence of direct selective pressures, which they encode (Jones & Marchesi 2007). Similarly TA modules encoded on chromosomes may stabilise neighbouring regions in a comparable fashion allowing transiently important functions, such as those required for colonisation or community development, to persist in the ecosystem (Pandey & Gerdes 2005).

1.5.2 Stress tolerance

Type II TA modules have consistently been associated with persistence and the survival of environmental stress which include nutrient deprivation and antimicrobial treatments, both of which are likely to be encountered during colonisation of the human gut. They were first identified as important in persistence through the HipAB module with expression of this module in the presence of certain antimicrobials leading to a portion of the population becoming metabolically inactive and resistant to the treatment (Moyed & Bertrand 1983). This would have significant implications for microbial gut community in terms of recovery following a period of antibiotic exposure.

This has since expanded to many other modules including the RelBE, MazEF, TisAB, MqsRA, VapBC and many homologues of these dispersed throughout prokaryotic genomics (Jayaraman 2008, Courcelle *et al*., 2001, Dorr *et al*., 2010, Unoson & Wagner 2008, Correia *et al*., 2006, Shah *et al*., 2006, Cooper et al., 2009). The MazEF module has been implicated in survival of bacterial populations to stressful conditions such as amino acid starvation, short-term

antibiotic treatments and high temperature by causing cellular suicide (Hazan *et al.*, 2004, Amitai *et al.*, 2004). This leads to approximately 95% of the bacterial cells eventually lysing and is proposed to provide nutrients for the remaining cells (Engelberg-Kulka *et al.*, 2006). Alternatively the RelBE loci of *E.coli* has been observed and described in detail inhibiting cell growth during amino acid starvation through reduction of translation without necessarily leading to cell death (Christensen *et al.*, 2001). This inhibition of cell growth allows cells to enter a stasis like phenotype, remaining viable until more favourable conditions return (Pedersen *et al.*, 2002). For species colonising the human gut, persistence through nutrient limitation would aid survival during periods of host starvation, both ancestrally and present day in regions where consistent food supply is still a significant problem.

1.5.3 Biofilm formation

The formation of a biofilm by a population of cells is a common method employed by bacteria to protect against unpredictable and unstable environments. Furthermore the formation of biofilm communities associated with food particles and the gut mucosa is generally considered to be an important method of persistence within the gut for both the commensal communities and potential pathogens (Kim *et al.*, 2009, Bollinger *et al.*, 2007, Kleessen & Blaut 2005). Biofilms are a mode of growth by which multicellular communities adhere to surfaces and produce an extracellular matrix composed of exo-polysaccharides, proteins and nucleic acid (Hall-Stoodley *et al.*, 2004, Kostakioti *et al.*, 2013). They are defined as an assemblage of surface-associated microbial cells that is enclosed in an extracellular polymeric sybstance matrix (Donlan 2002).

One of the first TA modules to be linked with biofilm formation was the MqsRA module. In the absence of stress MqsA represses rpoS however in the presence of stress MqsA is degraded by Lon, increasing levels of RpoS, which in turn leads to an increase in cellular cyclic diguanylate (c-di-GMP). The presence of this secondary messenger upregulates stationary phase gene expression and leads to synthesis of biofilm components such as cellulose and curli (Kasari *et al.*, 2010, Kim *et al.*, 2010). It is also possible that acitivation of MqsR cleaves mRNA leading to the production of persisters however what stimulates one pathway or another still remains unclear (Hong *et al.*, 2012). Additionally both RelBE and MazEF Type II modules and homologues of these have been shown to be highly expressed in cell populations forming biofilms with lysis of a sub-population of cells having been identified as crucial in stabilisation of the extracellular matrix because of the released cellular contents aiding formation (Allesen-Holm *et al.*, 2006).

1.5.4 Bacteriophage resistance

Bacteriophages outnumber bacterial cells ten-to-one with an estimated $\geq 10^{30}$ phage particles on the planet and an infection rate of approximately 10^{25} events per second (Suttle 2005, Wommack & Colwell 2000, Lima-Mendez *et al.*, 2007). Throughout evolutionary history, the virus-host interplay has led to bacteria generating a number of protective mechanisms against infection by phage including evading attachment and adsorption, preventing injection of genetic material, restriction modification systems to degrade incoming DNA, clustered regularly interspaced short palindromic repeats (CRISPRs) and providing a rudimentary immune system against phage infection and abortive infection (Abi, Sorek et *al.*, 2008, Chopin et *al.*, 2005). The last of which is now a confirmed function, which several TA modules are known to undertake. Abi systems are

generally cell death systems that are activated by phage infection to limit replication of these particles, providing a level of protection to the bacterial population rather than the individual cell (Dy *et al.*, 2014). With this in mind, TA systems may impact the gut microbiota by stabilising the community against bacteriophage infection.

Initially expression of the Type I *hok/sok* locus of the *E.coli* R1 plasmid was shown to exclude T4 phage infection (Pecota & Wood 1996) and later the chromosomally encoded Type II MazEF system of *E.coli* provided resistance to phage P1 (Hazan & Engelberg-Kulka 2004). However whilst both of these provide protection to a very narrow phage range, the recently discovered Type III ToxIN module provides resistance to a wide range of phage across multiple bacterial host backgrounds (Blower *et al.*, 2012). The mechanism of phage resistance by the ToxIN TA module above still remains unclear however Blower hypothesised that infection by phage results in the removal of ToxI antitoxin inhibition of the toxin leading to arrest of cell growth and the replication of the phage, eventually leading to bacterial cell death. Most recently a Type IV TA module has been discovered that is not an Abi system but helps regulates expression of an Abi system instead (Dy *et al.*, 2014). Of the five TA Types identified, four have systems that are confirmed to aid against phage infection.

1.5.5 Promotion of virulence

It has also been hypothesised that TA modules may have a role in virulence of certain organisms such as *Salmonella typhirmurium*. 11 Type II TA modules have been identified within the genomes of pathogenic *S.typhimurium* isolates whilst these have been lost in their closest related, benign counterparts (De la Cruz *et*

al., 2013). Carriage and subsequent expression of a HigAB-like module not only increased intracellular uptake but also increased survival in mesenteric lymph nodes in mouse model experiments, therefore suggesting that transient activation of this module (and perhaps others) provide a selective advantage favouring virulence within these environments. A later study further supports this hypothesis identifying a significant fitness advantage for *S.typhimurium* inside fibroblasts with expression of both Type I and II modules (Lobato-Marquez *et al.*, 2015). These studies did not go as far as to label TA modules as virulence factors, probably because the function of these systems may be multi-faceted promoting survival in many different stressful environments, however it may be possible to do so as Cross (2008) defines a virulence factor by "enabling an organism to replicate and disseminate within a host in part by subverting or eluding host defences". Regardless of this, TA systems have certainly been shown to promote virulence within at least one pathogenic species and the high prevalence of these in many more may hint at the possibility of a larger role within virulence.

1.5.6 Control of gut epithelial cell proliferation

To date, little direct evidence for TA modules playing a role in host-microbe interactions exists. However Jones (2010) hypothesised that the RelBE module harboured on the pTRACA22 plasmid and prevalent within the human gut microbiome may, when expressed by bacterial cells, lead to cell death of gut epithelial cells, possibly having a role in host-cell proliferation and the regulation of this. This hypothesis is based on the observation RelE toxins actively inhibit eukaryotic cells through the same mechanism that inhibits bacterial cell growth (Kristoffersen *et al.*, 2000, Andreev *et al.*, 2008), with expression of the *relE* gene within human cell lines leading to the induction of apoptosis (Yamamoto *et al.*,

2002). Notably cell death was controlled and not necrotic, an important feature for control over cell proliferation allowing reutilisation of cellular components and avoiding inflammation (Kerr *et al.*, 1972), suggesting regulation of the mechanism.

1.6 Aims and objectives

The overall aims of this study were to provide a comprehensive assessment of Type II toxin-antitoxin module distribution and abundance, confirm activity of TA modules identified as prevalent within the human gut and to explore putative roles these may play in gut associated bacteria and the human gut microbiome.

Specific objectives were to:

- Explore the distribution and abundance of all known Type II TA systems across a broad range of environments and discrete genetic units.

- Evaluate the ability of Type II TA systems to stabilise associated regions of DNA and encoded functions of gene neighbourhoods surrounding these systems.

- Investigate the effect of pTRACA22-RelBE TA expression on survival during gut-related and antimicrobial stress.

- Investigate the potential for gut-associated TA systems to modulate phage-microbe interactions and provide protection to bacteriophage infection.

- Assess the impact of gut-associated TA systems on health of the human intestinal Caco-2 cell line using an *in Vitro* co-culture model.

Chapter 2: General materials and methods

2.1 General microbiological methods

2.1.1 Bacterial strains, cultivation and growth media

Bacterial strains, plasmids, selection, growth requirements and sources used in this work are shown in Table 2.1. Strains were grown in 10 mL of Luria-Bertani (LB) broth (Fisher Scientific UK, Oxoid UK) or on LB agar plates at 37 ^{o}C. Agar plates were prepared by the addition of 1.25% (w/v) technical grade agar (Oxoid UK) to liquid medium prior to autoclaving. LB media is composed of tryptone (pancreatic digest of casein) 10 g/L, yeast extract 5g/L and sodium chloride 10 g/L (Miller 1987). Liquid cultures were aerated by shaking at 130 rpm. All cultures were grown in sterile, plastic universal tubes, with volumes ranging from 20-50 mL (VWR UK, Fisher UK), under conditions described above unless otherwise stated. Antibiotic selection, metabolite repressors and expression inducers were added to media where appropriate (See Table 2.1).

2.1.2 Storage of bacterial cultures

Bacteria were grown on LB agar plates (with any required selection) at 37 ^{o}C overnight. After 18 hours, the cells were harvested using sterile cotton swabs and re-suspended in 1 mL of LB broth supplemented with 8% (w/v) dimethyl sulfoxide (DMSO, Fisher UK) and stored at a temperature of -80°C in 2 mL screw cap vials (Fisher Scientific UK). Bacteria were revived by scraping a small shard of the frozen bacterial suspension from the tube, using a sterile plastic loop (Fisher Scientific UK), and this was used to inoculate 10 mL of fresh LB broth containing any required selection. A working stock of bacteria was maintained on agar plates and stored at 4 ^{o}C for up to 1 week.

Table 2.1: Bacterial species, strains and plasmids, antibiotic selection[1], growth conditions and their sources[2] used within this study.

Antibiotics were obtained from Fisher Scientific UK or Sigma Aldrich UK. Sources for each plasmid are specified below where available.

Species	Strain	Plasmid	Antibiotic selection[1]	Source & Supplementary information[2]
Escherichia coli	EPI300	pTRACA22	Km 50 µg/mL	(Jones & Marchesi 2007; Jones *et al.* 2010)
		pJ821	Km 30 µg/mL	(Giacalone *et al.* 2006), Supplied Xbrane Bioscience (Sweden)
		pJ821(TA)	Km 30 µg/mL	(Giacalone *et al.* 2006), Supplied by DNA2.0 (USA), rhamnose inducer, glucose metabolic inhibitor
		pJ821(ATx)	Km 30 µg/mL	(Giacalone *et al.* 2006), Supplied by DNA2.0 (USA), rhamnose inducer, glucose metabolic inhibitor
		pGEM-T Easy	Amp 100 µg/mL	Promega (USA)
		pGEM-T Easy(TA)	Amp 100 µg/mL	Promega (USA) & this study
		pBAD(Tx)	Amp 100 µg/mL	(Guzman *et al.* 1995a), arabinose inducer, glucose metabolic inhibitor
	One Shot® TOP10	pBAD(Tx)	Amp 100 µg/mL	(Guzman *et al.* 1995b) & this study, arabinose inducer, glucose metabolic inhibitor

Key: Km = kanamycin, Amp = ampicillin

2.1.3 Enumeration of viable bacterial cells

Viable counts were conducted on LB agar using either entire plate counts or the Miles and Misra method (Miles *et al.*, 1938). Bacterial samples were washed 3 times by centrifugation (4000 xg for 5 minutes) and resuspended in phosphate buffer saline (PBS, Oxoid UK). Final suspensions were serially diluted from 10^{-1} to 10^{-8}. For entire plate counts, LB agar plates, which had been thoroughly dried, were labelled and 100 µL of the diluted bacterial suspension was spread across the surface using a sterile, plastic L-spreader (Fisher UK). For the Miles and Misra method, LB agar plates were divided into sectors and labelled as appropriate. 3 x 10 µL drops of each dilution were placed onto the corresponding zones, ensuring space was left between each drop to prevent merging. All plates were allowed to dry and then incubated overnight at 37 ^{o}C. All counts were carried out in triplicate. The mean of the 3 (entire plate counts) or 9 (Miles & Misra) values were used to obtain viable counts and calculate the colony forming units per 1 mL of the initial inoculum (CFU/mL).

2.1.4 Assessment of growth by optical density

Bacterial growth, in liquid media, under experiment-specific culture conditions was monitored by measuring optical density. 100 mL of media, in a sterile glass conical flask, was inoculated with 1 mL of an overnight culture (1/100 dilution) and supplemented with antibiotic selection as required. The fresh 100 mL culture was then incubated at 37 ^{o}C with shaking for 8 hours. 1 mL samples were taken every 20 minutes and placed into a 1 mL polystyrene micro cuvette (1.6 mL capacity, 10mm light path, Fisher UK). The optical density was measured at a wavelength of 600 nm using a Jenway 6300 model spectrophotometer (Jenway Ltd UK). The

spectrophotometer was blanked using 1 mL of sterile media. The total OD_{600} per culture was calculated as OD_{600} readings per sample x dilution factor.

2.1.5 Statistical analysis

All experiments were carried out, at least in triplicate, with the data presented as mean values. Individual data points were used to generate the standard error of the mean (SEM). All statistical analyses conducted in this study were undertaken using Microsoft Excel 2010, Minitab15/16/17 (Minitab Ltd USA) or GraphPad Prism 6 (GraphPad Software USA). Significant differences between means were assessed using two-tailed t-tests, or one-way ANOVA when comparing 3 or more groups of samples at the 95% confidence interval where appropriate. The null hypothesis (H_o) for t-tests was no significant difference between means tested. For ANOVA this was that all group means being compared are equal. Where data was not normally distributed and broke this assumption of t-tests, a non-parametric Mann-Whitney U test was used in its place. Additionally when this assumption was also broken for one-way ANOVA, a Kruskal-Wallis test was used instead. Finally chi-squared tests were employed to investigate whether distributions of independent categorical variables differ from one another.

2.2 General molecular biology techniques
2.2.1 Measuring quantity and purity of nucleic acids

To measure purity and quantify the concentration of nucleic acids present in samples obtained, 1.2 µL of each sample was placed on the pedestal of a Nanodrop 2000 spectrophotometer (Thermo Scientific UK). The corresponding software was then set to either DNA or RNA and the nanodrop blanked with Tris-

buffer (TB, 10mM Tris-Cl, pH 8.5) or nuclease free water (NFW). Quantity was measured using the absorbance value at 260nm and the purity determined by the ratio between absorbance at 260 and 280nm (A260/A280).

2.2.2 Preparation of PCR template DNA

To prepare template DNA for initial diagnostic PCRs, the colony boil method was utilised. Using a sterile pipette tip, a very small quantity of cells from an individual colony was taken and suspended in 50 µL of sterile NFW by gentle pipetting. Colony suspensions were then heated to 95 ^{o}C for 20 minutes using a heating block and then kept on ice (short term) or at -20 ^{o}C for use in further work.

2.2.3 Plasmid extraction and purification

Preparation of plasmids from *Escherichia coli* was performed using Qiagen QIAprep Spin Miniprep kit (Qiagen UK). 5-10 mL of an overnight culture of *E.coli* harbouring the required plasmid was centrifuged at 4000 g for 10 minutes at room temperature (RT) to pellet and collect bacterial cells. The supernatant was then discarded and plasmid preparation was carried out according to the supplied protocol, and utilising all additional optional steps described in the handbook. All centrifugation steps from then onwards were performed at 17000 g unless otherwise stated. The captured plasmid DNA was eluted in 30 µL of either the supplied TB (pH 8.5) or sterile NFW with an elution time of 5 minutes at room temperature (RT). Purity and quantity of the extracted plasmid DNA was then measured using a nanodrop (Section 2.2.1) and stored at -20°C until required. Plasmids were visualised using gel electrophoresis (Section 2.2.5).

2.2.4 Polymerase chain reaction (PCR)

PCR was used to amplify regions of DNA from both plasmid and genomic templates. Each PCR reaction was performed in thin walled, 0.2 mL PCR tubes (Alpha Laboratories UK) using a BioRad icycler Thermocycler (Bio-Rad UK). All reagents, except for NFW, were stored at -20°C then thawed and maintained on ice during use. Negative control reactions had NFW added in place of template DNA and were included in all PCR runs in order to confirm purity of reagents used. Taq polymerase (Qiagen UK) was used throughout this study to amplify DNA regions ranging from 100 bp up to 10 kbp. Below is the standard protocol (including reaction component volumes and cycling conditions used) with any changes to this being stated with individual experiments. Completed PCR reactions were used immediately or stored at -20°C until required.

2.2.4.1 Taq polymerase

Taq polymerase has a non-template-dependent terminal transferase activity that adds a single deoxyadenosine to the 3' end of PCR products leaving sticky ends (Chien *et al.*, 1976). Individual reactions with a final volume of 25 µL were used, unless otherwise stated, and all reagents were obtained from Qiagen Taq Core Kits (Qiagen). Each reaction consisted of 13.3 µL NFW, 5 µL 5 x Q solution, 2.5 µL 10x buffer containing 15 mM MgCl2 (giving a working concentration per reaction of 1.5 mM), 0.75 µL forward primer (10 pmol), 0.75 µL reverse primer (10 pmol), 0.5 µL Deoxyribonucleotide triphosphates (dNTPs, containing 10 mM of each dNTP, giving a final concentration of 0.2 mM per reaction), 0.2 µL Taq polymerase (5 U/µL – giving 1U per reaction) and 2 µL template DNA (concentrations ranged 0.02-60 ng/µL and were specific to individual amplifications). Cycling conditions for standard Taq PCRs are shown in Table 2-2. Primers used and specific annealing temperatures (AT) can be found in method sections of individual data chapters.

2.2.5 Gel electrophoresis

Fragments of DNA were visualised using agarose gel electrophoresis. Separation of DNA fragments was achieved using the Fisher varigel maxi system (Fisher UK) or the Biorad sub-cell GT MINI electrophoresis tank (Bio-Rad UK) with the Biorad powerpack 300 (Bio-Rad UK). Standard agarose gels contained 1% agarose (w/v) (Sigma Aldrich UK or Fisher UK) in 1 x tris-acetate ethylenediaminetetraacetic acid (EDTA) buffer (TAE; 40 mM tris acetate, 1 mM EDTA) diluted from a 50x TAE solution (Fisher Scientific). If DNA fragments were >10 kbp, a 2% (w/v) agarose gel was used. Gels were set at RT and placed into gel tanks containing 1 x TAE as the running buffer. DNA samples were mixed with loading dye (Promega UK) at a ratio of 1:6 (dye:sample) and 5-10 µL of the resulting mixture was loaded into wells. Fragments were then separated at 70 – 85 volts over a period of 1.5 to 2.5 hours. DNA was subsequently stained with ethidium bromide (EtBr, Sigma Aldrich UK) in 1 x TAE at a final concentration of 0.6 µg/mL for 15-25 minutes at RT. Fragments were visualised using UV transluminescence in a Syngene INGENIUS Gel Documentation system (Syngene UK).

Table 2.2: Cycling conditions for a *Taq* polymerase PCR

Stage	Number of Cycles	Temperature (^{o}C)	Time
1	1	95	3 minutes
2	30	94	30 seconds
		Primer specific AT[1]	1 minute
		72	1 minute
3	1	72	5 minutes
		4	∞

[1]Primer specific AT refers to the temperature at which the primers would optimally anneal to the template DNA

The number of cycles, range of temperatures and length of time for each stage in a *Taq* polymerase PCR. A variable length of time can be used for the final step at 4°C.

2.2.6 DNA restriction digest

All restriction endonucleases were purchased from either New England Biolabs (UK) or Promega (UK). Each 40 µL reaction contained 4 µL 10x restriction buffer specific to the enzyme in use, 1 µL bovine serum albumin (BSA) (10 µg/mL, giving 0.25 µg per reaction), 1µL restriction endonuclease (10 U/µL, giving 0.25 U per reaction), a variable volume of DNA, giving a final quantity of ~1 µg per reaction, adding NFW to give a final volume of 40 µL. Standard incubation times for these enzymes ranged from 2 – 18 hours at a temperature of 37 ^{o}C (unless otherwise stated), after which they were heat inactivated according to the provided manufactures instructions. This was, usually, 65 ^{o}C for 15 – 20 minutes. Products were visualised using gel electrophoresis.

2.2.7 Extraction of DNA from agarose gels

Fragments of DNA, which had been separated by gel electrophoresis, were purified from the agarose gel matrix using the Qiagen QIAquick Gel Extraction kit (Qiagen UK) following the manufacturers protocol, including all optional steps in the kit handbook. Specific fragments of interest were excised from the gel using a sterile, disposable scalpel; ensuring minimal agarose gel was taken in the process, and transferred into sterile microcentrifuge tubes. 3 volumes of the provided QG buffer were added to 1 volume of gel (w/v) to dissolve the agarose and release the DNA. This was heated to 50 ^{o}C for 10 minutes to ensure complete dissolution. All centrifugation steps performed during this protocol were at 17000 g and all extracts were eluted in 30 µL of provided TB (pH 8.5) or NFW. Quantity and purity was measured using a nanodrop and integrity also confirmed by gel electrophoresis. Purified DNA was stored at -20 ^{o}C until required.

2.2.8 Purification of DNA

DNA from PCR reactions and restriction endonuclease digests were purified using QAIquick gel extraction kit (Qiagen UK) for use in subsequent molecular methods. This was done as in Section 2.2.7 but the PCR run DNA suspension was used in place of the excised gel slices.

2.2.9 Ligation of DNA fragments

Ligation of DNA fragments was accomplished using T4 ligase to catalyse the joining of vector and insert strands of DNA between the 5'-phosphate and 3'-hydroxyl groups of adjacent nucleotides. Ligation reactions were set up according to the manufacturer's instructions (Promega UK). A 20µL reaction contained 2µL of 10x ligase buffer, 1µL T4 ligase (1 U/µL) and differing volumes of vector and insert depending on the molecular ratio calculated. Amounts of DNA needed of each, to give molecular vector:insert ratios of 1:1, 1:3 and 3:1, were calculated using the equation below.

$$\frac{ng\ of\ vector\ x\ kb\ size\ of\ insert}{kb\ size\ of\ vector} \quad x \quad molar\ ratio\ of\ \frac{insert}{vector} = ng\ of\ insert$$

NFW was added to a final volume of 20µL. The ligation was then incubated at RT for 3 hours or 4°C overnight. The resulting mixture could then be used either directly for a transformation or an additional clean up step could be performed (section 2.2.8).

2.2.10 Chemical transformation of *E.coli*

Chemically competent *E. coli* EPI300 (Epicentre, USA) were transformed using the Epicentre chemical transformation protocol. Cells were gently thawed on ice and 100µL aliquots of these were then transferred to fresh, pre-chilled, 1.5 mL tubes. 5 µL of the required DNA at a concentration of between 25-100 ng/µL was added to the cell suspensions, mixed gently by pipetting and then incubated on ice for 30 minutes. Cells were then heat shocked in a water bath set at 42^oC for 30 seconds and then returned to ice for a further 2 minutes. 900 µL of sterile super optimal broth with catabolite repression (SOC broth) consisting of 5 g/L yeast extract, 20 g/L tryptone, 10 mM $MgSO_4$, 10 mM $MgCl_2$ and 20 mM glucose was added, mixed and the entire suspension transferred, with shaking at 130 rpm, to a sterile 15 mL universal tube. Transformed cells were then incubated for 1 hour at $37\ ^oC$. 100 µL of the transformed cell suspension was used in a serial dilution from neat - 10^{-4} and these, plus the neat, were spread on LB agar containing the appropriate selection and any other nutritional requirements using plastic L-spreaders.

2.2.11 Production of electrocompetent *E.coli*

5 mL of a log phase *E. coli* EPI300 (OD_{600} of 0.5, ~1.1×10^7 CFU/mL) culture was chilled on ice for 15 minutes with cells then harvested by centrifugation at 3000 g for 10 minutes at $4\ ^oC$. The supernatant was discarded and the cell pellets washed three times by re-suspending in ice cold 10% sterile glycerol and pelleting at the same speed and temperature as above. 100 µL aliquots were then transferred to pre-chilled sterile 1.5 mL microcentrifuge tubes and snap frozen using liquid nitrogen for storage at -80 ^oC and later use.

2.2.12 Electroporation of *E.coli*

Electrocompetent cells were electroporated following the manufacturer's protocol (Epicentre, USA). Frozen electrocompetent cells were thawed, on ice, and mixed gently by occasional flicking of the 1.5 mL tube. 100 µL of the suspension was immediately transferred to pre-chilled Electroporation cuvettes (Bio-Rad UK) on ice. 2 µL of the DNA to transform was added to the cells and mixed gently by pipetting up and down slowly, ensuring no bubbles were introduced. The cuvettes were then wiped using tissue paper to remove condensation on the electrodes and placed into the Gene Pulsar Xcell™ (Bio-Rad UK). Electroporation was carried out using the pre-set *E.coli* settings for 0.1 cm gap size cuvettes (1.8kV, 200Ω resistance and 25 µF capacitance). After electroporation, 900 µL of SOC broth was added to the cuvette, mixed, and transferred to a 15 mL sterile plastic universal. Cells were incubated for 1 hour with aeration at 37 $^{\circ}$C. 100 µL of the transformed cell suspension was used in a serial dilution from neat-10^{-4} and these, plus the neat, were spread on LB agar containing the appropriate selection and any other nutritional requirements using plastic L-spreaders.

2.2.13 RNA extraction

Cells were lysed and had total RNA extracted and purified using Qiagen RNeasy Mini kit (Qiagen UK). Before beginning, all workspace and equipment used were treated using RNAase ZAP (Ambion UK) to remove RNAse contamination and lower the chance of RNA degradation later in the process. 10 mL of LB broth, supplemented with any required selection, was inoculated with 500 µL of an overnight culture of the *E.coli* strain of interest and grown at 37 $^{\circ}$C in aerobic conditions until an OD_{600} of ~0.3 (10^7 CFU/mL). 500 µL of the bacterial suspension was added to a sterile 2 mL DNA LoBind microcentrifuge tube (Eppendorf

Germany) and 2 x volume of RNA protect (Qiagen UK) was added, mixed by vortexing for 5 seconds, and incubated at RT for 5 minutes to maximise stability of the RNA.

The resulting cell suspension was centrifuged for 10 minutes at 5000 g with the supernatant then discarded and residual supernatant around the rim of the tube removed by gently dabbing with a paper towel. Resulting pellets could then be used immediately or stored for up to 4 weeks at -80 ^{o}C. 100 µL TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) containing 15 mg/mL lysozyme (from egg white, Sigma UK) was mixed by vortexing for 10 seconds. The suspension was then incubated for 15 minutes at RT, with vortexing for 10 seconds every 2 minutes. All subsequent steps were performed as per manufacturer's instructions (Qiagen UK) and the resulting suspension was directly used in the RNeasy Mini Kit protocol 7: Purification of total RNA from bacterial lysate. All optional steps, except DNase digestion, were included and extracts were eluted in 30 µL of RNase Free water, quantified using the nanodrop and then samples placed back on ice. Residual DNA was then digested using Turbo DNA-free DNase (Ambion UK), using the routine DNase treatment provided with the manufacturer's instructions. DNA-free RNA samples were quantified using a nanodrop and stored at -80 ^{o}C.

RNA was reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen), which included a second genomic DNA wipeout stage. RNA (if stored) was thawed on ice whilst the gDNA wipeout buffer, Quantitect RT buffer, RT primer mix and RNAse-free water thawed at room temperature.

Genomic DNA wipeout reactions were prepared on ice following the protocol provided. 2 µL gDNA Wipeout buffer was added to 1-100 ng of template RNA and made up to a final reaction volume of 14 µL with NFW. This was incubated at 42 ^{o}C for 2 minutes then immediately placed on ice. A reverse transcription master

mix was then prepared, on ice, also according to the manufacturer's instructions. 1 µL Quantiscript Reverse Transcriptase, 4 µL Quantiscript RT 5x buffer, 1 µL RT primer mix and the entire 14 µL of the gDNA elimination reaction was added to a 1.5 mL DNA LoBind microcentrifuge tube (Eppendorf). This was incubated for 15 minutes at 42 ^{o}C and the enzyme then heat inactivated at 95 ^{o}C for 3 minutes. Reverse transcription negative controls were also made at this point by excluding the addition of the reverse transcriptase enzyme, and replacing this lost volume from the reaction mixture with RNAse-free water. Resulting cDNA was stored on ice until use or -20 ^{o}C for long-term storage. This cDNA was then utilized as a template for conventional Taq PCR (section 2.2.4.1) to identify the presence of gene expression.

2.2.14 pGEM-T$^{®}$ Easy DNA cloning system

DNA fragments were cloned into a pGEM-T plasmid vector (pGEM®-T Easy Vector systems, Promega) according to the manufacturer's instructions. The PCR fragments to be cloned were first purified using a QIAquick gel extraction kit (Qiagen) as described in Section 2.2.7 and eluted in NFW. Ligation reactions consisted of 1 µL T4 DNA ligase (3 U/µL), 5 µL 2x Rapid Ligation buffer, 1 µL of pGEM®-T easy vector (50 ng/µL), and the appropriate amount of target PCR product at either a 1:1 or 3:1 (insert: vector) molar ratio. NFW was added to a final volume of 10 µL. Reactions were performed in 0.2 mL PCR tubes and incubated overnight at 4°C.

Subsequent ligation mixtures were transformed into *E.coli* EPI300 chemically-compotent/electrocompetent cells either chemically (section 2.2.10) or by electroporation (section 2.2.12). Transformants were spread onto LB agar supplemented with 100 µg/mL ampicillin plates (100 µL per plate). LB plates used

had previously had 100 µL of 0.1 M Isopropyl β-D-1-thiogalactopyranoside (IPTG) and 20 µL of 50 mg/mL 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) spread on top and allowed to dry in a sterile environment for 30 minutes. Successful pGEM-T clones containing the target insert were visually identified as white colonies whilst unsuccessful transformants appeared blue.

A sample of white colonies were picked at random, streaked onto fresh LB plates supplemented with 100 µg/mL ampicillin selection and also grown in liquid cultures with the same selection. These were used to recover plasmids. The subsequently purified plasmids were then used to confirm correct insertion into the vector by Taq PCR and also through digestion with EcoRI, releasing the insert from the pGEM-T Easy vector. Products of both PCR and restriction digests were visualised by gel electrophoresis.

Chapter 3: Distribution and abundance of Type II toxin-antitoxin systems in the human gut microbiome

3.1 Introduction

3.1.1 Understanding the diversity and distribution of TA modules and the methodological limitations of this

In the last decade, large numbers of TA systems not identified during initial genome annotation have begun to be identified (Pandey & Gerdes 2005). Many of these newly identified TA systems appear to differ structurally and potentially functionally from known modules (Gerdes *et al.*, 2005). The previous failure to identify these is partially the result of limitations within the methodology for gene prediction and function classification. Algorithms used in these methods tend to be sub-optimal for the annotation of small genes, such as those of TA systems (Gerdes 2013), whilst also tending to exclude those genes that overlap or are internal to larger open reading frames (ORFs). For TA systems, both these examples are highly likely and fit with evidence supporting their role in vertical stabilisation of DNA; however, the fact remains that classification of TA systems and, by extension, an understanding of their distribution and abundance, has been significantly hindered because of this.

Toxin-antitoxin loci were initially discovered because of their ability to stabilise vertical transmission of plasmids through the mechanism of post segregational killing (PSK) (Gerdes *et al.* 1986, Hiraga *et al.*, 1986). This method of plasmid stabilisation is highly reliant on the altered stabilities of the toxin and the antitoxin (Ogura & Hiraga 1983; Gerdes *et al.*, 1986). Since then, a total of seven plasmid encoded TA loci families (and their relatives) have been described, all of

which stabilise plasmids, although structural and genetic organisation, as well as mechanism, of the toxin and the antitoxin can differ greatly (Pandey & Gerdes 2005). It has also been noted that several integrons, super-intergrons (fluid genomic platforms containing individual genes), gene cassettes and operons, of the Vibrionaceae also appear to carry TA modules (Rowe-Magnus & Guerout 2003, Christensen-Dalsgaard & Gerdes 2006, Szekeres *et al.*, 2007) possibly undertaking the same function: stabilisation of genomic regions, but within a different situation.

Homologues of TA systems have subsequently been found on chromosomes, with those of the Type II category being particularly widespread and often carried in multiple copies in bacterial/archaeal genomes (Makarova *et al*. 2009, Pandey & Gerdes 2005; Guglielmini *et al.*, 2008, Sevin & Barloy-Hubler 2007). Some of the most extreme examples of these can be seen in the genomes of *Nitrosomonas europeae* which has 42, *Mycobacterium tuberculosis* carrying 38 and *Sulfolobus tokodaii* with 32 (Gerdes *et al.*, 2005). It should also be noted that one of the most exhaustive searches for putative TA pairs, done in 2005, highlighted an abundance of these modules within free living bacterium (e.g. *M. tuberculosis*) but not those which require a eukaryotic host, i.e. those that form obligate intracellular relationships (Pandey & Gerdes 2005).

Another interesting level of diversity of TA systems in bacteria is the number of loci carried by individual isolates of the same species. Figure 3.1 highlights the presence and abundance of the nine families of Type II toxins across seven strains of *E.coli* with clear differences being visible (Melderen & De Bast 2009).
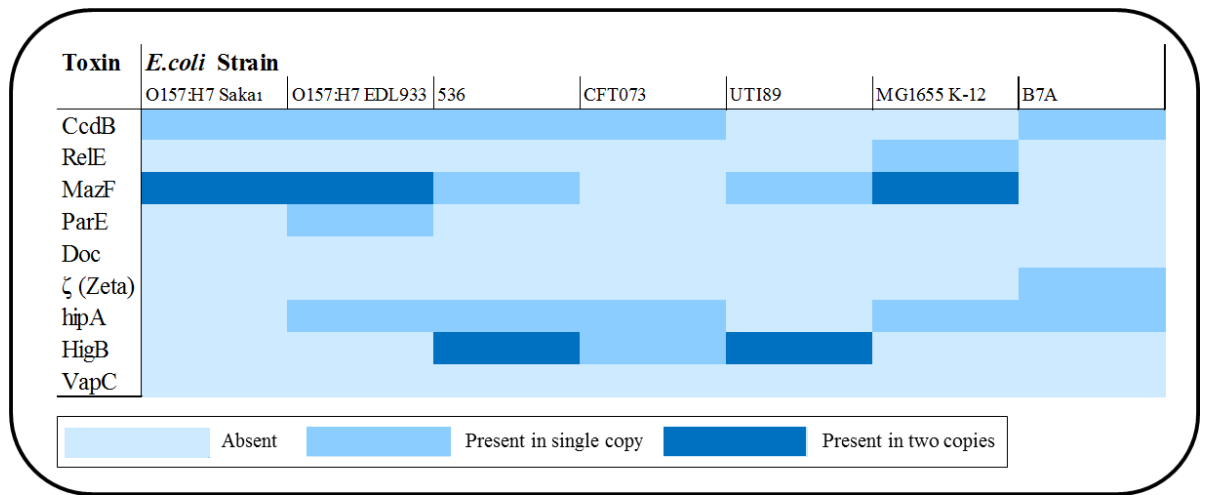
Figure 3.1: The abundance and diversity of Type II TA module toxins present in the chromosomes of 7 different strains of *E.coli*. Adapted from Van Melderen & De Bast 2009.

The differing frequencies of toxins between strains suggest recent events of horizontal gene transfer, therefore providing evidence that TA modules which are chromosomally located were originally part of the auxiliary/flexible genome, although, because of their nature and the possibility of multiple functionality, did not all remain that way. Very few species have been examined to the same level as *E.coli*; however, it is highly possible that a range of bacterial species exhibit multiple different TA modules, possibly in multiple copies within their genome.

With varying numbers of TA pairs in organisms across all bacterial phyla investigated, genetic analysis can be difficult, as observation of visible changes in phenotype often requires multiple deletions (Maisonneuve *et al.*, 2011). Due to this *E.coli* K-12 has regularly been used as a model organism purely due to convenience. This is not to say that TA modules are not being investigated in other species, just that it has been more difficult. With this in mind, the presence of bacterial TA systems in pathogenic model organisms is still regularly investigated such as in *M. tuberculosis, Streptrococcus pneumoniae* and *Salmonella enterica.* Excitingly, even multiple examinations of bacterial TA modules in Archaeal extremophiles have been undertaken.

3.1.2 Pathogens and TA systems

Interestingly, there appears to be a significant increase in the carriage of TA modules within highly pathogenic bacteria when compared to their closest non-pathogenic counterparts (Georgiades & Raoult 2011). This may be due to a role in virulence or, more simply, because the host pathogen was unable to lose them, i.e. a selfish genomic entity. In terms of pathogen evolution, when bacterial lineages transition from facultatively parasitic lifestyles to permanent host

associations they undergo significant loss of genetic material (Moran 2002). The PSK resulting from TA carriage may ensure the inability of the bacterial host to lose this module. Whilst this could also be the case for non-pathogenic prokaryotes, the small genome size of pathogens may not accommodate multiple copies of the same TA system, ensuring addiction and subsequently PSK. This concept of "selfish" genes has been proposed previously for TA systems; however, with more recent findings of new functionality (elaborated on in subsequent chapters), this now seem less likely.

3.1.3 Environmental prevalence of Type II TA systems

The prevalence and abundance of TA modules across different environments currently remains an area of deficiency. Perhaps the most robust, but by no means complete, study to look at the distribution of TA loci was done by Lennon and Jones (2011), which utilized a metagenomics approach to analyse and quantify functional genes involved in microbial dormancy. Looking at 4 TA pairs (HipAB, YafQ-DinJ, MazEF and RelBE) from the *E.coli* K-12's genome across 4 environments (ocean, soil, activated sludge and the human gut), they found the presence of TA module homologues were common within complex microbial communities, especially the human gut, with high levels of variation, the only exception being marine environments.

Focusing specifically on abundance of TA modules within the human gut, metagenomic analysis was also conducted by Jones *et al.*, (2010). Using transposon-aided capture analysis (TRACA), multiple plasmids were extracted from the human gut microbiome, one of these being the gut associated pTRACA22 which harbours a putative Type II RelBE toxin antitoxin module (p22-RelBE). Homologues of this module were found across all major phyla present in the gut microbiome, with the majority of these, and those with the highest

sequence identity, belonging to the Firmicutes. As one of the largest groups within the gut microbiome this result was unsurprising but in comparison Bacteriodetes, which are co-dominant within the gut, had relatively few TA homologues.

Also within this study (Jones *et al.*, 2010), the abundance of the p22-RelBE TA module within the gut compared to 3 other environments; mouse gut, Ocean, and soil, suggested the putative module was highly enriched within the human gut. Furthermore there was significantly higher abundance of p22-RelBE homologues present within the human gut, when compared to other Type II systems such as MazEF, ParDE and HigAB, providing early evidence of a gut associated TA system. Interestingly, there appeared to be a distinct lack of RelE within notable archaeal gut species (*Methoanobrevibacter smithii* and *Methanosphaera stadmanae*), with the authors hypothesising this may be due to differences in selective pressures affecting gut dwelling bacteria and archaea.

While these specific modules were not located in metagenomic datasets from the human gut, numerous TA systems have now been identified within archaea. In 1998 Grønlund & Gerdes found homologues of the RelBE TA locus in the genomes of *Methanocaldococcus jannaschii* and *Archaeoglobus fulgidus*, providing the first evidence of archaeal carriage of these systems (Grønlund & Gerdes 1999). Since then numerous more bacterial homologues, along with several archaea-specific modules, have been identified within archaea.

TA systems which function as translational inhibitors (for example the RelE toxin cleaves mRNA bound to the ribosomal A-site (Neubauer *et al.*, 2009) appear to be widely distributed throughout the archaeal domain (Makarova *et al.,* 2009). This is probably because we have a deeper understanding of bacteria and, specifically, this part of their physiology, which allows easier identification than those that interact with less understood archaeal processes. In parallel to this, the

conserved translational apparatus between the two kingdoms has made it possible to mechanistically study TA systems in both.

A Study conducted by Guo *et al.,* (2011) involved screening for the presence of the Type II TA loci super-families (*hicAB, vapBC and relBE*) using 124 completed archaeal genomes. The most dominant TA family of the thermophiles was VapBC, particularly organisms belonging to the order *Sulfolobales*, whilst RelBE and HicAB homologues were comparatively under represented, specifically in the *Crenarchaeota* (one of the most significant Phyla in the study). RelBE and HicAB were better represented in the Euryarchaeota, with approximately half their genomes harbouring between 1-9 copies of these. Frequency and distribution of the TA loci were examined based on environmental conditions (such as high temperature, salt concentrations, pressure, extreme pH and oxygen requirements), genome sizes and genomic location but firm conclusions were unable to be drawn, perhaps due to the under-representation of the *Thaumarchaeota*, *Korarchaeota*, *Aigarchaeota* and *Nanoarchaeota*.

The culmination of findings by Jones *et al.*, (2010) leads to interesting questions about the functionality of p22-RelBE. Does it play roles within the life cycles of those organisms that carry it or is it purely a selfish entity as previously suggested (Van Melderen & de Bast 2009)? Based on the wide prevalence of TA systems, it is more than plausible to speculate, and indeed some evidence already exists for, a potential role of these modules in cell survival, when exposed to stressors in their natural habitats. It is, therefore, important to understand the distribution and abundance of these modules (especially with respect to the host cell's habitat), to allow a robust investigation into the full spectrum of functions these may have within the prokaryotic cell and, on a larger scale, the prokaryotic population that occupies that environment.

This chapter will focus on providing a broad search of extensive metagenomic, NCBI prokaryotic chromosome, plasmid and phage datasets for homologues of the pTRACA22-RelBE TA module and all other Type II TA systems identified and uploaded to the Toxin-Antitoxin Database (TADB, Shao *et al*., 2011). It will also utilize a large scale PCR survey as well as quantitative PCR (qPCR), to investigate the presence and copy numbers of the individual toxins, antitoxins and in the case of p22-RelBE, complete module across 54 gut microbial metagenomes gathered from patients who are healthy, sufferers of adenomatous polyposis or colorectal cancer

3.2 Aims

The aims of work presented in this chapter were to explore the distribution and abundance of all known Type II toxin-antitoxin modules across a broad range of environments and discrete genetic units using an *in Silico* approach. Secondly to ascertain the presence/absence of the most abundant gut-related TA module toxins along with the p22-relB antitoxin and complete p22-RelBE module within metagenomic DNA samples from the human gut microbiome using a PCR based approach. Finally, to utilise quantitative PCR to identify the copy number of these abundant toxins within human gut metagenomic samples.

3.3 Materials and methods

3.3.1 Comparative metagenomic analysis

To ascertain the distribution and abundance of Type II TA modules, comparative metagenomic analysis of 781 metagenomic datasets, 4580 plasmids, 3919 bacterial chromosomes and 711 phage genomes or metagenomic fragments encompassing 12 distinct habitats was conducted. All datasets used can be seen in Table 3.1. These were searched using all known Type II TA systems present in the Toxin Antitoxin database available at: http://202.120.12.135/TADB2/index.php (Shao *et al.*, 2011).

All databases were initially searched using tBlastn with TA amino acid sequences (including toxin, antitoxin and regulator genes) used as queries to identify nucleotide sequences homologous to these queries. All hits with an identity of 35% or greater, 50% query coverage and an e-value of $1e^{-5}$ or lower were then passed on to a second round of validation and exclusion of redundant hits. This initial selection process for validating hits was an adaption of the methodology used by Jones *et al.*, (2010) which used an alignment length of 30 amino acids. These parameters were decided upon to ensure confidence in homology without being too restrictive, allowing for closely related but not identical sequences whilst also considering the fragmentary nature of the metagenomes being interrogated.

Table 3.1: Overview of datasets and sequences used in this study

Dataset type	Habitat/sequence type	Source[1]	Reference/comment
	Acid mine drainage	CAMERA	(Tyson *et al.*, 2004), http://data.imicrobe.us/
	Waseca County farm soil	CAMERA	(Tringe *et al.*, 2005), http://data.imicrobe.us/
	Washington lake	CAMERA	(Kalyyuzhnaya *et al.*, 2008), http://data.imicrobe.us/
	Marine (Marine microbes)	CAMERA	(Yooseph et al., 2007, Rusch et al., 2007), http://data.imicrobe.us/
	Marine (Global Ocean Sampling Expedition)	CAMERA	(Yooseph *et al.*, 2007, Rusch *et al.*, 2007), http://data.imicrobe.us/
Whole community metagenomes	Marine (Whale fall)	CAMERA	(Tringe *et al.*, 2005), http://data.imicrobe.us/
	Termite gut	CAMERA	(Warnecke *et al.*, 2007), http://data.imicrobe.us/
	Chicken caecum	CAMERA	(Qu *et al.*, 2008), http://data.imicrobe.us/
	Mouse gut	CAMERA	(Turnbaugh *et al.*, 2006), http://data.imicrobe.us/
	Human gut (Metahit: Danish & Spanish, n = 124)	EMBL	(Qin *et al.*, 2010), http://www.bork.embl.de/~arumugam/Qin_et_al_2010/

	Human gut (Japanese, n = 13)	HMCJ	(Kurokawa *et al.*, 2007), http://www.metagenome.jp
	Human gut (American, n = 2)	DDBJ	(Gill *et al.,* 2006), http://www.ddbj.nig.ac.jp/
	Human gut (HMP, n = 295)	HMP	http://hmpdacc.org/
	Human urogenital (HMP, n = 63)	HMP	http://hmpdacc.org/
	Human oral cavity (HMP, n = 292)	HMP	http://hmpdacc.org/
	Human skin (HMP, n =117)	HMP	http://hmpdacc.org/
Bacterial	NCBI chromosomes	NCBI	ftp://ftp.ncbi.nih.gov/genomes
chromosomes,	NCBI plasmids	NCBI	ftp://ftp.ncbi.nih.gov/genomes
plasmids and	NCBI bacteriophage genomes	NCBI	ftp://ftp.ncbi.nih.gov/genomes
phage genomes	Broad bacteriophage genomes	BI	http://www.broadinstitute.org/igv/Genomes

[1]Datasets and genome sequences utilised in this (and subsequent) studies were obtained from a range of publically accessible repositories:

CAMERA: Community Cyber-infrastructure for Advanced Microbial Ecology Research and Analysis. Homoepage: http://camera.calit2.ne

EMBL: Metagenomes comprising the MetaHIT dataset were obtained from the European Molecular Biology Laboratory database via the link provided in the table above. Homepage: http://www.ebi.ac.uk/.

HMCJ: Human Metagenome Consortium Japan. Homepage: http://www.metagenome.jp/. This site has closed since collection of the human gut metagenomic database, however the database is still available from the Genome Sequencing Center of Washington University: http://genome.wustl.edu/sub_genome_group_index.cgi?GROUP = 3.

DDBJ: DNA Databank of Japan, A collection of nucleotide sequence data freely available for research activity. Homepage: http://www.ddbj.nig.ac.jp/

HMP: Human Microbiome Project with the aim of a comprehensive charactisation of the human microbiome and its role in human health and disease. Multiple metagenomic datasets were obtained from this site. Homepage: http://hmpdacc.org/

NCBI: National Centre for Biotechnology Information. Phage, chromosomal and plasmid sequences deposited at NCBI can be obtained from the genomes ftp indicated in the table.

BI: Broad Institute. Homepage: http://www.broadinstitute.org/.

To avoid the production of redundant hits (i.e. multiple different queries hitting the same nucleotide sequence) and further refining the pool, a second phase of validation was undertaken. Prior to this all hits returned from the initial searches and meeting the criteria were screened to ensure they read from 5' to 3' so they could be fed correctly into the following analysis. For each individual sequence or scaffold, hits were listed in order of their bit score and hit start. With the highest bit score, the first hit was kept and assigned a unique identification number. Each subsequent hit had its hit start and end value compared to the first "non-redundant" hit until one was found that did not overlap by 51 nucleotides or greater at its hit start or end. When a hit meeting these conditions was found this was again assigned a unique identification number marking it as non-redundant. This entire process was repeated for each sequence hitting a query that met the preliminary criteria for all databases searched until a final non-redundant list of hits was established. These hits and were printed into .CSV files along with information regarding the original query and database searched, relative abundance (Hits/Mbp), hits per sequence, hits per sequence/average sequence length, hits per sequence count/Mbp and hits per Mbp/average sequence length.

As a positive control for studies undertaken in this chapter and the next, 54 RpoB genes, encoding the β subunit of the bacterial RNA polymerase were used as queries. Furthermore a second control consisting of 100 photorhodopsin genes were also used as queries as they were unlikely to be found in the gut environment and would act as a "gut-negative" control. This data was statistically interrogated using Chi-Squared statistical analysis. This entire process was automated by the use of a PERL (Practical Extraction and Report Language) script. The script was fully written by Dr Brian V Jones who granted permission to employ it for this project. Figure 3.2 summarises this pipeline.
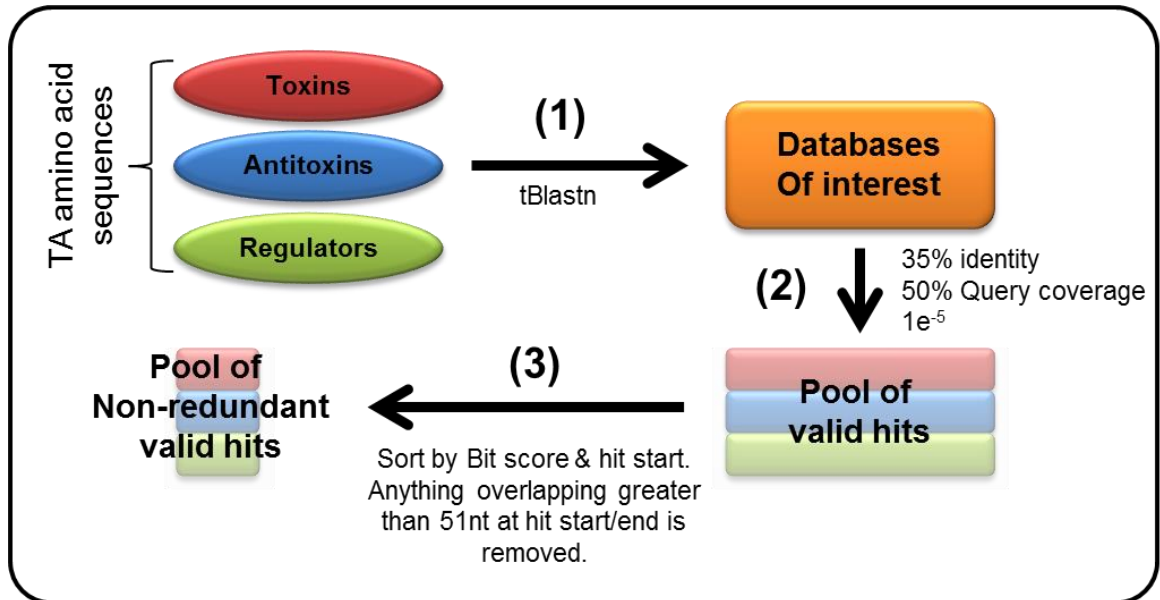
Figure 3.2: An overview of the process and pipeline used to achieve non-redundant, valid TA hits.

(1) Databases were searched with TA toxin, antitoxin and regulator queries using tBlastn.

(2) All hits with an identity of 35% or greater, 50% query coverage and an e-value of $1e^{-5}$ or lower were then passed on to a second round of validation and exclusion of redundant hits.

(3) Hits were listed in order of their bit score and hit start. Subsequent hit had its hit start and end value compared to the first "non-redundant" hit until one was found that did not overlap by 51 nucleotides or greater at its hit start or end, where a unique identification number was assigned, marking this hit as non-redundant.

3.3.2 Extraction of metagenomic DNA

Metagenomic DNA from the human gut microbiome of healthy patients, adenomatous polyposis and colorectal cancer sufferers was previously extracted as described in Ogilvie *et al.*, (2013) and utilised in PCR and qPCR studies undertaken in this chapter. The method used is briefly summarised below.

Bacterial cells from stool samples were recovered by thoroughly homogenising 10 g in 20 mL of PBS, centrifuged at 1000 xg for 5 minutes at 4^oC and the resulting supernatant removed to a fresh sterile tube. The faecal debris was then washed a further 3 timed with a single 5 mL PBS aliquot and pooled with the recovered supernatant. To separate bacterial cells from the resulting faecal slurry, 15 mL was layered onto a 9.75 mL cushion of Nycodenz solution (Axis-Shield, Norway) at a density of 1.3 g/mL $T^{10}E^1$ buffer. Bacterial cells were harvested by centrifugation at 10 000 xg for 6 minutes at 4^oC and pooled. 10% (v/v) 1 mL glycerol stocks were stored at -80^oC until required.

Stocks of Nycodenz extracted cells were thawed slowly on ice and centrifuged at 17 000 xg for 1 minute. Cells were lysed and pellets resuspended in 900 µL of TE buffer, 500 µL lysozyme (50 mg/mL, Sigma UK), 100 µL mutanolysin (1 mg/mL, Sigma UK) and incubated at 37^oC for 1 hour with occasional inversion. Further lysis was done using 200 µL proteinase K (>800 u/mL, Sigma UK) and incubated at 55^oC for 1 hour. The supernatant was then discarded and 800 µL of N-Lauryl Sarcosine solution (Sigma UK) was added to the cells and incubated for a further 15 minutes at 68^oC. Proteins were precipitated by the addition of 500 µL saturated ammonium acetate solution (Sigma UK), for 1 hour at RT. To extract DNA, an equal volume of chloroform (Fisher Scientific UK) was added, centrifuged at 12000 xg for 3 minutes and resulting extracts then transferred to a fresh tube and repeated. Resulting DNA was precipitated with ice-cold ethanol (absolute,

Fisher Scientific UK) dissolved in sterile nuclease free water and stored at -20°C until use.

3.3.3 Metagenomic PCR survey of human gut-abundant Type II toxins, p22-RelB antitoxin and the p22-RelBE complete module

To identify the presence or absence of the five most abundant Type II TA module toxins, the p22-RelB antitoxin and complete p22-RelBE module in the human gut microbiome, a PCR survey was undertaken utilising 54 metagenomic gut DNA samples from patients with adenomatous polyposis, colorectal cancer or healthy individuals. Using primers specific for the p22-RelBE complete module, p22-RelB antitoxin and 5 most abundant Type II toxins, which are provided in Table 3.2 along with primer specific annealing temperatures, PCR reactions were prepared following the standard *Taq* PCR protocol in Section *2.2.4.1* and cycling conditions in Table 2.2. Template DNA was kept consistently between 3-8 ng/µL throughout all PCR reactions.

3.3.4 Quantitative PCR

Before target gene copy number of gut abundant toxins could be determined using qPCR, standardised concentrations of each TA toxin to be analysed were produced. The same primer sets used previously from Table 3.2 were used to amplify the five most gut-abundant TA module toxins from metagenomic DNA that was found to be PCR positive for these toxins (Section 3.4.3). A PCR positive result refers to metagenomic template DNA that exhibited a clear band at the correct size for the TA toxin under investigation.

Table 3.2: Primers used during this study.

Primer pair	Sequence 5'-3'	AT(^{o}C)	Product & origin
Tx1internF1 Tx1internR1	CGTGATATCCGTGCTTCCGA TCTTGCCTGATCTTCTGCGA	52.1	336bp product internal to the putative Tx1 gut-abundant TA toxin, this study.
Tx2internF1 Tx2internR1	GTCGACATTTGGGAGGCATC ATGTGCAGTATGGGGTAGGG	57.8	377bp product internal to the putative Tx2 gut-abundant TA toxin, this study.
pTRACA22ORF6F1 pTRACA22ORF6R1	CCCGTCCACTTCGTAGATCA GCGGCTTTGATATTCCTTTG	53.3	160bp product internal to the p22 putative RelE-like toxin (Tx3) ORF, this study
Tx4internF1 Tx4internR1	ATTGGATGGCGTGAGAGACA CTGCAGCACCAAGACCTAAA	57.1	257bp product internal to the putative Tx4 gut-abundant TA toxin, this study.
Tx5internF1 Tx5internR1	TGAAGATGCAGGTAGTCCCC GCCAATTCCTCGGTGTCTTC	57.8	385bp product internal to the putative Tx5 gut-abundant TA toxin, this study
pTRACA22ORF7F1 pTRACA22ORF7R1	CCTGATTGCTTCGACGGTTT TCGTATGGACGCAGACCTGA	54.3	168bp product internal to the p22 RelB-like antitoxin ORF, this study.
RBCF1 RBCR1	GGGTAAACGGCCAAGGAATA ACTGCGCGATCCTCTCTTTAT	52.6	678bp product encompassing the entire p22-RelBE TA module, this study
LessAB1F1 LessAB1R1	CGGTTATATTAGGCCATTACG ATACGGATGATTGGACCATCC	54.1	311bp product internal to a putative TA toxin, approximately 100x less abundant than Tx5, this study.
LessAB2F1 LessAB2R1	CGGATTAGCGATAGGCATTG ATGGACGGAGGTCTTAAGCG	52.9	296bp product internal to a putative TA toxin, approximately 10x less abundant than Tx5, this study.
27F 1492R	AGAGTTTGATCMTGGCTAG TACGGTACCTTGTTACGACTT	55.3	~1500bp product encompassing the 16S rRNA chromosomal region (Lane 1990).

A list of primers, their sequence, annealing temperature (AT), product size and origin (if available) used within this study. All primers listed above were obtained from Eurofins MWG Operon.

This was also repeated for one toxin gene that was approximately 10x lower in abundance than the 5 most abundant toxins found in section 3.4.1. For the 3rd most gut abundant toxin, the putative p22-RelE toxin, plasmid DNA from pTRACA22 was used as a template instead, as this was readily available for use.

The resulting DNA fragments were gel extracted (Section 2.2.7) and cloned into the pGEM-T Easy vector (see Section 2.2.14) using either a 1:1 or 1:3 vector to insert ratio following the manufacturer's instructions. After confirmation of insertion by PCR and restriction digest, serial 5-fold dilutions of insert-containing pGEM-T Easy DNA were used to produce standard curves with concentrations ranging from 10^3 to 10^7 copies. Copy number of all standards for each toxin was calculated using the following equation:

$$1)\ (bp\ of\ vector + Insert)(330\ Da\ \times 2\ nucleotide\ per\ bp) = g/mol$$

$$2)\ \frac{(g\ /\ mol)}{Avogadro's\ Constant: 6.02214199 \times 10^{23}} = g/molecule = copy\ number$$

$$3)\ \frac{Concentration\ of\ plasmid\ (\ g\ /\ \mu L)}{copy\ number} = molecules\ /\ \mu L$$

Subsequently, copy number variation of putative TA toxins was analysed using qPCR. Amplification was performed on a Rotor Gene Q thermocycler (Qiagen UK) in 0.1 mL PCR tubes (Qiagen UK) using a Rotor Q SYBR Green PCR kit (Qiagen UK). Each 25 µL reaction contained 12.5 µL SYBR Green PCR Master Mix, 7.5 µL nuclease free water, 0.5 µL forward primer (10 pmol), 0.5 µL reverse primer (10 pmol), 3 µL 25mmol MgCl$_2$ (giving a final concentration of 1 mmol) and 30-50 ng of template DNA using the cycling conditions visible in Table 3.3. To verify specificity of the resulting amplicon, melt curve analysis was performed on each run ensuring an efficiency of approximately 100% and an M-value of between -3.4 to -3.7.

Table 3.3: The cycling conditions for Rotor Q SYBR green quantitative PCR reactions.

Stage	Number of Cycles	Temperature (^{o}C)	Time
1	1	95	5 minutes
2	40	95	30 seconds
		Primer specific AT[1]	45 seconds
		72	45 seconds

[1]Primer specific AT refers to the temperature at which the primers optimally anneal to the template DNA

Copy number in individual DNA samples was calculated using the Rotor Gene Q software series and expressed as copies per ng/DNA. Resulting data was tested for normality and interrogated with one-way ANOVA analysis.

3.4 Results

3.4.1 Environmental distribution and abundance of Type II TA modules

Homologues to TA amino acid sequences were identified in a range of datasets using tBlastn. Valid hits for TA toxin, antitoxin and regulator queries were found in all 12 distinct environments investigated. The marine environment was found to have the highest number of toxin and antitoxin queries producing hits of all habitats investigated within this study, with approximately 75-80% of all queries searched for being hit within this environment. Interestingly, not a single regulator of the 4 available within the TADB was hit for any non-host associated environment. In fact hits to regulator queries were only seen within mammalian related habitats. The percentage of total queries producing hits in these broad environmental categories can be seen in Figure 3.3A.

Further processing to acquire non-redundant hits for each query was undertaken and used to calculate relative abundance. Significant differences in relative abundance values for toxin and antitoxin queries were seen for the mammalian gut and human microbiome when compared to the other 5 broad environmental categories used for this study. These differences can be seen in Figure 3.3B. This included significantly higher abundances of both Type II TA toxins and antitoxins than avian gut, marine and soil environments ($P<0.001$) and lower abundances than acid mine drainage, fresh water and termite gut ($P<0.001$). What few valid hits were identified for regulators across all environments did not show any significant differences ($P\geq0.05$). It was also noted that non-mammalian associated environments tended to have either minimal differences between toxin and antitoxin number or a significantly higher abundance of toxins ($P<0.05$). This is in comparison to the mammalian gut and human microbiome, which had significantly higher abundances of antitoxins ($P<0.001$).
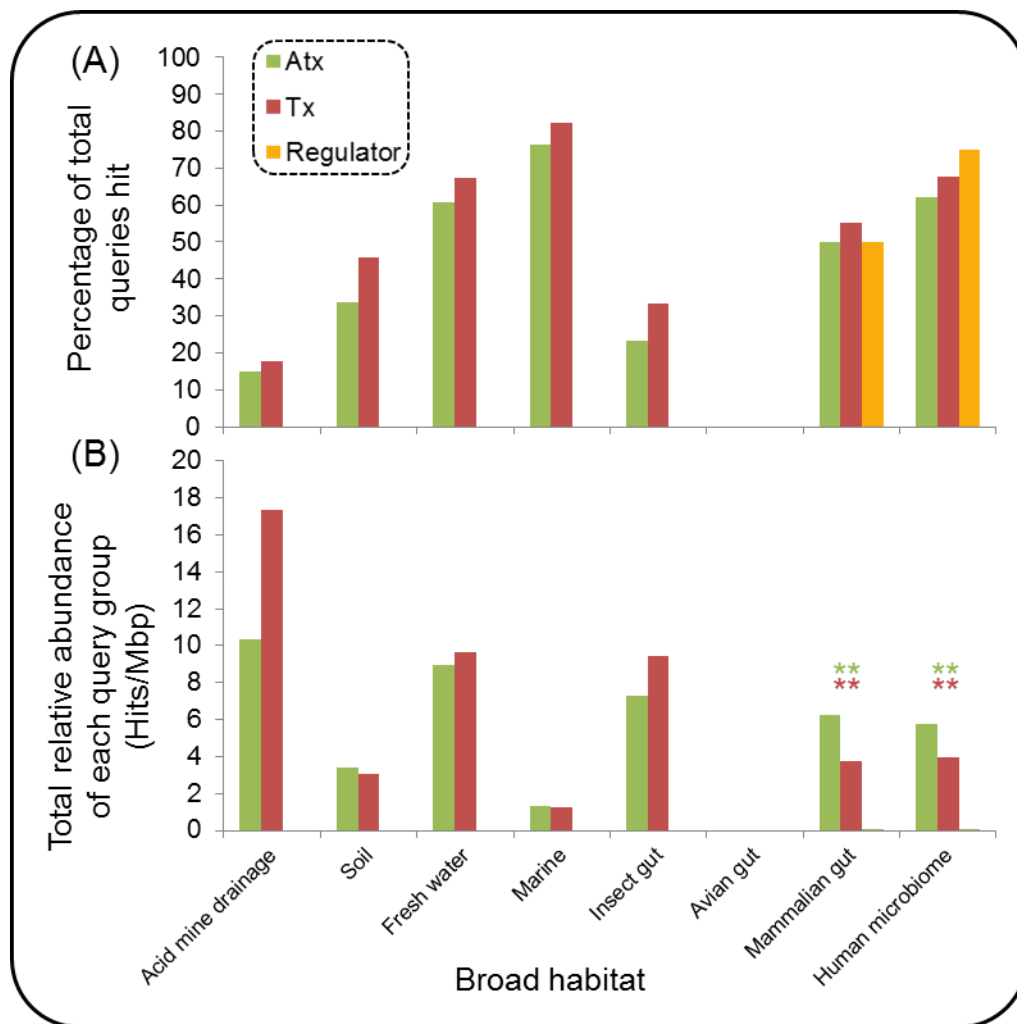
Figure 3.3: Total percentage queries with hits across a range of host- and non-host-associated habitats and total relative abundance of the 3 query groups.

(A) Data used is still redundant and did not specify best hits per region to ensure all valid hits were incorporated.

(B) Total hit counts for TA "toxin", "antitoxin" and "regulator" queries were used to calculate the total relative abundance of each group across 7 broad environments. Data was interrogated using Chi-squared analysis.

** = Significant difference in relative abundance compared to all other environments (P<0.001 or lower).

The Simpson's diversity index was used to investigate the diversity Type II TA toxins, antitoxins and regulators within each environment they were detected. This can be seen in Figure 3.4. All environments with the exception of chicken caecum (from which no homologues for any query were identified) had an extremely high diversity of toxins and antitoxins, with no value dropping below 0.98. Conversely diversity of regulators was minimal with only the human gut and oral cavity harbouring any homologues to these, and each of these environments have an index value of below 0.5.

Figures 3.5 and 3.6 depict the top 10% of toxins and antitoxins in terms of abundance across the 781 metagenomic datasets explored. With fewer original regulator queries available, all hits for these across all environments investigated are shown in Figure 3.7. By grouping datasets into categories including; non-gut associated and gut associated, clear differences and similarities between these environments become visible. When focusing on the human gut environment, there was a visible difference between the Japanese (denoted as "JP" on Figures 3.5 and 3.6) datasets and others. Valid hits for the regulator queries were much more limited and were found only in the human gut and oral cavity habitats.

Of the four human microbiome related environments (gut, oral cavity, skin and urogenital) the gut and oral cavity appeared to have higher abundances across a wider range of queries than the skin and urogenital environment. Conversely while the latter two environments harboured a less diverse query count, those queries that were represented tended to have a higher abundance than the gut and oral cavity, with the exception of the top toxins and antitoxins hit (see Figure 3.5 and 3.6).
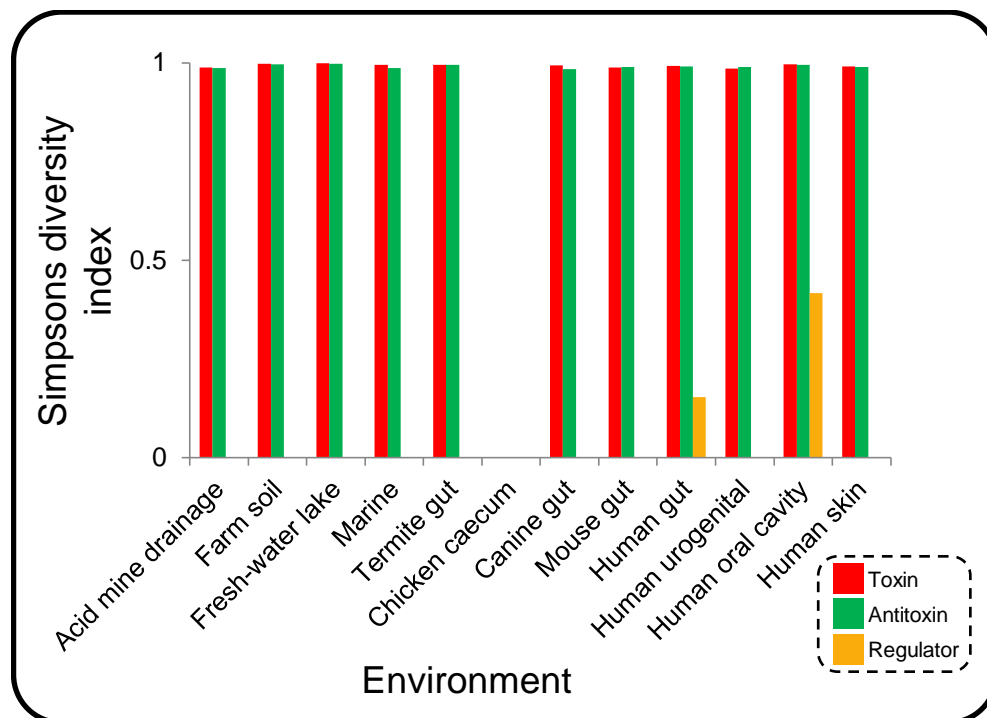
Figure 3.4: Simpson's diversity index values for the 12 broad environments investigated within this study. A value of 0 represents no diversity within an environment whilst a value of 1 indicates infinite diversity.
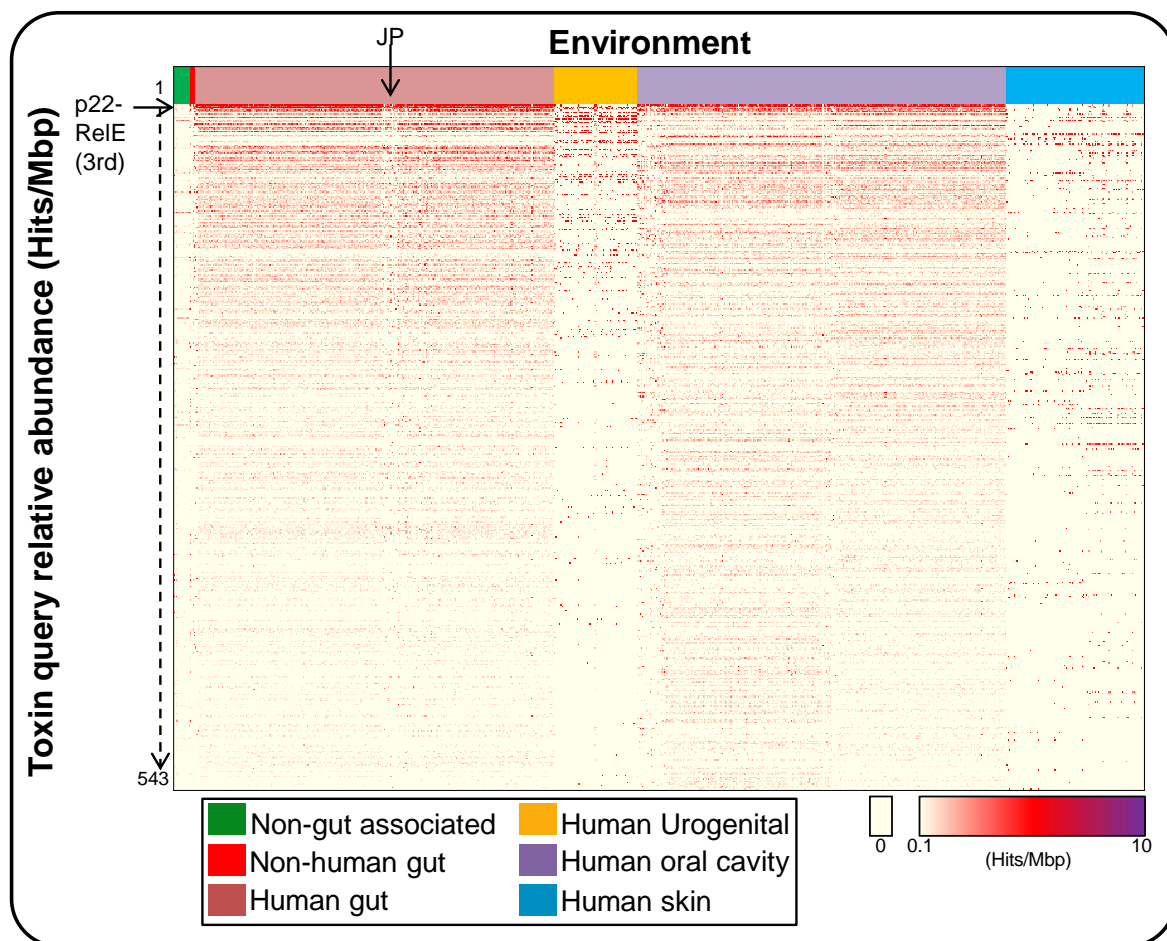
Figure 3.5: Relative abundance of sequences homologous to the 5 most abundant Type II TA module toxin genes across 781 metagenomic datasets. Each individual plot point within the heatmap represents the abundance of a specific toxin query in an individual dataset. Data was organised in descending order from highest total abundance (the sum of all environments) to lowest total abundance. Datasets have then been grouped into environments to help visualise any trends that appear. As the number of toxin queries searched was extensive, the heatmap has been cut-off at a total relative abundance value of 1.0, which leads to only the highest 543 of 5305 queries being shown.
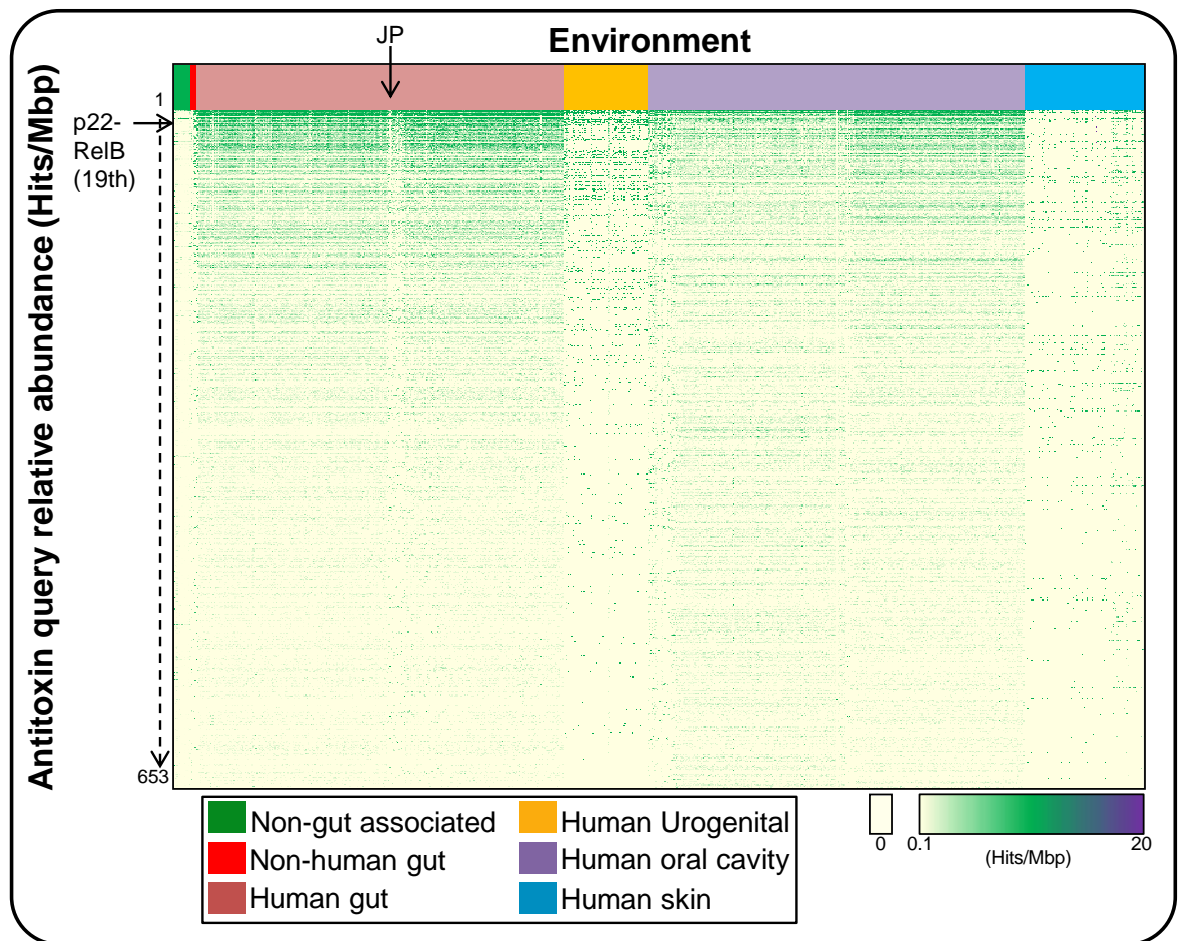
Figure 3.6: Relative abundance of sequences homologous to the 5 most abundant Type II TA module antitoxin genes across 781 metagenomic datasets. Each individual plot point within the heatmap represents the abundance of a specific antitoxin query in an individual dataset. Data was organised in descending order from highest total abundance (the sum of all environments) to lowest total abundance. Datasets have then been grouped into environments to help visualise any trends that appear. As the number of antitoxin queries searched was extensive, the heatmap has been cut-off at a total relative abundance value of 1.0, which leads to only the highest 653 of 5215 queries being shown.
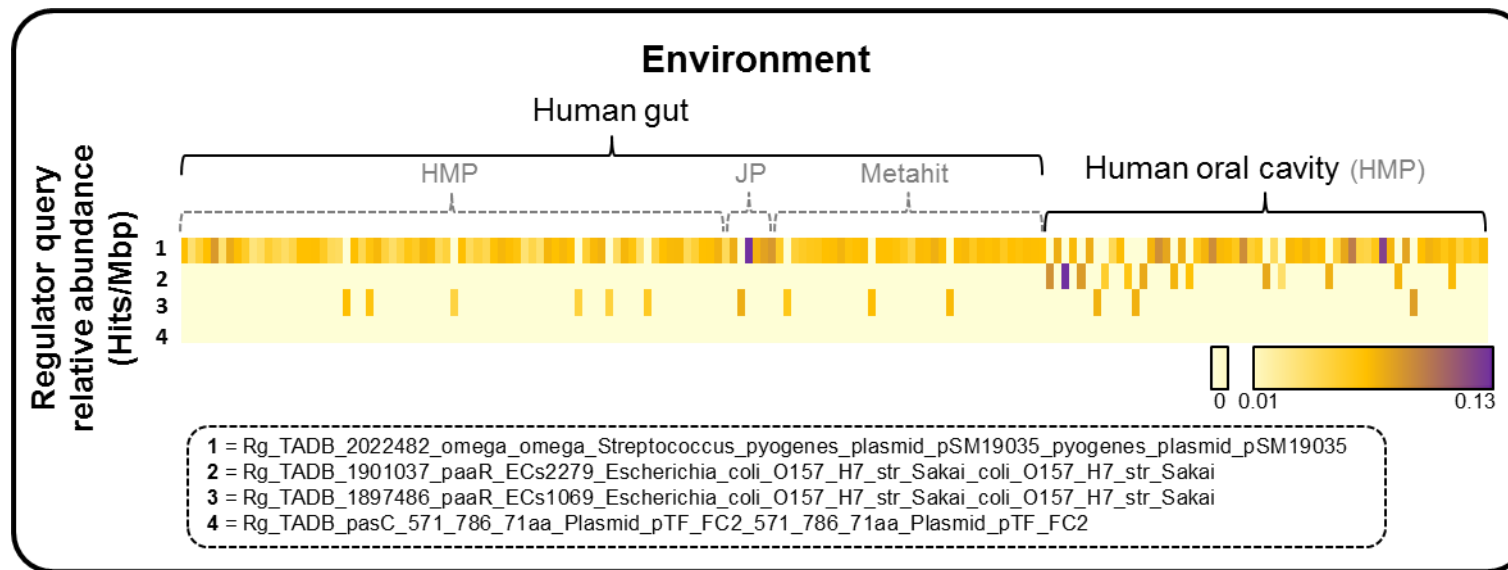
Figure 3.7: Relative abundance of sequences homologous to the 5 most abundant Type II TA module regulator genes across 781 metagenomic datasets. Each individual plot point within the heatmap represents the abundance of a specific regulator query in an individual dataset. Data was organised in descending order from highest total abundance (the sum of all environments) to lowest total abundance. Datasets have then been grouped into environments to help visualise any trends that appear. Only 4 regulators were available from the TADB and only 3 of which returned valid hits meeting all criteria (35% sequence identity, an alignment length of 50aa or greater and an e value 1e^{-5}). All of these hits were within the human gut or human oral cavity environment.

Table 3.4 Summary of the five most gut-abundant Type II TA module toxins and antitoxins.

Assigned Number	Query database reference	Related toxin family	Comments
Tx1	Tx_TADB_3680100_NC_007498 _1285752_1286780_Pcar_1093_ Pelobacter_carbinolicus_DSM_23 80	**FicT** (FicTA TA system)	Forces inefficient binding of ATP to DNA gyrase and topoisomerase IV reducing cellular metabolism (Harms *et al.*, 2015).
Tx2	Tx_TADB_6444557_NC_009512 _4108311_4108754_Pput_3670_ Pseudomonas_putida_F1	**GNAT** (Xre potential antitoxin)	An acetyltransferase effecting cell wall biosynthesis, often seen in studies related to antibiotic resistance (Makarova *et al.*, 2009).
Tx3	Tx_pTRACA22_ORF6	**RelE** (RelBE system)	Ribonuclease cleaving mRNA at the ribosomal A-site (Jones *et al*, 2010. Pedersen *et al.*, 2003).
Tx4	Tx_TADB_5773739_NC_009012 _3185146_3185496_Cthe_2699_ Clostridium_thermocellum_ATCC _27405	**PemK** (PemIK system)	Potent ribonuclease with preference for pyrimidines (Agarwal *et al.,* 2010)
Tx5	Tx_TADB_3732194_NC_007514 _323822_324835_Cag_0294_Chl orobium_chlorochromatii_CaD3	**FicT** (FicTA TA system)	Forces inefficient binding of ATP to DNA gyrase and topoisomerase IV reducing cellular metabolism (Harms *et al.*, 2015).
Atx1	ATx_TADB_3496963_NC_00733 3_818333__818782_Tfu_0708_T hermobifida_fusca_YX	**Xre**	Transcriptional regulator shown, possible antitoxin for multiple hypothetical TA module toxins (Makarova *et al.,* 2009).
ATx2	ATx_TADB_7332089_NC_00999 7_2379780__2380067_Sbal195_ 1992_Shewanella_baltica_OS195	**HigA** (HigAB system)	Initially found on Proteus vulgaris plasmid, often cotranscribed with HigB toxin (Fivian-Hughes & Davis 2010).
ATx3	ATx_TADB_3414349_NC_00710 9_187908__188189_RF_0168_Ri ckettsia_felis_URRWXCal2	**CopG**	Found previously partnered to the MazF toxin, abundant in firmicutes (Chopra et al., 2013)
ATx4	ATx_TADB_8112630_NC_01055 4_2258397__2258741_PMI2091_ Proteus_mirabilis_HI4320	**Xre-like protein** (COG3636)	Possible Transcriptional regulator, possible antitoxin for multiple hypothetical TA module toxins (Makarova *et al.*, 2009).
ATX5	ATx_TADB_7694153_NC_01033 3_73303__73623_Caul_5360_Ca ulobacter_sp__K31	**AbrB**	Identified to behave as antitoxins for MazF, Kis & PemK toxins (Makarova *et al.*, 2009).

Investigation of sequence data harbouring valid (and non-redundant) toxin hits within human gut metagenomic datasets revealed that on average 43.74% of these sequences also harboured 1 or more valid hits to an antitoxin query. This may suggest the presence of novel and as of yet undescribed antitoxins pairing with these modules or, alternatively, many toxin genes that are regulated by antitoxins (or other molecules) from other genomic locations.

3.4.2 Examination of the highest gut-abundant TA toxins and antitoxins

To investigate the most human-gut abundant Type II TA toxins and antitoxins in more depth, valid, non-redundant hits for all queries from human gut-related datasets were ranked according to relative abundance within this overall environment and the top 5 examined further. Table 3.4 summarises the top 5 most gut-abundant toxins and antitoxins. Furthermore, where possible the toxin or antitoxin hits are assigned to the family they are most closely related based on the original sequence database entry.

Of the 5 most abundant putative toxins present within the human gut microbiome two, the first and fifth most abundant, were related to FicT a Type II toxin which forces inefficient binding of ATP to DNA gyrase and topoisomerase IV reducing cellular metabolism (Harms *et al.*, 2015). The second most abundant did not belong to any specific toxin family, but did contain a GNAT motif, which is often associated with TA module toxins (Makarova *et al.*, 2009). This was followed by the p22-RelE toxin and the fourth was related to PemK, a ribonuclease (Agarwal *et al.*, 2010). These are referred to as Tx1-FicT, Tx2-GNAT, Tx3-RelE, Tx4-PemK and Tx5-FicT.

With regards to specific antitoxin families found, firstly 2 of the 5 most gut abundant (ATx1 and ATx4) were related to Xre, a transcriptional regulator protein predicted to neutralise several toxins including those containing GNAT motifs (Makarova *et al.*, 2009), such as Tx2-GNAT. The HigA antitoxin gene was found to also have a high relative abundance within the human gut and neutralises the HigB toxin, which mediated translation dependant mRNA cleavage (Hurley & Woychik 2009). Furthermore CopG and AbrB were found to be the third and fifth most abundant respectively. Both have been identified as possible antitoxins to the MazF toxin (Chopra *et al.*, 2013); however additionally AbrB has also been shown to neutralise PemK toxin activity (Makarova *et al.*, 2009), which was similarly highly abundant within the human gut environment.

To further analyse this data, all datasets were grouped by environment of origin and relative abundance was calculated based on the total size of that environment (in Mbp) rather than individual datasets (Figure 3.8). Valid hits for at least 1 of the previously identified 5 toxins and 5 antitoxins (See Table 3.4) were found in all environments with the exception of Acid mine drainage and Chicken caecum. Figure 3.7 shows heatmap representing these findings. The housekeeping gene RpoB (which encodes β subunit of bacterial RNA polymerase and was used a control due to the necessity of its presence in bacterial genomes) produced no valid hits within the farm soil, chicken caecum and the mouse gut environments. For the proposed 'gut-negative' photorhodopsin genes (which were expected to be lacking representation within the human gut microbiome) no valid hits were found for any environment, including all gut habitats, with the exception of marine environments. Comparatively, all 5 toxins and antitoxins were significantly lower in non-gut associated environments than the human gut environment ($P<0.05$).

Examination and comparisons of mammalian gut environments revealed significant differences in relative abundance for Tx1-FicT (human and mouse), Tx2-GNAT (human and canine), Tx4-PemK (human and canine) and Tx5-FicT (Human, mouse and canine, P<0.05). Differences were also apparent between ATx1-Xre, ATx2-HigA, ATx3-CopG and ATx5-AbrB putative antitoxins for human and canine gut environments while only Tx4 was significantly less abundant within the mouse gut microbiome than the human gut (P<0.05).

Comparisons between different sites of the human body also revealed differences with Tx2 and Tx4 being significantly less abundant in the human oral cavity. The examination of mammalian gut environments revealed significant differences in relative abundance for Tx1 (human and mouse), Tx2 (human and canine), Tx4 human gut (P<0.001). Similarly Tx4 and 5 in the urogenital tract were also less abundant than the human gut environment (P<0.05). As with toxins, antitoxin relative abundance also differed to the human gut when specifically focusing on the human microbiome (P<0.05). Those specifically were: ATx1-4 (human skin), ATx1-3 (human oral cavity) and ATX5 (human urogenital).
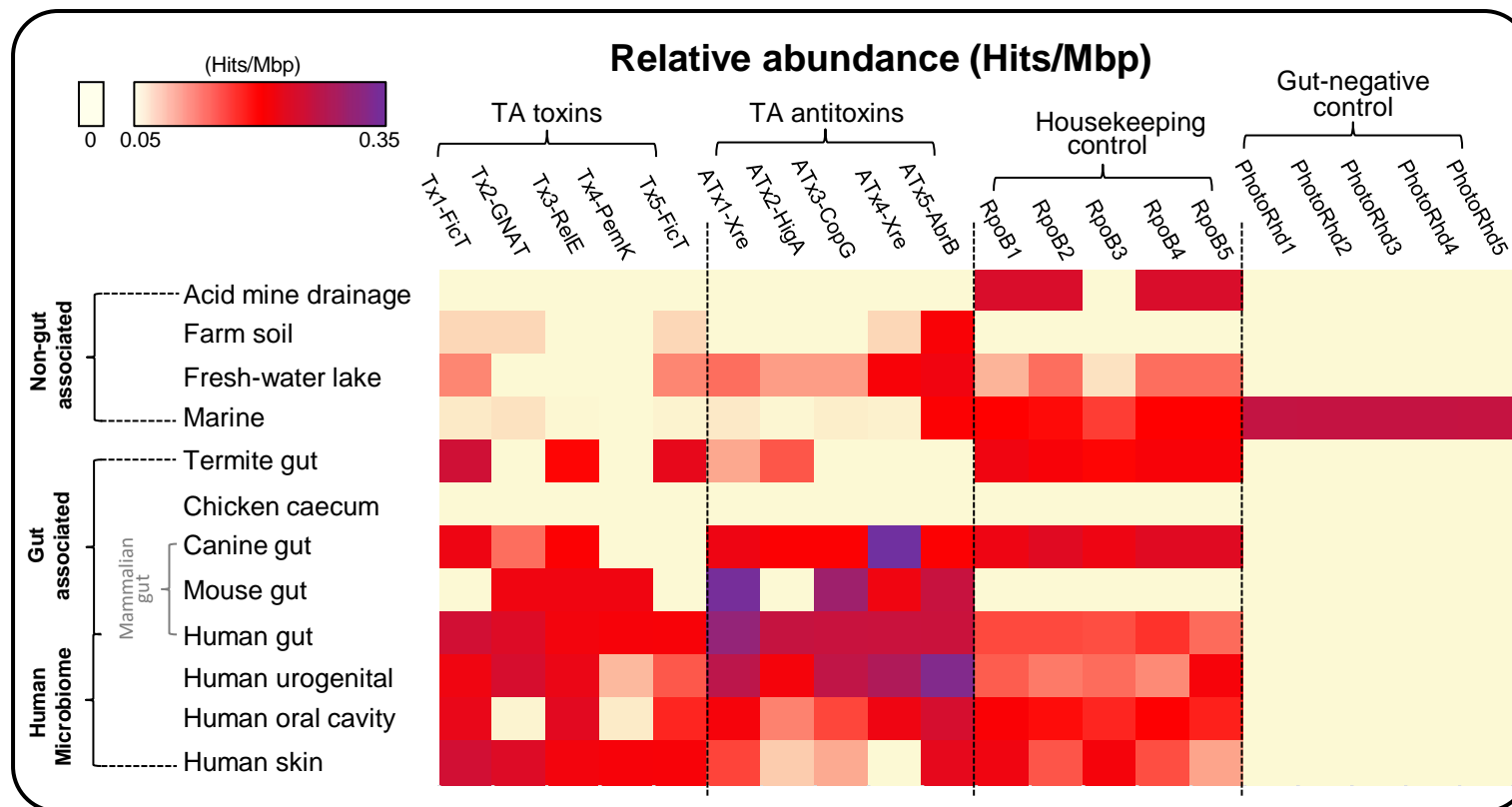
Figure 3.8: Relative abundance of sequences homologous to the 5 most abundant Type II TA module toxins and antitoxins across 12 unique environments. These were further compared to homologues of RpoB housekeeping genes and photorhodopsin gene which were included as a further control as these were not expected to be well represented within the human gut microbiome.

A heat map generated to illustrate the relative abundance of sequences homologous to 5 most abundant Type II TA module toxins and antitoxins in Hits/Mbp of DNA across a range of combined metagenomic datasets. A total of 10,524 TA module query sequences were used with 781 metagenomic datasets searched spanning 12 distinct habitats which have been categorised into, gut, non-gut or human microbiome associated. Within the gut subgroup, a further division between mammalian gut and others has also been noted.

As controls, a selection of 100 RpoB housekeeping genes and 54 photorhodopsin 'gut-negative' genes were also used as queries and relative abundance of these for all datasets was identified.

Data was normalised by the overall size of the dataset searched. The intensity of shading corresponds to an increase in value as indicated by the scale above the heat map panel.

3.4.3 Carriage rate and genetic distribution of gut-abundant Type II module toxins and antitoxins

In order to explore the carriage of the 5 most gut-abundant toxin and antitoxin components of Type II TA modules on distinct genetic units (chromosomes, plasmids and phage) secondary NCBI bacterial chromosome, plasmid and phage datasets were processed as for metagenomic datasets. Data is presented in Figure 3.8. In terms of carriage (hits per individual genetic unit, Figure 3.9A) it was clear that bacterial chromosomes harboured the greatest levels of both toxins and antitoxins with these 5 gut-related toxins appearing to be much more abundant in the NCBI datasets than gut-related antitoxins. Interestingly when examining the relative abundance (Figure 3.9B) of these ORFs, Tx3 (p22-RelE), ATx4-Xre and ATx5-AbrB were more abundant on plasmids than chromosomes ($P<0.05$). It was also noted that carriage of gut-related TA toxins and antitoxins appeared to be significantly less well represented than the housekeeping RpoB genes on chromosomes ($P<0.001$). Gut related TA toxins and antitoxins appeared to be completely absent from NCBI phage genomes, which did not differ from housekeeping or photorhodopsin control gene findings.
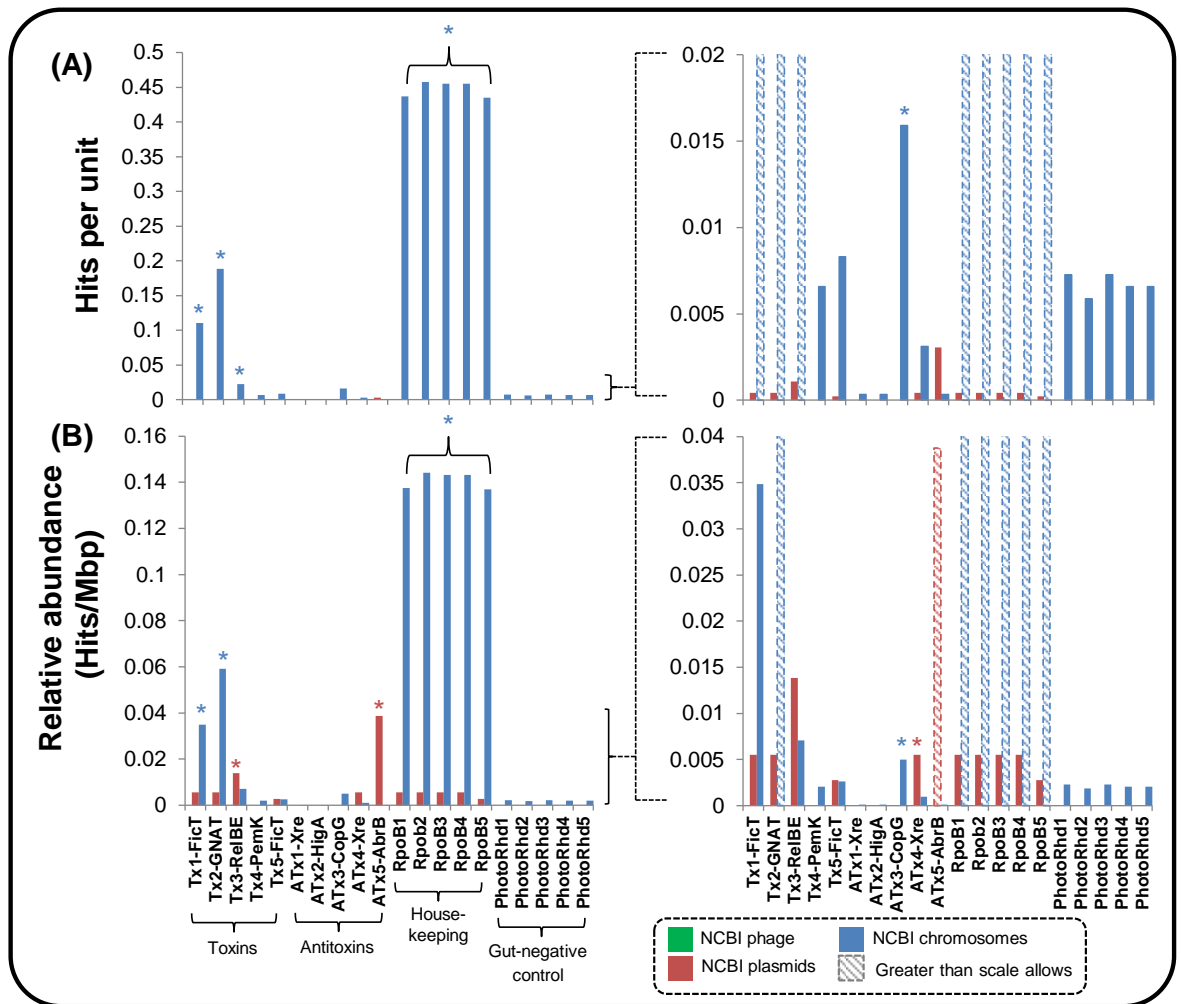
Figure 3.9: Carriage analysis of the 5 most gut-abundant TA module toxins and antitoxins on distinct genetic units. This comprised of 612 bacteriophage genomes, 4580 bacterial plasmids and 2891 bacterial chromosomes.

(A) Carriage of toxin, antitoxin, RpoB gene and photorhodopsin homologues within distinct genetic units (bacteriophage, plasmids and chromosomes), expressed as average number of hits per unit.

(B) Relative abundance (Hits/Mbp) of toxin, antitoxin, RpoB gene and photorhodopsin homologues in distinct genetic units.

* = Significant difference in carriage between genetic units compared to housekeeping and gut-negative controls (P<0.05).

3.4.4 PCR survey of Type II toxins in human gut microbial metagenomes

To support data from the comparative metagenomic approach undertaken, a PCR survey utilising 54 human gut metagenomic DNA samples (extracted from faecal samples collected from patients from the UK and Czech Republic) was conducted. Patients were either healthy or sufferers of adenomatous polyposis (AP) or colorectal cancer (CRC). The PCR survey used internal primer sets specific to the 5 most gut-abundant toxins (which included the p22-RelE ORF) as well as p22-RelB antitoxin ORF and complete p22-RelBE module. All samples were first shown to be amplifiable using 27F/1492R universal 16S rRNA primers.

Toxins found to have high relative abundance within the human gut, based on *in Silico* searches, also showed significantly greater detection rate than toxins with low relative abundance within these searches (P<0.05, Figure 3.10A). All 5 toxins of interest had a percentage presence within the metagenomic samples of approximately 80-90%. Tx3 (p22-RelE) was also detected significantly more than the p22-RelB antitoxin or module in a complete form, which were present in approximately 70% of samples examined (Figure 3.10B). It was also apparent that presence of one or both of the pTRACA22 TA components did not necessarily ensure the presence of a complete module in a given sample. For example, samples P7, 003, 004, 005, 014, 040, 053 and 057 all detected both the toxin and the antitoxin yet the complete TA system was not (Figure 3.10C).

Figure 3.10: A PCR survey identifying the presence or absence of the 5 most gut-abundant TA module toxins and the p22-RelBE complete module and individual components. 54 metagenomic samples from human gut microbiomes of healthy and

unhealthy patients encompassing adenomatous polyposis (AP) and colorectal cancer (CRC) sufferers from the United Kingdom and Prague, Czech Republic. Relative abundance values from previous metagenomic analysis are provided on bars where available. Values at the top of each bar refer to the relative abundance of each putative TA component based on *in Silico* work undertaken previously in this chapter.

(A) The percentage of PCR positive reactions for the 5 most gut-abundant TA module toxins, which included p22-RelE. These were also compared to two other toxins with 10 (Low abundance control TxA) and 100 (Low abundance control TxB) times lower relative abundance identified during the earlier *in Silico* studies.

(B) The percentage of PCR positive reactions for the p22-RelBE toxin and antitoxin individual components as well as the complete intact module.

(C) Presence/absence chart displaying which samples which harboured the 5 putative TA modules toxins, the p22-RelB antitoxin and the p22-RelBE complete module. Blue indicates the presence of the toxin (PCR positive) whilst red indicates absence (PCR negative).

* = significant difference in presence compared to both controls (A) and the p22-RelB/p22-RelBE complete module (B) ($P<0.05$).

3.4.5 Abundance of the most gut-abundant Type II toxins in human gut microbial metagenomes

Given the increased representation of Tx1-5 in gut metagenomic DNA surveyed; Copy number of these genes between distinct subgroups (healthy, adenomatous polyposis sufferers, colorectal cancer sufferers and geographic location) was assessed using qPCR. Wide inter-individual variation in copy number was seen ranging between 1.58×10^5 -7.61×10^7 per ng DNA. As shown in Figure 3.11A, all 5 toxins of interest had significantly higher copy number per ng DNA when compared to the low abundance control toxin included ($P < 0.05$). Interestingly Tx3 (P22-RelE) had a significantly higher overall copy number in DNA samples than Tx1, 4 and 5 while Tx1 and 2 were significantly higher than Tx5.

Comparative analysis of geographic locations the samples were recovered and disregarding health status (Figure 3.11B) highlighted a significant increase in copy number of Tx5 for patients from the Czech Republic ($P < 0.05$). Tx1-3 along with the low abundance control did not show any significant difference.

To shed further light on copy numbers of the RelE toxin within these samples, data was interrogated in terms of health status (Figure 3.11C). Tx1-FicT, Tx3-RelE and Tx5-FicT showed no significant differences between healthy, AP and CRC sufferers whilst Tx2-GNAT showed a significant reduction of copy number in those suffering from CRC ($P < 0.05$). CRC sufferers were also different when analysing Tx4-PemK however copy number was significantly higher in CRC sufferers ($P < 0.05$). It should be noted that this data was not normally distributed due to a small sample size for CRC sufferers of just 6 (n = 6).
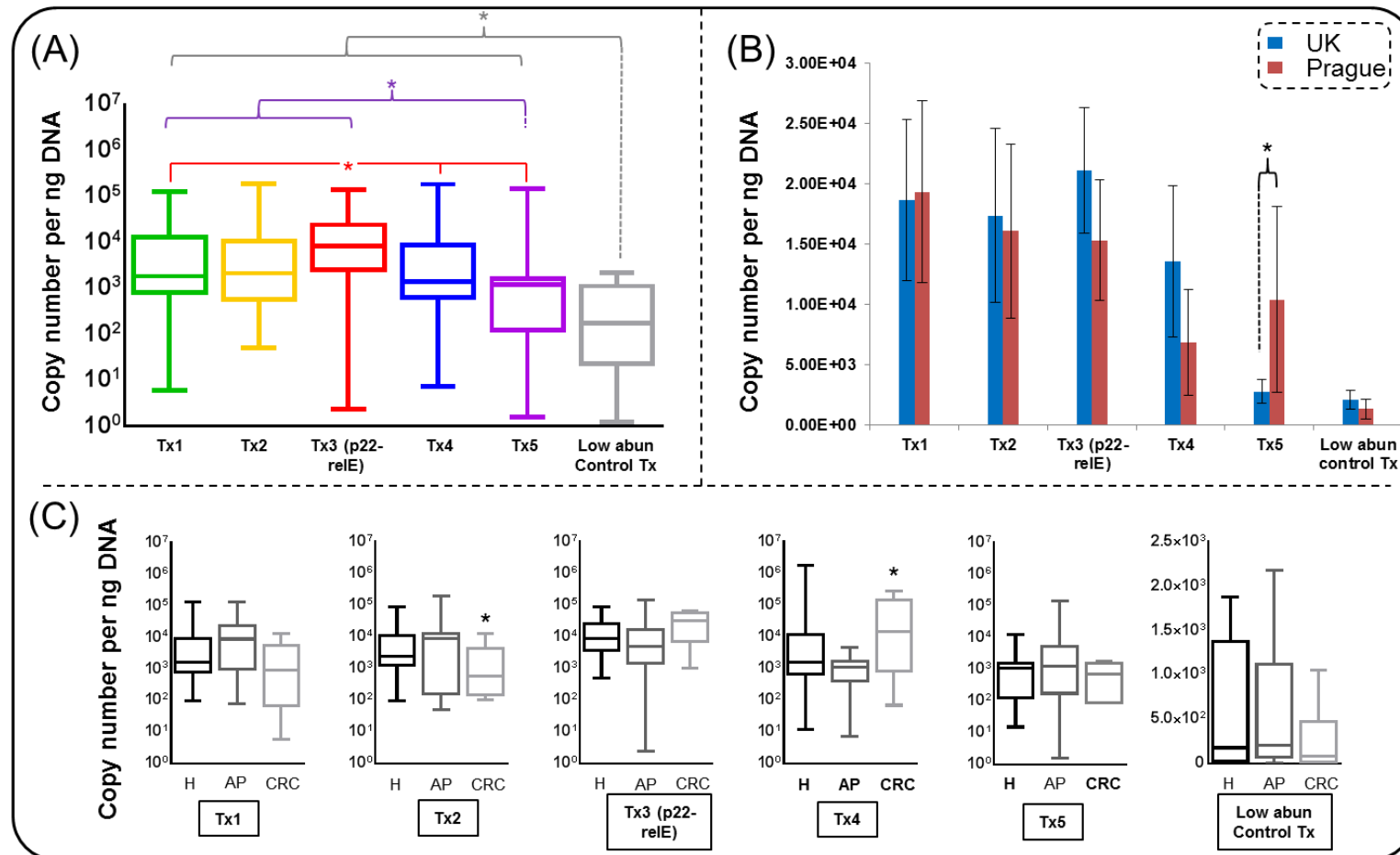
Figure 3.11: Analysis of the 5 most human gut-abundant TA module toxins copy number using metagenomic DNA from 54 human gut microbial metagenomes. Samples were obtained from healthy and unhealthy patients who encompassed adenomatous polyposis (AP)

and colorectal cancer (CRC) sufferers from the United Kingdom and Prague, Czech Republic. Data is presented in the form of box plots, with the central line representing the median whilst upper and lower quartiles identified by "whiskers" respectively. (A) Comparison of copy number per ng of DNA of the 5 most abundant TA module toxins within the human gut environment.

(B) A secondary comparison, grouping samples by geographic location rather than patient health group. Data was normally distributed but of unequal variance. United Kingdom n = 35, Czech Republic n = 19.

(C) Toxin copy number for Healthy, AP and CRC cohorts were compared for each of the most abundant gut-TA module toxins and also one found to be less abundant in previous *in Silico* studies within this chapter. Data was not normally distributed. Healthy n = 20, adenomatous polyps n = 21 and colorectal cancer n = 6.

All experiments were undertaken in triplicate and results shown are the mean ± SEM of three independent experiments.

* = A significant difference in copy number compared to the healthy cohort (P<0.05). Unless specifically displayed otherwise, coloured brackets signify which particular dataset is different to others it connects to.

3.5 Discussion

3.5.1 Environmental distribution and abundance of Type II TA systems

In this study we have undertaken a comparative metagenomic approach to identify sequences homologous to all known Type II TA modules module across 12 different habitats as well as chromosomal, plasmid and bacteriophage datasets. This work was then further supported by an extensive PCR survey of 54 gut microbial metagenomes, along with quantitative analysis of the 5 most human gut-abundant toxins using qPCR to discriminate between samples, geographic location and different health groups (healthy, AP or CRC). Sequences homologous to the p22-RelE toxin were found to be highly abundant within the human gut microbiome, when compared to other environmental metagenomic datasets.

Until recently, the cellular functions of TA modules were thought to be consigned to vertical transmission of plasmids and their maintenance through the mechanism of PSK (Gerdes 2000). In this system, loss of the plasmid from daughter cells and subsequent loss of antitoxin production ultimately leads to cell death. With this in mind, TA systems may contribute to their own higher prevalence within the human gut microbiome; however, this does not explain the observed lower abundance in other environments.

The grouping of both toxin and antitoxin relative abundances by dataset provided a number of new insights into TA distribution. The most obvious is that there appears to be a higher abundance of toxins and antitoxins in the human gut and human oral cavity than all other environments investigated, including other portions of the human microbiome such as the urogenital tract and skin. Additionally there appears to be some similarities between both these habitats in terms of which toxins/antitoxins are abundant and which are not. This is perhaps

not that surprising as the oral cavity is the route through which microbes colonise the GI tract. At birth the human foetus is considered sterile with the initial seeding of both the skin and gut microbiome occurring during delivery. Generally (if a normal birth) this includes *Enterobacteriaceae*, streptococci and staphylococci from the mother's vagina and faeces (Morelli 2008). From this link, similarities between the microbiota, their distinct metagenomes and possibly abundance in TA genes might be expected. However the initial microbial colonisers tend to represent a tiny portion of the adult gut microbiome and the differences in Type II TA relative abundance may be explained by the development of the gut microbiome and replacement of these colonising species by more mature 'enteric' gut microbiota (Sommer and Backhed 2013). Furthermore the processing and sequencing of metagenomic DNA may have a bias towards particular genus; suggesting that while the datasets searched within this study are relevant; they may not represent the entire community and its genomic content (Brooks et al., 2015).

The significant difference in relative abundance profiles between the American/HMP/Metahit and the Japanese gut dataset many be a result of differences in diet. Diet has been shown to influence microbiome diversity, being perhaps the largest factor effecting composition considering how resilient the gut microbiome is (Xu & Knight 2015). Whilst this is likely a factor, it is also possible that differences in methodologies used to obtain and sequence these metagenomes may also affect the outputs seen in this study. The American and Japanese datasets utilised Sanger sequencing technology to generate both datasets which is considered highly accurate, but provides a relatively low read coverage. Alternatively the Metahit and HMP datasets utilised next generation sequencing techniques (including both Illumina and 454) which have a much

higher read depth but are less accurate and provide shorter reads (Shendure & Ji 2008).

Out of the 25 non-human metagenomes examined, the canine and mouse gut dataset were similar to the human gut for both the toxin and antitoxin relative abundance. This similarity is understandable as both are mammals and share similar gastrointestinal (GI) anatomy and physiology (Smeets-Peeters et al. 1998). In parallel to this, as previously found, the average gut microbiome of a dog is similar to humans in terms of phylogenetic structure, with the Phyla Bacteriodetes and Firmicutes being co-dominant, together comprising ~70% of the microbial load within this environment (Swanson et al. 2011). The practice of keeping dogs as pets also increases similarities between human and dog dietary intake (Swanson & Schook 2006) and sharing of habitats would undoubtedly increase the convergence of dog and human microbiomes.

Of the remaining gut-associated habitats explored, the termite gut showed significant similarities in relative abundance for Tx1-FicT, Tx3-RelE (p22-RelE) and Tx5-FicT. Hits for all environments were normalized by the size of the dataset to remove any bias created by the dataset size; however this would not have removed the effect a low depth of coverage and differences in initial sampling processes may have had on the dataset when it was originally gathered.

Each dataset used within this study was comprised of variable numbers of metagenomes. For example, to generate the human gut dataset, 439 individual metagenomes were used with a sum of 28265.11 Mbp of DNA whereas, in comparison, acid mine drainage was comprised of just 1 metagenome of 61 Mbp. The diversity of acid mine drainage communities has been demonstrated to be considerably lower than those found in other habitats therefore requiring less sequencing effort to get a depth of coverage comparable to more complex

microbiomes despite being subject to greater levels of sequencing. While not a limitation of this dataset, it should also be noted that the lower diversity found in AMD environments would affect the relative abundance of TA modules, due to a select few species being more abundant and either harbouring this module or not. In parallel to this, low diversity may also affect, either positively or negatively, horizontal gene transfer (Bond & Druschel *et al.*, 2000; Bond & Smriga *et al.*, 2000; Baker & Banfield 2003).

The finding that pTRACA22-RelE (Tx3) toxin was in such a high abundance within the human gut environment supports previous work by Jones *et al.,* 2010 which found this module at a higher abundance in the human gut than murine gut, soil and marine metagenomes, albeit on a small scale. Additionally it was also more abundant than several other TA modules (MaezEF, ParDE and HigAB). The second most gut-abundant toxin (Tx2) that was identified in studies undertaken in this chapter was a Gcn5-related N-acetyltransferase or GNAT protein. This had previously been identified through comparative genomic analysis as a new putative toxin when investigating TA systems and how they are related to mobile stress response systems in prokaryotes (Makarova *et al.*, 2009). GNAT-like TA toxins were predicted to utilise Xre, a family of transcriptional regulators, as an antitoxin, and interestingly 2 of the 5 most abundant antitoxins (ATx1 and ATx4) found in the human gut dataset metagenomic analysis are predicted to belong to this family of proteins.

'Xre-like' proteins have been suggested to behave as antitoxins for several different putative TA module toxins due to its conserved Helix-turn-helix (HTH) motif used to bind nucleic acid (Brennan & Matthews 1989, Makarova *et al.*, 2009). This non-specific ability to neutralise and in some cases repress multiple toxins appears to be a somewhat common feature for most of the highly abundant

antitoxins within the human gut microbiome with the 3rd (ATx3) most abundant, CopG, and 5th (ATx5) most abundant, AbrB, also suggested to have this potential (Makarova *et al.*, 2009 Chopra *et al.*, 2013). This is not a new concept as several studies to date have addressed mutli-level interactions between both homologous and non-homologous, non-cognate systems which co-exist within bacterial genomes but also smaller replicons such as mobile genetic elements (MGEs) or plasmids (Masuda *et al.*, 1993, Goeders *et al.*, 2013, Nolle *et al.*, 2013). From data presented in this chapter it is possible to conclude that broad-spectrum Type II TA ATx appear to be abundant within the human gut microbiome and to hypothesise that while Tx functions are more specific in terms of mechanism ATx might be more general allowing one family to regulate and repress multiple Tx. The finding that only 43.74% of sequences harbouring homologues to known TA toxins also carried homologues of known TA module antitoxins also supports this hypothesis.

Another potential benefit from carriage of antitoxins that can neutralise several different TA toxins would be protection from parasitical mobile genetic elements harbouring TA systems. As shown in Figure 3.9B and 3.9C it is possible for toxins to be encoded away from the corresponding antitoxin. This would enable cells to avoid the limiting effects of free toxin without the further expenditure of energy as it would be possible to maintain and express the antitoxin separate (and without risk). If this were the case, carriage of broad spectrum ATx within the gut microbial community provides an already present countermeasure to incoming mobile genetic elements harbouring TA systems. These ATx could prevent the initiation of PSK, allowing unhindered loss of the element from the population without repercussions, i.e. an anti-addiction system. This would have to be a delicate balance however as balanced toxin-antitoxin ratios have been shown to

be crucial for survival with perturbation leading to an excess of free toxin and subsequent inhibition of growth and/or cell death (Goeders & Van Melderen 2014).

The finding that the second most abundant putative ATx within the human gut environment was a HigA antitoxin was surprising based on previous studies by Jones *et al.*, (2010). In this chapter homologues of the HigA antitoxin (ATx2) were significantly higher in the human gut datasets when compared to p22-RelB (the 19th most abundant ATx found), whilst Jones *et al.*, (2010) found it to be significantly lower than the RelB antitoxin. Somewhat contradicting the broad-spectrum findings of Xre-like Antitoxins within this environment, evidence suggests that the HigA antitoxin is unable to regulate other genes outside of its own operon, at least within the background of *Mycobacterium tuberculosis* (Fivian-Hughes & Davis 2010). While relevant, the neutralising spectrum of HigA has only been investigated within one host, a host that harbours, to date, approximately 79 TA modules and occupies a very niche environment (Sala *et al.*, 2014). Additionally, the majority of these TA systems were related to VapBC, MazEF or RelBE. Furthermore no experimental evidence of the HigB toxin being neutralised by other antitoxins has been noted to date. If, in the study undertaken in this chapter, HigB homologues also had a high relative abundance, it could be argued that the data also concluded HigA was not broad spectrum in its activity. However, the finding that the closest HigB homologue had a relative abundance of 0.001 (Hits/Mbp) and was the 415th most abundant, combined with the lack of diversity in TA modules located with *M.tuberculosis* would suggest this is not the case. Further study into the activity range of HigA is required.

It is difficult to draw solid conclusions from results relating to Type II TA regulators due to the small pool of data to draw from, unlike other TA components. While some Type II regulators have been found, this is relatively minimal (Smith &

Rawlings 1997, Hallez *et al.*, 2010, Bordes *et al.*, 2011) and they are more commonly seen associated with Type I TA systems (Brantl 2012). Of the 4 regulator queries available within the TADB, only 3 returned valid and non-redundant hits. These were located within the human gut, and oral cavity datasets. The most abundant of these was the ω regulator of the ω-ε-ζ module. This is unsurprising as out of the four regulators within the TADB, ω on its own is responsible for repression of the ω-ε-ζ module i.e. the antitoxin and toxin-antitoxin complex are not involved in this process (Unterholzner *et al.*, 2013). Excitingly no homologues of ε were found during metagenomic searches of the human gut and oral cavity datasets, however the ζ toxin was. Firstly it can be hypothesised that the regulator alone, can repress the expression of this toxin and secondly that the ζ toxin can be neutralised by other, non-related, antitoxin molecules. The final two regulators identified within the human gut and oral cavity datasets were related to the PaaA-ParD TA system. The regulator generally precedes the antitoxin and toxin gene (in its normal operon architecture) and both the antitoxin and regulator are required for strong repression (Mruk & Kobayashi 2014). As with ω-ε-ζ module, no antitoxin homologues for ParD were identified within these datasets suggesting, again, that this toxin is regulated by other TA system antitoxins within that environment.

3.5.2. Genetic carriage and distribution of 5 most gut abundant toxins and antitoxins

Using 9510 genetic units (comprised of 3919 bacterial chromosomes, 4880 plasmids and 711 phage genomes/metagenomic fragments) separated into distinct datasets the average number of gut-abundant toxins, antitoxins and control genes per unit as well as relative abundance was identified. The high carriage rate

of hits per unit seen predominately on chromosomes is, very likely, due to their size when compared to plasmids. TA modules are fairly ubiquitous throughout prokaryotic genomics; however, numbers per chromosome and plasmid appear to be affected by species habitat, lifestyle, niche and function (Van Melderen 2010). The significantly higher number of Tx1-FicT, Tx2-GNAT, Tx3-RelE and ATx4-Xre loci on chromosomes, could be interpreted as there being 'more' carried on this discrete genetic unit, however, this can be misleading.

The method of normalisation here did not involve the size of the entire dataset but instead was based on the number of units (i.e. chromosomes, plasmids or phage) present within each dataset and it was found that per individual chromosome searched, there were more toxins and antitoxins than per single plasmid. This is unsurprising when the size of a bacterial chromosomes compared to that of a plasmid is considered (for example *E.coli* K-12 has a genome size of 4.6Mbp comprising 4377 predicted genes, and 42 TA loci (Yamaguchi & Inouye 2011) compared to the plasmid pTRACA22 which is 5.9kb in length with 9 predicted ORFs, two of these being the RelBE module (Jones *et al*. 2010)). Chromosomes are larger genetic entities than plasmids and there does seem to be a significant correlation between genome size and TA numbers (Makarova *et al*. 2009), with smaller genomes such as the obligate host associated bacterium mycoplasma and obligate intracellular parasite Ricketsia containing very few systems (Pandey & Gerdes 2005; Makarova *et al*. 2009). This is likely consequential of the closed environments occupied by these species and therefore low horizontal gene transfer rates or may be simply due to genome size reduction, known to happen in obligate host associated organisms. However, due to the extreme levels of adaption from these organisms, they are not ideal comparisons to the majority of other prokaryotic organisms.

When normalizing the number of hits by dataset size to give relative abundance rather than number of units, it was apparent that, for the most part there was no difference between 'hits per Mbp' and 'hits per units' values. Three exceptions were the Tx3-RelE (p22-RelE toxin), ATx4-Xre and Atx5-AbrB where there was a greater rate of carriage of homologous sequences identified on plasmid DNA. Focusing specifically on the Tx3-RelE toxin, belonging to the p22-RelBE module, which was initially found on plasmid DNA using the TRACA system to capture plasmids in the human gut microbiome from stool samples (Jones & Marchesi 2006). Later analysis, using PCR, revealed that, of 46 plasmids isolated using this technique, 60.8% harboured these genes (Ogilvie *et al.* 2012). All plasmids captured ranged in sizes from 3.7-10.8kb, with similar G+C contents to the major bacterial divisions that comprise the human gut microbiota (Jones *et al.* 2010). However, it is unknown whether the method of recovery of plasmids by TRACA may have resulted in a pool biased for plasmids of this size or harbouring these TA systems. The abundance of the module on plasmids may represent a function(s) more usually undertaken on plasmids than chromosomes, such as vertical plasmid stabilisation. It may also simply reflect the effectiveness of the module to be transferred between mobile elements and behave addictively. Plasmids within the gut environment may be involved in the maintenance of ecosystem functions, as was proposed by Jones *et al.* (2010), and could aid retention of these genes, even when there was an absence of selective pressure on the functions that they encode.

The lack of hits to the most gut-abundant toxins and antitoxins for any bacteriophage genomes or genome fragments derived from metagenomes was unexpected as there has been compelling evidence suggesting TA modules can be carried on bacteriophage (Lehnherr *et al.* 1993; Romero *et al.* 2009). It can be

hypothesised that this lack of TA hits within phage genomes may, in part, be because they are frequently employed by bacteria as anti-phage factors. Work done by Pecota & Wood has linked Type I TA modules (hok/sok) with phage resistance (specifically that of the T4 phage) and more recent evidence provided, showed discovery of the Type III module ToxIN acting as a phage abortive infection system (Abi) (Fineran *et al.* 2009; Blower *et al.* 2009; Pei *et al.* 2011; Blower *et al.* 2012). Of course it has to be taken into account that these 5 putative toxins and 5 putative antitoxins only represent those most abundant in the gut and hits to phage/virome databases did occur but these may be phage related to other environments and/or bacterial species not found within the human gut.

3.5.3. Presence and copy number of the 5 most gut abundant TA toxins, p22-RelB antitoxin and complete p22-RelBE module.

Using conventional PCR techniques to identify the presence (or absence) of the top 5 gut-abundant toxins including p22-RelE, p22-RelB (19th most abundant ATx in *in Silico* studies) and the complete p22-RelBE module, we tested metagenomic DNA samples extracted from 54 human gut microbiomes from patients who were either healthy or suffering from AP or CRC. The failure to detect the toxin, antitoxin or entire module is not proof of absence, as conditions were initially optimised using the pTRACA22 plasmid as template and a single metagenomic sample (H1), therefore not allowing for variation of quality between 53 metagenomic samples. The detection limit of all 3 p22-related PCRs was identified using pTRACA22 DNA (0.001 ng/µL) with 0.002-0.010 ng/µL being sufficient to see a clear product for each of them. H1 was used to determine the detection limit for the remaining 4 gut-abundant Tx (0.01 ng/µL) with 0.1-5 ng/µL being sufficient.

Approximately 85% or more of the 54 gut metagenomic samples were positive for the presence of the 5 most gut abundant toxins identified during the previous *in Silico* study and this was significantly higher than two low-relative abundance toxins. TxA and TxB were chosen as they had significantly lower relative abundances (100 and 10 times respectively) than the five highest and it was hypothesised that these would be significantly lower in the PCR survey, supporting *in Silico* findings.

A significantly higher incidence (referring to the number of metagenomes in which the toxins, p22-RelB antitoxin and p22-RelBE full module were detected) of the p22-RelE toxin than the antitoxin and complete module was found within these samples. These findings are supported by other recent work done by Leplae *et al.*, (2011), who utilised an *in Silico* approach to survey a database containing 2181 genome sequences (comprised of 794 bacterial genomes, 964 plasmids and 420 phage genomes) for homologous sequences to 8 toxin and 9 antitoxin sequences, representing different TA super families. The criteria used by Leplae *et al.*, (2011) to generate a valid hit included sequence alignments being excluded if they had less than 50% alignment, smaller or greater than 40 or 650 amino acids respectively, with an e-value threshold of $1e^{-3}$. These criteria were less stringent than that used in Section 3.3.1 of this chapter.

Leplae *et al.*, (2011) found a much higher proportion of hits returned for the RelE toxin than the RelB antitoxin and Figure 3.12, an adaption of their supplementary data demonstrates this. This, to some extent contradicts findings by Jones *et al.*, (2010), however it must be considered that while Jones *et al.*, (2010) used metagenomic datasets subdivided into environments, Leplae *et al.*, (2011) utilised genomes and plasmids from the NCBI.
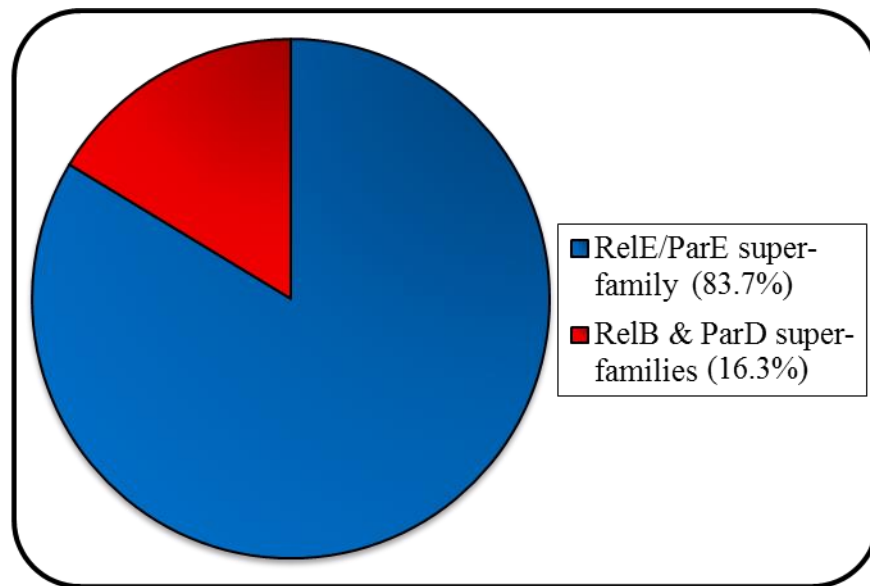
Figure 3.12: Percentages of valid hits to the RelB/ParE toxin and RelB/ParD antitoxin super-family in NCBI plasmids and genomes. Using the Chi-squared test, a significant difference can be seen between the two with a p-value of <0.001. Super-families were designated by Van Melderen *et al*, 2011. Data was adapted from Van Melderen *et al*, 2011.

Furthermore, when comparing general trends in presence of the p22 toxin and antitoxin seen by PCR survey and the abundance *in Silico* study there appears to be some discrepancies. The *in Silico* metagenomic analysis, derived from the MetaHit, HMP, Japanese and American datasets, identified higher antitoxin abundance (0.26 Hits/Mbp) than the toxin (0.07 Hits/Mbp). In comparison the PCR survey undertaken, which utilised metagenomic DNA samples, collected by the laboratory of Dr Brian Jones indicated the opposite. It is possible however that the simplest reason for descrepencies between both studies was that some components of the p22-RelBE system were below the detection threshold for PCR in some of the samples.

In parallel to the PCR survey undertaken, quantitative PCR was used to measure the copy number of the p22-RelE toxin and other 4 gut-abundant toxins per ng of metagenomic DNA, for each of the 54 samples. No significant difference could be seen in copy number for Tx1-FicT, Tx3-RelE (p22-RelE) and Tx5-FicT between healthy and AP/CRC sufferers, however Tx2-GNAT and Tx4-PemK CRC samples were significantly different to healthy samples.

Multiple studies have provided evidence of the involvement of gut bacteria in the development of CRC through mechanisms such as production of superoxide radicals, which damage DNA, and induction of cell proliferation and carcinogenic pathways (Wu *et al.*, 2011; Cuevas-Ramos *et al.*, 2010). It may be that this notable increase/decrease in abundance of both these toxins within the gut microbiome could just be related to population shifts in the normally stable adult microflora (Dethlefsen *et al.*, 2006; Dethlefsen *et al.*, 2007; Costello *et al.*, 2009) but, in light of these recent advances in our understanding of links between gut commensals and CRC, it is not inconceivable that differences in toxin copy number may have

either a causative, exacerbative or even preventative role for CRC, whether on large or small scale.

Although no evidence using live bacteria expressing the RelBE TA system or co culture modules exists as of yet, the ability of RelE toxins to inhibit growth or induce apoptosis in Eukaryotic cells (Kristoffersen *et al.* 2000; Yamamoto *et al.* 2002; Audoly *et al.* 2011) could suggest a regulatory or preventative role in the homeostasis of the intestinal epithelium, which, in turn, may link with tumourgenesis. It is, infact, becoming ever more apparent that gut microbes play some role in the events leading to tumourgenesis within the gut.

For instance, animal models have allowed elucidation of carcinogenic properties from several bacterial species, such as the pro-inflammatory proteins of Streptococcus infantarius which stimulated the release of growth factors whilst promoting pre-neoplasic lesions in rat models (Biarc *et al.* 2004) and the development of adenomas protruding into the colonic lumen after bacterial infection of ApcMin/+ mice with Citrobacter rodentium (Newman *et al.* 2001). Moreover, CRC patients appear to exhibit bacteria adhering to tumours (Swidsinski *et al.* 1998), whilst there is also indirect evidence of bacterial invasion (Abdulamir *et al.* 2009; Gupta *et al.* 2010). Even more recently, the identification of gut commensals, such as *E. coli* K-12 and Nissle, regulating epithelial cell differentiation factors demonstrates clear routes for bacteria to promote tumourgenesis (Becker et al. 2013). It is therefore not impossible to envisage a TA module toxin, known to have an effect on eukaryotic cells, having a role also.

The other possibility relates to recent findings that TA modules, especially those of the RelBE family, play a role in survival when stressed by factors such as nutrient deprivation (Gerdes 2005). If the CRC environment were a more hostile habitat than that of a healthy human gut, it would be logical to harbour more of

these persistence genes to enable successful colonisation and continued existence. Recently there have been a large number of studies providing evidence that certain 'pathobionts' encompassing Proteobacteria, Bacteriodetes and Fusobacteria and recovered from tumour microenvironments produce harmful metabolites and this correlates to increased inflammation of the gut epithelium and reduced antimicrobial peptide secretion (Louis *et al*., 2014, Konstantinov *et al*., 2013). Indirectly related to the above hostile environment hypothesis is the finding that non-TA-related toxin gene expression has been found to be increased in CRC transcriptomes (Dutilh *et al*. 2013). Increased production of free radicals and other toxic molecules by other species (Huycke *et al*. 2002), may make survival under these altered conditions more difficult, requiring more "persistence" genes such as TA systems.

Differences in copy number for Tx5-FicT was unexpected as both locations (UK and Czech Republic) are European countries, having westernised diets with similar environmental conditions and seasons. The seemingly large variance in copy number between the individual AP samples could suggest an intermediate step from a healthy colon, to that of a CRC sufferer, and this has in fact been suggested previously to be the case (Vogelstein *et al*, 1988).

3.6 Conclusions

- Of all TA toxin, antitoxin and regulator queries searched for, the marine environment had the highest diversity, whilst acid mine drainage, termite gut and avian gut were the least diverse.

- Toxins and antitoxins found highly abundant within the human gut were also found to be abundant within other mammalian gut environments and other regions of the total human microbiome.

- Carriage of gut abundant toxins and antitoxins on chromosomes, plasmids and phage genomes was explored and a distinct lack of these was seen in phage genomes.

- Chromosomes appeared to be the primary location for gut-abundant toxins and antitoxins, possibly due to their size in comparison to other units investigated.

- The presence of both the p22-RelE toxin, and p22-RelB antitoxin does not necessitate the presence of the complete module within human gut metagenomic samples.

- qPCR analysis of the 5 gut-abundant Type II toxins highlighted Tx5 to have a higher copy number in gut metagenomic DNA samples from the Czech Republic. Additionally Tx2 had a lower copy number in metagenomic DNA samples from CRC sufferers than healthy or those with AP whilst conversely Tx4 copy number was higher in CRC sufferers.

Chapter 4: Functional stabilisation by Type II TA modules in the human gut microbiome

4.1 Introduction

Plasmid (and potentially all MGE)-located TA modules have a very apparent primary function: to stabilise both vertically and horizontally the transmission of their nucleic host. They may also undertake other important physiological functions for the cell; however this is true for all TA modules. The function of TA systems that are located within bacterial chromosomes seems less clear, especially with regards to stabilising this genetic material and the functions it encodes; however it has been suggested that they may be involved in the prevention of large scale genome reductions (Gerdes 2013). The stabilising effect of TA systems seen in some mobile genetic elements suggests these modules may have the potential to stabilise specific functions within the gut microbiome metagenome which to date remains poorly explored and understood.

4.1.1 Type II TA modules as addiction systems and plasmid stabilisation

Very low copy number and large plasmids are often spread by conjugation or through mobilisation with help from other conjugative plasmids, representing a substantial portion of the flexible (or mobile) genome and contributing to prokaryotic evolution (Van Melderen & De Bast 2009). These plasmids very commonly harbour toxin-antitoxin systems where they function, at least partly, as an effective method of increasing the stability of their plasmid host (Jensen & Gerdes 1995). TA systems increase the plasmid prevalence (defined as the number of plasmid-containing cells/total number of cells in a population) in growing

bacterial populations through a mechanism known as post-segregational killing or PSK (Gerdes *et al.*, 1986), where daughter cells which did not inherit a copy of the plasmid carrying the TA module are selectively killed during cell division (Jaffe et al., 1985, Yarmolinsky 1995). PSK has been discussed in depth within Chapter 1.

While historically TA systems stabilising plasmids within populations and environments has focused on vertical transmission, more recent studies have shown TA modules may also aid horizontal transmission of plasmids also utilising TA modules and their addictive nature. Plasmids have been shown to disseminate efficiently and rapidly through heterogeneous bacterial populations (Dionisio *et al.*, 2002) however one major obstacle they must overcome is the presence of resident plasmids already within populations and the possibility of incompatibility. Plasmid incompatibility is usually defined as the failure of two co-resident plasmids to stably be inherited together in the absence of external selection (Novick *et al.*, 1976). When an invading plasmid belongs to the same incompatibility group competition ensues and will result in one of these plasmids being lost, potentially from the entire cell population. Usually this is decided by which plasmid has the least cost to the host bacteria (Haft *et al.*, 2009), however when PSK systems such as TA modules are carried this is not always applicable.

Cooper & Heinemann (2000) identified that TA systems function in plasmid-plasmid competition and therefore horizontal stabilisation of plasmids and their encoded functions. Plasmids that were PSK+ were able to outcompete identical replicons belonging to the same incompatibility group that were devoid of a TA system (PSK⁻). This suggests that TA systems increase the relative fitness of their host DNA and eliminate competitor molecules (plasmids in this case) in bacterial progeny through PSK. Furthermore it was identified that this competitive advantage was only present when host cells were sensitive to the effects of the

plasmid-encoded TA system (i.e. no chromosomal or other mobile genetic elements harboured the same module (Cooper *et al.*, 2010). This may be partially why plasmid-encoded TA systems have been so evolutionary successful. Whether vertical or horizontal, stabilisation of these genomic entities within populations would undoubtedly lead to stabilisation of functions associated with these. It is possible this is also the case with other mobile genetic elements but also chromosomally.

4.1.2 Stabilisation of other mobile genetic elements and functions by Type II TA modules

The carriage of TA systems on other mobile genetic elements, whilst found, has been less frequently noted since their initial discovery on plasmids. This has included conjugative transposons and temperate bacteriophage. The SXT conjugative transposable element of Vibrio cholera (Waldor *et al.*, 1996, Toma *et al.*, 2005) was shown to harbour TA systems homologous to the tad-ata module of *Paracoccus aminophilus*. These homologues were the first genes in each operon within this element (Dziewit *et al.*, 2007) and perhaps more interestingly all were demonstrated to encode functional toxins when expressed within *E.coli*. These TA systems were the first to be linked with stabilisation of integrative and conjugative elements, which can exist integrated into a host chromosome or as circular episomal elements (Burrus & Waldor 2003, Dziewit *et al.*, 2007).

Bacteriophages are another mobile genetic element responsible for genetic transfer between bacterial species and genera. Phage N15 and P1 are temperate *E.coli* phages. The genome of the former can be stably maintained as a linear double stranded molecule whilst the latter is maintained as a circular plasmid once

inserted into the host cell. Phage N15 encodes a TA system homologous to the tad-ata module and has been hypothesised to act as a means of stabilising the genetic material of N15 when behaving in a lysogenic manner (Dziewit *et al.*, 2007). Similarly and with more data supporting this, the phd (prevent host death) and doc (death on cure) TA module of phage P1 has been repeatedly shown to stabilise the genome of P1 (Lehnherr *et al.*, 1993). The discovery and early work on phd/doc and its ability to stabilise prophage P1 was based on an extremely low loss rate of approximately 10^{-5} phage genome particles per cell per generation. (Lobocka *et al.*, 2004). In this case, loss rate is defined as the frequency with which prophage negative cells appear within the population. Homologues of this module have since been found on phage P7 as well and these have not been shown to be functional TA modules, it is assumed they also serve a similar function for this phage as in P1 (Lehnherr *et al.*, 1993, Zhao & Magnuson 2005).

A study undertaken in 2009 comparatively analysing the genomes of temperate phage from *Streptococcus pneumoniae* identified a homologue of the MazEF Type II TA system in phage Spn_6 (Romero *et al.*, 2009). Further analysis revealed the presence of this module in a defective prophage and also in EJ-1 a mosaic bacteriophage of *Streptococcus mitis* (Romero *et al.*, 2004). In addition to this two TA systems; MazE F and PemIK were recently identified on the defective prophage LJ771 of *Lactobacillus johnsonii* and it was found they behaved as a typical addiction system when cloned into *E.coli* (Denou *et al.*, 2008). Carriage of a TA module within the genome of Spn_6 has not yet been functionally related to stabilising the genetic material of this phage; however with knowledge that the MazEF TA system is a highly effective plasmid addiction system, similarities can be drawn.

Finally Goh *et al.*, in 2013 when investigating the ability of Phage φC2 to transduce Tn6215 between *Clostridium difficile* strains, identified two separate homologues to known or predicted TA pairs (Goh *et al.*, 2013). These were GCN5-related N-acetyltransferase (GNAT)-Xre and ω-ε-ζ systems. It was noted that whilst the GNAT-Xre system seemed to be intact, the ζ toxin was truncated to 52aa in length, whilst 79aa was previously found to be required for this toxin to remain functional (Zielenkiewicz & Ceglowski 2005). The authors suggest this redundant TA module may be retained within the transposon (and therefore phage genome) as a method of stabilising host cells during infection by the phage which may trigger other TA toxin activity, thus allowing stable transfer into that host (Goh *et al.*, 2013). This appears possible as a similar situation was seen in phage T4 (Otsuka & Yonesaki 2012). As with all hypothesised functions for TA modules, those found on mobile genetic elements are proposed to be acting solely as selfish entities (Van Melderen & De Bast 2009) however this selfish behaviour may reduce the deletion rate of the host molecule from bacterial genomes (Magnuson 2007).

4.1.3 Chromosomally located TA modules and genomic stabilisation

TA systems found within bacterial chromosomes are often embedded in prophages or other mobile genetic elements. Superintegrons are chromosomal genetic elements containing assemblies of genes, each flanked by a recombination sequence (attC site) targeted by the integron integrase (Fluit & Schmitz 2004). These attC sites are highly abundant within superintegrons, which would suggest an intrinsic instability; however paradoxically they appear to be highly stable (Lawrence *et al.*, 2001). This stability implies either the existence of selective pressures to maintain gene cassettes harboured or alternatively mechanisms exist that favour their persistence within the bacterial chromosome,

even in the absence of selection. With this in mind, Rowe-Magnus *et al.*, in 2003 suggested that the addictive properties of TA modules could act against the loss of chromosomal regions (Rowe-Magnus *et al.*, 2003). Subsequent to this hypothesis the combination of ParDE and RelBE TA systems from the *Vibrio vulnificus* superintegron was shown to reduce large DNA deletions in different genomic contexts (Szekeres *et al.*, 2007). Furthermore bioinformatic searches of superintegron-containing genomes for TA system from seven known TA families revealed larger superintegrons contained multiple TA systems, whilst smaller ones were devoid (Szekeres *et al.*, 2007). The ability of TA modules to stabilise genomic DNA was also examined in a 165kb dispensable region of *E.coli* β2155 background, which exhibited large-scale deletions in the absence of these modules (Szekeres *et al.*, 2007).

Similarly the 3 ParE-containing systems encoded within the superintegron of *Vibrio cholerae* chromosome II may also act in a stabilising way, maintaining the integrity of the superintegron and perhaps additionally to its primary function prevent the loss of chromosome II (Szekeres *et al.*, 2007). Conflicting with this finding was evidence suggesting that these TA systems may in fact contribute to DNA degradation in cells lacking chromosome II but were found not to be involved in other phenotypic abnormalities seen in these cells (Yuan *et al.*, 2010, Yuan *et al.*, 2011). Deghorain *et al.*, (2013) suggest that these ParE TA system may function as part of a more complex, and chromosome II-specific stabilisation system due to their ability to mediate PSK-like phenomenon, as seen for plasmid maintenance however further investigation is needed (Gerdes 2013).

Although still poorly understood, it does appear that chromosomal stabilisation is a possible function for TA modules, perhaps under specific conditions. To date much of the study directed on this possible function of

chromosomal TA modules has been focused on the physical nucleic acid and not the encoded functions surrounding these addiction systems. In this chapter we employ a large-scale bioinformatical approach to search for and identify these functions, and investigate whether certain classes seem more prevalent on nucleic acids harbouring TA modules. It may then be possible to infer whether some functions are more likely to be stabilised by TA systems than others.

4.2 Aims

The aims of the work presented in this chapter are to identify the encoded functions of sequences harbouring TA module components across the human gut environment and ascertain whether these components are associated with particular functions.

4.3 Materials and methods

4.3.1 Bioinformatic identification of functional content of human-gut genomic neighbourhoods of Type II TA module components

The process described below was automated by the use of a PERL (Practical Extraction and Report Language) script. The script was fully written by Dr Brian V Jones who granted permission to employ it for this project. Figure 4.1 summarises this pipeline.

For each unique blast hit within all sequences generating valid hits to Type II TA system components (see Chapter 3, Section 3.2), 2500 nucleotides flanking either side of the alignment coordinates were captured and analysed further. In this analysis all metagenomic, draft chromosomes and phage sequences were treated as linear molecules, whilst plasmids and finished chromosomes were treated as circularly permuted molecules. The recovered regions of interest (ROI) were processed to predict and identify putative open reading frames (ORFs) by Metagene (Noguchi *et al.*, 2006) using the WebMGA server (Wu *et al.*, 2011) which is accessible using this link: http://weizhong-lab.ucsd.edu/metagenomic-analysis/. Finally the FASTA metagene output was searched and annotated for antibiotic resistance genes, mobile genetic elements, conserved domains and other functions. Databases searched can be seen in Table 4.1.

Due to the linearity of some sequences and differences in valid hit length, many of the ROI also varied in hit length. To address the returned hits generated from clusters of orthologous groups, resistance determinants and mobile genetic element analysis, were normalised by the average sequence length for each of the four datasets (TA, BSH, RpoB and Random).

Table 4.1: Databases and servers used to annotate function of ORFs on sequences carrying Type II TA module homologues.

Database	Description	Source
Resistance Determinants Database (REDDB)	A non-redundant repository of resistance genes from public databases including EMBL, DDBJ and Genbank.	http://www.fibim.unisi.it/REDDB/Default.asp
A Classification of Mobile Genetic Elements database (ACLAME)	ACLAME; a collection of classified mobile genetic elements from various sources comprising all known phage genomes, plasmids and transposons.	http://aclame.ulb.ac.be/ Leplae *et al.*, 2010.
Conserved Domains Database (CDD)	A collection of well-annotated multiple sequence alignment models for protein domains.	http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml Marchler-Bauer & Bryant 2004, Marchler-Bauer *et al.*, 2015.
Clusters of orthologous groups (COG)	Phylogenetic classification of proteins encoded in complete genomes.	http://weizhong-lab.ucsd.edu/metagenomic-analysis/server/cog/ Tatusov *et al.*, 1997, Wu *et al.*, 2011.
Virulence factor database (VFDB)	A comprehensive database of virulence factors associated with bacterial pathogens	http://www.mgc.ac.cn/VFs/ Chen *et al.*, 2005.
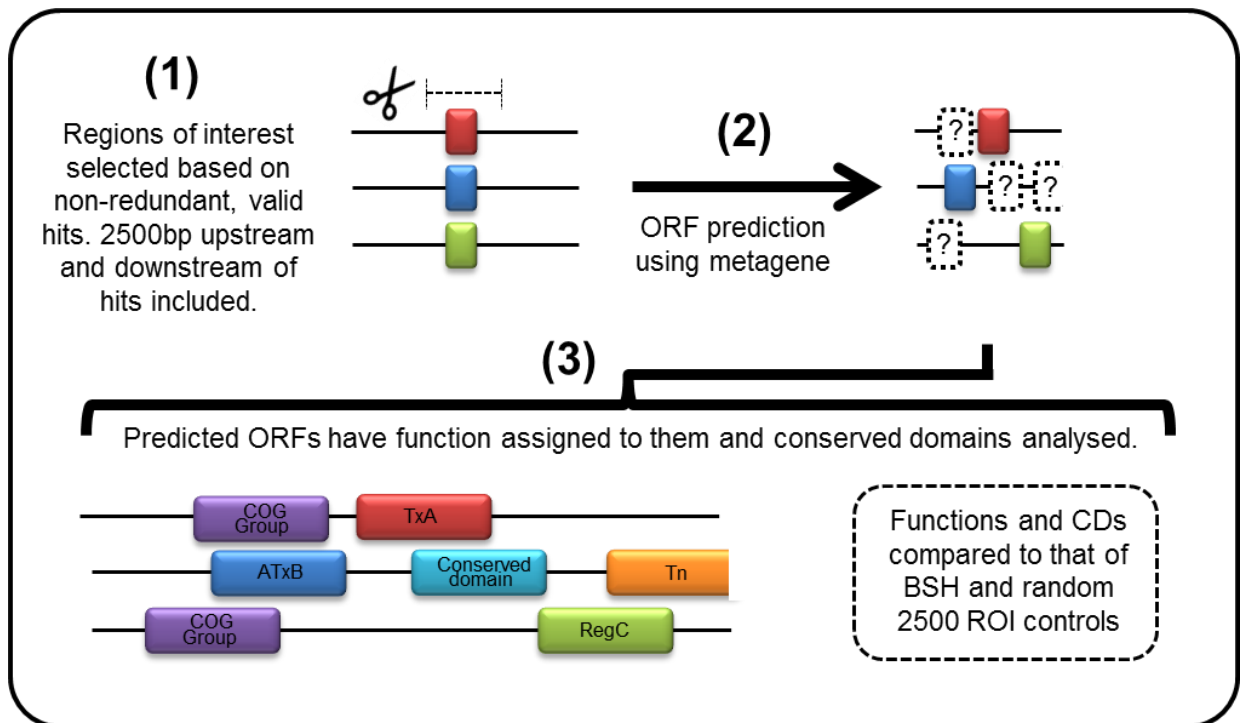
Figure 4.1: An overview of the process and pipeline used to achieve non-redundant and valid TA hits, predicted functions and conserved domains for genomic neighbourhood analysis undertaken in this chapter.

Data related to conserved domain and virulence factor searches was normalised by database size in Mbp. Data was statistically interrogated using one-way ANOVA and Chi-Squared analysis where appropriate.

4.3.2 Generation of control datasets

To ensure associations seen between TA components and functions were real three control ROI datasets were generated. The first and second control datasets were generated by selecting 2500 ROI either side of alignment coordinates of unique blast hits to 413 bile salt hydrolase (BSH) genes or 100 β-subunit RNA polymerase (RpoB) genes. The final control dataset consisted of randomised 2500 nucleotide ROI from human gut metagenomic datasets. As with the experimental TA dataset above, the recovered regions of interest for all were processed to predict and identify putative open reading frames by Metagene using the WebMGA server. The FASTA metagene outputs were searched and annotated for antibiotic resistance genes, mobile genetic elements, conserved domains and other functions. Databases searched can be seen in Table 4.1. All three control datasets were also generated using the PERL script by Dr Brian V Jones with permission.

4.4 Results

4.4.1 Average sequence length of ROI captured from each dataset

The process by which ROI were captured from linear sequences harbouring valid TA hits allowed these ROI to vary in length depending on the coordinates of hits within these sequences. To address this, the average sequence length was calculated for datasets, which would require normalising by values obtained. These can be seen in Figure 4.2. TA ROI were significantly different in length for all 3 databases analysed (P<0.0001).

4.4.2 Functional analysis of TA-harbouring regions of interest through clusters of orthologous groups (COG) and conserved domain (CD) searches

To classify functions encoded by predicted ORFs surrounding putative TA genes, Clusters of Orthologous Groups or COG analysis was undertaken. Results from this are displayed in Figure 4.3 and statistical interrogation of the data is presented in Table 4.2. Significant differences in the representation of COG classes between TA ROI datasets and control datasets were noted. Only COG classes for RNA processing and modification (A), chromatin structure and dynamics (B) and Nuclear structure (Y) did not show any differences between the experimental TA ROI and 3 control ROI datasets.

ORFs surrounding putative TA genes showed a significantly increased number of ORFs encoding functions related to: cell cycle/division and chromosome portioning (COG class D), nucleotide transport and metabolism (F), transcription (K), replication/recombination/repair (L), Cell motility (N), general function prediction only (R), function unknown (S) and signal transduction mechanisms (T).

110
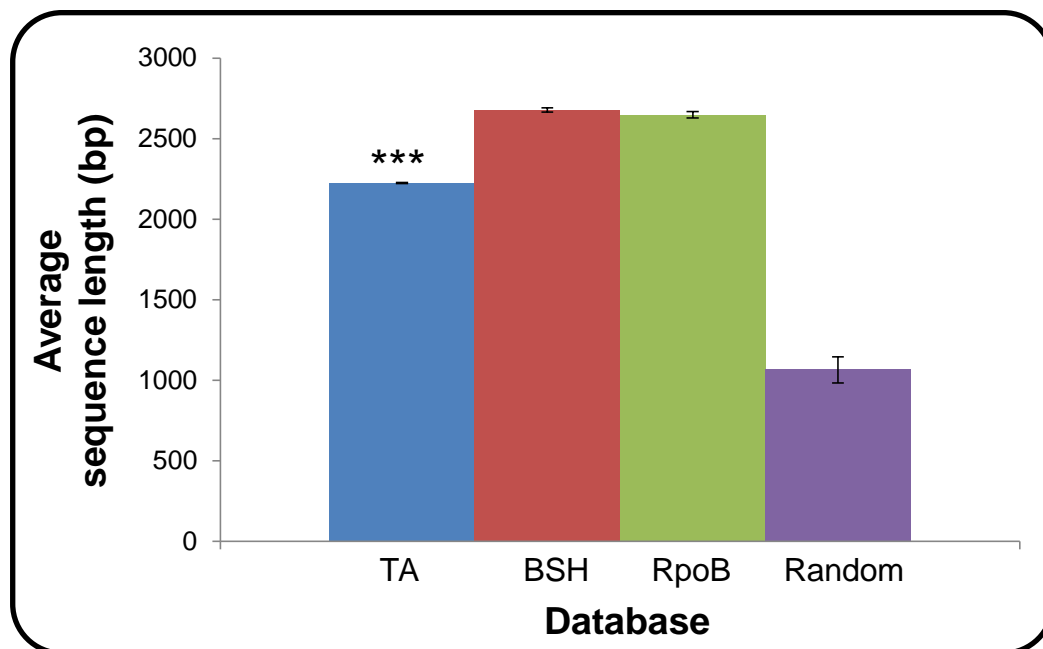
Figure 4.2: Average sequence length (ASL) of ROI surrounding blast hits to Type II TA, BSH, RpoB genes and those randomly generated from human gut metagenomic datasets. The ASL values above were used to normalise COG, ACLAME and REDDB functions predicted within this study.

*** = P value ≤0.0001 when comparing TA ROI sequence lengths to BSH, RpoB and Random ROI sequence lengths.
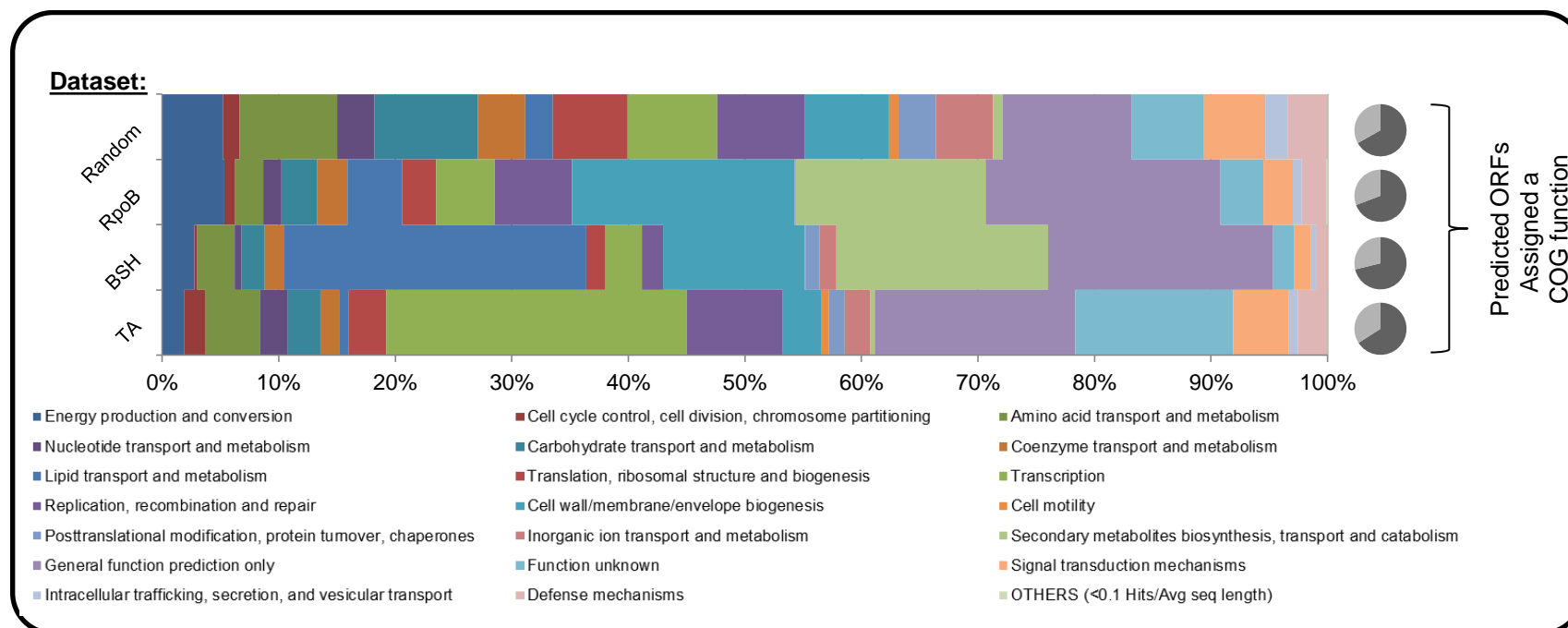
Figure 4.3: Functional profile of regions of interest surrounding putative TA genes using clusters of orthologous groups analysis to classify encoded functions. Open reading frames were predicted for regions of interest (ROI) ranging up to 2500bp upstream and downstream of putative TA genes using metagene before having functions assigned using COG. The proportion of assignable ORFs affiliated to distinct categories in the COG database are by pie charts to the right of each dataset. Light grey is the unassigned proportion whilst dark grey is the assigned proportion The "OTHERS" category consists of hits to COG classes: (A) RNA processing and modification, (B) Chromatin structure and dynamics, (Y) Nuclear structure and (Z) Cytoskeleton. Values are presented as hits/average sequence length of the ROI query each hit was identified on and statistical differences have been tabulated below in Table 4.2.

Table 4.2: Statistical interrogation of COG analysis undertaken and presented in section 4.4.2.

COG Class	Significance		
	TA vs Random Generated	TA vs BSH	TA vs RpoB
Energy production and conversion	<***	-	<***
Cell cycle control, cell division, chromosome partitioning	>***	>***	>*
Amino acid transport and metabolism	<***	>**	>***
Nucleotide transport and metabolism	-	>***	-
Carbohydrate transport and metabolism	<***	-	-
Coenzyme transport and metabolism	<**	-	-
Lipid transport and metabolism	<***	<***	-
Translation, ribosomal structure and biogenesis	<***	>**	-
Transcription	>***	>***	>***
Replication, recombination and repair		>***	-
Cell wall/membrane/envelope biogenesis	<***	<***	<***
Cell motility	-	>**	>*
Posttranslational modification, protein turnover, chaperones	<***	-	-
Inorganic ion transport and metabolism	<***	-	-
Secondary metabolites biosynthesis, transport and catabolism	<***	<***	<***
General function prediction only	>**	-	<*
Function unknown	>***	>***	>***
Signal transduction mechanisms		>***	-
Intracellular trafficking, secretion, and vesicular transport	<***	-	*
Defense mechanisms	<***	>**	-
OTHERS	-	-	-

* = P value <0.05

** = P value <0.001

*** = P value <0.0001

> = Indicates a significant increase of this functional class in TA ROI datasets compared to the control dataset being analysed.

< = Indicates a significant decrease of this functional class in TA ROI datasets compared to the control dataset being analysed.

- = No significant difference

Conversely significant decreases in ORFs encoding functions related to: energy production and conversion (C), carbohydrate transport and metabolism (G), coenzyme transport and metabolism (H), lipid transport and metabolism (I), cell wall membrane/envelope biogenesis, post-translational modification and protein turnover (O), inorganic ion transport and metabolism (P), Secondary metabolite biosynthesis/transport/catabolism and intracellular trafficking/secretion/vesicular transport (U) were also seen. Interestingly amino acid transport and metabolism (E), translation/ribosomal structure and biogenesis (J) and defence mechanisms showed significant decreases in predicted functions when compared to the randomly generated control gut ROI dataset, but showed significant increases when compared to the BSH gut-specific control ROI dataset.

In most cases COG values were uniform in their difference between the TA ROI dataset and controls with exceptions to this being COG class E, J, R and V. The TA ROI showed significantly less amino acid transport and metabolism (E) related functions than the random ROI dataset however significantly more than BSH and RpoB. This was also the case for Translation, ribosomal structure and biogenesis (J) and Defence mechanisms (V) with the exception that the values for the RpoB dataset were not significantly different from the TA ROI dataset. Finally, significantly fewer functions were assigned to (R) for the random control dataset than the experimental TA ROI dataset.

The largest proportion (26%) of functions identified in ROI surrounding putative TA genes belonged to COG class K and are linked to processes involved with transcription. When exploring this class further and sub-dividing COG families, it revealed 123 unique families. Many of the ORFs were predicted to function as transcriptional regulators with Xre family helix-turn-helix (HTH) domain, which is often associated with TA module toxins and antitoxins. These

transcriptional regulators encompassed approximately 70% (1st – 5th) of functions within COG class K and were 50x more prevalent in TA associated ROI in comparison to the 3 control datasets. Interestingly the 6th most frequently classified COG family within COG class K for TA related ROI was COG3617, a prophage antirepressor. This was found to be 2.36% of the entire COG output for TA ROI. In comparison, this function was found to be between 40 (random ROI) and 72 (RpoB) fold less abundant in control ROI datasets, suggesting an association between this function and genomic sequences surrounding putative TA genes.

Conserved domains (CDs) are recurring polypeptide units that relate to the function of the protein they belong to. Identification of these is employed as a tool for predicting the potential function of the overall protein. Using the same methodology described above for other functional analysis, the presence and abundance of all known conserved domains on ROI surrounding Type II TA genes was examined by searching the NCBI CCD using predicted ORFs from ROI.

23578 individual protein domains were identified across all ROI datasets searched with many of the most abundant being domains found in or closely related to TA module toxins or antitoxins. Figure 4.4 shows the 50 most abundant CD in relation to those most abundant in ROI datasets surrounding TA genes. The first and second most abundant CDs were HTH-Xre super-families, a motif commonly seen in many Type II TA toxins. Unsurprisingly these were significantly higher in TA ROI datasets ($P<0.05$) as might be expected as sequences and ORFs on these regions were generated due to their proximity to putative TA module genes.
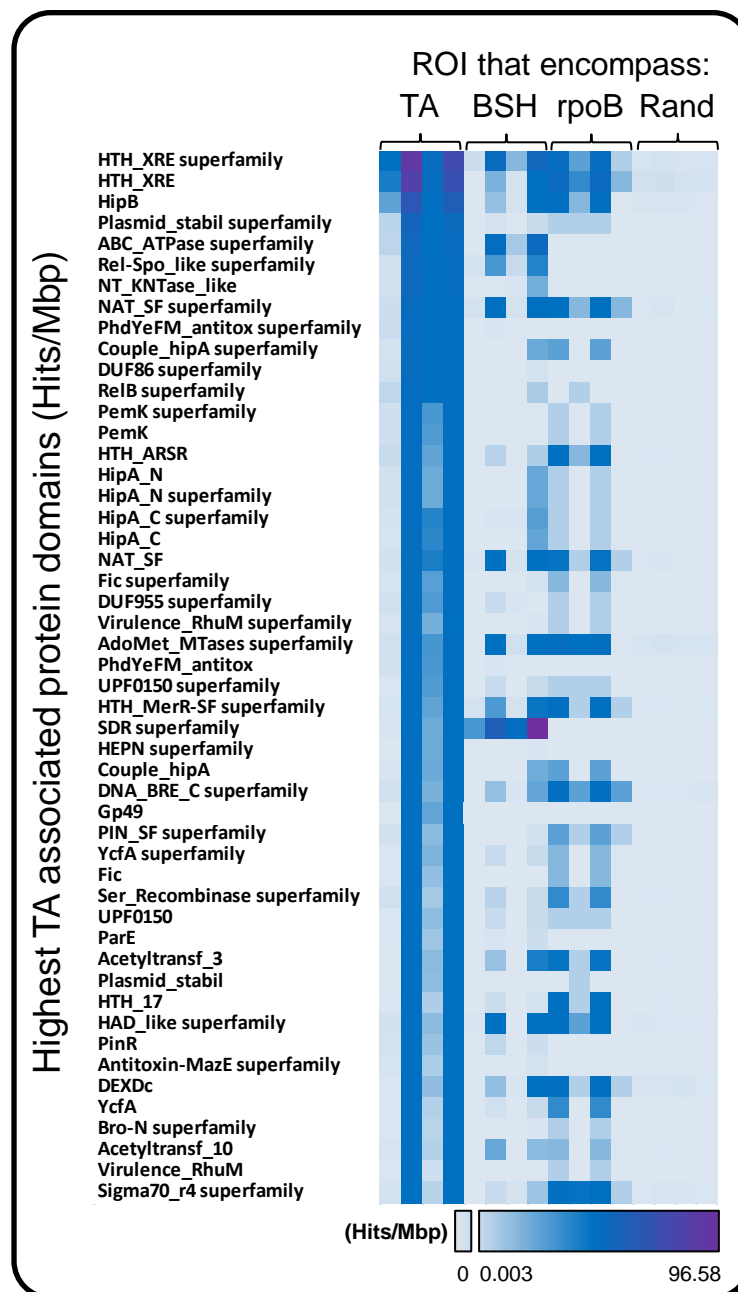
Figure 4.4: Relative abundance of conserved protein domains on regions of interest from human gut metagenomes encompassing predicated TA module, BSH and RpoB genes as well as randomly generated regions of interest from the same metagenomic datasets.

Conserved domains are ranked by highest relative abundance within the Type II TA regions of interest dataset Data is presented in hits/Mbp and each subset of ROI datasets are comprised of sequence data from the Japanese, American, Metahit and Human Microbiome Project gut metagenomic datasets detailed in Table 3.1. Data was interrogated statistically using Chi squared analysis.

Other highly abundant CDs included HipB (a TA antitoxin), Rel-Spo superfamily, encompassing proteins with sequence and folding similiaries to the RelA and SpoT protiens of *E.coli* which are involved in the stringent response regulation, specifically in conjunction to the RelBE TA module. Interestingly, the third most abundant CD was a plasmid stability superfamily, which was almost 100x more abundant in TA ROI datasets compared to all other control ROI datasets.

4.4.3 Association of mobile genetic elements with putative TA genes located in gut metagenome regions of interest

The ability of TA systems to stabilise plasmid DNA has been thoroughly documented for both Type I and Type II TA systems and TA presence within prophage genomes have also been identified. To further investigate the association between Type II TA modules and mobile genetic elements, specifically within the human gut environment, predicted ORFs from the human gut TA ROI dataset were used as queries to search the ACLAME database. Figure 4.5 and Table 4.3 shows the percentage of hits per average sequence length of the dataset for each ROI dataset and also the proportion of assignable ORFs using the ACLAME database. A significantly higher percentage of assigned ORFs for the TA ROI dataset were seen when compared to the RpoB and random control datasets (P≤0.0001) whilst the BSH ROI dataset was comparable. The TA ROI harboured significantly more plasmid, prophage and viral-like elements than all control datasets, even after normalisation by the average sequence length for each dataset (P≤0.0001).
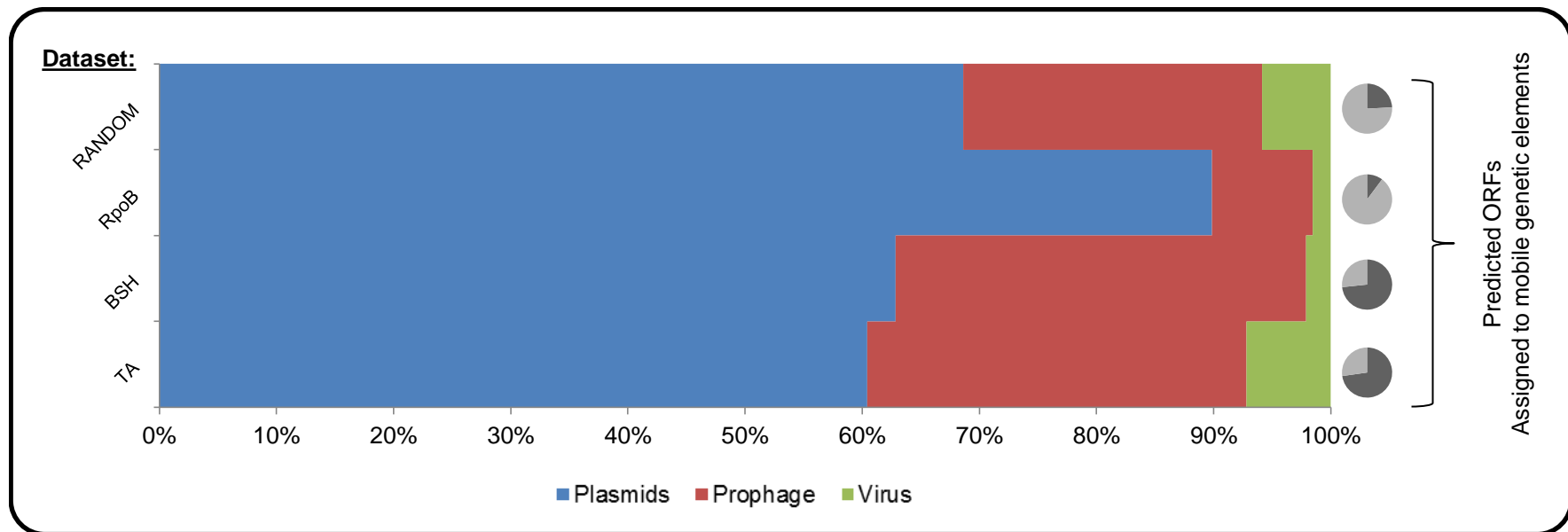
Figure 4.5: Mobile genetic elements identified on regions of interest surrounding putative TA genes using the ACLAME database.
Open reading frames were predicted for regions of interest (ROI) ranging up to 2500bp upstream and downstream of putative TA genes using metagene before identifying putative mobile genetic elements using the ACLAME database. The proportion of assignable ORFs affiliated to distinct categories in the ACLAME database are visualised by pie charts to the right of each dataset. Light grey is the unassigned proportion whilst dark grey is the assigned proportion. Values are presented as hits/average sequence length of the ROI query each hit was identified on and statistical differences have been tabulated below in Table 4.3.

Table 4.3: Statistical interrogation of mobile genetic element association analysis undertaken and presented in section 4.4.2.

Mobile genetic element	Significance		
	TA vs Random generated	TA vs BSH	TA vs RpoB
Plasmid	>***	>***	>***
Prophage	>***	>***	>***
Virus	>***	>***	>***

*** = P value <0.0001

> = Indicates a significant increase of this type of mobile genetic element in TA ROI datasets compared to the control dataset being analysed.

4.4.4 Association of resistance determinants in regions of interest surrounding putative TA genes

TA modules have been repetitively shown to stabilise resistance markers preventing loss of these traits from a population. It is also possible this may aid populations in retaining genes encoding resistance when no selective pressure is in place. In this study searches for resistance determinants on ROI surrounding putative TA module genes within the human gut microbiome were undertaken and results from this can be seen in Figure 4.6 whilst and statistical interrogation of the data is presented in Table 4.4.

When comparing ORFs predicted on ROI encompassing putative TA genes to control datasets significantly more β-lactam resistance determinants can be identified ($P<0.0001$) This was also true for aminoglycosides comparing the TA ROI dataset to the randomly generated gut dataset ($P<0.05$). Additionally significant decreases in quinolone and glycopeptide resistance determinants can be seen when comparing the experimental TA ROI dataset with the randomly generated gut dataset ($P<0.05$) for the former determinant, and RpoB housekeeping-gene control dataset ($P<0.05$) for the latter. Specific β-lactam determinants seem to be much more prevalent in regions of DNA surrounding putative Type II TA genes, than putative BSH and RpoB genes as well as random ROI.

The most commonly found resistance determinants for β-lactams predicted was an *E.coli blaTEM2* gene known to encode a β-lactamase that can hydrolyse penicillins and first generation cephalosporins (Shaikh *et al.*, 2015).
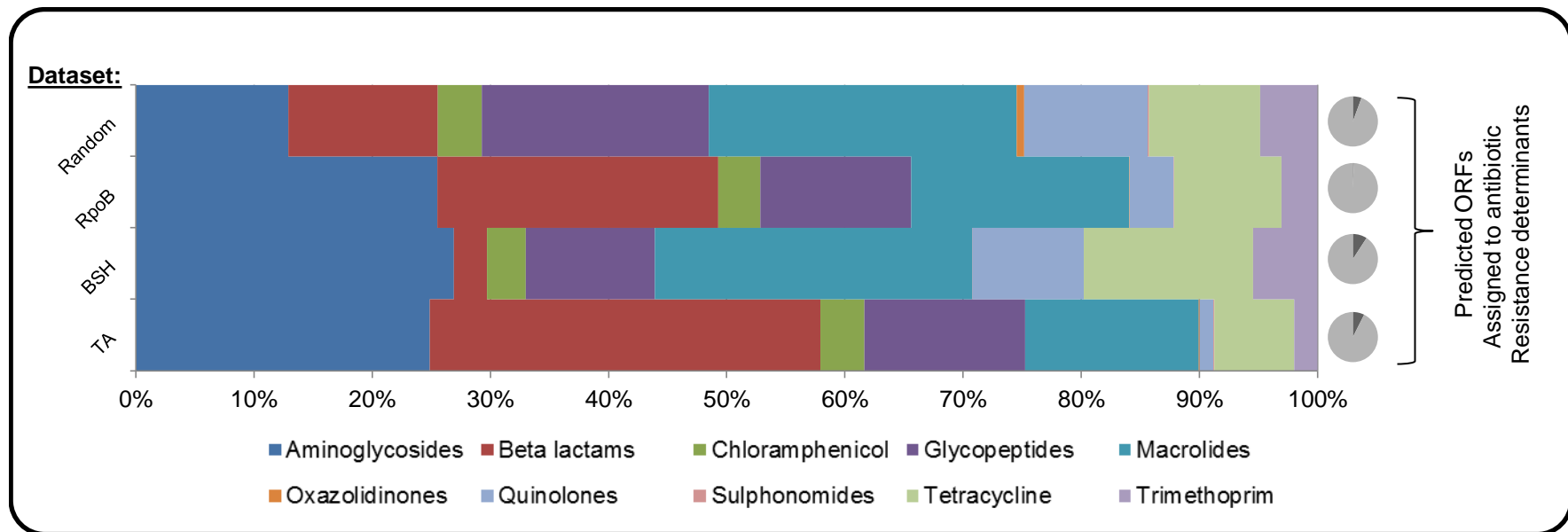
Figure 4.6: Resistance determinants profile on regions of interest surrounding putative TA genes. Open reading frames were predicted for regions of interest (ROI) ranging up to 2500bp upstream and downstream of putative TA genes using metagene allowing any resistance determinants to be identified when using these ORFs as queries to search the Resistance Determinants Database (REDDB). The proportion of assignable ORFs affiliated to specific antibiotic resistance determinants are displayed as pie charts to the right of each dataset. Light grey is the unassigned proportion whilst dark grey is the assigned proportion. Values are presented as hits/average sequence length per ROI dataset and statistical differences have been tabulated below in Table 4.4.

Table 4.4: Statistical interrogation of resistance determinants present on ROI undertaken and presented in section 4.4.2.

Antibiotic class associated with resistance:	Significance		
	TA vs Random generated	TA vs BSH	TA vs RpoB
Aminoglycosides	<*	-	-
Beta lactams	>***	>***	>***
Chloramphenicol	-	-	-
Glycopeptides	-	-	<*
Macrolides	-	-	-
Oxazolidinones	-	-	-
Quinolones	<*	-	-
Sulphonomides	-	-	-
Tetracycline	-	-	-
Trimethoprim	-	-	-

* = P value <0.05

** = P value <0.001

*** = P value <0.0001

> = Indicates a significant increase of this functional class in TA ROI datasets compared to the control dataset being analysed.

< = Indicates a significant decrease of this functional class in TA ROI datasets compared to the control dataset being analysed.

- = No significant difference

Predicated blaTEM2 genes were found to be 5.3x more prevalent on ROI in proximity to a putative TA gene than ROI not specifically associated with anything and 4.1x more than ROI surrounding BSH genes. Additionally, in ROI incorporating putative RpoB housekeeping genes the blaTEM2 determinant was completely absent. The next 3 more commonly identified β-lactam resistance determinants in TA ROI had homology to resistance markers found in *Staphylococcus aureus*. These were the *hsdM* gene which was 14.5 fold more abundant in ROI sequences harbouring TA genes when compared to controls, *hsdR* (10x more prevalent) and *ccrB* gene which was identified approximately 5x more in the experimental TA ROI. *hsd* genes behave as restriction modification systems, protecting host cells from foreign pieces of DNA whilst, *ccr* genes encode chromosomal recombinases allowing DNA cassettes to be inserted into bacterial chromosomes.

4.4.5 Association of virulence factors in regions of interest surrounding putative TA genes

With multiple pathogenic species shown to harbour large numbers of TA systems within their genome it can be hypothesised that these modules may help retain virulence factors (VF). Using the same methodology described above for resistance determinants and clusters of orthologous groups analysis, the presence and abundance of previously documented virulence factors on ROI surrounding Type II TA genes was examined by searching the VFDB using predicted ORFs from ROI. A total of 1152 different putative virulence factors were identified across all ROI datasets. Figure 4.7 shows the 50 most abundant VF in relation to those most abundant in ROI datasets surrounding TA genes.
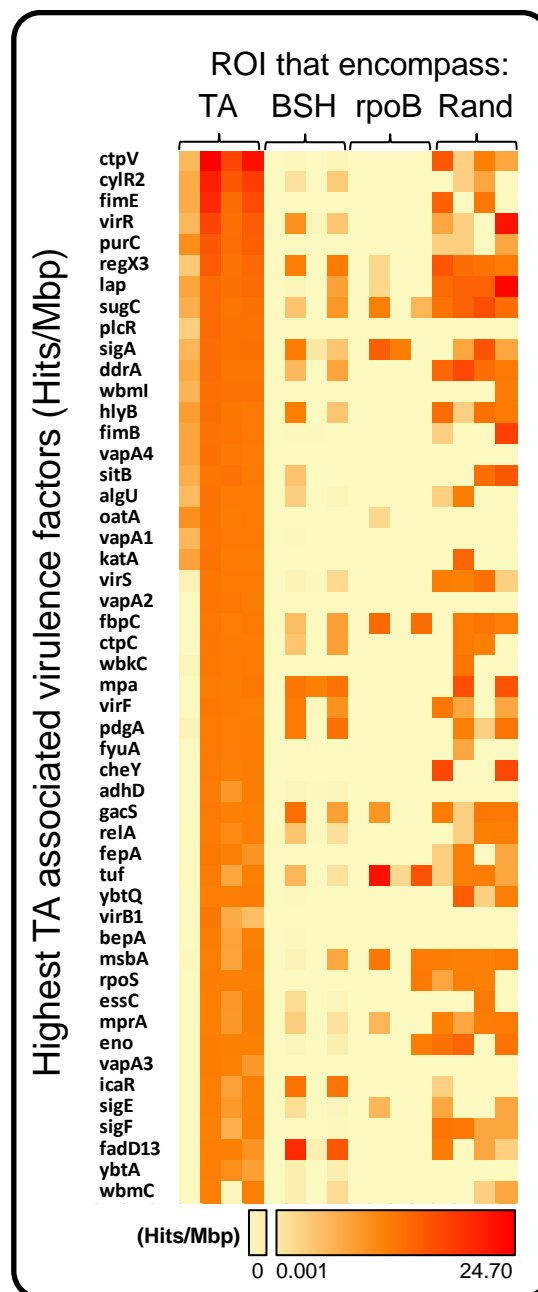
Figure 4.7: Relative abundance of virulence factors on regions of interest from human gut metagenomes encompassing predicated TA module, BSH and RpoB genes as well as randomly generated regions of interest from the same metagenomic datasets.

Putative virulence factors are ranked by highest relative abundance within the Type II TA regions of interest dataset. Data is presented in hits/Mbp and each subset of ROI datasets are comprised of sequence data from the Japanese, American, Metahit and Human Microbiome Project gut metagenomic datasets detailed in Table 3.1. Data was interrogated statistically using Chi squared analysis.

When ranking relative abundance of virulence factors in relation to those most abundant in ROI datasets surrounding TA genes, *ctpV*, a cation transporting ATPase belonging to the *Mycobacterium* genera was found to be the most highly abundant virulence factor in ROI surrounding TA genes and was significantly more abundant than in all control ROI datasets ($P<0.001$). This was also the case for *cylR2* (A transcriptional regulator for enterococcal exotoxin; cytolysin), *fimE* (a regulatory fimbrial protein of *E.coli*) and *plcR* (a Bacillus transcriptional regulator involved with expression of other virulence factors within this genera) ($P<0.05$). For rpoB ROI datasets appeared to have relatively few virulence factors and tended to be lower in abundance when they were present with the exception of *tuf*. This encodes human compliment regulator binding protein in *Streptococcus pneumoniae* (Mohan *et al.*, 2014) and had a significantly higher abundance in rpoB ROI than TA ROI. BSH ROI also appeared to harbour less of these particular virulence factors.

4.5 Discussion

4.5.1 General function prediction using COG and conserved domain searches

Using predicted ORFs to search the clusters of orthologous groups (COG) database and conserved domain (CD) databases has previously been used by to asses functional diversity within gut environments, such as for Phage genome signature-based recovery (PGSR) phage (Ogilvie *et al.*, 2013). COG functional prediction for regions of interest surrounding TA genes revealed a decrease in abundance of functions related to energy production/conversion, carbohydrate; coenzyme and lipid transport and metabolism, cell wall and membrane biogenesis, post translation modification and protein turn over, inorganic ion transport and metabolism, secondary metabolite biosynthesis/transport/catabolism and secretion/vesicular transport.

The reduced abundance these functions may be linked to where the TA modules they surround are most commonly carried. Many of these functions, including carbohydrate metabolism and secondary metabolite catabolism are vital functions within the human gut, especially for *Bacteroides spp.* one of the major phyla within this environment (Qin *et al.*, 2010, Kurokawa *et al.*, 2007, Xu *et al.*, 2007). Because of the necessity of these functions, it would be expected that many would be encoded on sequence which is highly conserved due to the important nature of these functions to continued survival within a healthy human gut environment (Qin *et al.*, 2010, Kurokawa *et al.*, 2007, Flint *et al.*, 2012). TA systems have shown to be associated with plasmids and other mobile genetic elements however tend to be more abundant on chromosomes (data shown in chapter 3). The accumulation of these systems within bacterial chromosomes (and spread throughout communities) would need to occur through the movement of

mobile genetic elements which are known to carry accessory genes not commonly found among housekeeping genes (Frost *et al.*, 2005). In this case these housekeeping genes are likely to be related to core functions involved in survival in the human gut, such as carbohydrate metabolism. Additionally, sites exist within bacterial chromosomes that are more susceptible to incoming foreign DNA, which would also impact on functions located around TA systems (Wang *et al.*, 2013).

It is also possible that this distinct lack of these functions in ROI surrounding TA genes may be more complex than this. Unlike plasmid-borne TA systems, chromosomes may, and often do, encode multiple TA systems, even multiple homologues of the same TA system (Yamaguchi & Inouye 2011). This can often lead to redundancy in function, with antitoxins from other TA systems, which are located on the bacterial chromosome, neutralising the residual toxin when the section of DNA harbouring the TA module is lost. Furthermore there is evidence to suggest that even disparately related TA antitoxins sharing motifs able to neutralise/sequester other TA module toxins (Yang *et al.*, 2010). Figure 4.8 is a diagrammatical representation of this. Mobile genetic elements tend to carry accessory functions not found in the bacterial chromosome. Due to the effect of TA systems on mobile genetic element stabilisation and the presence of TA systems on plasmids and other elements, they are likely to be commonly associated with functions harboured by these elements. In contrast, when chromosomally encoded TA modules and functions commonly seen surrounding modules are considered this same stabilisation effect may not hold true because of TA redundancy.

Interestingly functions involved in transcription were significantly increased in ROI surrounding putative TA genes compared to all 3 control datasets along with unknown and general function predictions as well. When exploring the

transcription COG class in more detail through family analysis, the 5 most frequently classified were transcriptional regulators however, the 6th was COG3617; a prophage antirepressor. Many bacterial species harbour prophage within their genomes firstly as part of a temperate lifecycle but also as these can often carry genes which may benefit the bacterium. However, when induced, these phage can convert to infectious particles and become lytic (Krupovic *et al*., 2011).

This is detrimental to the bacterial population, and on a larger scale, the community as a whole; however, it can also be beneficial in terms of gene dissemination and often linked to the spread of bacterial virulence genes (Huddleston 2014). This mechanism is tightly controlled by the use of prophage repressors, with prophage induction usually caused by RecA-stimulated proteolysis of the repressor (Crowl *et al*., 1981.). An alternative strategy to this was identified in 2011 where the encoding of antirepressors which inactive the repressor rather than cleave it (Lemire *et al*., 2011).
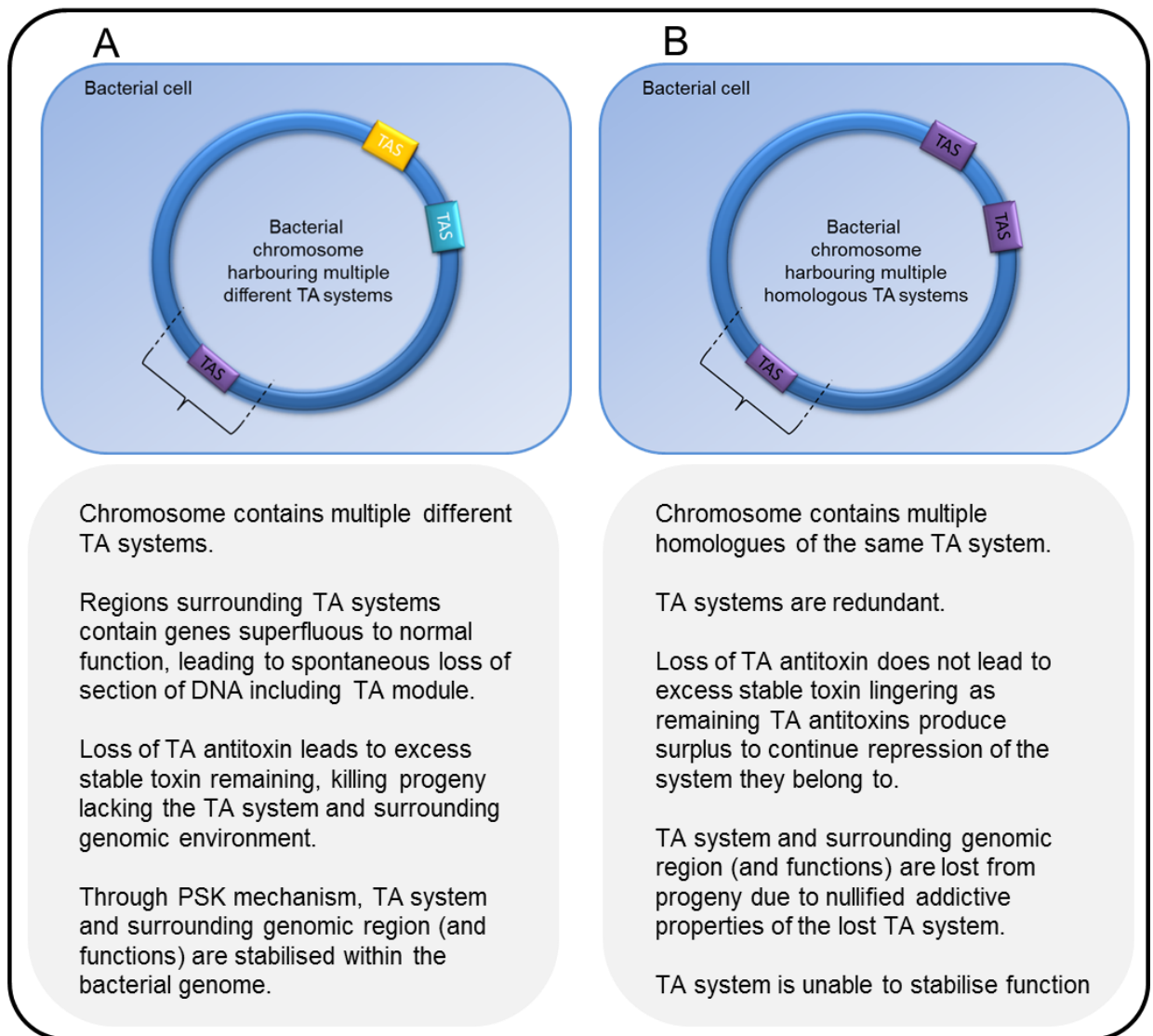
Figure 4.8: Redundancy of multiple TA systems on bacterial chromosomes.

(**A**) Multiple TA systems can be found on a single bacterial chromosome. If these are all unrelated and individual components are not able to cross-interact, if the genomic region containing one of these TA systems is lost, PSK would eliminate any progeny not still harbouring the section of DNA with the TA system present, stabilising the region.

(**B**) If however there are multiple copies/homologues of the same TA system, or individual components of these system can interact, TA systems may become redundant. Loss of a TA system (and any regions of DNA surrounding it) through deletion/mutation can result in permanent loss of function from a population.

The concept shown above is also applicable to other genetic units such as plasmids.

While stability of the gut microbial population is vital both from the higher host's perspective and the gut community, horizontal gene transfer is an important tool in survival, especially during times of stress such as antimicrobial treatments. Transduction as a significant mechanism of horizontal gene transfer has been traditionally underestimated. However metagenomic analysis of viromes has suggested functional bacterial genes (encompassing a broad range of functions) exist in upwards of 50% of bacteriophages (Dinsdale *et al.*, 2008). The most likely reason for finding this function associated with TA systems is stabilisation of the phage as a whole, allowing it to be retained within a bacterial population or community. If TA modules were more specifically stabilising this prophage antirepressor (as a function rather than the phage as an entity) two different conclusions can be drawn. Firstly, that TA modules are (as has been previously discussed in Chapter 1) purely selfish entities which act like genetic parasites, primarily focussing on their own dissemination throughout populations. Alternatively, a function of these modules within the gut environment is specifically to aid and stabilise sequence transduction within this community, thus increasing genetic variability and function distribution.

By using the conserved domains database, we were able to predict functions based on protein sequence and predicted structure that exist on ROI surrounding putative TA genes. Conserved domains are homologous portions of sequences that are encoded in different genetic contexts often encoding self-contained protein entities that have a similar function but when viewed as part of the larger overall protein may have vastly different biological functions (Ponting & Dickens 2001).

HTH_XRE conserved domains were the most highly abundant of all the 23578 individual protein domains that were identified in close proximity to putative

TA genes or the 3 controls used (BSH, RpoB and Random ROI). It is very likely that the abundance of these CD is due to their presence in TA systems. A previous study, which undertook comparative-genomic analysis of Type II TA systems, found a very high proportion of HTH motifs associated with TA toxins and many Xre motifs associated to TA antitoxins (Makarova *et al*., 2009). While the conserved domain findings in this chapter were expected, they cannot just be discarded as results of solely TA related motifs. HTH_Xre CD are DNA binding motifs of approximately 50-60 residues and are also commonly found in transcriptional regulators present in both prokaryotic and eukaryotic genomes (Aravind *et al*., 2004).

Unfortunately this may mean that any HTH_XRE like conserved domains not related to TA systems that are being stabilised within these regions are likely masked by the presence of the same motif within TA system proteins. In addition, this may also be why such a high proportion of transcription COG families were found. The 4th most abundant CD was a plasmid stability superfamily that encompasses both RelE/ParE, which are known TA toxins suggesting these toxins are also the reason for the highly elevated abundances of this CD. When considering this in light of ACLAME searches of ROI, approximately 60% of assigned functions appeared to be plasmid related. It is unsurprising then that one of the most abundant CDs found surrounding TA systems was plasmid related. However, TA ROI had significantly less ORFs associated with plasmids than control ROI datasets. This significance may be due to differences in the number of hits which had functions assigned. TA and BSH had approximately 70% assigned whilst the random data set had 13% and the random ROI just 8%, suggesting that while RpoB ROI had almost 90% of the assigned functions being related to plasmids, hardly any ORFs identified in this control ROI could be assigned using

the ACLAME database. Noticeably, approximately 39% of ORFs assigned function using ACLAME were prophage or virus related. This was significantly more than all control ROI datasets further supporting the hypothesis that TA systems may be stabilising phage (or phage functions) within the human gut community.

4.5.2 Abundance of β-Lactam resistance determinants in close proximity to putative TA genes

β-Lactams are the most commonly used antibiotic family worldwide and include penicillins, carabpenems, monobactams and cephalosporins (Elander 2003). Several studies have linked antibiotic treatments such as ampicillin to the development of *Clostridium difficle* infections through induced dysbiosis of important gut microbial commensal species (Sullivan *et al.*, 2001, Jernberg *et al.*, 2007, Freeman 2008). Carriage of β-Lactam resistance determinants in the gut microbiome has been noted repeatedly by both more traditional culture-based studies and more recently by metagenomic analysis, including the finding of several novel determinants (Sommer *et al.*, 2009, Sommer *et al.*, 2010, Schmieder & Edwards 2012, Cheng *et al.*, 2012). It was therefore not unexpected to find β-Lactam resistance determinants within ROI, however the significantly increased abundance of those in ROI surrounding putative TA modules was novel.

The simplest explanation for increased β-Lactam as well as aminoglycoside resistance determinants associated with TA systems is that functional TA genes within close proximity act to stabilise these genes by virtue of their addictive properties. However this does not explain why only these two specific antibiotic resistance determinants have significantly increased relative abundance in TA ROI. For pathogenic bacteria, gene loss and retention is an important part in

energy efficiency with obligate pathogens showing larger loss than facultative counterparts (Ochman & Davalos 2006). This is postulated to be due to either pathway-specific loss or genome wide reduction. For the latter possibility, and if this is applicable to host-associated when compared to free-living, a mechanism for avoiding loss of important functions which are currently not being selected for would be a valuable asset (Ochman & Moran 2001, Moran 2002, Andersson & Andersson 1999).

The Core Genome Hypothesis was first used in 1996 (Lan & Reeves 1996) and was used to distinguish between a fraction of the genome termed 'the core' which is shared by all members of a population and the fraction located only in a subset of this population ('the auxiliary'). Core genes tend to encode essential metabolic housekeeping functions whilst auxiliary genes generally encode supplementary biochemical pathways, aiding adaption to local environmental pressures (Feil 2004, Cohan 2002). Resistance determinants would be classed as auxiliary genes, not vital for fitness unless the 'norm' changes in the environment. The presence of a higher abundance of β-Lactams and aminoglycoside resistance determinants on TA ROI suggest that within the gut environment TA systems may be able to help stabilise these functions without the antimicrobial compound being present, counteracting genome reduction for metabolic benefits.

Whether this is intended or purely an additional benefit of TA systems addictiveness cannot be said with certainty although the distinct lack of other classes of resistance determinants (even significantly less than random controls for some) may provide some evidence in support of intended purpose. It was also interesting to note that ROI surrounding the RpoB housekeeping gene homologues were completed devoid of β-Lactam resistance determinants. It would be expected that this core genes would be located within highly conserved

chromosomal locations due to the important nature of their function and this further supports the hypothesis that TA systems may be stabilising auxiliary (rather than core) gene regions within the gut bacterial community.

Specific β-lactam determinants seem to be much more prevalent in regions of DNA surrounding putative Type II TA genes, than putative BSH and RpoB genes as well as random ROI. The most commonly found resistance determinants for β-lactams predicted was an *E.coli blaTEM2* gene known to encode a β-lactamase that can hydrolyse penecillins and first generation cephalosporins (Shaikh *et al.*, 2014). A recent metagenomic study investigating the gut microbiome as a mobile reservoir for antibiotic resistance between different continents found increased β-lactamases without the use of antibiotics, and this suggests that these resistance determinants are able to persist within the gut community without active selection (Bengtsson-Palme *et al.*, 2015). The next three more commonly identified β-lactam resistance determinants in TA ROI had homology to resistance markers found in *Staphylococcus aureus*. These were the hsdM gene, hsdR and ccrB gene. *hsd* genes behave as restriction modification systems, protecting host cells from foreign pieces of DNA whilst, *ccr* genes encode chromosomal recombinases allowing DNA cassettes to be inserted into bacterial chromosomes. How specifically a ccr gene is classed as a resistance determinant is questionable, other than its role in facilitating insertion of other resistance determinants into the genome in question, however it does provide a link between TA systems and chromosomal based insertion of resistance determinants. Until this point, its highly possible that much of the data gathered may have been within the boundaries of mobile genetic elements rather than chromosomally.

4.5.3 Gut related virulence factors share a genomic neighbourhood with putative TA genes

A large number of virulence factors (as assigned by the VFDB) were found across all regions of interest including TA, BSH, RpoB and those randomly generated from gut metagenomes. When examining the most highly abundant putative virulence factors across all ROI datasets, *bsh cylG* and *fabZ* homologues were the most abundant, specifically in ROI encompassing putative BSH genes. Perhaps unsurprisingly the inclusion of BSH genes here raises the questions, are BSH really virulence factors and does this distort the findings? Bacterial virulence factors enable a bacterium to replicate and disseminate within with in a host by subverting or eluding the host's defences (Cross 2008). The most basic example of what microbial virulence is being: pathogenicity of avirulent microbes in immunocompromised hosts compared to virulent pathogens in immune hosts. That is to say a microbe does not necessarily have to be virulent to take advantage of immunocompromised hosts (Casadevall & Pirofski 2001). Virulence (and the factors attributing to it) has always been difficult to define and as such there is no uniformity in definitions of it.

This lack of agreement on definition stems from virulence being a microbial property; however it is only expressed within susceptible hosts designating it a dependent variable (Casadevall & Pirofski 1999, Isenberg 1988, Casadevall & Pirofski 2001). This paradox means it is difficult for a microbial gene and the resulting product to be classified as a virulence factor as their presence during interactions between microbe and immunocompromised host does necessarily mean this is the case during interactions with immune hosts. In addition to this there the possibility for a virulence factor to be subjective. Is it necessarily a factor of virulence or perhaps colonisation or even commensalism (Wassenaar &

Gaastra 2001). It is also important to consider the database that was searched with the ROI predictions. The VFDB, while aiming to curate all known virulence factors, has included any bacterial toxins, cell surface proteins that mediate bacterial attachment, cell surface carbohydrates and proteins that protect a bacterium, and hydrolytic enzymes that may contribute to the pathogenicity of the bacterium. Many of these may not directly contribute to the virulence of an organism however their involvement in gene products that do may lead to their inclusion in the VFDB.

In addition to these findings, when organising relative abundance of virulence factors in relation to those most abundant in ROI datasets surrounding TA genes an extremely different abundance profile was seen. *ctpV*, as mentioned above is a cation transporting ATPase belonging to the mycobacterium genera and is closely linked to virulence for *Mycobaterium Tuberculosis* (Ward *et al.*, 2010). Ward *et al.*, suggest that not only did this transporter aid initial colonisation and continued survival of this species within the host, but it was also able to increase virulence compared to ΔctpV mutants. *Mycobacteria* are known residents of the human gut with dysbiosis of the gut microflora often leading to chronic infections of species from this genera and inflammatory diseases (Yokoyama *et al.*, 1994, Tomasello *et al.*, 2011). It is possible to hypothesise that the close proximity of *ctpV* to TA genes may not be due to a role in virulence per say, but instead aiding colonisation and survival within the gut environment, as while the ORFs present are related to Mycobacterial sequences, they are not necessarily from them.

More expected in this environment but interesting nevertheless was the finding that putative TA genes appear to be associated with *cylR2*, a transcriptional regulator for Enterococcal exotoxin; cytolysin, and *fimE*, a

regulatory fimbrial protein of *E.coli* (Rumpel *et al.*, 2004, Hinde *et al.*, 2005). *Enterococcus faecalis* is one of the major causes for hospital-acquired antibiotic-resistant infections. Cytolysin is lethal for a wide range of Gram positive bacteria and is toxic to higher organisms as well. Coburn & Gilmore (2003) suggested that this exotoxin may be for virulence but may also have an alternative function in competition in complex microbiota communities as well. This hypothesis fits well with findings from this chapter, suggesting the high abundance of cylR2 on TA ROI may be related to interspecies competition within the human gut environment rather than enabling virulence against the higher host. Fimbriae in *E.coli* are a vital physical structure for many pathogenic strains including Enterohemorrhagic *E.coli* 0157:H7 (Riley *et al.*, 1983) however they are also critical for avirulent gut commensal strains as well (Rendon *et al.*, 2007). Rendon *et al*, (2007) found that both pathogenic and commensal strains use a common pilus adherence factor for epithelial cell colonisation and it is possible that other elements that are considered virulence factors are important for colonisation of an environment which can then subsequently lead to virulence.

4.6 Conclusions

- Regions of interest surrounding putative toxin-antitoxin system genes have different functional profiles to control ROI surrounding bile salt hydrolyses, *rpoB* genes and those that were randomly generated from gut metagenomic datasets.

- TA ROI have a higher proportion of predicted functions involved in cell cycle/division and chromosome portioning, nucleotide transport and metabolism, transcription, replication/recombination/repair, Cell motility, general function prediction only, function unknown, and signal transduction mechanisms than control ROI.

- TA ROI have a lower proportion of predicted functions involved in energy production and conversion, carbohydrate transport and metabolism, coenzyme transport and metabolism, lipid transport and metabolism, cell wall membrane/envelope biogenesis, post-translational modification and protein turnover, inorganic ion transport and metabolism, Secondary metabolite biosynthesis/transport/catabolism, intracellular trafficking/secretion/vesicular transport and cytoskeleton when compared to control ROI.

- β-lactam resistance determinates have a higher abundance on sequences harbouring putative TA genes than control dataset

- TA ROI had a higher abundance of *ctpV, cylR2* and *fimE* virulence factors when compared to control ROI, however these may be functioning as colonisation factors within the gut microbiota community instead.

Chapter 5: Regulation of gut-related stress responses by the p22-RelBE toxin-antitoxin module

5.1 Introduction

The abundance of several Type II TA systems within the human gut, including the putative pTRACA22 RelBE module found in the previous chapter, has two possible explanations. Firstly, that these modules which initially entered this habitat through early microbe colonisation have simply persisted and replicated through the microbial host's inability to lose them, acting as solely selfish entities (Van Melderen 2010). The second possibility is that carriage and expression of these systems provides some beneficial function for the microbial hosts within the gut environment. The latter is the currently favoured hypothesis within the field; however the function of TA systems within individual bacterial species, the wider gut microbiome and the whole human supra-organism currently remains unclear.

5.1.1 Discovery of TA systems as stress response elements

It was first observed by Christensen *et al.* (2001), through examination of the stringent response, that TA systems are involved with metabolic regulation and survival of the bacterial cells during times of stress. The stringent response is defined as the pleotropic physiological changes triggered by amino acid (aa) starvation (Cashel *et al.*, 1996). This is exemplified by the rapid increase in concentration of guanosine tetraphosphate (ppGpp) in eubacteria (Cashel *et al.*, 1996). Notable effects of this response include immediate termination of rRNA and tRNA synthesis, therefore reducing energy consumption and stimulation of increased expression of genes involved in aa biosynthetic pathways (Cashel *et al.*, 1996, Chatterji & Kumar 2001). The stringent response was also found to increase

production of the starvation sigma factor (σ^S), further reducing gene expression (Gentry *et al.*, 1993). With regards to the relE toxin, it was apparent that it became activated during this nutritional stress, independent of other stress response elements, resulting in a global reduction of translational rates from between 5-10% of the pre-starvation level (Christensen *et al.*, 2001).

As suggested by Christensen & Gerdes (2003), it seems plausible that cells which are subject to these extreme changes in amino acid concentrations would benefit from systems and mechanisms which can rapidly and efficiently adjust global rates of both protein and DNA synthesis, such as certain TA loci. The RelE toxin of the RelBE TA module has been shown to be one of these global translational inhibitors through cleavage of mRNA codons between the second and third base positioned at the ribosomal A-site, ensuring mRNA cannot be fully translated into functional proteins (Pedersen *et al.*, 2003, Christensen & Gerdes 2003).

5.1.2 Dormancy, persistence and Type II TA systems

The existence of unpredictable and unstable environments throughout nature force organisms to grow and reproduce in sub-optimal conditions, often encouraging them to enter reversible states of reduced metabolic activity. For bacteria, this is often referred to as dormancy, an umbrella term used for both the cellular and population level processes: sporulation, biofilm formation and bacterial persistence (Lennon & Jones 2011). TA systems are implicated in all three of these major dormancy processes within bacteria and Table 5.1 summarises the key features and gives examples of the role of TA systems.

Table 5.1: A summary of dormancy and the role played by TA modules within dormancy of bacterial populations

Mechanism of dormancy	Description	Relevance of TA modules	Relevance to the human gut
Sporulation	A complex sequence of cell division, differentiation and assembly events leading to formation of highly resistant spores which can reactivate in more favourable conditions (Veening *et al.*, 2009, Teleman *et al.*, 1998, Burton *et al.*, 2007, Henriques & Moran 2007).	The Type II MazEF TA system appear involved in myxobacterial fruiting body formation and cell lysis during the release of spores (Nariya 2008). Also, a relative of the MazF toxin, NdoA, has been shown to increase the rate of sporulation in *Bacillus subtilis* (Wu *et al.*, 2011).	Sporulation is a less documented mechanism of dormancy within the normal gut microflora, more often associated with *Clostridium difficle* associated diarrheas (Paredes-Sabja *et al.*, 2014) or when normally soil associated organisms are ingested such as *B.subtilis* (Tam *et al.*, 2006)
Biofilm formation	A mode of microbial growth by multicellular communities that adhere to both biological and non-biological surfaces (Hall-Stoodley *et al.*, 2004). These communities produce a self-produced extracellular martix composed of exo-polysaccharides, proteins and DNA (Kostakioti *et al.*, 2013).	Biofilm formation and maturation consists of a complex sequence of stages involving distinct changes in gene expression and subsequent phenotypic alterations. TA systems, specifically MazEF and RelBE (and their homologues), have been reported to be involved within both early biofilm formation and late biofilm dispersal through translational repression of other genes (Kim *et al.*, 2009). Lysis of a portion of cells through TA activation has also been identified further	The formation of microbial biofilms begins with the initial colonisation of a newborn infant gut (de Vos 2015). Identification of biofilm formation in the colonic microbiome was shown almost a decade ago through microscopic analysis of food particles and continuous flow systems (Macfarlane *et al.*, 1997, Macfarlane & Macfarlane 2006, Macfarlane & Dillon 2007). While evidence of luminal biofilm formation exists, mucosal biofilms have yet to be confirmed and are hypothesised to be largely

		aiding biofilm formation through release of cellular contents crucial in stabilisation of the matrix (Allesen-Holm *et al.* 2006, Thomas *et al.* 2008).	prevented by the speed by which the human colonic mucosa grow and the density of the mucus layer (Lebeer *et al.*, 2011, Gustafsson *et al.*, 2012).
Bacterial persistence	Used to describe the surviving proportion of a cell population when exposed to a lethal stress (Bigger 1944, Balaban *et al.*, 2004) . They achieve this through existence in a slow growing state. Two forms of persistence have been documented; stochastic and determanistic (Lewis 2010, Keren *et al.*, 2004, Dorr *et al.*, 2009) .	Mutations in the Type II HipAB (<u>hi</u>gh <u>p</u>ersistence) loci were found to produce high numbers of persisters (Moyed & Bertrand 1983) whilst ectopic expression of Type II TA systems was shown to induce a reversible persister-like state (Pedersen *et al.*, 2002). It has also been demonstrated that persister cells contain elevated levels of HigAB and RelBE transcripts (Keren *et al.*, 2004, Shah *et al.*, 2006). In paralell, progressive and cumulative deletions of the 12 TA systems present on *E.coli* K-12's genome appeared to be correlated with reducing persister numbers (Maisonneuve *et al.*, 2011).	Bacterial persistence within the human gut environment is often discussed in the context of population level resistance to antimicrobial treatments (Francino 2016). The microbiome has been shown to return to a similar composition as pre-exposure once the antimicrobial compound has been removed (Dethlefsen *et al.*, 2008, Dethlefsen & Relman 2011).

5.1.3 Non-immunological microbial barriers the gastrointestinal tract creates

The physiology of the human digestive tract and the constant changing of composition play an important role in microbial colonisation of this environment. The GI tract of a healthy adult is approximately 5.5 meters in length and consists of the alimentary canal (Oesophagus, stomach, small intestine and large intestine) as well as several accessory organs including those of the oral cavity, liver, gall bladder and pancreas (Martini & Nath 2009). These accessory organs and the parietal cells of the stomach secrete enzymes, acid and bile, which aid the mechanical digestion also taking place along the tract (Martini & Nath 2009).

In addition to humoral and cellular immunity, it is generally accepted that the non-immunological defence system of the human GI tract is an important first line of defence against pathogens (Sarker & Gyr 1992). It was shown during the 1970's that even individuals completely lacking secretory immunoglobulins, a major component of mucosal immunity, could still maintain a perfectly functioning gut (McLaughlin *et al.*, 1978) indicating just how effective this system could be. However, this effectiveness is non-discriminatory, excluding both pathogenic bacteria but also those normal gut commensals, which could potentially be beneficial to the host. The mechanisms of this non-immunological defence system are: gastric acidity, detergent activity of bile, secretion of pancreatic enzymes, motility of the intestine/epithelial turnover and the normal intestinal microflora. While little evidence exists of TA involvement in persistence to gut related stressors, their previously identified role as stress regulators may indicate a contribution to persistence for some of those mentioned above. Figure 5.1 depicts the human GI tract, sites of notable stress for microbes within this and a summary of the bacterial composition seen in each distinct site.
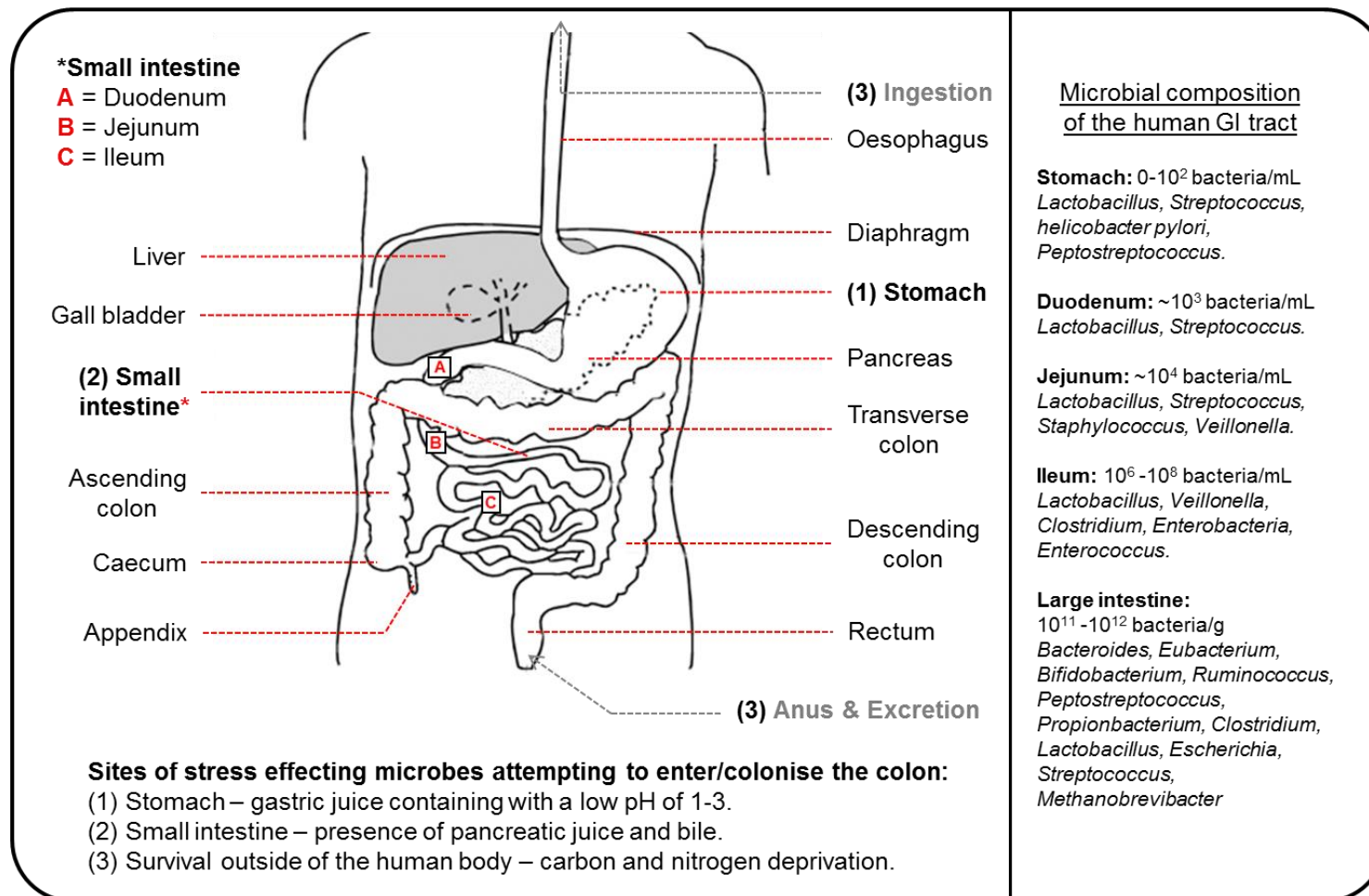
Figure 5.1: Anatomical characteristics, sites of heavy stress and the microbial composition within the human GI tract. Data displayed was taken from Ridlon *et al*., 2006, Sartor 2008, Eckburg & Relman 2007, Frank *et al*., 2007.

5.1.3.1 Low pH

The low pH of gastric juice plays a vital role not only in facilitating digestion of dietary nutrients and augmenting iron and calcium absorption but also as a major defence factor against exogenous microorganisms and potential pathogens (Smith 2003). In conjunction with this defensive behaviour, it is able to supress colonisation of the proximal bowel by oropharyngeal and faecal commensal flora (Howden & Hunt 1987). The low pH of gastric juice (<3.0), due to hydrochloric acid production by parietal cells, is responsible for the bactericidal properties of this fluid and is achieved by high concentrations of H^+ ions which interact with macromolecules (including lipids, carbohydrates and DNA) affecting their chemical properties and structure (Hingorani & Gierasch 2013). Studies conducted by Giannella *et al.*, (1972) and more recently by Tennant *et al.*, (2008) demonstrated firstly that this low pH ensured exogenous bacteria introduced are usually destroyed within the first 15 minutes of inoculation and secondly, that mice with supressed acidity (due to mutations in a gastric H^+ and K^+ ATPase) had a significantly higher rate of infection by pathogens such as *Yersinia enterocolitica* and *Salmonella enterica*.

With such an effective method of killing in place, for those bacterial cells ingested this represents a potent obstacle to overcome whilst in transit through the upper GI tract. Studies involving probiotic *Lactobacilli*, *Bifidobacterium* and *E.coli*, have highlighted that these species are able to survive periods of 3 hours in highly acidic environments (pH 1-3), some even up to 24 hours with 5×10^4 CFU being recovered (Coldewey *et al.*, 2007, Berrada *et al.*, 1991, Lankapurthra & Shah 1995). Notably this 3 hour period appears to be the approximate window of time that the majority of solid food ingested in one sitting takes to pass from the stomach into the small intestine (Camilleri *et al.*, 1989). Acid tolerance involves

complex changes at the level of protein expression, some of these known to be involved in the rpoS (RNA polymerase sigma S) or SOS system, thus regulating the general stress response (Jorgensen *et al*., 1999, Arnold *et al*., 2001). Finally it also has to be taken into consideration that whilst pH is clearly an important factor when studying survival and transit through the stomach, other gastric juice components such as pepsin and lysozyme (albeit in small concentrations) may also factor in. Although there is no direct evidence linking TA modules to persistence during acid shock, it is conceivable, based on their potential to be regulated by the general stress response, that they could be involved.

5.1.3.2 Bile

The liver secretes about 1 litre of bile into the GI tract every day, which represents a significant challenge for gut commensals to overcome. Bile is a biological digestive detergent involved in the solubilisation and emulsification of lipids and is therefore able to affect phospholipids and proteins within the cell membrane, disrupting the homeostasis of the cell (Succi *et al*., 2005, Begley *et al*., 2005, Ridlon *et al*., 2006). Bile is composed of several components including ions, proteins, pigments, cholesterol and a variety of bile salts, the last of which have been shown to impede bacterial growth (Inagaki et al. 2006). The two primary products of bile acid synthesis in humans are cholic acid and chenodeoxycholic acid and are known as primary bile acids. These are produced and conjugated with taurine and glycine within the liver before being stored within the gallbladder (Jones 2011, Staels & Fonseca 2009). Upon ingestion of a meal, the gallbladder contracts releasing primary conjugated bile acids into the intestinal lumen. Through bacterial enzymatic action, these are de-conjugated and the free primary bile acids can be converted to deoxycholic acid and lithocholic acid, ensuring solubility at a wider range of ionic strengths (Hofmann & Mysels 1992, Hofmann

1999 and Okoli *et al.*, 2007). Additionally, intestinal bacteria further metabolize lithocholic acid into the tertiary bile salt ursodeoxycholic acid (Hay & Carey 1990).

Bile is found at high concentrations in the proximal ileum, but declines as transit occurs through the human hut, leading to much lower concentrations in the colon. This is achieved by passive diffusion across the entire length of the gut and termed enterohepatic circulation (Carey & Duane 1994, Begley 2006). With the shown antimicrobial activity of bile it can be hypothesised that this is a major barrier to colonisation of the ileum and the prevention of microbial overgrowth. The commensal relationship of humans and the gut microbiome would suggest that microbial cells need to pass through this highly antimicrobial environment in order to colonise the colon without harming the higher host. Therefore systems which permit survival but not resistance to the barrier provided by bile would be a favourable trait and one we are keen to explore with regards to TA functionality.

5.1.3.3 Carbon and nitrogen starvation

Colonisation of the human gut of infants, those recently perturbed by infection and antimicrobial treatment is a major part of any gut commensal life cycle but survival outside of the human gut in order to colonise a new host, as well as within this environment during times of host starvation provides a major difficulty for microbes to overcome (Kau *et al.*, 2011, Xia *et al.*, 2014, Elsas *et al.*, 2011). This nutritional deprivation of bacterial cells/populations would result from a lack of carbon source, nitrogen source or both depending on the microbe's current environment (Devkota & Chang 2013). It is therefore logical that prokaryotes would develop a mechanism to temporarily survive this. One example could be periodic metabolic inactivity, a function which several TA systems, including the RelBE system, have been shown to be significantly involved with.

5.1.4 The pTRACA22 RelBE loci

With the numerous stresses bacterial gut commensals are faced with outside the body, during initial colonisation and throughout prolonged existence within the human gut, it has been shown that those that chose to inhabit such an environment have developed innovative mechanisms to persist. Examples include aa decarboxylases (Hong *et al.*, 2012), chaperone protection of transmembrane proteins and low-pH induced protonation which alters folding of these proteins (Foit *et al.*, 2013, Zhang *et al.*, 2011). The presence and abundance of putative Type II RelBE TA loci within the human gut microbiome suggests a role for this particular module in colonisation or survival within the human gut environment.

The pTRACA22 (p22) plasmid which harbours a putative RelBE TA module has a total of 9 ORFs; including two predicted to be involved with plasmid replication and mobilisation, two for segregational stability (the suspected TA module) and five of unknown function. See Figure 5.2 for a map visualising the 5903bp plasmid with positions of all predicted ORFs and their orientation. p22 was isolated using the TRACA method for the which is used for the acquisition of plasmids from metagenomic DNA and allows maintenance in a surrogate host (Jones & Marchesi 2007).

The p22 plasmid has been shown to have high homology to the draft genome of *Blautia hydrogenotrophica*, a member of the *Firmicutes* division, with all ORFs having between 98-100% aa identity to those identified in this species (Jones et al., 2010). A major obstacle for further investigation of the putative pTRACA22-RelBE module (p22-RelBE) is the presence of multiple other ORFs of unknown function also carried on the plasmid. It would not be possible to identify which genes would be responsible for any observed effects and so this would need to be confirmed through either mutagenesis or complementation approaches.
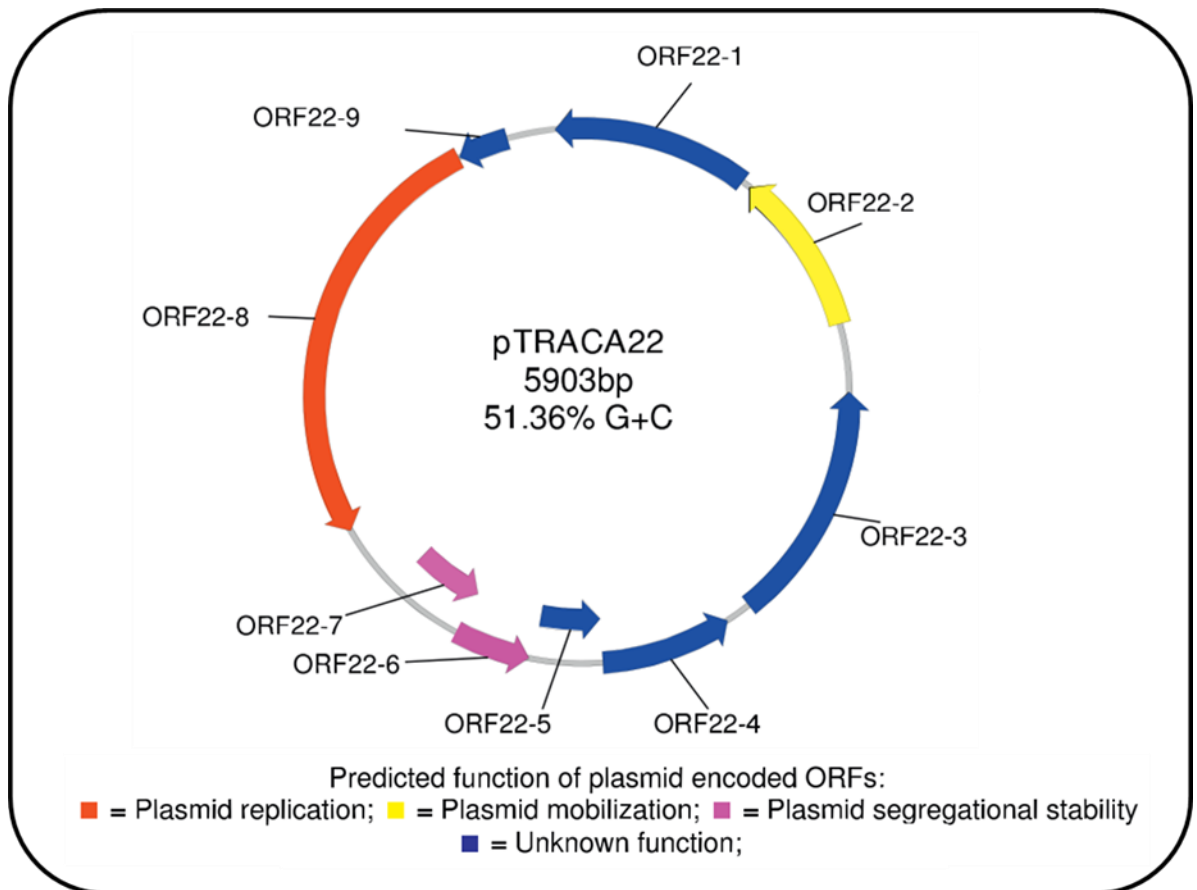
Figure 5.2: Physical maps of the complete nucleotide sequence from pTRACA22 indicating the 9 predicted ORFs, which are colour coded according to predicted function. ORF6 and 7 are the putative genes encoding the RelE toxin and RelB antitoxin respectively. Notably they are in the usual Type II TA organisation with the antitoxin being upstream and a slight overlap of the two genes. Reproduced with permission (Jones *et al.*, 2010).

With this in mind the functionality of the p22-RelBE module was identified and the role this TA system may play within gut stress was examined.

5.2 Aims

The aims of the work presented in this chapter were i) to clone the p22-RelBE toxin, antitoxin and complete module under the control of inducible promoters, ii) confirm p22-RelBE toxin activity within an *E.coli* EPI300 background and iii) ascertain the role of this gut TA associated system in survival of environmental stresses encountered during GI colonisation including low pH, bile, carbon and nitrogen starvation.

5.3 Materials and methods

5.3.1 Construction of the inducible pBAD-TOPO+RelE toxin expression vector

To clone the pTRACA22-RelE toxin gene under the control of an inducible promoter (araBAD: pBAD), the pBAD TOPO® TA Expression Kit (Life Technologies, UK) was used following the manufacturer's instructions. Initially, primers were designed to amplify the entirety of p22-ORF6, including the start codon (ATG), to prevent fusion of the N-terminal leader peptide and to remove the V5 epitope as well as the polyhistidine region, as per the manufacturer's instructions. Using the primer set 'ORF6TOPO' which can be seen in Table 5.2, along with the specified annealing temperature, Taq PCR was performed using the standard protocol provided in Section 2.2.4 with the addition of 2 µL of $MgCl_2$ (25 mM, giving a total working concentration of 2 mM per reaction). A 272bp product was produced, separated by gel electrophoresis on a 1% agarose gel and extracted using the protocol provided in Section 2.2.7. DNA concentration was then measured using a Nanodrop 2000 Spectrophotometer (Thermo Scientific, UK).

The cloning reaction was prepared using the following components and in the order described below. All reagents were stored, as directed, at $-20^{o}C$ until use and thawed on ice. A ratio of 1:1 insert: vector was used with 2 µL of the insert (0.384 ng/µL), 1 µL of salt solution, 1 µL of NFW and finally 1 µL of the pBAD TOPO vector (10 ng/µL) were added and gently mixed using a pipette. The reaction was then incubated for 5 minutes at RT, placed on ice and used directly to transform competent cells.

Table 5.2: Primers used during this study detailing their sequence, annealing temperature (AT), product size and origin. All primers shown below were obtained from Eurofins MWG Operon.

Primer pair	Sequence 5'-3'	AT (°C)	Product & origin
ORF6TOPOF1	GAGGAATAATAAATGTATAACATAAGACCAAC	56.3	272bp product including shine delgarno, start codon and p22 RelE-like toxin ORF. This study.
ORF6TOPOR1	CTAAAATAAATCGCTATGCG		
p22ORF67PF1	TGGGTAAACGGCCAGGAATA	56.3	680bp product: p22 antitoxin, toxin and 200bp upstream to capture suspected promoter region. This study.
p22ORF67PR1	ACTGCGCGATCCTCTCTTTA		

For transformations, 2 μL of the ligation reaction was added to an individual vial of OneShot® TOP10 cells and gently mixed avoiding bubbles. This was then incubated on ice for 30 minutes before heat shock treatment at $42^{o}C$ for 30 seconds. Both tubes were immediately placed on ice and 950 μL of SOC medium was added. Vials were then sealed and incubated at $37^{o}C$ horizontally for 1 hour with shaking (200 rpm). Half the transformation suspensions were diluted 1/2 and 100 μL aliquots of both the neat and diluted suspensions spread across pre-warmed LB plates containing 100 μg/mL ampicillin and incubated overnight at $37^{o}C$.

A total of 10 transformants were picked, inoculated individually onto LB+100μg/mL ampicillin liquid and solid medium and incubated overnight at $37^{o}C$ with shaking (200 rpm) for liquid cultures. Preliminary confirmation of correct insertion and transformation was achieved using a colony boil (Section 2.2.2) and 2 μL of each used as template for Taq PCR using the same conditions original cloning primers. PCR products were then separated by gel electrophoresis on a 1% agarose gel. Final confirmation of correct construct generation was achieved by plasmid extraction (Section 2.2.3), digest with the restriction endonuclease BSE RI (New England Bioloabs, USA, Section 2.2.6) as well as Taq PCR (Section 2.2.4) with the original cloning primers; same conditions as above and extracted plasmids as template. A physical map of the plasmid containing the PCR product can be seen in Figure 5.3. Finally the correct plasmid was extracted and transformed by electroporation (Section 2.2.13) into an *E.coli* TransforMax EPI300™ (Epicentre, UK) background, the same strain harbouring the pTRACA22 plasmid.
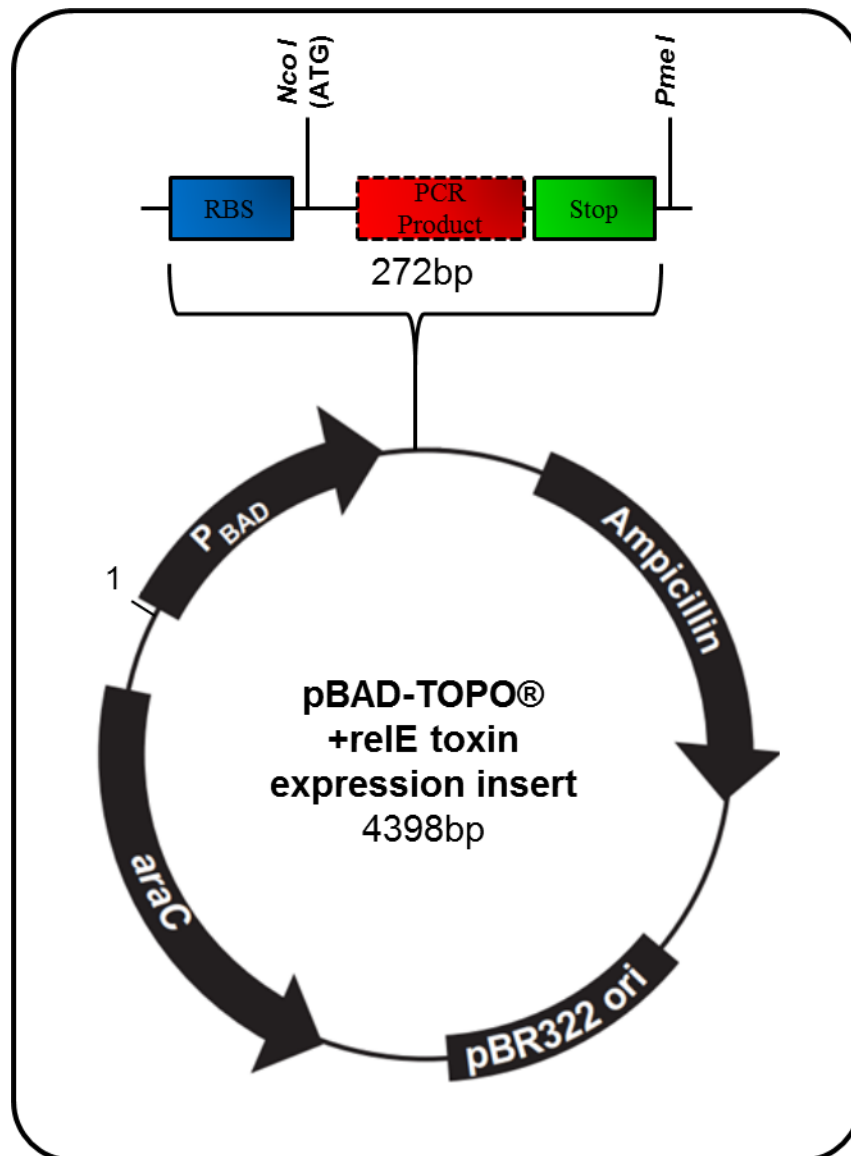
Figure 5.3: Physical map of the 4398bp pBAD-TOPO® vector and RelE expression insert. Sequence coordinates are provided in the 5'-3' orientation with "1" signifying the start of the sequence when linear. This indicates the positions of the inducible araBAD promoter and regulatory elements (4-276bp), ampicillin resistance gene (1013-1873bp), pBR322 origin of replication (2018-2691bp), AraC (4100-3222bp) ORF and insertion site of the PCR product (328-600bp, which also included a ribosomal binding site (RBS), a start codon and two stop codons to ensure termination). Figure was an adaption of the plasmid map from the pBAD TOPO® TA Expression Kit handbook.

5.3.2 Design of the inducible pJ821 RelB antitoxin and complete RelBE module constructs

The USA based gene synthesis and bioengineering company, DNA 2.0 (https://www.dna20.com/) were used to generate inducible constructs of the p22-RelB antitoxin and p22-RelBE complete module. Small alterations to the sequence were made to ensure the most efficient codon usage for *E.coli* expression. These were cloned into pJ821 low copy number expression vector with kanamycin selection under the control of the inducible promoter RhaBAD and expression of these genes was confirmed in *E.coli*. Figure 5.4 provides a physical map of the pJ821 vector with inserts. For the complete TA module, a second RhaBAD promoter was also attached to the toxin gene to ensure equal expression of both genes (i.e. no polarity in expression). A physical map of the pJ821 expression vector containing the two inserts of interest can be seen in Figure 5.4.

5.3.3 Construction of the pGEM-T Easy + RelBE plasmid

The pTRACA22 RelBE complete module including 200bp upstream to capture the suspected promoter region was amplified by Taq PCR performed using the p22ORF67P primer set displayed in Table 5.2 and standard protocol provided in Section 2.2.4 with the addition of 2 μL of $MgCl_2$ (25 mM, giving a total working concentration of 2 mM per reaction). A 680bp product was produced, separated by gel electrophoresis on a 1% agarose gel and extracted using the protocol provided in Section 2.2.7. DNA concentration was then measured using a Nanodrop 2000 Spectrophotometer (Thermo Scientific, UK). A ligation reaction between the amplified insert and the pGEM-T Easy vector was prepared follow protocol outlined in Section 2.2.15 and used directly for electroporation. A physical map of the pGEM-T Easy(TA) plasmid can be seen in Figure 5.5.
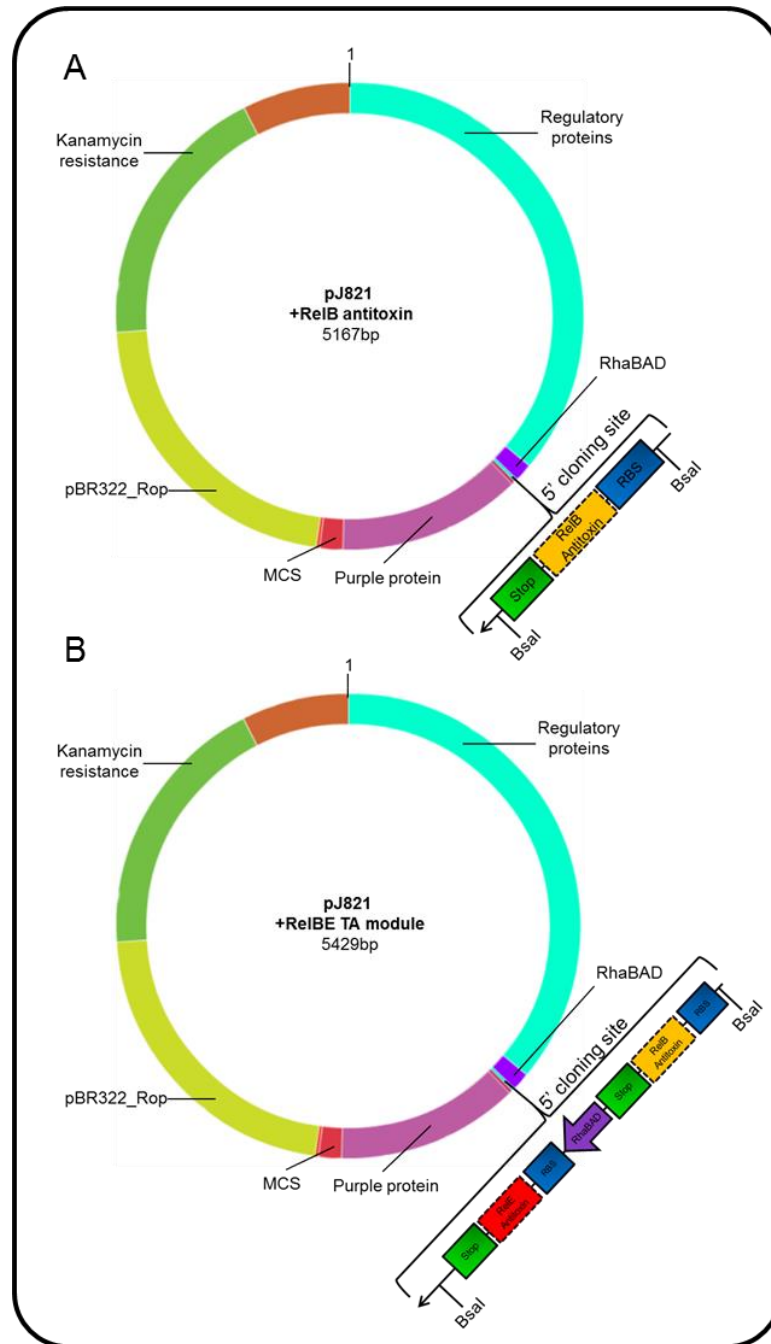
Figure 5.4: Physical map of (A) the 5167bp pJ821 vector and the RelB antitoxin, (B) the 5429bp pJ821 vector and both the RelB antitoxin and RelE antitoxin. Sequence coordinates are provided in the 5'-3' orientation with "1" signifying the start of the sequence when linear. This indicates the position of regulatory proteins (1-1962bp), RhaBAD promoter (1963-2032bp), 5' cloning site, PrancerPurple protein (2059-2748bp), 3' multiple cloning site (2749-2837bp), pBR322-Rop origin of replication and terminator (2849-3772bp), kanamycin resistance gene (3838-4861bp).
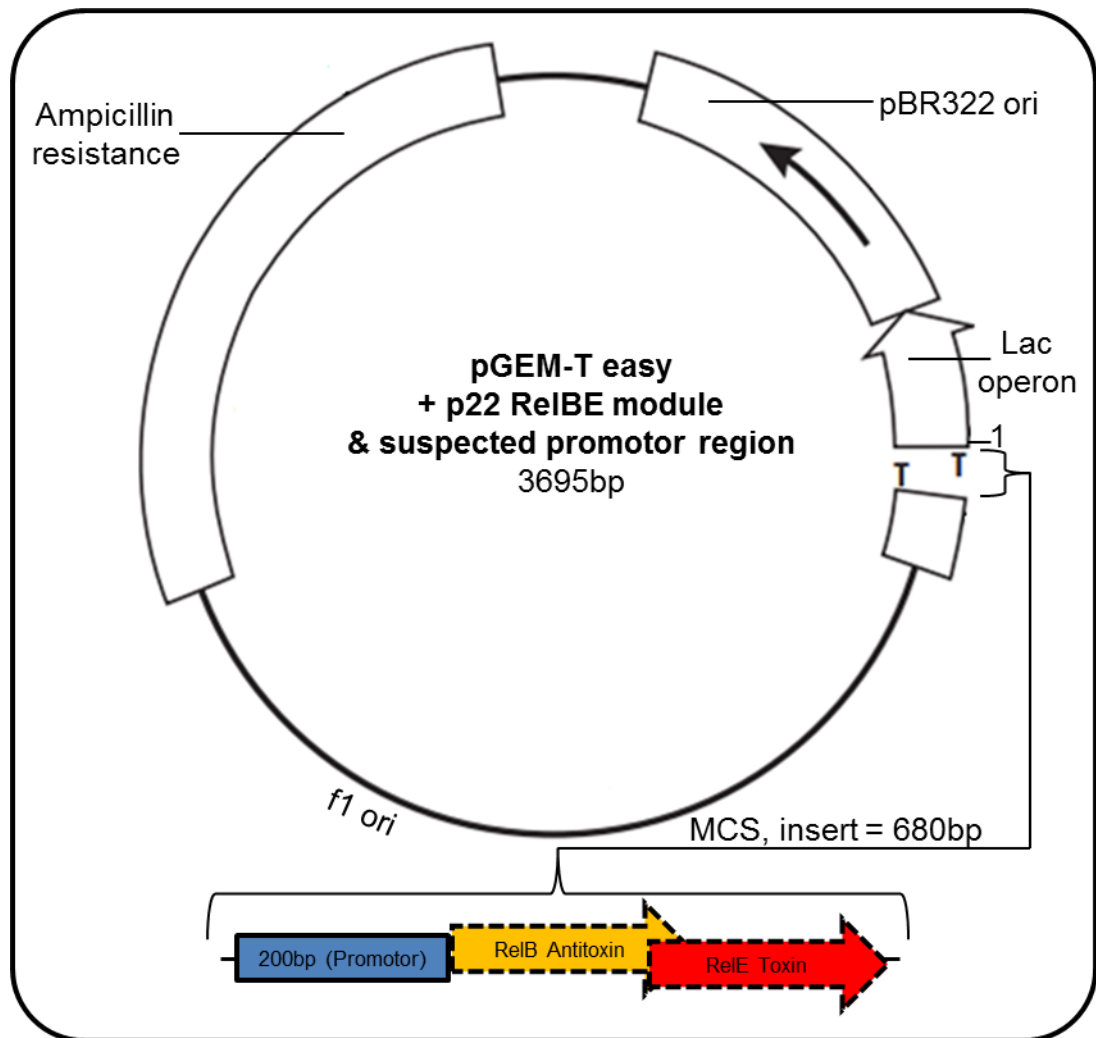
Figure 5.5: physical map of the 3695bp pGEM-T Easy(TA) plasmid containing the full p22-RelBE TA module in its natural confirmation. Sequence coordinates are provided in the 5'-3' orientation with 1 signifying the start of the sequence when linear. This indicates the multiple cloning site, Lac operon (151-380, 2821-2981bp), pBR322 origin of replication (564-1183bp), Ampicillin resistance gene (1338-2198bp) and F1 origin of replication (2513-2819bp).

5.3.4 Transformation of the pJ821 RelB antitoxin and complete RelBE module inducible constructs

The pJ821 RelB antitoxin (pJ821(ATx)), pJ821 RelBE complete module (pJ821(TA)) and pGEM-T Easy(TA) plasmids were transformed by electroporation into *E.coli* TransforMax EPI300™ background following protocol in Section 2.2.13.

5.3.5 Proof of pBADTOPO-RelE toxin activity in *E.coli* EPI300

To confirm the activity of the p22-RelE toxin (pBAD(Tx)) within *E.coli* EPI300, standard growth curves using OD reading at a wavelength of 600nm were employed using media with and without the addition of the inducer, 2% arabinose at the 4 hour mark for one replica and sterile water added to the other. These were done over a period of 12 hours, for 1 of the transformants giving the correct digestion pattern and positive PCR products from extracted plasmid Taq PCRs done in Section 5.3.3.

5.3.6 Reverse transcription to confirm expression of pTRACA22, pBAD(Tx), pJ821(ATx), pJ821(TA) and pGEM-T Easy(TA)

To confirm expression of the toxin and antitoxin in the native plasmid pTRACA22 pBAD(Tx), pJ821(ATx), pJ821(TA) and pGEM-T Easy(TA), cells were grown and induced using their respective inducers. Cells were then lysed during log phase, their total RNA extracted, reverse transcribed following protocol in Section 2.2.14, and used as template for Taq PCR (Section 2.2.4) confirmation of the presence of mRNA for the two genes. The PCR reaction was prepared as in Section 1.2.4.1 and the pTRACAORF6 and pTRACAORF7 internal primer sets were used from Table3.2.

All strains were grown for 18 hours in LB broth with the correct selection and additional supplements needed (Table 2.1) for inhibited expression of the toxin, antitoxin and complete module. 2 mL of the overnight culture was then inoculated into 98 mL of fresh LB medium containing the same selection (1/50 dilution) but replacing glucose (if it was required as a metabolic inhibitor) with 2% arabinose for EPI300 harbouring the pBAD+ORF6 plasmid and 100mM rhamnose for the two pJ821 constructs and grown until an OD600 of ~0.3. The resulting log phase cultures were then used for RNA extraction.

5.3.7 Growth curves to ensure reliability between viable counts and fluorescence live/dead staining methodology

Standard growth curves were completed following methodology in Sections 2.1.3 and 2.1.4 with dilutions of individual time point samples done directly and immediately plating these. The media used contained 0.2% (w/v) glucose to ensure metabolic repression of inducible constructs with glucose also added to control strains to ensure comparable results. These growth curves were repeated with samples stored on ice until the concluding time point and finally diluted and plated together.

5.3.8 Starvation, bile, low pH and gastric juice viability assays

Cultures of *E.coli* EPI300: wild type (WT), pTRACA22, pGEM-T Easy(empty), pGEM-T Easy(TA), pBAD(Tx), pJ821(empty), pJ821(ATx) and pJ821(TA) were inoculated and grown overnight with appropriate selection and other required supplements (Table 2.1) with shaking at 150 rpm. These overnight cultures were diluted to an OD_{600} of 1.0 and 1 mL of each added to a separate 50 mL of LB broth

containing no selection and grown to an OD_{600} of 0.5. Entire cultures were centrifuged at 4500 xg for 10 minutes, the supernatant discarded, and the remaining pellet resuspended in the stresses summarised below in Table 5.3.

5.3.8.1 Carbon and nitrogen starvation

10x M9 salts consist of dibasic sodium phosphate (60 g/L), monobasic potassium phosphate (30 g/L) and ammonium chloride (10g /L) at a pH of 7.4. To produce M9 minimal media, 100mL of 10x M9 salts were added to 889mL of deionised water and autoclaved. 1mL of filter sterilised 1M magnesium chloride was added and the final volume made up to 1L by including/excluding 2% (w/v) Casamino acids and 0.2% (w/v) D-glucose or sterilised deionised water. If expression of the constructs were required inducers were added at the same concentrations as Section 5.3.5. Cultures were then incubated at $37^{o}C$ whilst being shaken at 150 rpm for 24 hours and 1mL samples taken at the 0, 1, 2, 4, 6, 8, 16 and 24 hour time points. Cells were pelleted by centrifugation at 17000 xg for 2 minutes, washed 3 times in PBS using the same centrifugal speed and finally re-suspended in the same volume of PBS. A serial dilution was then done in PBS from neat to 10^{-8}.

Table 5.3: A summary of important characteristics of the individual stresses to be examined within this chapter.

Stress	Notable characteristics	Reference/source
Carbon starvation (M9 minimal media)	1x M9 salts pH 7.4 2% (w/v) casamino acids	Sambrook & Russell 2006, modified to exclude glucose
Nitrogen starvation (M9 minimal media)	1x M9 salts pH 7.4 0.2% (w/v) D-glucose	Sambrook & Russell 2006, modified to exclude amino acid source
Carbon and Nitrogen Starvation (M9 minimal media)	1x M9 salts pH 7.4	Sambrook & Russell 2006, modified to exclude glucose and amino acid source
Porcine bile	LB broth supplemented with 25% (w/v) porcine bile	Sigma Aldrich, UK
Low pH (2.0)	LB broth acidified with 5M HCl to pH 2.0	N/A
Artificial Gastric Juice	pH 2.0 0.83 g/L proteose peptone 0.10 g/L lysozyme	Corcoran *et al.*, 2007

5.3.8.2 Artificial gastric juice and low pH

Artificial gastric juice (AGJ) was formulated according to Corcoran *et al.*, 2006 comprised of 0.83g/L proteose peptone, 3.5g/L D-glucose, 0.37g/L sodium chloride, 0.6g/L potassium phosphate, 0.11g/L calcium chloride, 0.37g/L potassium chloride, 0.05g/L bovine bile, 0.10g/L lysozyme, 0.0133g/L pepsin and adjusted to a pH 2.0. If expression of the constructs were required inducers were added at the same concentrations as Section 5.3.5. Cultures were then incubated at 37°C whilst being aerated at 150rpm for 5 hours and 1mL samples taken at the 0 min, 15 min, 30 min, 1, 2, 3, 4 and 5 hour time points. These samples were treated as in Section 5.3.7.1. For low pH alone (2.0) cells were resuspended in LB broth acidified with 5M HCl and treated as above.

5.3.8.3 Porcine bile

Bile assays were done in an identical fashion to artificial gastric juice (Section 5.3.7.2) but with the replacement of gastric juice with LB broth supplemented with 25% (w/v) porcine bile (Sigma Aldrich, UK).

5.3.9 Viability counts

Immediately after serial dilutions from Section 5.3.7, each dilution was plated onto LB plates using the Miles & Misra technique (Miles *et al.*, 1938) containing the required selectivity for the plasmid being examined and incubated for 18 hours at 37°C. CFU/mL was calculated and used to determine the viability of cells within these bacterial populations before and after treatment with the six stresses.

5.3.10 BacLight Live/Dead staining and confocal microscopy

Immediately after serial dilutions from Section 5.3.7 live/dead fluorescence staining was performed on each sample taken. The LIVE/DEAD® BacLight Bacterial Viability Kit was used (Life Technologies, UK). Equal volumes of component A (SYTO 9 dye at a concentration of 3.34 mM) and component B (Propidium iodide at a concentration of 20 mM) were added to a 1.5mL micro centrifuge tube and mixed. Per mL of bacterial cell suspension and dilutions, 3 µL of the dye mixture was added and mixed thoroughly. Samples were wrapped in foil to exclude light and incubated for 15 minutes at room temperature. 5 µL of stained bacterial suspension was then trapped between a 26 x 76 x 1 cm microscope slide (Fisher, UK) and a coverslip. Images were captured using Leica TCS SP5 confocal microscope with percentage live and dead cells calculated per field of view (FOV). Injured cells were recorded separately (those which appeared a light orange colour using a fluorescence microscope), but recorded as "live". A total of three replicate slides were prepared and visualised from each sample tested, with 10 randomly selected FOV captured and analysed for live/dead counts per slide.

5.4 Results

5.4.1 Confirmation of p22-RelE toxin and p22-RelB antitoxin expression in pTRACA22 and expression constructs within *E.coli* EPI300

To confirm expression of p22-RelBE in various constructs reverse transcription PCR was undertaken using internal p22ORF6 (toxin) and p22ORF7 (antitoxin) primer sets. Table 5.4 lists the various constructs, the TA gene(s) they carry and expression systems used. Expression of the p22-RelE toxin was confirmed in pTRACA22, pGEM-T Easy(TA), pBAD(Tx) and pJ821(TA). Expression of the p22-RelB antitoxin was also confirmed in pTRACA22, pGEM-T Easy(TA), pJ821(TA) and pJ821(ATx). Figures 5.6, 5.7 and 5.8 show electrophoresis gels confirming expression of both TA components by RT PCR.

Table 5.4: p22-RelBE constructs designed/obtained throughout this study, TA genes harboured and the expression system and promoters utilised by each.

Abbreviated Construct name	Plasmid	TA gene(s)	Expression system	Source
p22	pTRACA22	RelB, RelE	Under control of natural promoter	Jones & Marchesi 2007
pGEM-T Easy (TA)	pGEM®-T Easy Vector	RelB, RelE	Under control of natural promoter	Promega (USA)
pJ821(TA)	pJ821 inducible expression vector	RelB, RelE	*RhaBAD*, inducible promoter, individual promoter for each gene	Giacalone *et al.* 2006 (Sweden)
pJ821(ATx)	pJ821 inducible expression vector	RelB	*RhaBAD*, inducible promoter	Giacalone *et al.* 2006 (Sweden)
pBAD(Tx)	pBAD-TOPO® vector	RelE	*araBAD*, inducible promoter	Guzman *et al.* 1995, Thermofisher (UK)
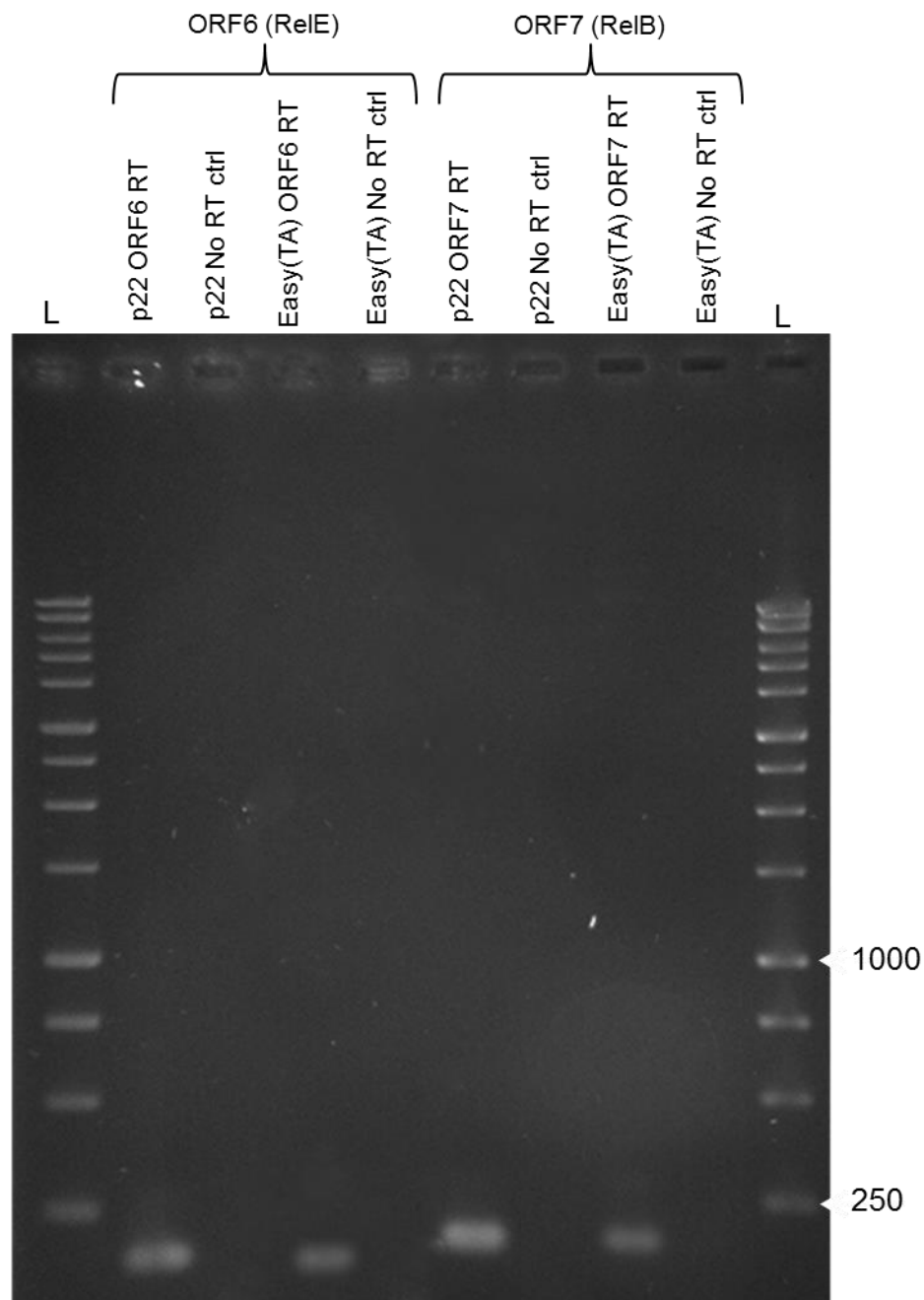
Figure 5.6: Confirmation of pTRACA22 and pGEM-T Easy(TA) TA expression in *E.coli* EPI300. RT PCR products from reverse transcription and subsequent PCR amplification using 10ng of total RNA per reaction (detailed in Section 5.3.6) visualised on a 1% agarose gel using gel electrophoresis.

ORF6 RT = p22-ORF6 (RelE toxin) RT product

ORF7 RT = p22-ORF7 (RelB antitoxin) RT product

No RT ctrl = Negative RT controls

L = 1kb Promega DNA ladder
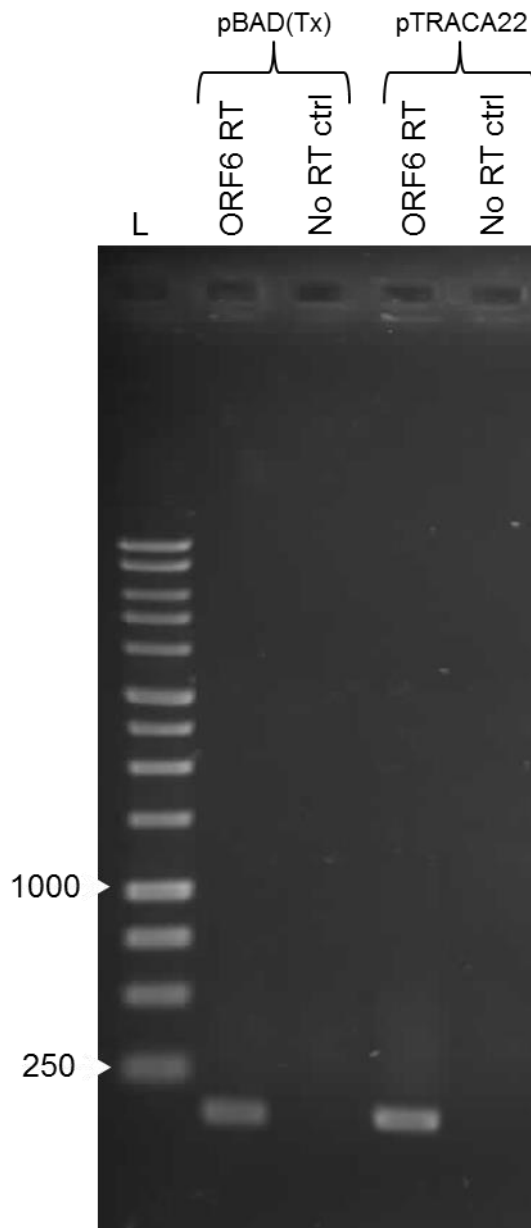
Figure 5.7: Confirmation of pBAD(Tx) toxin expression in *E.coli* EPI300. RT PCR products from reverse transcription and subsequent PCR amplification using 10ng of total RNA per reaction (detailed in Section 5.3.6) visualised on a 1% agarose gel using gel electrophoresis. pTRACA22 was included as a positive control.

ORF6 RT = p22-ORF6 (RelE toxin) RT product

No RT ctrl = Negative RT controls

L = 1kb Promega DNA ladder
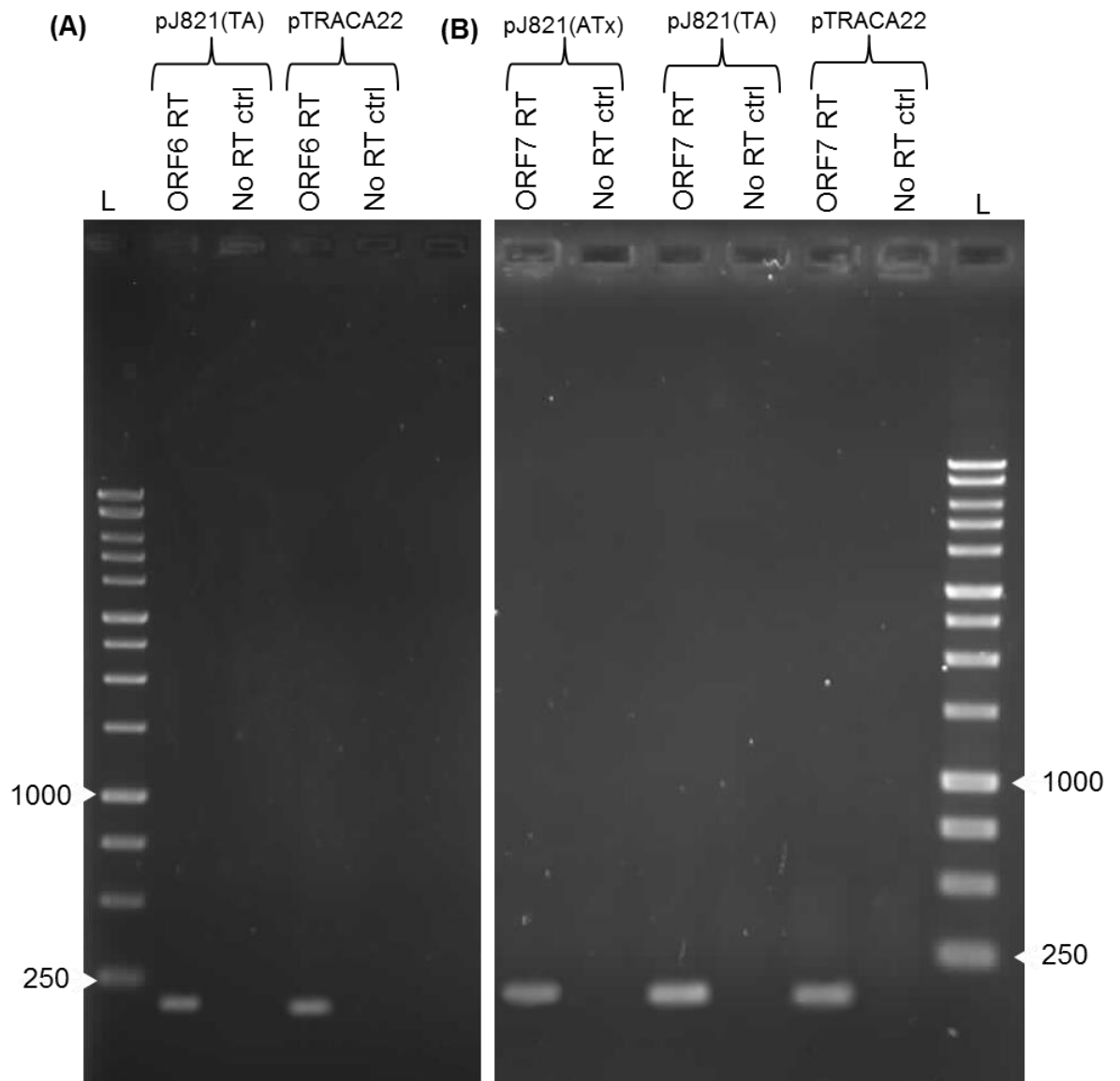
Figure 5.8: Confirmation of (A) pJ821(TA) toxin expression and (B) pJ821(ATx) and pJ821(TA) antitoxin expression in _E.coli_ EPI300. RT PCR products from reverse transcription and subsequent PCR amplification using 10ng of total RNA per reaction (detailed in Section 5.3.6) visualised on a 1% agarose gel using gel electrophoresis. pTRACA22 was included as a positive control.

ORF6 RT = p22-ORF6 (RelE toxin) RT product

No RT ctrl = Negative RT controls

ORF7 RT = p22-ORF6 (RelB antitoxin) RT product

No RT ctrl = Negative RT controls

L = 1kb Promega DNA ladder

5.4.2 Confirmation of RelE toxin activity in *E.coli* EPI300

To confirm activity and function of the RelE toxin encoded on the pBAD TOPO vector (pBAD(Tx), a simple growth curve over using OD_{600} as an approximate measurement of cell growth was employed. Gene expression was induced by the addition of 2% arabinose at the 4-hour time point. A significant difference was seen by 6 hours (p value <0.05), with OD_{600} plateauing from this point onwards, confirming both function and activity of the p22-RelE toxin in *E.coli* EPI300 (Figure 5.9).
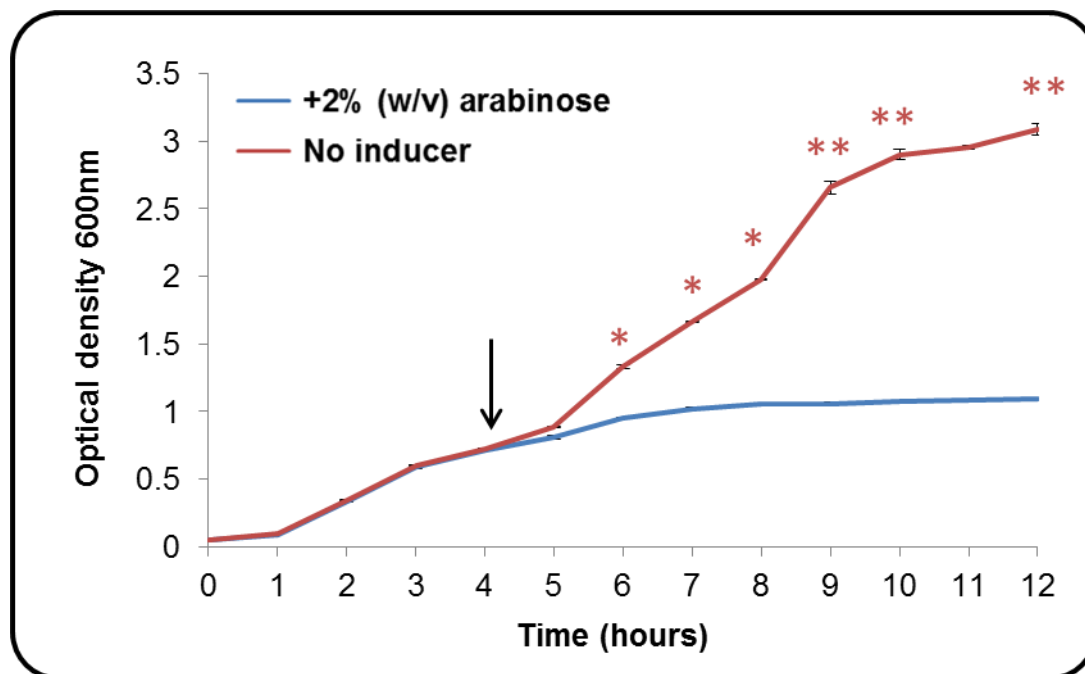
Figure 5.9: OD600 measurements representing growth of _E.coli_ EPI300 carrying the pBAD(Tx) construct over a 12 hour period. Expression of RelE toxin was induced at the 4-hour time point by addition of 2% arabinose (with the arrow representing this event). For the control culture the same volume of sterile H_2O was added instead of arabinose. All experiments were undertaken in triplicate and results shown are the mean ± SEM of three independent experiments.

* = $P<0.05$.

** = $P<0.001$

Significant differences in OD_{600} for the control compared to the induced Tx culture were found 2 hours after the addition of the inducer. All time points after this were also significantly different.

5.4.3 Characterisation of *E.coli* EPI300 growth with and without the presence of arabinose and rhamnose

Growth in LB broth supplemented with 0.2% glucose of *E.coli* EPI300 WT and EPI300 harbouring pTRACA22, pGEM-T Easy(TA), pBAD(Tx), pJ821(ATx), pJ821(TA), the empty pGEM-T Easy vector and pJ821 vector was monitored over a period of 6 hours. With no induction of expression and the presence of glucose to further metabolically repress expression, values obtained did not differ significantly from one another for all time points ($P>0.05$, Figure 5.10A). With the addition of 100mM rhamnose (inducer for the pJ821 vector) or 2% arabinose (inducer for the pBAD vector) at the 4 hour time point, CFU/mL was significantly lowered for EPI300 harbouring the pJ821(TA) and pBAD(Tx) constructs compared to controls ($p<0.001$) as shown in Figure 5.10B.
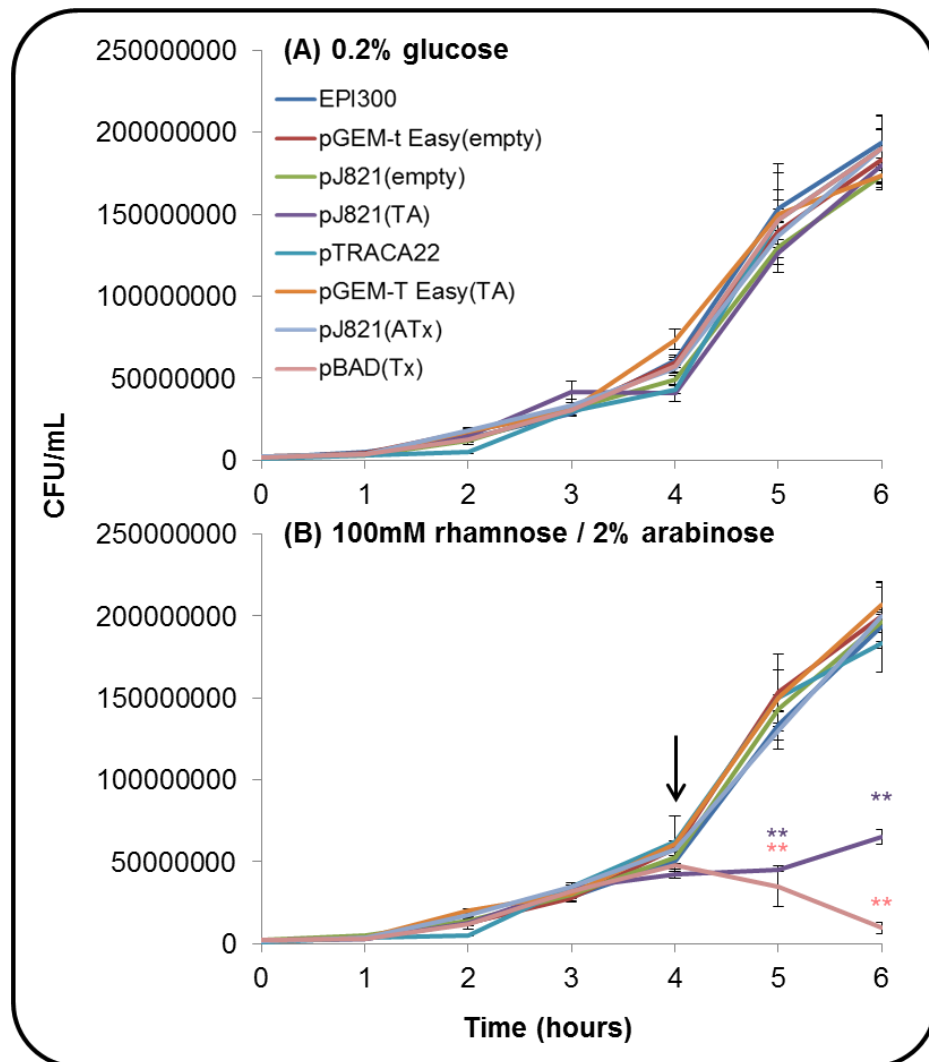
Figure 5.10: Growth characteristics of *E.coli* EPI300 harbouring the p22-RelBE TA plasmid constructs. Cultures were grown for 6 hours with samples taken every hour. At 4 hours either 100mM rhamnose or 2% (w/v) arabinose was added to the culture and samples taken for the remaining 2 hours. All experiments were undertaken in triplicate and results shown are the mean ± SEM of three independent experiments.

(**A**) Growth curve of *E.coli* EPI300 harbouring p22-RelBE constructs and controls without induction of expression at the 4-hour time point.

(**B**) Growth curve of *E.coli* EPI300 harbouring p22-RelBE constructs and controls with induction of expression at the 4-hour time point.

** = A significant difference in CFU/mL at the 5 and 6 hour time points between pJ821(TA)/pBAD(Tx) and control datasets (P<0.001).

5.4.4 Impact of the p22 TA system on cell viability during exposure to gut related stress

Using viable counts, the percentage survival was calculated for *E.coli* EPI300 WT and *EPI300* harbouring pGEM-T Easy(empty), pJ821(empty), pJ821(TA), pTRACA22, pGEM-T Easy(TA), pJ821(ATx) and pBAD(Tx). To confirm these results and to determine if changes in viable CFU counts represented cell death or entry into a viable but non-cultivable state, the BackLight® Live/Dead fluorescence staining kit was used in conjunction with confocal microscopy to examine the percentage of cells live, dead, and injured. Cultures of *E.coli* EPI300 harbouring these plasmids were exposed to carbon, nitrogen and carbon/nitrogen deprivation, acidified broth (pH 2), artificial gastric juice, porcine bile and all of these combined.

5.4.4.1 Carbon and nitrogen starvation

Without induction of TA expression both the nitrogen-only and nitrogen-carbon starvation experiments (Figures 5.11 and 5.12), results showed very little variation between construct harbouring *E.coli*, with the exception of pTRACA22 and pGEM-T Easy(TA). For EPI300 carrying both these plasmids this decrease was less steep resulting in significantly more (~20%, $P<0.05$) survival by the 8, 16 and 24 hour time points. When TA expression was induced at the 0 hour time point, EPI300 harbouring the pJ821(TA) construct also demonstrated this marked increase percentage survival. This was supported by live/dead staining which identified significantly higher percentages of live cells with carriage of the pJ821(TA) construct when TA expression was induced and pTRACA22/ pGEM-T Easy(TA) both with and without TA expression. Notably, induction of the pBAD(Tx) construct and subsequent expression of the RelE toxin significantly lowered the percentage survival of EPI300 when using viable counts, however the percentage

of live cells when using live/dead staining did not differ from the wild type or cells carrying the empty vectors.

For carbon-only starvation (Figure 5.13), all TA constructs with the exception of pJ821(Tx), both with and without expression of TA genes, decreased gradually over the 24 hours. The percentage of live cells counted using fluorescence staining supported these results. Survival began to decrease drastically in cultures harbouring the pBAD(Tx) when RelE toxin expression was induced ($P < 0.05$), however live/dead counts remained comparable to controls. Results gained from this series of experiments suggest that the p22-RelBE TA module is able to facilitate persistence under during nitrogen starvation.
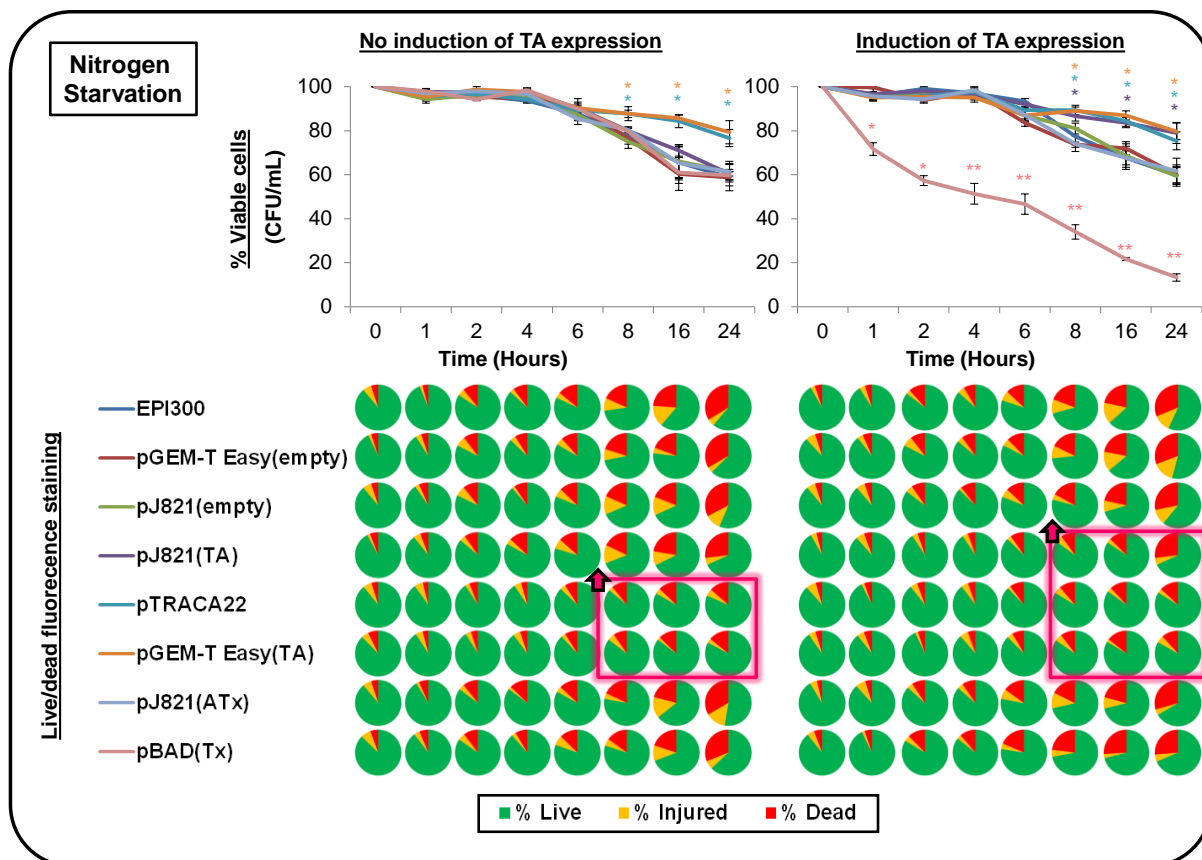
Figure 5.11: Effect of the p22-RelBE TA system on *E.coli* EPI300 survival during nitrogen starvation. Cell suspensions in M9 minimal media lacking casamino acids were incubated for 24 hours and used for both viable counts and live/dead fluorescence staining. 0.2% (w/v) glucose was added immediately at time point 0 to cultures in which cells were not induced. For those to be induced 100mM rhamnose or 2% (w/v) arabinose was added. Graphs refer to percentage viable cells (CFU/mL), whilst pie charts represent percentage live/dead/injured. All experiments were undertaken in triplicate and results shown are the mean ± SEM of three independent experiments.

* = P<0.05, ** = P<0.001 and represents a significant difference in percentage viable cells compared to control constructs.

For percentage live data, those significantly different from the control constructs are highlighted in a pink box (P<0.05) with the arrow representing whether the % live cells is higher or lower.

Figure 5.12: Effect of the p22-RelBE TA system on *E.coli* EPI300 survival during carbon and nitrogen starvation. Cell suspensions in M9 minimal media lacking casamino acids and glucose were incubated for 24 hours and used for both viable counts and live/dead fluorescence staining. 0.2% (w/v) glucose was added immediately at time point 0 to cultures in which cells were not induced. For those to be induced 100mM rhamnose or 2% (w/v) arabinose was added. Graphs refer to percentage viable cells (CFU/mL), whilst pie charts represent percentage live/dead/injured. All experiments were undertaken in triplicate and results shown are the mean ± SEM of three independent experiments.

* = P<0.05, ** = P<0.001 and represents a significant difference in percentage viable cells compared to control constructs.

For percentage live data, those significantly different from the control constructs are highlighted in a pink box (P<0.05) with the arrow representing whether the % live cells is higher or lower.

Figure 5.13: Effect of the p22-RelBE TA system on *E.coli* EPI300 survival during carbon starvation. Cell suspensions in M9 minimal media lacking glucose were incubated for 24 hours and used for both viable counts and live/dead fluorescence staining. 0.2% (w/v) glucose was added immediately at time point 0 to cultures in which cells were not induced. For those to be induced 100mM rhamnose or 2% (w/v) arabinose was added. Graphs refer to percentage viable cells (CFU/mL), whilst pie charts represent percentage live/dead/injured. All experiments were undertaken in triplicate and results shown are the mean ± SEM of three independent experiments.

* = P<0.05, ** = P<0.001 and represents a significant difference in percentage viable cells compared to control constructs.

For percentage live data, those significantly different from the control constructs are highlighted in a pink box (P<0.05) with the arrow representing whether the % live cells is higher or lower

5.4.4.2 Low pH and artificial gastric juice and porcine bile

A sharp decrease in percentage survival was seen for *E.coli* EPI300 when resuspended in LB broth acidified to a pH of 2.0 (Figure 5.14) and artificial gastric juice (AGJ, Figure 5.15) regardless of construct harboured or TA gene expression. Approximately 0% of cells were viable for all TA constructs by 120 minutes in acidified broth whilst AGJ displayed the same effect by 30 minutes and no significant differences were seen when TA gene expression was activated ($p>0.05$). These results were reflected by live/dead fluorescence staining and confocal microscopy demonstrating no significant differences between the percentage of live cells with or without induction of TA gene expression.

In LB broth supplemented with 25% (w/v) porcine bile (Figure 5.16), decreases in percentage viable and percentage live cells were less uniform; however, no significant differences could be identified between those carrying p22-RelBE TA module components and those not, regardless of TA gene induction ($p>0.05$). By the 240-minute time point, differences could be seen between the two experimental methods. Viable count data suggested that there were almost no viable cells remaining whilst live/dead staining identified approximately 10% of cells within these treated cultures were still alive ($p<0.05$).
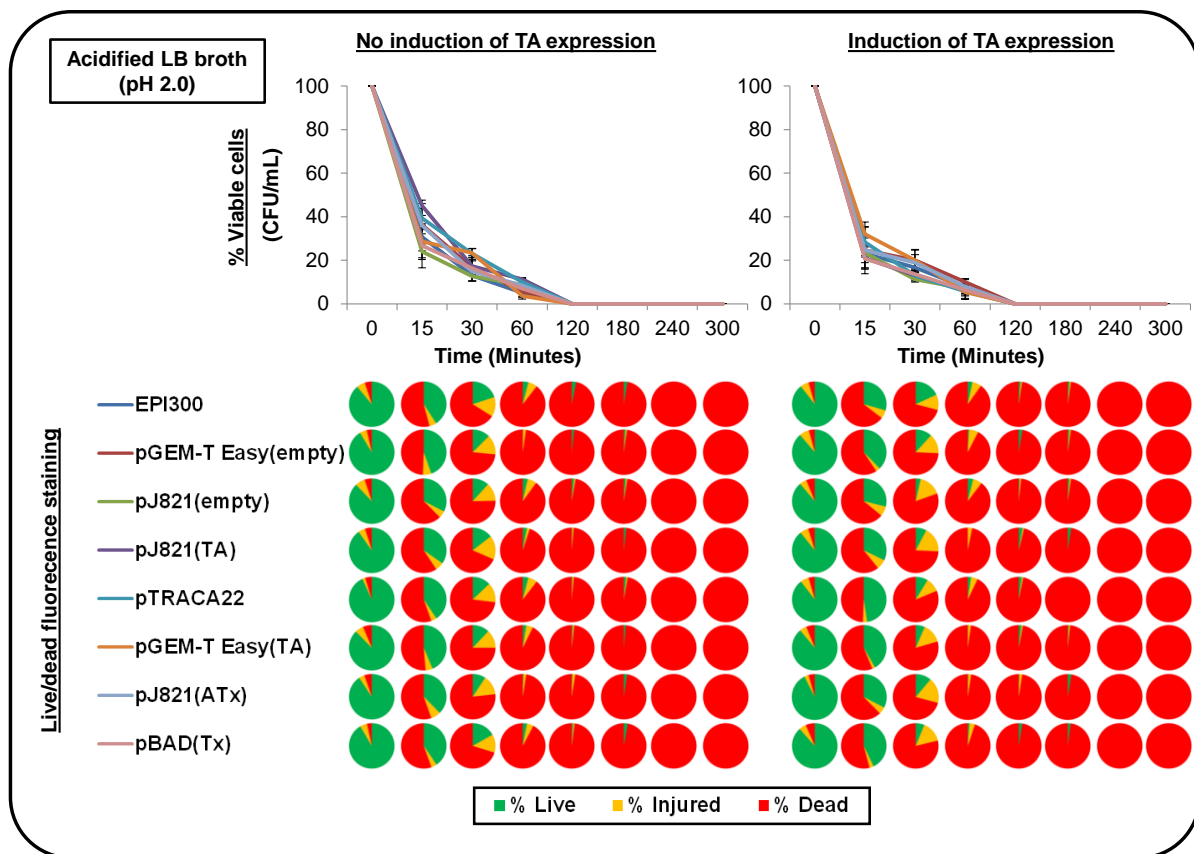
Figure 5.14: Effect of the p22-RelBE TA system on *E.coli* EPI300 survival during exposure to a pH of 2.0. Cell suspensions in LB broth acidified to pH 2.0 were incubated for 5 hours and used for both viable counts and live/dead fluorescence staining. 0.2% (w/v) glucose was added immediately at time point 0 to cultures in which cells were not induced. For those to be induced 100mM rhamnose or 2% (w/v) arabinose was added. Graphs refer to percentage viable cells (CFU/mL), whilst pie charts represent percentage live/dead/injured. All experiments were undertaken in triplicate and results shown are the mean ± SEM of three independent experiments.
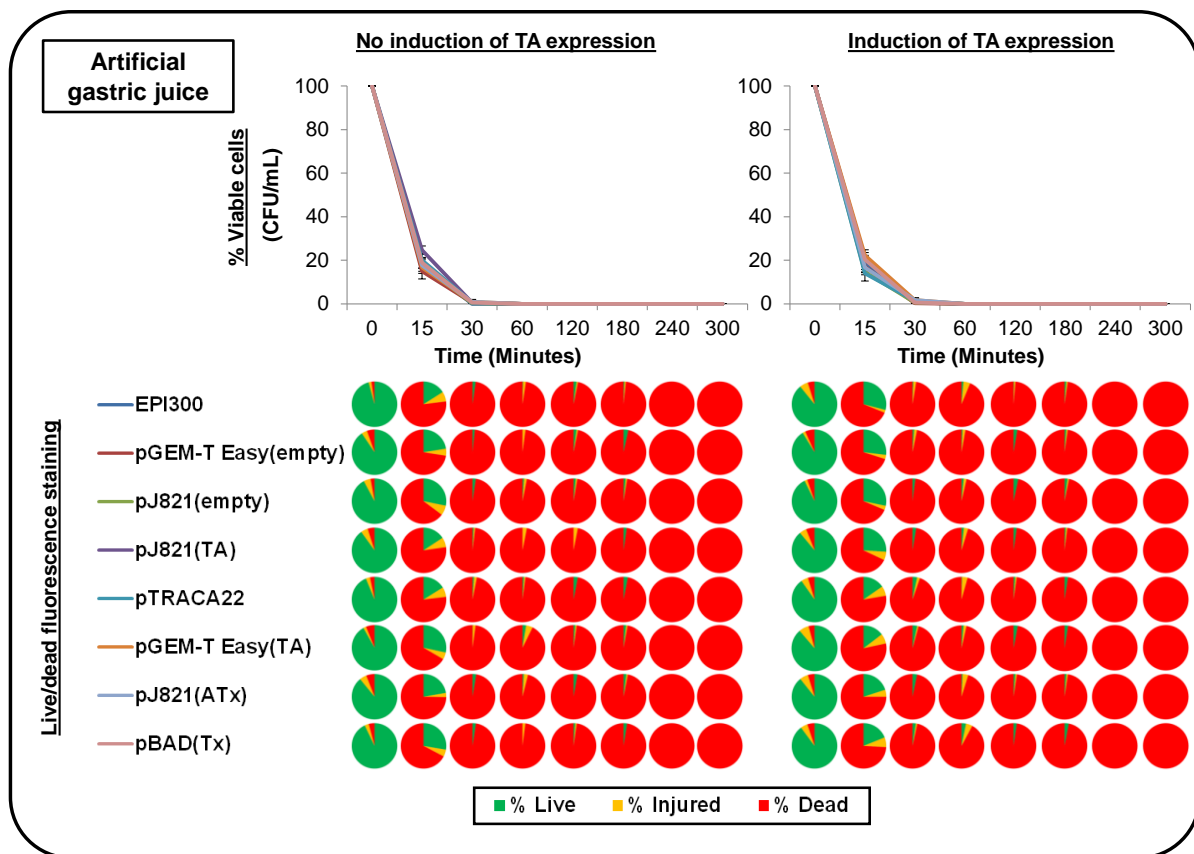
Figure 5.15: Effect of the p22-RelBE TA system on *E.coli* EPI300 survival during exposure to artificial gastric juice. Cell suspensions in artificial gastric juice were incubated for 5 hours and used for both viable counts and live/dead fluorescence staining. 0.2% (w/v) glucose was added immediately at time point 0 to cultures in which cells were not induced. For those to be induced 100mM rhamnose or 2% (w/v) arabinose was added. Graphs refer to percentage viable cells (CFU/mL), whilst pie charts represent percentage live/dead/injured. All experiments were undertaken in triplicate and results shown are the mean ± SEM of three independent experiments.
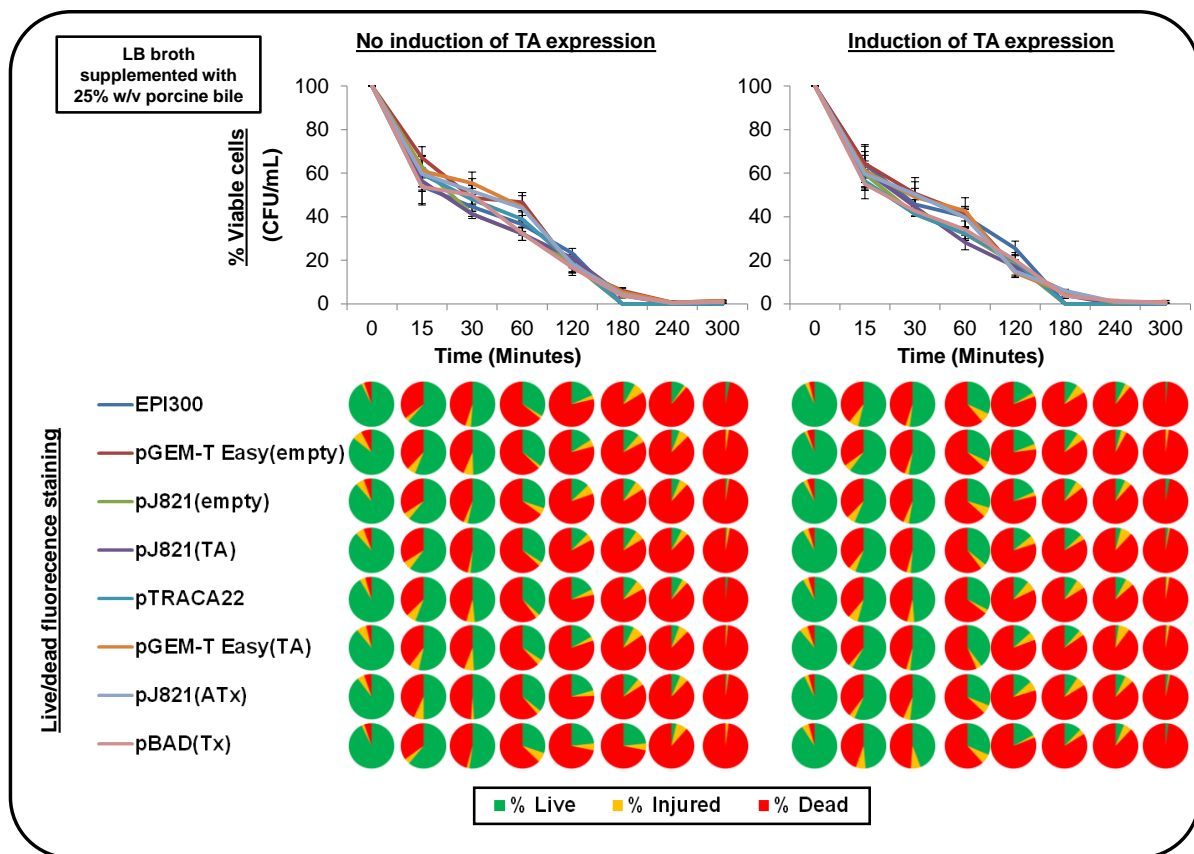
Figure 5.16: Effect of the p22-RelBE TA system on *E.coli* EPI300 survival during exposure to 25% (w/v) porcine bile. Cell suspensions in LB broth supplemented with 25% (w/v) porcine bile were incubated for 5 hours and used for both viable counts and live/dead fluorescence staining. 0.2% (w/v) glucose was added immediately at time point 0 to cultures in which cells were not induced. For those to be induced 100mM rhamnose or 2% (w/v) arabinose was added. Graphs refer to percentage viable cells (CFU/mL), whilst pie charts represent percentage live/dead/injured. All experiments were undertaken in triplicate and results shown are the mean ± SEM of three independent experiments.

5.4.4.3 Combinatorial stress

To investigate the ability of the p22-RelBE TA module to effect the global stress response, *E.coli* EPI300 carrying the TA module constructs were firstly challenged with carbon and nitrogen deprivation for 8 hours, followed by suspension in AGJ for 1 hour and finally, suspension in LB supplemented with 25% porcine bile for 2 hours (Figure 5.17). With induction of TA expression, by 8 hours EPI300 harbouring pTRACA22, pGEM-T Easy(TA) and pJ821(TA) had a significantly higher percentage of viable cells than EPI300 harbouring control constructs, comparable to results seen in Section 5.4.4.1 ($P<0.05$). EPI300 harbouring the pBAD(Tx) construct when induced also behaved in a similar fashion with percentage viable cells dropping significantly ($P<0.05$).

With resuspension in AGJ the percentage of viable and percentage live cells rapidly began to decrease; however cells harbouring the pTRACA22, pGEM-T Easy(TA) and pJ821(TA) (when induced) plasmids had significantly higher viable counts and percentage live cells after the first 15 minutes post-resuspension ($p <0.05$). This trend did not continue with values rapidly dropping to similar levels seen by EPI300 carrying the control constructs, with or without TA gene expression. Final resuspension of cultures in LB broth supplemented with 25% porcine bile did not reveal anything as both percentage survival and percentage live cells had already decreased to a value of approximately 0.
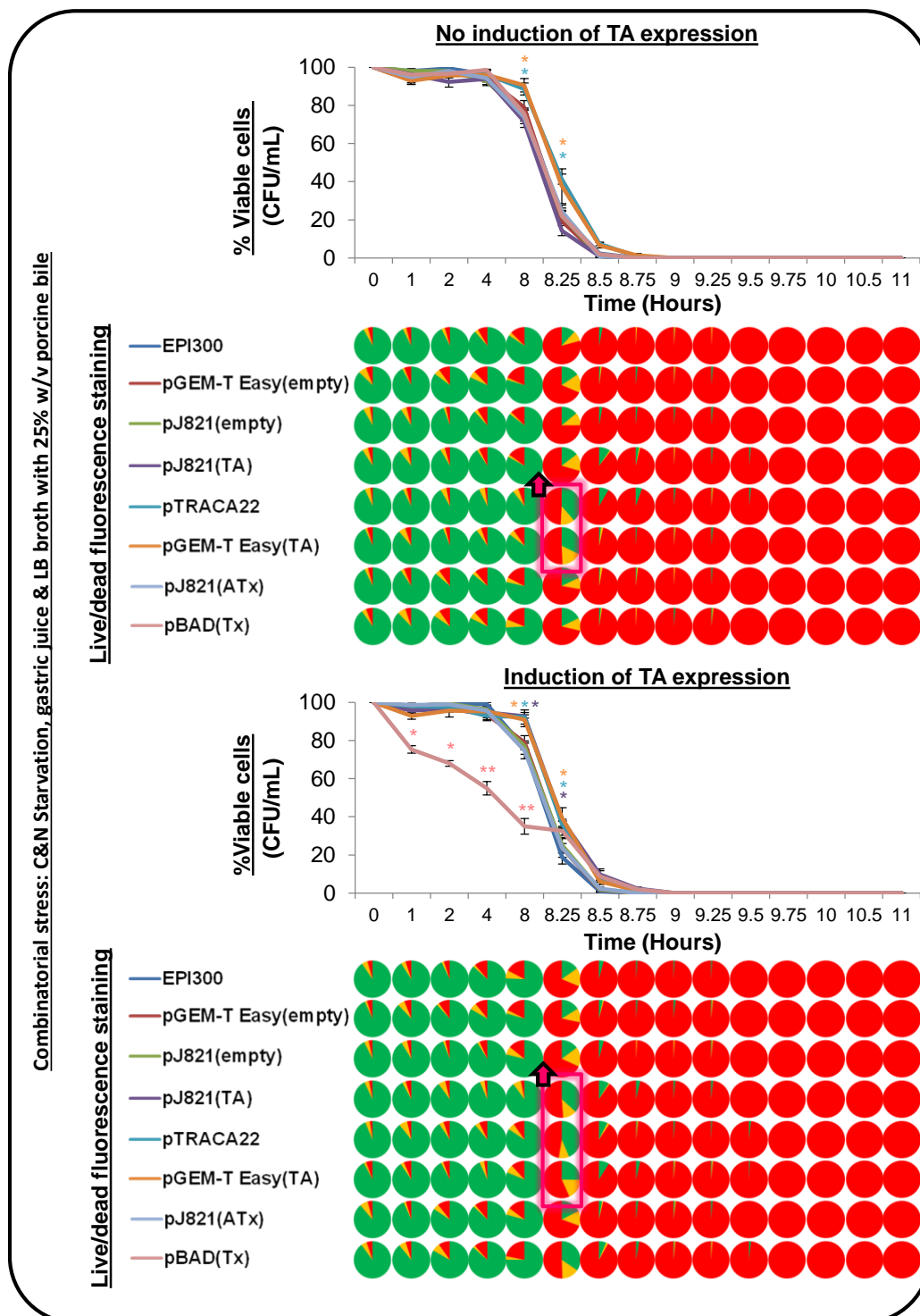
Figure 5.17: Effect of the p22-RelBE TA system on *E.coli* EPI300 survival during exposure to carbon and nitrogen starvation, artificial gastric juice and 25% (w/v) porcine bile. Cell suspensions in M9 minimal media lacking glucose and casamino acids were incubated for 8 hours, centrifuged and resuspended in AGJ for 1 hour and then finally centrifuged and resuspended in LB broth supplemented with 25% porcine bile for 2 hours. Samples taken were used for both viable counts and live/dead fluorescence staining. 0.2% (w/v) glucose was added immediately at time point 0 to cultures in which cells were not induced. For

those to be induced 100mM rhamnose or 2% (w/v) arabinose was added. Graphs refer to percentage viable cells (CFU/mL), whilst pie charts represent percentage live/dead/injured. All experiments were undertaken in triplicate and results shown are the mean ± SEM of three independent experiments.

* = $P<0.05$, ** = $P<0.001$ and represents a significant difference in percentage viable cells compared to control constructs.

For percentage live data, those significantly different from the control constructs are highlighted in a pink box ($P<0.05$) with the arrow representing whether the % live cells is higher or lower

5.5 Discussion

5.5.1 Design and expression of the p22-RelBE TA constructs

Carriage of a putative RelBE Type II TA locus on a plasmid that appears to be highly abundant within the human gut, when compared to both other environments, is intriguing in terms of why but also how it became so prominent. This p22-RelBE like module may have accumulated purely due to its addictive nature (by acting as a parasitic genetic entity) or may also be ancestral, harboured by ancestors of the modern gut flora, persisting and expanding within the population due to its addictive nature. While this may be the case, it is possible the p22-RelBE module may also have a beneficial impact on the human gut microbiome, providing a fitness advantage that warrants their continued presence within this community.

To allow accurate investigation into the potential functions of the p22-RelBE module, p22-RelBE was divorced from the pTRACA22 plasmid background and constructs were made of the individual toxin and antitoxin ORF as well as the entire module, both under the control of its natural promoter and tightly regulated inducible ones. Interestingly ORF5 from pTRACA22, in regards to orientation and genetic positioning, has similar characteristics to that of a regulator gene, which helps regulate expression of TA systems (Kawano 2012). These regulators are usually cis-encoded; overlapping genes and translation of toxins of modules that utilise regulators generally rely heavily on paired translation with their regulators. Consequently, many of these 3 gene systems utilize post-transcriptional regulation with the antitoxin inhibiting translation of the regulator, thus preventing translation of the toxin (Thisted & Gerdes 1992). Isolation of the p22-RelBE TA genes from this ORF5 (potential regulator) therefore seemed both necessary and intriguing as if expression of the module did require the presence of it, this would provide

evidence of a TA component in Type II systems mostly commonly associated with Type I TA systems (Kawano 2012). To date regulators have not been described when discussing relBE related loci.

In this study, we have shown expression of both p22-RelBE components and the entire module in a surrogate host: *E.coli* EPI300. Work done by Jones *et al*., (2010) identified significant homology between pTRACA22 and sequences from the draft genome *Blautia hydrogenotrophica* DSM 10507, a member of the *Firmicutes* division and isolated from human faeces. All species within this division are Gram positive and phylogenetically distant to the Gram-negative phyla *Proteobacteria*, to which *E.coli* belongs. This difference is of importance as it raises the possibility of alterations in codon usage (Courvalin 1994) and an inability for the *Firmicute*-related p22-RelBE TA module to be expressed within *E.coli*. While p22-RelBE is likely to have originated from the *Firmicutes,* one of the two dominant phyla within the human gut (along with the *Bacteriodetes*) and a Gram positive host may have been preferable because of this, *E.coli* was chosen instead due to its relevance as an important gut commensal. Furthermore, *E.coli* is a resident in all known vertebrate GI tracts (Tenaillon *et al*., 2010), a medically relevant intra- and extra-intestinal pathogen (Kosek *et al*., 2003, Russo & Johnson 2003) and has a large number of genetic and molecular techniques available.

In conjunction with the above, the p22-RelE toxin appeared to be active within the surrogate *E.coli* EPI300 host with growth retardation on plates and within liquid culture being phenotypically visible when the toxin was expressed. Plate counts done in parallel to OD measurements suggested cells were not being killed, with CFU/mL remaining at a constant level after expression of the toxin was induced. This suggests that the population of cells expressing the toxin were entering into a metabolically inactive state which supports previous findings by

Pedersen *et al.*, (2003) who investigated the mechanism of the RelE toxin on a cellular level and work done by Christensen *et al.*, (2001) exploring RelE's effect on cell populations during amino acid starvation.

5.5.2 Expression of p22-RelBE within *E.coli* EPI300 and its effect on growth

With addition of inducer to EPI300 harbouring the pJ821(TA) and pBAD(Tx) inducible constructs, i.e. those which contained the toxin, and the subsequent expression of genes carried, a significant reduction in growth was found. Whilst it was expected that the toxin only construct would have a negative effect on cell growth it was surprising to see this phenotypic change in cells carrying both the inducible toxin and antitoxin, especially as the module under control of the native promoter did not produce this result. Several hypotheses may explain this: - i) the strong and sustained level of expression had a negative effect on other cell metabolic processes, inhibiting cell growth, ii) continued expression of both the toxin and antitoxin led to an eventual accumulation of the more stable toxin, or iii) a combination of both the above. Effects from all hypotheses above would be exacerbated by removal of both genes from their natural promotor and stoichiometry, affecting the modules self-regulatory mechanisms and further enabling free toxin to interact with cellular machinery.

In the case of the potential metabolic burden generated by over expression of RelBE, the regulatory mechanisms in place must be examined. Metabolic burden is defined as the amount of resources (raw material and energy) that are withdrawn from the host metabolism for maintenance and expression of foreign DNA (Bentley *et al.*, 1990). This reduction in resources available can significantly decrease cell growth and division (Paulsson *et al.*, 1998) to give a phenotype

similar to that of RelE toxin induction (Griffin *et al*., 2013), a metabolically stationary cell population whilst avoiding cellular death. The occurrence of metabolic burden is most often seen in biotechnological and industrial production of recombinant proteins for purification. These proteins are often large and foreign to the *E.coli* cell (Sørensen & Mortensen 2005), whilst in comparison, the RelBE TA system is small (90 and 93aa respectively) and commonplace both within *E.coli*'s genome and mobile genetic elements this species carries (Yamaguchi & Inouye 2011). Moreover, the relative instability of the relB antitoxin and eventual degradation of RelE-RelB complexes through Lon protease activity on relB (Christensen *et al.,* 2001) would enable recycling of resources to a certain degree. However the volume of TA proteins being produced may partially explain the negative impact on growth observed during prolonged expression of both the RelE toxin and RelB antitoxin.

Whilst the RelB antitoxin within the cell is known to exist as a dimer, $relB_2$ (Overgaard et al., 2009), there is currently some discrepancy as to the exact complex formed between both the toxin and antitoxin proteins. Li *et al*., (2008) reported the repressor complex had a $RelB_2$-$RelE_2$ stoichiometry, whilst Overgaard *et al*., (2008) reported a $RelB_2$-RelE stoichiometry. When considered in the context of the potential metabolic burden imposed by RelBE overexpression, these possible differences in TA complex stoichiometry are of particular relevance. Should the $RelB_2$-$RelE_2$ stoichiometry in fact be correct and prevalent, two times as much antitoxin would be required to neutralise the toxin protein. This leads smoothly onto the second hypothesis of free toxin accumulation within cells with the continued expression of the inducible module.

Christensen *et al*., (2001) reported that relBE expression was driven by a P_{lac} promotor. The RelBE module was induced and as expression of the antitoxin

is higher during the induction period (due to being physically closer to the promotor) the higher antitoxin levels ensured translation was not inhibited by free toxin. At zero time the inducer was removed and aa starvation induced which resulted in severe inhibition of global translation within the population. The identified the ability of the RelE toxin to be freed from the repressor complex and also suggests that unless persistent expression of the antitoxin (at higher levels than the toxin) occurs, bound toxin will eventually be released and interact with mRNA positioned in the ribosomes (Yarmolinsky 1995). It is likely that the use of individual inducible promoters for each TA component, as done in studies within this chapter, would remove any auto-regulatory systems in place for the p22-RelBE module, allowing continued, constant production of both protein products. This can either lead to a general accumulation of the stable toxin and/or induce a starvation-like phenotype leading to cleavage of the antitoxin, releasing further toxin. Figure 5.18 depicts the RelBE self-regulatory mechanism which is often used as a model for Type II module regulation and also a hypothesised regulatory model for the constant expression of both the toxin and antitoxin in the pJ821(TA) construct used within these experiments.
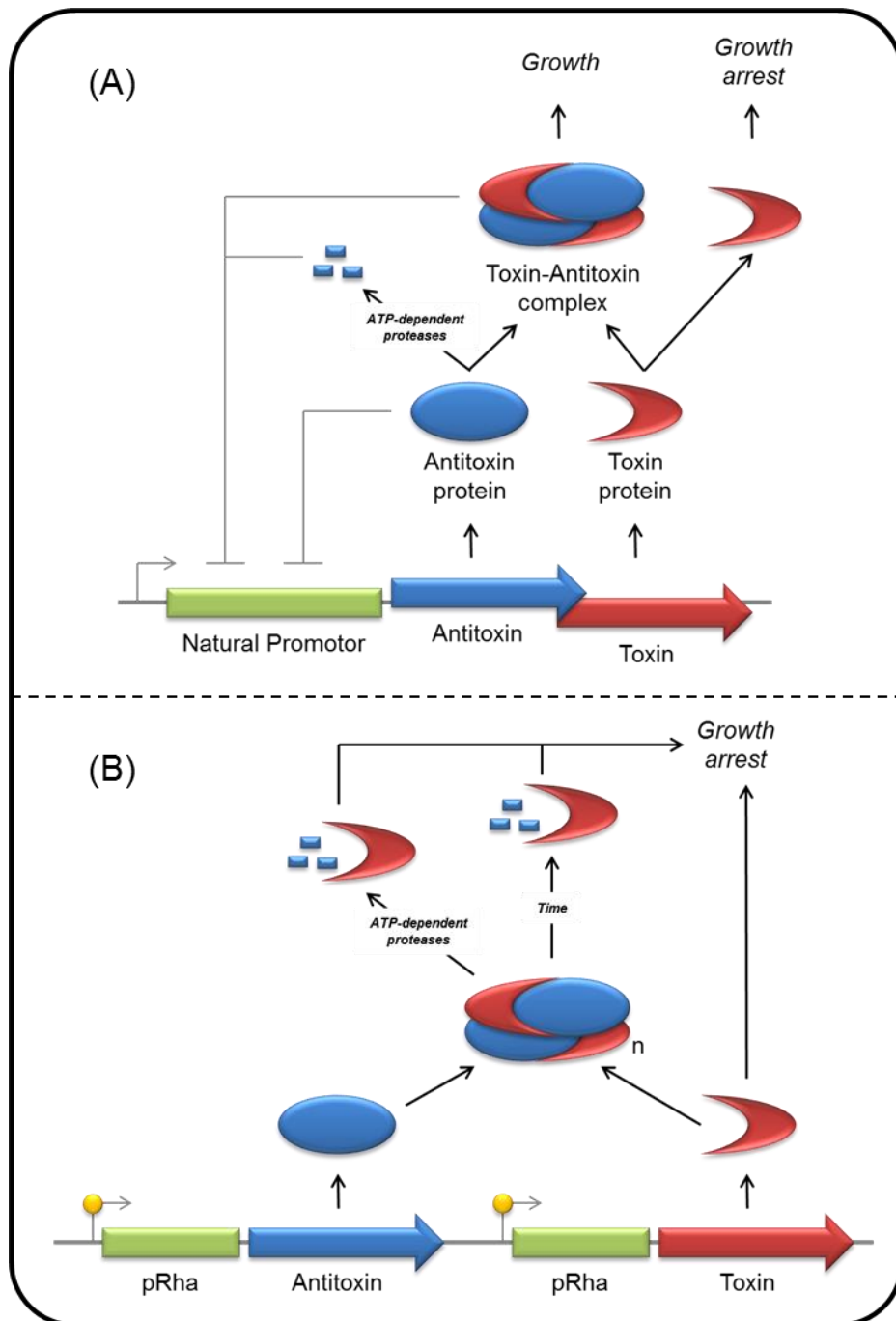
Figure 5.18: A schematic of the Type II RelBE toxin-antitoxin module self-regulation mechanism and an adaption of this for the inducible pJ821(TA) construct. pRha refers to the inducible promoter used for inducible expression within the pJ821 expression vector.

(A) RelBE toxin-antitoxin module regulation: Antitoxin and toxin mRNAs are synthesised from the same promoter and both are translated into proteins. Antitoxin forms a heterodimeric polypeptide complex with the toxin to inhibit toxicity and to auto-regulate the module. Antitoxins are subjected to cleavage under stress conditions by ATP-dependent proteases. The antitoxin itself also regulates the system but more weakly. The toxin is released from the complex and

can interact with its cellular target leading to cell growth arrest. Adapted and modified from Yamaguchi *et al.*, 2011.

(B) Hypothesised regulation of the pJ821(TA) RelBE system: Individual promoters, when induced, transcribe the two components separately which are then translated. There is no auto-regulatory system in place with the use of the pRha promoter and constant production of both components continues. This can either lead to a general accumulation of the more stable toxin, and/or become a metabolic burden and may induce a starvation-like phenotype, causing antitoxin cleavage and releasing the toxin. Both possibilities would eventually lead to growth arrest.

5.5.3 Carriage and expression of pTRACA22 and p22-RelBE constructs under gut related stress

Using conventional viability plating, Live/Dead fluorescence staining and confocal microscopy the ability of p22-RelBE, when carried and expressed by *E.coli* EPI300 to aid survival in the presence of gut related stress was investigated. These stresses included low pH, AGJ, porcine bile, carbon starvation, nitrogen starvation and a combination of the above. These were chosen, as all were environmental stresses that are likely to be encountered and overcome during GI tract colonisation (Begley *et al.*, 2005, 2013, Kanjee & Houry 2013).

The acidic pH of the stomach is one of the first and foremost non-immunological barriers to microbial cells attempting to enter the lower digestive tract of mammals and is effective due to the non-specific nature in which it kills (Sarker & Gyr 1992, Smith 2003). Acid acts by altering ionization of glutamate and aspartate side chains, disrupting ionic bonds used to maintain the tertiary structure of proteins and initially affecting those that span the cell membrane (Smith 2003, Dormmett *et al.*, 2005). This leads to loss of membrane integrity subsequently allowing protons to leak into the cytosol and effect internal protein structure as well. Based on the mechanism of acid on cell function it was unsurprising that p22-RelBE expression did not significantly increase survival of *E.coli* EPI300 in the presence of low pH (2.0). The RelE toxin is an mRNAse with no evidence to date suggesting it can interact and stabilise protein tertiary structure, especially those located at the cell membrane. Similar results were also seen with p22-RelBE (and individual TA component) expression not providing any increase in cell survival in *E.coli* EPI300 when in the presence of 25% (w/v) porcine bile. Like acid, although through a different mechanism, bile's antimicrobial properties primarily come from its effect on cell membranes with disruption of the membrane causing intracellular

leakage implying bile alters membrane integrity/permeability (Leverrier *et al.*, 2003, Valdez *et al.*, 1997, Noh & Gilliland 1993, Begley *et al.*, 2005). It was therefore unsurprising that a TA module (RelBE) that modulates global gene expression had no protective effect on cells exposed to bile.

The lack of impact of RelBE TA expression on membrane and protein disrupting stress does not mean TA modules do not play regulatory roles in response to environmental stress that effect and denature membranes and cellular proteinaceous structures, merely the RelBE module does not appear to in *E.coli*. Another stress that alters cell membranes and damages proteins is high temperatures (Quinn 1988, Somero 1995) and TA modules have been shown to provide some level of protection against this. The crenarchaeon *Sulfolobus solfataricus* has at least 26 *vapBC* TA loci within its genome and transcriptional analysis of this organism during heat shock (temperature shifting from 80-90°C) revealed many of these *vapB*C genes were triggered by a thermal shift (Cooper *et al.*, 2009). Furthermore, knocking out specific loci substantially changed the transcriptome and even rendered the crenarchaeon heat-shock labile. Unfortunately there has been no further investigation into this discovery but it can be hypothesised, based on known stress mitigation systems involving TA modules and VapC's ribonuclease activity, that this module plays a role in archaeal RNA management systems, especially during times of stress.

Of all the gut related stressors examined, carbon and nitrogen starvation and nitrogen only starvation showed a significant increase in the survival of *E.coli* EPI300 when expression of the p22-RelBE module was induced. This increase was seen not only for the viable counting methodology but also when confirming this through live/dead fluorescence staining. Additionally by the 24-hour mark there appeared to be a trend emerging with *E.coli* populations expressing the

module having higher percentage survival during carbon starvation; however, at this point these differences were not significant. It can be hypothesised, based on these results, that a proportion of the cell population has become metabolically inactive due to global inhibition of translation, but are still viable and could be resuscitated with the return of amino acid availability (Christensen & Gerdes 2004, Gotfredsen & Gerdes 1998).

Transcription of TA-coding genes including *relBE*, *MazEF* and *hicAB* are strongly induced during amino acid starvation (Chistensen & Gerdes 2003, Christensen *et al.*, 2001, Jorgensen *et al.*, 2009). Specifically focusing on the RelBE module this effect has been noted by several lab groups. Christensen *et al.*, in 2001 provided evidence of the RelE toxin acting as a global ppGpp-independent translational inhibitor when nutritionally stressed, with their work identifying a significantly reduced rate of translation (~10%) in *E.coli* with carriage of the *relBE* locus. Concomitantly, during this study it was also found that cellular RelB content was reduced, possibly leading to release of RelE to further decrease translation. Interestingly, they also established that carbon limitation induced relBE transcription as well, which suggests that this module may have a more generalised effect during nutritional limitation and perhaps stress responses in general. While results shown in this chapter do not show a significant increase in survival during carbon starvation, there does appear to be a trend emerging when expression of the entire p22-RelBE module is induced. This could be investigated further by using a larger experimental time frame. With this in mind and as briefly mentioned above, other cases of TA modules acting as stress response loci to non-gut related stresses have been reported, such as the VapBC locus of *Sulfolobus* (Cooper *et al.*, 2009).

Tashiro *et al.*, in 2012 published evidence of RelE mediated dormancy in high cell density populations of *E.coli* K-12 MG1655. They generated an *E.coli* strain expressing the RelE toxin with the presence of rhamnose and found with expression of the toxin increased numbers of persister cells within either high density populations and during amino acid starvation. Moreover, the persister cells produced due to density and aa starvation appeared to be less susceptible to antibiotics such as ampicillin, ciprofloxacin and tobramycin (Anderl *et al.*, 2003, Walters *et al.*, 2003), a potentially medically important and gut colonisation relevant by-product of persistence during nutrient deprived periods. This appears especially true as a strong link between RelE and persister cell formation has already been described (Keren *et al.*, 2004, Maisonneuve *et al.*, 2011).

From evidence collected in this chapter and that of Tashiro *et al.*, (2012) amino acid starvation (and perhaps nutrient deprivation in general) appears to increase the cellular activity of RelE. These changes in expression alone do not explain the increased impact on survival and the pJ821(TA) and pBAD(Tx) constructs fit with this model due to the way TA genes are expressed. This does not mean that RelBE transcription may not play a role, as experiments involving the full RelBE module (with both natural and artificial promoters) also had significantly higher survival percentages, but it does suggest that this transcriptional regulation of the p22-RelBE system may not be required for the persistent phenotype.

5.6 Conclusions

- Plasmid constructs of the complete p22-RelBE TA module, individual toxin and individual antitoxin have been generated under the control of both their natural promoter and inducible promoters.

- The p22-RelBE TA module was shown to be expressed within an *E.coli* EPI300 background.

- The p22-RelE toxin is active within *E.coli* strain EPI300.

- *E.coli* EPI300 harbouring inducible constructs grew no differently to controls without induction of TA expression; however when induced, cells expressing the full module had growth significantly reduced. This is possibly due to deregulation of the module at the transcriptional level.

- Expression of the p22-RelBE TA module enabled an increased percentage of cells to survive nitrogen starvation. This implies p22-RelBE has a function as a stress response element within the human gut environment.

Chapter 6: Regulation of antibiotic stress responses by the p22-RelBE toxin-antitoxin module

6.1 Introduction

Antimicrobials are one of the most successful forms of chemotherapy throughout the history of medicine, significantly contributing to the control of infectious diseases that previously led to the loss of countless human lives each year. An antimicrobial agent is a natural or synthetic chemical that kills or inhibits the growth of microorganisms and use of these compounds has become an established and important tool in modern medicine to control and treat bacterial infections. Since the initial discovery of penicillin in 1928 by Alexander Fleming and subsequent isolation of the antibiotic 10 years later by Florey and Chain, medicine has become more and more reliant on the bacteriostatic and bactericidal activity of these compounds. Over the last 77 years our antimicrobial arsenal has increased dramatically through both new discovery and chemical manipulation of existing compounds. The main classes of antibiotics that have been deployed in human medicine can be seen in Table 6.1.

6.1.1 Effect of antimicrobial use on the gut flora

Antimicrobial therapies are used to treat infection and target specific pathogens however the oral administration of therapeutic doses also disturbs the normal gut community and in doing so influences the functional output. While normal function of this community is maintained during treatment, it has been repeatedly shown that a return to approximate normal levels of function can be recovered with cessation of treatment (De La Cochetiere *et al.*, 2005, Panda *et al.*, 2014).

Table 6.1: A summary of antimicrobial compound classes, their mode of action and examples of antibiotics within each class. Those in red were utilized within this study.

Class	Sub-class	Mode of action	Example
β-Lactams	Penicillins	Inhibit peptidoglycan biosynthesis by binding to the active site of PBPs preventing transpeptidation (Fisher *et al.*, 2005).	ampicillin
	Cephalosporins (Generations 1-5)	*See Penicillins*	Cephalexin
	Carbapenems	*See Penicillins*	Doripenem
Monobactams		*See Penicillins* (Georgopapadakou *et al.*, 1983).	Aztreonam
Aminoglycosides		Irreversibly binds to the 30S ribosome subunit interrupting protein synthesis (Mingeot-Leclercq *et al.*, 1999, Hammes & Neuhaus 1974).	Gentamicin
Polyketides		Inhibit protein synthesis by inhibiting binding of aminoacyl-tRNA to the mRNA-ribosome complex (Chopra & Roberts 2001).	Tetracycline
Rifamycins		bind to the β-subunit of bacterial RNA polymerase interfering with transcription (Adachi & DuPont 2006).	Rifaxamin
Macrolides		Inhibits protein synthesis by binding to 50S ribosome subunit and blocking peptide chain progression (Tenson *et al.*, 2003).	Clarithromycin
Ketolides		*See Macrolides*	Telithromycin
Lincosamides		*See Macrolides*	Clindamycin
Glycopeptides		Inhibit peptidoglycan biosynthesis by binding to acyl-D-alanyl-D-alanine preventing addition of new units (Watanakunakorn 1984).	Vancomycin
Lipopeptides		Disrupt cell membrane by insertion and aggregation within the phospholipid membrane Steenbergen *et al.*, 2005).	Daptomycin
Streptogramins		Inhibit protein synthesis by binding to 50S ribosome subunit preventing elongation and releasing incomplete chains (Allington & Rivey 2001).	Quinupristin
Sulphonamides		Inhibit DNA synthesis by acting as a competative antagonist of para-aminobenzoic acid disrupting folic acid synthesis (Henry 1943, Hitchings 1973).	Sulphamethoxazole
Oxazolidinones		Disrupt translation of mRNA by preventing formation of the initiation complex (Swaney *et al.*, 1998).	Linezolid
Quinolones	(Generations 1-4)	Inhibit DNA synthesis by interfering with DNA gyrase (primarily Gram negative) and topoisomerase IV (primarily Gram positive) (Bergan 1988, Schaumann & Rodloff 2007).	Ciprofloxacin
Others	Metronidazole	Inhibits nucleic acid synthesis and disrupts DNA through reduction of their nitro group (muller 1983).	-
	Polymixins	Disrupt cell membrane through interaction with phospholipids (David & Rastogi 1985, Evans *et al.*, 1999).	Colistin E
	Trimethoprim	Inhibits DNA synthesis by binding to dihydrofolate reductase (Hitchings 1973, Gleckman *et al.*, 1981).	-
	Chloramphenicol	Inhibits protein synthesis by inhibiting peptidyltransferase activity of ribosome (Jardetzky 1963).	-

As with any microbial community, development of strategies involved in persistence through periods of environmental stress would be highly beneficial and necessary. The ecological balance between the human host and associated microorganisms is significantly shifted during antibiotic treatment and often results in reduced colonisation by commensal microbiota, loss of competitive colonisation resistance, emergence of antibiotic resistant strains and a shift in metabolite production (Bartosch *et al.*, 2004, de la Cochetieare *et al.*, 2005, Panda *et al.*, 2014, Jump *et al.*, 2014). The extent to which the gut microflora is disturbed varies between the antimicrobial used, its dose, pharmacokinetic and pharmocodynamic properties, rate of internal inactivation within the human body and incomplete absorption (Vollaard & Clasener 1994, Sullivan *et al.*, 2001).

Disease states, caused by oral use of antibiotics, range from the acute, such as self-limiting diarrhea (Sullivan *et al.*, 2001) to more serious pseudomembranous colitis (Beaugerie & Petit 2004). Additionally alterations in the intestinal microbiota, and subsequent changes to the chemical transformations it mediates suggest antibiotic disruption may also factor in the formation of cancer and obesity (O'Keefe *et al.*, 2007, Mcgarr *et al.*, 2005, Ley *et al.,* 2005, Backhed *et al.,* 2004). For most cases however, a return to pre-treatment conditions is achieved within weeks after cessation of the antimicrobial treatment (Modi *et al.*, 2014, Panda *et al.*, 2014). Initially, functional characterisation of the culturable community members and use of techniques with low phylogenetic resolution such as gel electrophoresis showed varied changes ranging from significant decreases in Enterobacteria after treatment with cephalosporins to increases in aerobic Gram positive cocci after beta-lactam exposure (Sullivan *et al.*, 2001, Edlund & Nord 1993, Floor *et al.*, 1994, Lode *et al.*, 2001).

More recently, deep sequencing methodologies including array-based pyrosequencing technologies providing a much more expansive depth of coverage have been employed on patient samples before and after treatment of antibiotics to investigate both the short and long term effects of orally administered antimicrobials on the gut community. Panda *et al.*, (2014) revealed that in the short term β-lactams and fluoroquinolones (both broad spectrum antibiotic classes) significantly decreased the microbial diversity, increased the Bacteriodetes: Firmicutes ratio and also increased the proportion of unknown taxa within the Bacteriodes genus while β-lactams were found to almost double the microbial load differing from previous studies. The author suggests that the discrepancies they report could arise from differences in antibiotics used/dosage administered but may also be related to the use of animal models in previous studies (for example Antonopoulos *et al.*, 2009) while theirs involved data collected from human patients. Another study exploring the long-term effects of ciprofloxacin treatment found gut communities disrupted closely resembled their pre-treatment state by 4 weeks after cessation of treatment however several taxa including *Clostridiales*, had not recovered by 6 months (Dethlefsen *et al.*, 2008). These findings are indicative of factors promoting community resilience and persistence within this environment.

6.1.2 Persister cells: A survival strategy

Antibiotic resistance has increased dramatically since the introduction of penicillin along with its extensive use in medicine and observations that bacteria could degrade these antimicrobial compounds were noted as early as 1940 (Abraham & Chain 1940). Often when discussing mechanisms of antimicrobial resistance the bullet-target concept is referred to with the bullet being an

antimicrobial molecule and the target being the intended cellular target of this molecule (Aminov 2010). With this in mind antibiotic resistance is achieved either intrinsically or through acquisition of new mechanisms. The mechanisms of antibiotic resistance can generally be clustered into 7 discrete categories: (i) antibiotic target is protected from modification by mutation, (ii) antibiotic target is modified by an enzyme, (iii) antibiotic target is replaced by another molecule/process, (iv) target is protected at a population level through the formation of a protective barrier, (v) the antibiotic is modified reducing efficiency, (vi) antibiotic is destroyed and (vii) antibiotic is pumped out of the cell via efflux pump mechanisms (Aminov 2010, Alekshun & Levy 2007).

For many antibiotics active growth is required to see a lethal effect of the drug. A prime example of this being; Beta-lactams inhibit peptidoglycan biosynthesis by binding to the active site of Penicillin Binding Proteins (PBPs) preventing transpeptidation (Fisher *et al.*, 2005). This is a vital process in the formation of the bacterial cell wall during replication and division and beta-lactams can only target this when actively occurring. Switching to a metabolically dormant phenotype would therefore be a reliable way of escaping many antimicrobial molecules and the effects these have. A summary of bacterial dormancy can be seen in section 4.1.2. Persister cell populations were first reported in 1944 (Bigger 1944), however it was not until their association with biofilm drug tolerance was discovered that investigation into mechanisms involved in their formation took place (Spoering & Lewis 2001). Many chronic infections in immune-comprised patients are associated with the ability of the pathogen to resist bactericidal antimicrobial therapies without any notable drug tolerance mechanisms (Costerton *et al.*, 1999).

Type II toxin-antitoxin modules are consistently linked to the formation of persister cells and by extension, may play a role in antimicrobial escape of a sub-population of a community. Bactericidal antibiotics often function by altering their targets, which in turn then behave in a toxic fashion to the cell. Examples of these include aminoglycosides, which cause mistranslation and misfolding of peptides (Davies et al., 1986) and fluoroquinolones, which bind to DNA gyrase and topoisomerase inhibiting the relation step (Hooper 2001). It was proposed in 2004 by Keren *et al.* that the exhibited (often multidrug) drug resistance was due to inactivity of antimicrobial targets within the bacterial cells. This study employed a simple ampicillin lysis method of a culture-enabled collection of a persister cell transcriptome, which identified high expression of TA modules (Keren *et al.*, 2004). Through a ribosomal promoter-GFP fusion experiment an *E.coli* population was sorted based on fluorescence intensity. Cells entering dormancy had a diminished level of protein synthesis and thus low intensity whilst also showing increased antimicrobial tolerance (Shah *et al.*, 2006) suggesting a mechanism of reducing this synthesis drastically.

Mycobacterium tuberculosis, a pathogen that is renowned for ability to persist through many conventional antibiotic treatments, has at least 62 TA modules within it genome (Ramage *et al.*, 2009). Approximately 10% of the TA modules identified within the genome of *M.tuberculosis* were homologues of MazEF, a system known to interfere with the efficiency of antibiotics such as fluoroquinolones, and it has been hypothesised that it is because of these and possibly other TA families present that it is so hard to eradicate (Korch *et al.*, 2009, Tan *et al.*, 2012, Meredith *et al.*, 2015). It was also suggested that the high number of individual modules (many of them VapBC, MazEF and RelBE) may exist either because of the parasitic nature of the module or to ensure redundancy (Ramage

et al., 2009). A more recent study by the Gerdes Laboratory presented evidence that a deletion of at least 5 separate TA modules from the genome of E.coli K-12 was needed before a decrease in persister formation could be seen (Maisonneuve et al., 2011) suggesting a level of redundancy between modules, persister formation and subsequent resistance to antibiotics. One possible explanation for the multiplicity of TA modules within genomes is that each plays a role in persister formation upon exposure to different environmental conditions.

6.1.3 The role of TA modules in persister cell formation

Due to the nature of TA modules and the mechanism(s) of specifically the toxin component, TA systems have not been linked with a direct mechanism of antibiotic resistance. Instead (as discussed in chapter 4) their involvement and ability to modulate the general stress response has been widely noted and investigated especially from the perspective of persister cell formation. The primary characteristic of persister cells being a small population which can revert to their original phenotype once the antimicrobial stress has been removed (Singh et al., 2009) which TA systems have frequently been shown to do, at least in respect to non-antimicrobial stresses.

When defining the genetic basis for persister cell formation, TA models are not only implicated but have been shown to be primarily responsible due to their induction of a state of dormancy allowing portions of a cell population to escape the effects of antibiotics (Jayaraman 2008). The effect of the Type I TisAB module on persister levels appears to be directly linked to the bacterial SOS response (Courcelle *et al.*, 2001, Kowano *et al.*, 2007) with experiments using the fluoroquinolone; ciprofloxacin (a known inducer of the SOS response) linking this module to persister formation under the conditions of SOS induction (Dorr *et al.*,

2010). Persister levels observed for this module during time-dependent killing assays with ampicillin and streptomycin, both of which do not cause DNA damage, did not change in tisAB deletion strains. In the same study it was also shown that the TisB toxin was only active in the presence of a functional RecA protein (essential for the repair and maintenance of DNA) confirming dependency of function on the SOS pathway (Dorr *et al.*, 2010).

Ciprofloxacin kills cells primarily by converting target proteins (DNA gyrase and topoisomerase IV) to endonucleases, which are dependent on ATP for activity. The TisB toxin has been reported to reduce levels of cellular ATP with ectopic expression studies providing evidence for this (Unoson & Wagner 2008). It has been hypothesised that this drop in ATP availability not only reduces cellular metabolism but also prevents topoisomerases/gyrases from damaging DNA (Drlica et al., 2008). Importantly whilst this module provides resistance to fluoroquinolones the dormancy it also provides a mechanism for resistance to other antibiotics as well.

In terms of Type II TA systems, the first module to be implicated in, and subsequently named after, its effect on persister formation was the Type II HipAB (high persistence) loci (Moyed & Bertrand 1983). When over-expressed, HipAB was found to produce high numbers of persister cells (Moyed & Bertrand 1983). These cells were more resistant to both early and late stage inhibitors of peptidoglycan synthesis such as phosphomycin, cycloserine and ampicillin all of which require an actively growing cell to have effect. Further investigation into the role of the HipAB module identified its ability to increase resistance to other antimicrobials such as the quinolone naladixic acid, which is an inhibitor of DNA synthesis (Scherrer & Moyed 1988).

The increase in tolerance to multiple antibiotics has since been shown to be ubiquitous with this particular TA system and overexpression of the HipA toxin, leading to increased frequencies of persister cells within a population (Falla & Chopra 1998, Correia *et al.*, 2006) whilst deletion of both the toxin and full module did not produce any phenotypic change (Korch *et al.*, 2003, Hansen *et al.*, 2008).

Another TA system that has been well documented for its relatedness to persister cell formation is the *mqsRA* loci in *E.coli.* Deletion of the full module or toxin alone significantly decreases persistence whilst increased expression also increased persister numbers (Kim *et al.*, 2010). Additional findings which link the MqsR toxin to bacterial persistence include *MsqR* being the most highly expressed gene in persister cells when compared to non-persisters (Shah *et al.*, 2006) and the module itself being one of the most highly expressed operons during kanamycin stress (Kohanski *et al.*, 2007). This is not a phenomenon solely linked to this module either, many other TA module toxin genes (including *relE*, *higB*, *MazF*, *YafQ* and *yeoB*), are also highly induced within persister cells in several species such as *E.coli* and *Pseudomonas aeruginosa* (Keren *et al.*, 2004, Kohanski *et al.*, 2009). However to date, the MqsRA system is unique in terms of function as it remains the only Type II system that severely reduces persister formation upon deletion in planktonic populations. In biofilm communities YafQ has also shown a similar phenotype when deleted (Kohanski *et al.*, 2009).

Much of the work done on elucidating the role of TA modules in bacterial persistence has been conducted in an *E.coli* background which contains to date 36 complete TA modules (17 Type I and 19 Type II, Yamaguchi *et al.*, 2011) and there appears to be a certain level of redundancy in the formation of persister cells when in the presence of antibiotic pressure. Loss of function of a single module, appears to have no significant deleterious effect (Dorr *et al.*, 2010) however

progressive and cumulative deletions of 12 TA systems present on E.coli K-12's genome eventually correlates with a successive reduction in persister numbers (Maisonneuve *et al.*, 2011).

The RelBE module is one of the most widely understood Type II modules yet very little information is available with regard to the effect of expression of this module on cells exposed to antimicrobial stresses. With *In silic*o findings in Chapter 3 identifying the pTRACA22 RelBE module as highly abundant within the human gut, the importance of the gut microbiome to the higher host and the role TA modules appear to play in antimicrobial survival further investigation of the pTRACA22 RelBE TA module with regards to survival of *E.coli* EPI300 exposed to antimicrobial pressure will be examined.

6.2 Aims

The aim of work presented in this chapter was to identify the effect expression of the pTRACA22 RelBE TA module has on survival of *E.coli* EPI300 when treated with a range of antibiotics from multiple classes and where possible, gut relevant. These were: Ampicillin, Mecillinam, Doripenem, Cephalexin, Colistin, Rifaximin, Gentamicin, Clarithromycin, Ciprofloxacin, Sulphamethoxazole, Tetracycline, Fosfomycin, Trimethoprim and Chloramphenicol.

6.3 Materials and methods

6.3.1 Antibiotics used within this study

Antibiotics used within this study along with solvents and suppliers can be seen in Table 6.2.

6.3.2 Minimum inhibitory concentration assessment of antibiotics on *E.coli* EPI300

To establish the minimum inhibitory concentrations (MIC) of each antibiotic used a high throughput 96 well plate assay method was employed. LB broth was freshly prepared just prior to the experiments containing 0, 0.25, 0.5, 1, 2, 8, 16, 32, 64, 128, 256 and 512 µg/mL of the antibiotics listed in table 6.2. The outside ring of wells, of each 96 well plate, were filled with 100 µL sterile water whilst the inner 80 wells were filled in columns with 100 µL of antibiotic containing LB broth at the concentrations above. This was done in triplicate with positions of the column concentrations being randomised to minimise any error introduced from evaporation from these experimental wells.

A 50 mL culture of *E.coli* EPI300 WT was inoculated and grown overnight in LB broth with shaking (150 rpm) at 27°C. This overnight culture was diluted to an OD_{600} of 1.0 and 1 mL added to separate 50 mL LB broth containing no selection and grown to an OD_{600} of 0.5. 5 µL of standardised culture was then added to all experimental wells and plates were placed into loosely sealed bags containing damp tissue paper to keep the environment humid, again, to reduce the effect of evaporation. Plates were then incubated at 37°C for 20 hours and bacterial growth recorded using spectroscopy at an OD_{600}.

Table 6.2: Antibiotics and solvents used and preparation within this study.

Antibiotic	Class	Solvent*	Supplier
Ampicillin	Beta-lactam	Water	Fisher Scientific UK
Mecillinam	Beta-lactam	Water	Sigma Aldrich UK
Doripenem	Beta-lactam (carbapenem)	Water	Sigma Aldrich UK
Cephalexin	Beta-lactam (cephalosporin)	1M NH$_4$OH	Sigma Aldrich UK
Colistin	Polymixin	Water	Sigma Aldrich UK
Rifaximin	Rifamycin	100% Ethanol	Sigma Aldrich UK
Gentamicin	Aminoglycoside	Water	Sigma Aldrich UK
Clarithromycin	Macrolide	Acetone	Sigma Aldrich UK
Ciprofloxacin	2nd gen Fluoroquinolone	0.1M HCl	Fisher Scientific UK
Sulphamethoxazole	Sulfonamide	Acetone	Sigma Aldrich UK
Tetracycline	Polyketide	100% Ethanol	Sigma Aldrich UK
Fosfomycin	Phosphonic acid derivative	Water	Sigma Aldrich UK
Trimethoprim	N/A	100% Ethanol	Sigma Aldrich UK
Chloramphenicol	N/A	100% Ethanol	Sigma Aldrich UK

*Solvents were purchased from Fisher Scientific UK or Sigma Aldrich unless using sterile water or from a source otherwise stated.

Results were then used to calculate and average per antibiotic concentration. The lowest concentration showing no significant difference in OD to the control wells (0 µg/mL) was taken as the MIC for *E.coli* EPI300 for that antibiotic.

6.3.3 Antimicrobial viability assays

Cultures of *E.coli* EPI300: WT, pTRACA22, pGEM-T Easy(empty), pGEM-T(TA), pBAD(Tx), pJ821(ATx) and pJ821(TA) were inoculated and grown overnight with appropriate selection and other required supplements with shaking (150 rpm) in LB broth. These overnight cultures were diluted to an OD_{600} of 1.0 and 1 mL of each added to separate 50 mL LB broth containing no selection and grown to an OD_{600} of 0.5. Entire cultures were then centrifuged at 4500 g for 10 minutes, the supernatant discarded and the pellet resuspended in LB broth supplemented with 0.2% (w/v) glucose (controls) or 2% arabinose/100mM rhamnose (during expression) containing antibiotics at MIC based on results outlined in Table 6.3. Cultures were then incubated at $37^{o}C$ whilst being shaken at 150 rpm for 5 hours and 1 mL samples taken at the 0, 0.25, 0.5, 1, 2, 3, 4 and 5 hour time points. Cells were pelleted by centrifugation at 17000 xg for 2 minutes, washed 3 times in PBS using the same centrifugal speed and finally re-suspended in the same volume of fresh PBS. A serial dilution was then done in PBS from neat to 10^{-8}.

6.3.3.1 Enumeration of viable cells

Immediately after serial dilutions each dilution was treated identically to those in Section 5.3.9 for all antibiotic treatments tested.

6.3.3.2 BacLight Live/Dead staining and confocal microscopy

Immediately after serial dilutions live/dead fluorescence staining was done on each sample taken as in Section 5.3.10. Injured cells were recorded separately

(those which appeared a light orange colour using a fluorescence microscope), but recorded as "live". A total of three replicate slides were prepared and visualised from each sample tested, with 10 randomly selected FOV captured and analysed for live/dead counts per slide.

6.4 Results

To investigate the ability of the pTRACA22 relBE TA module to increase survival of *E.coli* EPI300 to a variety antibiotic stress, viability assays were undertaken involving both viability counts and BacLight Live/Dead fluorescence staining in conjunction with confocal microscopy.

6.4.1 Identification of *E.coli* EPI300 antibiotic minimum inhibitory concentrations

For use in subsequent experiments the MIC values of all antibiotics included in Table 6.3 for *E.coli* EPI300 WT were identified. This was achieved through a high throughput 96 well plate growth inhibition assay, containing these antibiotics at a range of concentrations in liquid media. OD was then used as a measurement of growth in comparison to control wells containing no antibiotics. Results of this assay can be seen in Table 6-3. MICs were taken as the lowest concentration that inhibited growth and resulted in OD values comparable to inoculated control wells (containing media and antibiotics only). Antibiotics were used at this MIC in all subsequent experiments.

Table 6.3: Minimum inhibitory concentrations of a selection of antibiotics for

E.coli **EPI300.**

Antibiotic	Minimum inhibitory concentration (μg/mL)
Ampicillin	64
Mecillinam	128
Doripenem	0.5
Cephalexin	0.5
Colistin	64
Rifaximin	32
Gentamicin	16
Clarifthromycin	256
Ciprofloxacin	0.5
Sulphamethoxazole	256
Tetracycline	16
Fosfomycin	8
Trimethoprim	64
Chloramphenicol	8

6.4.2 Impact of the p22 TA system on cell viability during exposure to antibiotics

Using viable counts, the percentage survival was calculated for *E.coli* EPI300 WT and EPI300 harbouring pGEM-T Easy(empty), pJ821(empty), pJ821(TA), pTRACA22, pGEM-T Easy(TA), pJ821(ATx) and pBAD(Tx). To confirm these results and to determine if changes in viable CFU counts represented cell death or entry into a viable but non-cultivable state, the BackLight® Live/Dead fluorescence staining kit was used in conjunction with confocal microscopy to examine the percentage of cells live, dead, and injured. Cultures of *E.coli* EPI300 harbouring these plasmids were exposed to the antibiotics listed in Table 6.2 at the MICs listed in Table 6.3.

6.4.2.1 Beta-lactams: Ampicillin, Mecillinam, Doripenem and Cephalexin

For ampicillin (Figure 6.1), *E.coli* EPI300 carrying the pTRACA22 plasmid showed significantly higher viable cell counts (~25%, $P<0.05$) and percentages of live cells at the 30 and 60 minute time points when expression of the full TA module had not been induced. By 120 minutes, viable cell and live-stained cell counts of EPI300 harbouring the pTRACA22 plasmid had returned to WT levels. With RelE gene expression of the pJ821(TA) construct, significantly higher viable cell and live-stained cell counts were also noted at the 30 and 60 minute time points with return to WT levels by 120 minutes. The pGEM-T Easy(empty), pGEM-T Easy(TA) and pBAD(Tx) plasmids were unable to be used for this assay as all 3 carry an ampicillin resistance gene. In contrast EPI300 in the presence of both mecillinam and doripenem showed no significant difference between constructs regardless of induction of expression and data can be seen in appendix 1, Figure 1 and 2.
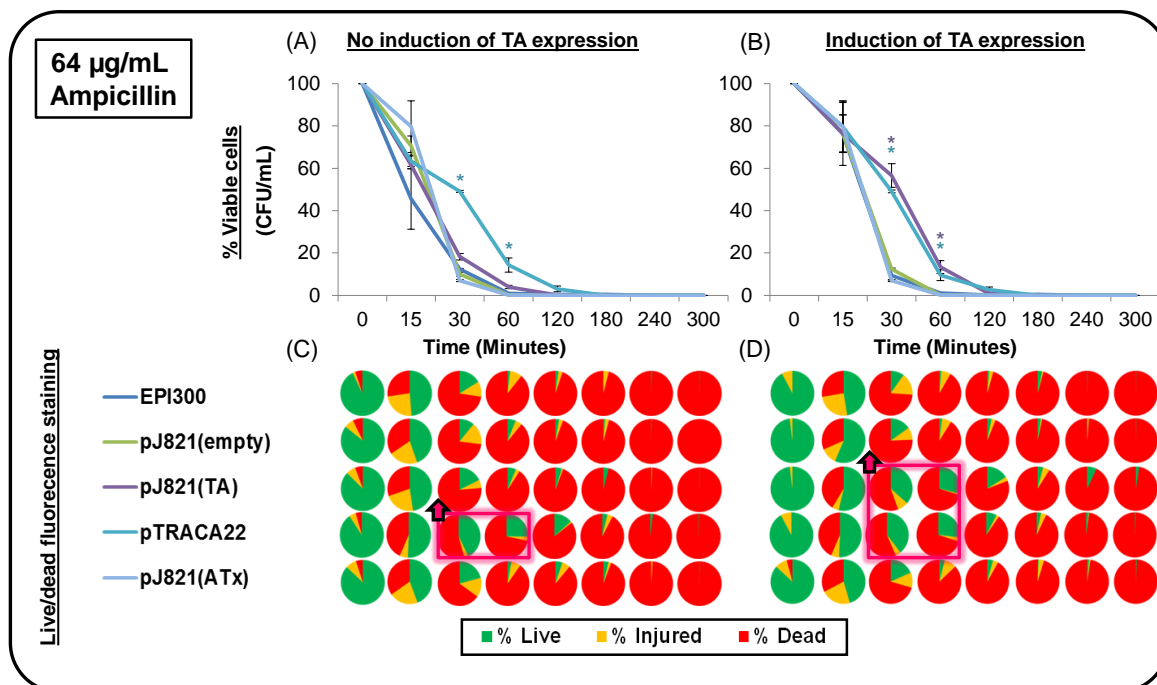
Figure 6.1: The effect of the p22-RelBE TA system on *E.coli* EPI300 survival during treatment with 64 µg/mL ampicillin. Cell suspensions in LB broth supplemented with 64 µg/mL ampicillin were incubated for 5 hours and used for both viable plate counts and live/dead fluorescence staining. 0.2% (w/v) glucose was added at time point 0 to cultures in which cells were not induced. For those induced, 100mM rhamnose or 2% (w/v) arabinose was added. A & B) graphs refer to percentage viable cells (CFU/mL), whilst C & D) pie charts represent percentage live/dead/injured. All experiments were undertaken in triplicate and results shown are the mean ± SEM of three independent experiments.

* = $P < 0.05$ and represents a significant difference in percentage viable cells compared to control constructs.

For percentage live data, those significantly different from the control constructs are highlighted in a pink box ($P < 0.05$) with the arrow representing whether the % live cells is higher or lower.

When treated with cephalexin (Figure 6.2), expression of the RelE toxin from pBAD(Tx) resulted in a significant decrease in viable cell counts, in comparison to the WT control at the 15 minute time point. Whilst not significant ($P>0.05$) this trend could also be seen in other pTRACA22-RelE carrying plasmids. EPI300 carrying pTRACA22 and pGEM-T Easy(TA) showed an increased percentage survival compared to WT and empty vector controls, similar to that seen with the ampicillin stress. Noticeably the significant increase in viable cell counts was also seen at the 120-minute time point ($P<0.05$). When the individual TA toxin gene in (pBAD(Tx) or the complete module within pJ821(TA)) were expressed, an increase in viable cell counts was also seen during the 30-120 minute time frame ($P<0.05$). Live/dead fluorescence staining showed a significantly higher percentage of live cells at the 30, 60 and 120 minute time points EPI300 harbouring pTRACA22 and pGEM-T Easy(TA), and with induced expression of the RelBE module, this was also seen for pJ821(TA) and to a lesser extent pBAD(Tx) ($P<0.05$). Both percentage survival and live/dead staining cell counts returned to levels of the WT by 180 minutes.
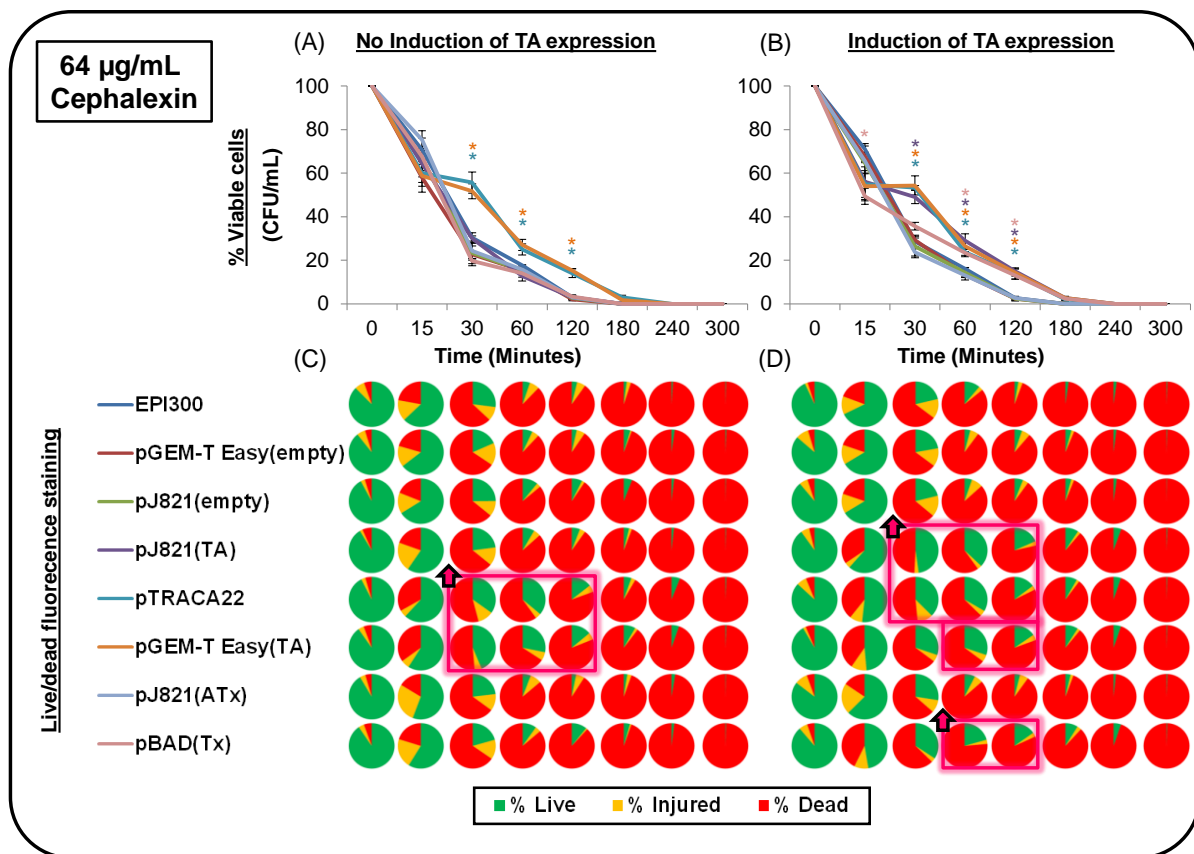
Figure 6.2: The effect of the p22-RelBE TA system on *E.coli* EPI300 survival during treatment with 64 µg/mL cephalexin. Cell suspensions in LB broth supplemented with 64 µg/mL cephalexin were incubated for 5 hours and used for both viable plate counts and live/dead fluorescence staining. 0.2% (w/v) glucose was added at time point 0 to cultures in which cells were not induced. For those induced 100mM rhamnose or 2% (w/v) arabinose was added. A & B) graphs refer to percentage viable cells (CFU/mL), whilst C & D) pie charts represent percentage live/dead/injured. All experiments were undertaken in triplicate and results shown are the mean ± SEM of three independent experiments.

* = $P<0.05$ and represents a significant difference in percentage viable cells compared to control constructs.

For percentage live data, those significantly different from the control constructs are highlighted in a pink box ($P<0.05$) with the arrow representing whether the % live cells is higher or lower.

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6.4.2.2 Macrolides: Clarithromycin

Without induction of TA expression and in the presence of the macrolide clarithromycin at 256 µg/mL, *E.coli* EPI300 harbouring the pTRACA22 and pGEM-T Easy(TA) plasmids showed a significantly reduced percentage survival from viable counts from 15 minutes until 240 minutes (P<0.05, Figure 6.3). In parallel, cultures harbouring both these plasmids also showed a significantly lowered percentage of live cells (based on live/dead staining) from 30 to 300 minutes when compared to the WT and empty vector controls (P<0.05). Whilst viable cell counts steadily declined to almost 0 by 300 minutes, this was not seen with fluorescence microscopy live/dead percentages with approximately 40-50% of cells still alive by the 300-minute time point.

When expression of the RelE toxin gene on pJ821(TA) and pBAD(Tx) was induced by the addition of rhamnose or arabinose, EPI300 carrying the pJ821(TA) plasmid also showed a similar decrease in viable cell counts seen above whilst the pBAD(Tx) construct showed an even more significant drop to approximately 20% by the 30 minute time point. Additionally with expression of the toxin live/dead microscopy counts also displayed the same significant decrease in comparison to the WT (P<0.05).
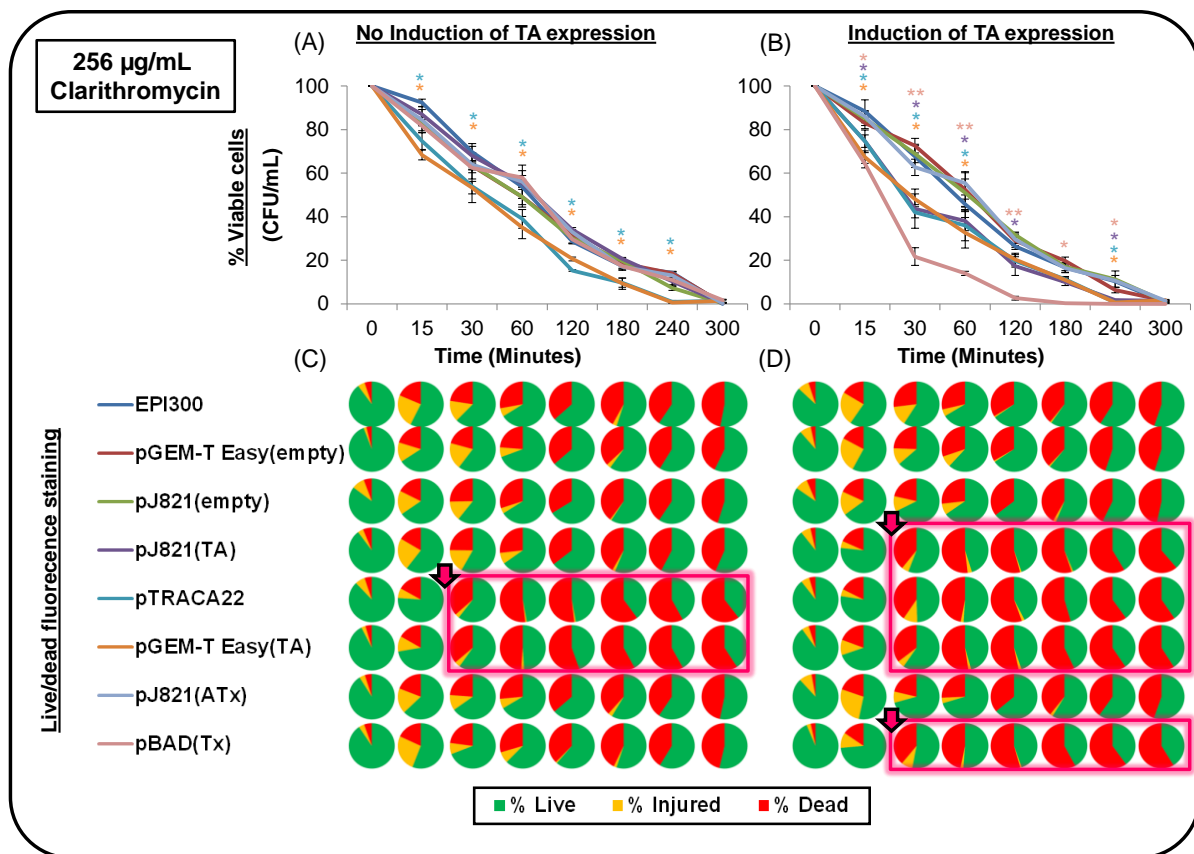
Figure 6.3: The effect of the p22-RelBE TA system on *E.coli* EPI300 survival during treatment with 256 µg/mL clarithromycin. Cell suspensions in LB broth supplemented with 256 µg/mL clarithromycin were incubated for 5 hours and used for both viable plate counts and live/dead fluorescence staining. 0.2% (w/v) glucose was added at time point 0 to cultures in which cells were not induced. For those induced 100mM rhamnose or 2% (w/v) arabinose was added. A & B) graphs refer to percentage viable cells (CFU/mL), whilst C & D) pie charts represent percentage live/dead/injured. All experiments were undertaken in triplicate and results shown are the mean ± SEM of three independent experiments.

* = $P<0.05$, ** = $P<0.001$ and represents a significant difference in percentage viable cells compared to control constructs.

For percentage live data, those significantly different from the control constructs are highlighted in a pink box ($P<0.05$) with the arrow representing whether the % live cells is higher or lower.

6.4.2.3 Polyketides: Tetracycline

E.coli EPI300 harbouring the pTRACA22 and pGEM-T Easy(TA), in the presence of 16 µg/mL tetracycline (Figure 6.4), showed a significantly reduced percentage survival from viable counts during the 30-60 minute period ($P<0.05$). Induction of TA expression also reduced viable cell counts for pJ821(TA) and the pBAD(Tx) cultures ($P<0.05$). Interestingly, whilst the viable cell counts demonstrated a rapid drop in percent survival, this was not supported by the live/dead data collected. An initial drop of approximately 10-15% of live cell counts was observed during tetracycline treatment for all constructs with and without expression. This decreased by a further 15-20% over the 5-hour experimental period. At the 5 hour time point approximately 50-55% of cells were still viable whilst plate-based viable counts displayed almost no growth.
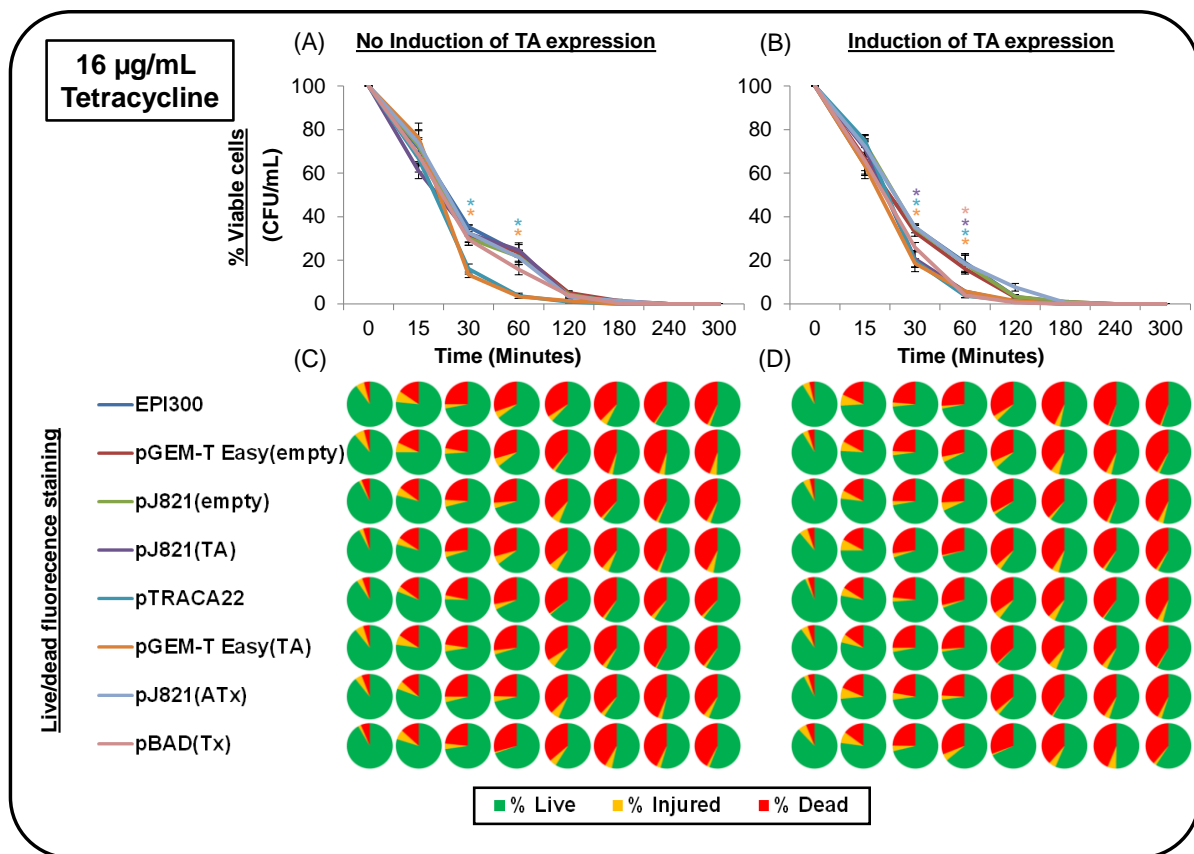
Figure 6.4: The effect of the p22-RelBE TA system on *E.coli* EPI300 survival during treatment with 16 μg/mL tetracycline. Cell suspensions in LB broth supplemented with 16 μg/mL tetracycline were incubated for 5 hours and used for both viable plate counts and live/dead fluorescence staining. 0.2% (w/v) glucose was added at time point 0 to cultures in which cells were not induced. For those induced 100mM rhamnose or 2% (w/v) arabinose was added. A & B) graphs refer to percentage viable cells (CFU/mL), whilst C & D) pie charts represent percentage live/dead/injured. All experiments were undertaken in triplicate and results shown are the mean ± SEM of three independent experiments.

* = $P<0.05$ and represents a significant difference in percentage viable cells compared to control constructs.

6.4.2.4 Chloramphenicol

When exposed to chloramphenicol (µg/mL), a steady decline in percentage survival was seen for all EPI300 cultures regardless of plasmids and TA genes carried (Figure 6.5). Notably the pGEM-T Easy(TA) construct presented a more substantial decrease in viable cell counts at the 15 ($P<0.05$), 30 ($P<0.001$) and 60 ($P<0.001$) minute time points when compared to the empty PGEM-T Easy empty vector and WT. Regardless of this initial difference, the pGEM-T Easy(TA) construct behaved similarly to the others for the remaining duration of the experiment. EPI300 harbouring pJ821(TA) and pJ821(ATx) did not deviate from the WT or empty control vectors when expression of TA genes were induced. In contrast expression of the RelE toxin from pBAD(Tx) reduced viable cell counts significantly ($P<0.05$) from 15 to 300 minutes.

The percentage of cells fluorescing green (living) for all constructs decreased gradually across the entire 5 hour experimental window to between 60-50% with the exception of pGEM-T Easy(TA) and the pTRACA22 constructs. For pGEM-T Easy(TA), the percentage of live-stained cells values reached 50% by the 60 minute time point and then decreased further for the remaining 3 hour period. This was significantly lower than the WT and empty vector control ($P<0.05$). Furthermore at 300 minutes significantly less live cells were detected for *E.coli* carrying the pTRACA22 plasmid than the WT ($P<0.05$). When expression of the RelE toxin was induced, *E.coli* harbouring pJ821(TA), and pBAD(Tx) also showed a rapid reduction, to approximately 50% live-stained cells, by 60 minutes similar to that detected for pGEM-T Easy(TA) compared to the WT and empty vector controls.
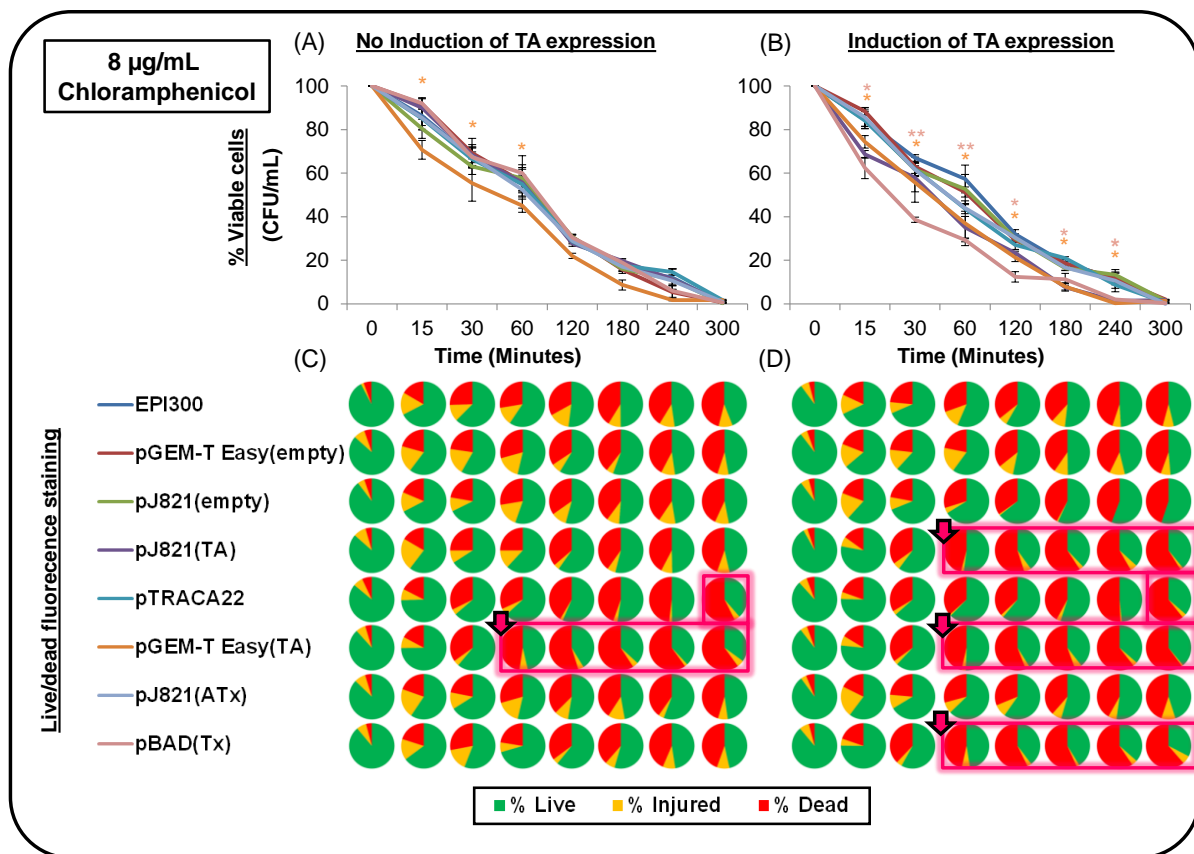
Figure 6.5: The effect of the p22-RelBE TA system on *E.coli* EPI300 survival during treatment with 8 µg/mL chloramphenicol. Cell suspensions in LB broth supplemented with 8 µg/mL chloramphenicol were incubated for 5 hours and used for both viable plate counts and live/dead fluorescence staining. 0.2% (w/v) glucose was added at time point 0 to cultures in which cells were not induced. For those induced 100mM rhamnose or 2% (w/v) arabinose was added. A & B) graphs refer to percentage viable cells (CFU/mL), whilst C & D) pie charts represent percentage live/dead/injured. All experiments were undertaken in triplicate and results shown are the mean ± SEM of three independent experiments.

* = $P < 0.05$ and represents a significant difference in percentage viable cells compared to control constructs.

For percentage live data, those significantly different from the control constructs are highlighted in a pink box ($P < 0.05$) with the arrow representing whether the % live cells is higher or lower.

6.4.2.5 Polymixin: Colistin, Rifamycin: Rifaximin, Aminoglycoside: Gentamicin, Fluoroquinolone: Ciprofloxacin, Sulfonomide: Sulphamethoxazole, Phosphonic acid derivative: Fosfomycin and Trimethoprim

When exposed to colistin E (64 µg/mL) rifaximin (32 µg/mL), gentamicin (16 µg/mL), ciprofloxacin (0.5 µg/mL), sulphamethoxazole (256 µg/mL), fosfomycin (8 µg/mL) and trimethoprim (64 µg/mL), *E.coli* EPI300 harbouring plasmids encoding the p22-RelBE module or the individual toxin/antitoxin gene showed no significant differences in viable cell counts or live/dead staining cell counts when compared to the WT or empty vector controls. This was also the case when expression of the TA module and individual genes was induced. The data can be seen in Figure 3-9 of Appendix 1.

6.5 Discussion

6.5.1 Antibiotics of interest

Antibiotics used within this study were chosen firstly to include a broad range of available classes and modes of action and secondly for their spectrum of activity, specifically against Gram negatives.

6.5.1.1 Cell wall and membrane disruption

The most commonly used antibiotics that can be placed within this group are the beta-lactams. Compounds in this superfamily include penicillins, carbapenems, monobactams cephalosporins, all of which contain an amide beta-lactam ring within their molecular structure which may be fused to 5 or 6-membered heterocyclic rings (James & Gurk-Turner 2001). With members of this class being the most widely prescribed antibiotics across the globe (Elander 2003), inclusion of these within work done in this chapter was a necessity but also interesting from a mechanistic perspective as well.

Beta-lactam antimicrobial function comes from their bacteriocidal ability to distrupt cell wall biosynthesis. This is accomplished through inhibition of peptidoglycan synthesis, an important polymer of sugars, consisting of alternating residues of β-linked N-acetylglucosamine (NAG) and N-acetylmuramic (NAM) acid, with an attached peptide chain, consisting of 3-5 amino acids (Schleifer & Kandler 1972). Figure 6.6 is a graphical representation of peptidoglycan formation. The polymer chains can then be cross-linked forming a strong mesh-like structure, approximately 8 nm thick in Gram negative bacterium and between 20-80 nm in thickness for Gram positives (Madigan *et al.*, 2009).
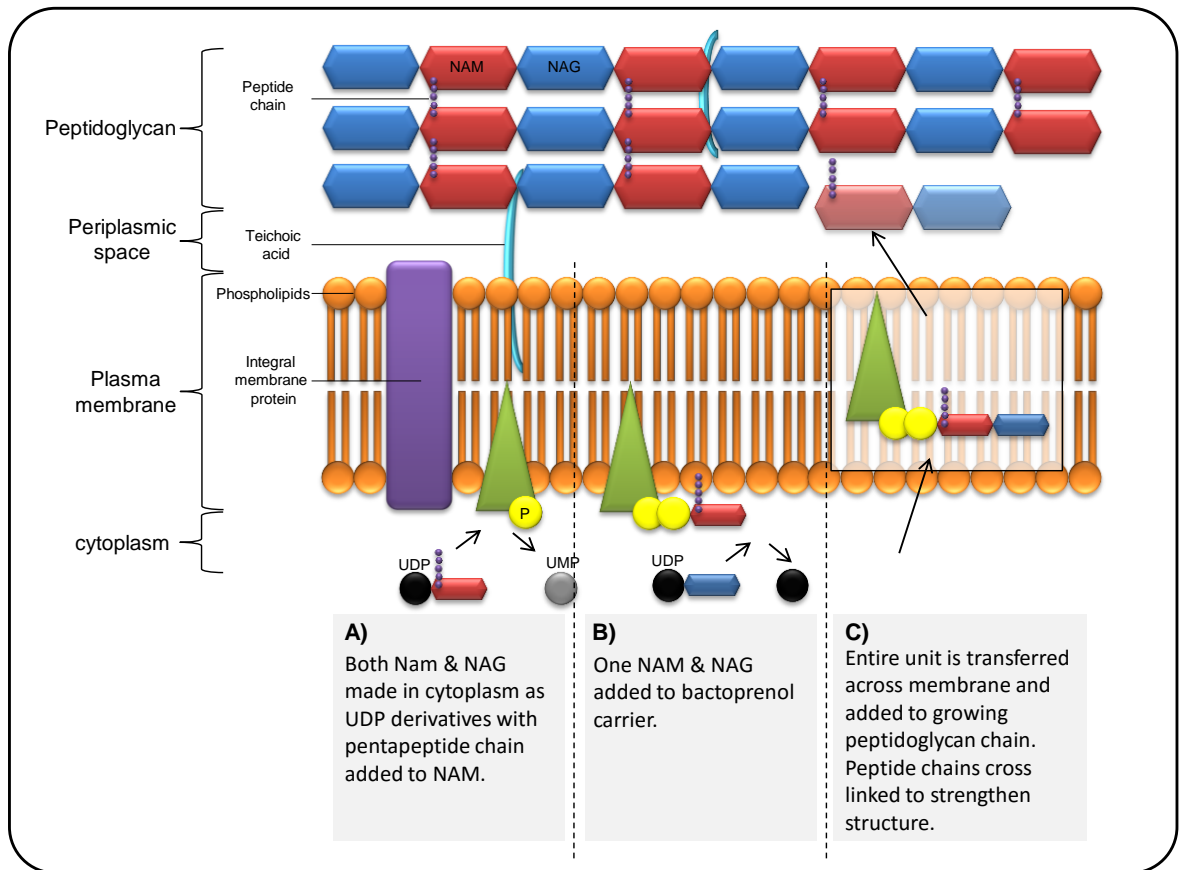
Figure 6.6: A simple schematic depicting the biosynthesis of peptidoglycan.
A Gram-positive cell has been used as an example. A) Bactoprenol is a hydrophobic
alcohol that bonds to both NAG and NAM/pentapeptides, the peptidoglycan precursors,
one subunit at a time. Both of these are synthesised in the cells cytoplasm. B) These
subunits are UDP derivatives and attach to the bactoprenol individually until a full
peptidoglycan unit (one NAM+pentapeptide and one NAG) is formed. C) Bactoprenol then
transports these precursors across the cytoplasmic membrane by rendering them
sufficiently hydrophobic. Once across glycolases insert these precursors into the growing
cell wall by catalysing glycosidic bond formation. Finally transpeptidation occurs by
crosslinking between muramic acid residues in adjacent peptide chains (Madigan *et al.*,
2009).

The final step of synthesis is facilitated by DD-transpeptidases, also known as penicillin-binding proteins (PBPs) and these vary in their affinity for binding penicillin or other beta-lactam antibiotics (Schleifer & Kandler 1972). The number of PBPs may also vary between bacterial species (Schleifer & Kandler 1972, Vollmer & Bertsche 2008). Inhibition of peptidoglycan is achieved because beta-lactam antibiotics are analogues of D-alanyl-D-alanine, the two terminal amino acid residues of NAM/NAG peptide subunits. Beta-lactams bind to the ser403 residue of the PBP active site causing irreversible inhibition of the PBP, preventing transpeptidation and creating a weakened peptidoglycan layer which can no longer fully maintain cell rigidity and tolerance of osmotic stress (Bycroft & Shute 1985). The process of transpeptidation requires the cell to actively be growing and metabolising, therefore anything that interrupts either this specific process or cellular metabolism, growth and division as a whole would reduce the efficiency of Beta-lactam antibiotics.

The RelE toxin of the RelBE TA module behaves as a global translational inhibitor by cleaving mRNA indiscriminately when positioned at the ribosomal A-site between the second and third nucleotide (Pedersen 2003). It can be hypothesised that carriage of one or more of these modules by cell populations in the presence of inhibitory concentrations of beta-lactam antibiotics may provide increased resistance, even if only transient. Studies undertaken in this chapter identified that firstly, *E.coli* EPI300 carrying the p22-RelBE module under control of its natural promoter in a gut-associated and control (pGEM-T Easy) plasmid background had significantly higher proportions of viable cells compared to the WT and empty vector controls when treated with both ampicillin and cephalexin. This increase suggests transient (and moderate) increase to persistence over a period 30-120 minutes before returning to WT levels. Interestingly constructs harbouring

the entire p22-RelBE module and individual toxin gene under the control of an inducible promoter also showed this same increase when expression was initiated, suggesting the TA module, or more specifically the p22-RelE toxin was responsible for the phenotypic change. This was further supported by the isolated p22-RelB antitoxin expression not demonstrating this increase in persistence and not deviating from the empty vector control or WT.

The aforementioned earlier work done by Moyed & Bertrand in 1983 and later by Falla & Chopra in 1998 on the HipBA loci identified this modules ability to significantly increase persister formation with expression when in the presence of cell wall acting antibiotics and of particular interest; ampicillin. Notably, studies investigating the HipBA module found that the antibiotics used (chosen as they inhibited peptidoglycan production) all effected survival of the cell population similarly. It should also be mentioned here that initially, killing of cell populations was equally rapid however with carriage of the HipBA locus significant increases in survival were seen after 30 minutes. This is in-line with results found for ampicillin and cephalexin within this chapter. One comparison between results was the increase in viable cells seen in Falla & Chopra (1998) lasted up to 4 hours (the length of the experiment) whilst results seen from the pTRACA22-RelBE module are less prolonged and eventually returned to WT levels and it is possible these differences may be due to the mechanism of the HipA toxin.

HipA, the toxin component, is a phosphatidylinositol kinase which binds to ATP with high affinity and is confirmed to phosphorylate elongation factor 'Tu' (Shumacher 2009). The altered state of Tu causes shut down of cellular metabolism (Shumacher 2009). Tu mediates anino-acyl-tRNA binding to the ribosome. When phosphorylated, Tu cannot alter its confirmation halting translation (Weijland *et al.*, 1992). This is a very different method of inhibiting

translation to that exhibited by the RelE toxin, which halts translation by cleaving and thus destroying the mRNA. These differences in toxin mechanism may account for differences seen in the results, especially as dosages used by Moyed & Betrand (1983) were higher than those used in our own. This could be due to RelE's (although reversible) binding to the ribosomes A site ensuring any mRNA which enters is cleaved at certain residues and unable to be properly translated (Neubauer et al., 2009) whilst HipA only inhibits free tRNA from entering the ribosome (Correia *et al.*, 2006). This may allow some translation to continue and therefore some cellular activity as well. The same logic is also applicable to the comparable cephalexin data.

In contrast when *E.coli* EPI300 expressing the p22-RelBE module was exposed to Doripenem (a carbapenem) and mecillinam (an extended spectrum beta-lactam) it did not show increase in survival. Mecillinam has been shown to be highly effective against *E.coli* clinical isolates (much more so than other penicillins), especially those isolated from urinary tract infections (Mazzulli *et al.*, 2001). It functions similarly to other beta-lactams except specifically targets PBP2 involved with cell shape regulation (Neu 1985). Of the 12 PBP identified in *E.coli,* inhibition of PBP2 has been shown to be least proficient in autolytic activation which leads to loss of cell wall integrity (Neu 1985). Beta-lactams which target and inhibit PBP1 and 3 (such as ampicillin and cephalexin) have also been shown to stimulate the SOS response (Spratt 1975, Kitano & Tomasz 1979, Plata *et al.*, 2013), a process known to upregulate Lon protease activity which in turn degrades the relB antitoxin leading to release of the RelE toxin (Christensen *et al.*, 2001, Christensen *et al.*, 2003). This would allow the free toxin to interact with the ribosome and cleave mRNA reducing global translation (and subsequent cellular metabolism). As inhibition of PBP2 has not been shown to induce this same

response, it may not be as effective in stimulating global inhibition and the increase in persistence seen for ampicillin and cephalexin would not be mirrored by mecillinam treatment. Similarly Doripenem has also shown high affinity to PBP2 in *E.coli* and so the same hypothesis may also be applicable this antibiotic (Davies *et al.*, 2008).

Certain gut commensals have been strongly linked with the formation of urinary tract infections (UTI's) and this is especially true for *E.coli* with extra-intestinal pathogenic strains (ExPEC) being divided into uropathogenic (UPEC) and septicaemic groups (Katouli 2010). Fosfomycin is a commonly used antibiotic for UTIs caused by *E.coli* and *P.aeruginosa* by inhibiting peptidoglycan synthesis and thus cell wall biogenesis. This antibiotic functions by inhibiting the enzyme UDP-N-acetylglucosamine-3-enolpyruvyltransferase also known as 'MurA' which catalyses the committed step in peptidoglycan synthesis, the linking of the glycan and peptide portions of peptidoglycan together (Brown *et al.*, 1995). Fosfomycin has bacteriocidal activity against both gram positive and negative species with specific examples of use being *E.coli* and *P.mirabilis*. It is also a highly used antimicrobial for treatment of UTIs because of its retained activity in low pH environments and excretion in its active form into urine (Falagas *et al.*, 2008). With this link between *E.coli* uropathogenic infections and the relBE module's ability to increase persistence in peptidoglycan targeting antimicrobials, it is possible to predict that expression of the p22-RelBE module could provide some increase in survival of *E.coli* when exposed to this antibiotic. However, this was not the case.

Colistin is a polymixin antibiotic that consists of a cyclic peptide with a long hydrophobic tail (Kwa *et al.*, 2007). This is able to disrupt the bacterial cell membrane by interacting with phospholipids (Kwa *et al.*, 2007). For gram negatives, Colistin binds to the LPS and phospholipids in the outer cell membrane

and competitively displaces divalent cations (Ca^{2+} and Mg^{2+}) from the phosphate groups of membrane lipids (Schindler & Osborn 1979, Groisman *et al.*, 1997). This leads to disruption and leakage of intracellular contents and bacterial death (Li *et al.*, 2006, Evans *et al.*, 1999). With knowledge of this mode of action it was expected that expression of the p22-RelBE module, even individual components, would not significantly increase EPI300 survival. The RelE toxin is an mRNAse with no evidence to suggest that it can move across, interact or stabilise the phospholipid cell membrane.

The first Type V TA module, the GhoTS system, was identified in 2012 and it was shown that low expression of the ghoT toxin significantly increased mqsR toxin (part of the mqsRA Type II TA module)-mediated persistence in *E.coli* to ampicillin and, interestingly, to kanamycin, an aminoglycoside (Wang *et al.*, 2012). However at higher doses the mqsR toxin was shown to induce cell death Brown *et al.*, (2009). Remarkably the toxin is a 57 aa in length and is highly hydrophobic, specifically causing membrane damage by forming pores and subsequently leading to cell lysis. It was suggested that low cellular levels of free GhoT may induce persistence through a loss of membrane potential, which in turn induces expression of other pathways leading to eventual persistence (Wang *et al.*, 2012). It is also possible that carriage of this module may have a negative effect on cell survival during treatment with polymixin antibiotics, with both causing membrane damage rapidly leading to cell death as *E.coli* strains have been shown to harbour this TA module in their genome (Wang *et al.*, 2012).

6.5.1.2 Protein synthesis inhibition

Clarithromycin is a member of the macrolide antibiotic class and a protein synthesis inhibitor. This is done by binding reversibly the P site of the 50S ribosomal subunit and preventing amino acid elongation, by blocking transfer and incorporation of new amino acids from tRNA (Kirillov *et al.*, 1997, Tenson *et al.*, 2003). It has also been suggested that some members of this class can induce premature dissociation of the peptidyl-tRNA from the ribosome, further inhibiting translation (Tenson *et al.*, 2003). From a clinical perspective macrolides are considered bacteriostatic antibiotics and are of significant interest as they are actively concentrated within leukocytes and are therefore transported to the site of infection (Bailly *et al.*, 1991).

With p22-RelBE decreasing survival in the presence of clarithromycin, it is hypothesised that the reduction in survival may be due to an additive effect of both the antibiotic, specifically targeting the P-site of the 50S ribosomal subunit, and the RelE toxin which binds to the A-site of the same subunit. A study in 2001 investigating amino acid starvation and RelBE expression identified a drop in global translation of approximately 90-95% and with clarithromycin also present this may explain the drop in survival with TA expression. However when percentage survival was close to 0% using viable plate counts, the percentage of cells stained as living using live/dead fluorescence staining was still at approximately 50% with no expression of the p22-RelBE module and approximately 40% with expression. These differences in results from both experimental procedures can be explained through cells entering a "viable but non-culturable" (VBNC) state which could be predictable considering the mode of action of the antibiotic (Oliver 2005).

Other than clarithromycin several antibiotics that interfere with protein synthesis were also investigated. With a similar mode of action to the macrolides class, chloramphenicol prevents protein chain elongation by inhibiting peptidyltransferase activity of the 50S ribosomal subunit leading to prevention of peptide bond formation (Hahn et al., 1955, Jardetzy 1963). This is in contrast to macrolides, which as described above sterically block progression of the growing peptide chain. Tetracycline, a polyketide antibiotic, inhibits binding of aminoacyl-tRNA to the mRNA-ribosome complex by bindings to the 30S ribosomal subunit (Chopra & Roberts 2001). This therefore interferes with a separate process to the RelE molecule whilst achieving a similar outcome. As with other protein synthesis inhibitors the drop in survival with expression of the p22-RelBE may be due to the effect of both the toxin and tetracycline not mutually excluding one another and behaving in an additive fashion.

6.5.1.3 DNA synthesis Inhibition

Several classes of antibiotics have modes of action which affect DNA synthesis and processes related to this vital part of the cell cycle. In this chapter three DNA synthesis inhibitors, Sulphamethoxazole, trimethoprim and ciprofloxacin, were investigated with respect to expression of the p22-RelBE TA module providing increased survival to these antibiotics, however, no significant increases in percentage survival or live cells were found. Trimethoprim and sulphamethoxazole (a sulphonamide) act by interfering with the biosynthesis of Tetrahydrofolic acid (THF), the active form of folic acid, a vital substrate for the transfer of one carbon units to nucleotides (Henry 1943, Hitchings 1973). Specifically this is an essential precursor to thymidine synthesis (Ahrmad *et al.*, 1998). Within microbes dihydrofolate (DHF) is synthesised de novo from *p*–aminobenzoic acid whilst in

mammalian cells it is a dietary requirement and this allows for selective toxicity to cells dependent on synthesising folic acid making both useful antimicrobials (Bermingham & Derrick 2002). Sulphamethoxazole is a structural analogue and competitive antagonists of para-aminobenxoic acid (PABA), a precursor molecule in this pathway, which blocks synthesis of DHF (Hitchings 1973). Trimethoprim binds to dihydrofolate reductase, which reduces DHF to THF. Again this antibiotic has selective toxicity against bacterial cells by having an affinity to bacterial dihydrofolate reductase several thousand times higher than the human enzyme (Hitchings 1973, Gleckman *et al.*, 1981).

Ciprofloxacin, a second generation fluoroquinolone, is a bactericidal broad spectrum antibiotic used to treat a range infections including UTIs, GI tract infections and abdominal infections encompassing both gram positives and gram negatives. They are quinolones, which have had their potency increased through addition of a fluorine atom at position 6 and rapidly obstruct DNA synthesis through inhibition of DNA gyrase and topoisomerase IV activity. In general DNA gyrase is the primary target of ciprofloxacin in gram-negative bacteria, more so when complexed with DNA. Quinolones are able to stabilise the quinolone-enzyme-DNA complex at this point, resulting in double stranded DNA breaks and prevention of re-ligation (Chen et al., 1996). Similarly for gram positives, again with some exceptions, topoisomerase IV is the major target, which results in disruption of DNA daughter strand separation (Pan & Fisher 1997). The bactericidal effects on gram positives tends to occur more slowly due to the enzyme being located further behind the replication fork (Hawkey 2003).

With the mode of action of ciprofloxacin (and other agents of this class) being direct damage to DNA it seems unsurprising that cells expressing a TA module toxin component which behaves as an mRNAse when bound to the 50S ribosomal

subunit displayed no phenotypical change to the WT. This result differs somewhat to studies involving the HigAB and TisAB loci, which found persister formation increased when *E.coli* expressed this module in the presence of ciprofloxacin (Dorr *et al.*, 2010). It has been hypothesised that the TisB toxin reduces levels of cellular ATP (which both DNA gyrase and topoisomerase IV require for function) preventing some damage to DNA (Drlica 2008). On the other hand RelE toxin globally inhibits translation, essentially releasing ATP, which can then utilised by the newly converted endoribonucleases (Christensen *et al.*, 2001). Due to the global reduction of metabolism by free RelE and that both sulphamethoxazole and trimethoprim affect metabolism of an entirely separate cellular process to the RelE molecule, expression of the p22-RelBE module was unlikely to have an impact on survival in the presence of these antibiotics. This was supported by data collected in this chapter.

6.5.2 TA modules may aid re-colonisation of the human gut

The human colonic microflora, the metabolic processes it undertakes and it's stable colonisation of this environment are a necessity for the well-being of the human host through its effect on immune and neural development, production of metabolites and prevention of infection (Edlund & Nord 1999, Sullivan *et al.*, 2001, Backhed *et al.*, 2005, Ley *et al.*, 2006, Heijtz *et al.*, 2011). Although the microbial colonic ecosystem is generally stable it can be disturbed by the administration of antimicrobial agents when administered both orally or intravenously and being exposed to this via incomplete absorption, secretions from the salivary glands and intestinal mucosa or in bile from the liver (Nord et al., 1984a, Nord et al., 1984b, Arvidsson et al., 1988, De La Cochetiere et al., 2005). The changes which occur within this community as a result of exposure to active antimicrobials appear to

vary; however, it is generally accepted that they can result in a shift in population with the suppression or elimination of some organisms and overgrowth by organisms that are not susceptible to these agents (Rafii *et al.*, 2009, Panda *et al.*, 2014, Modi *et al.*, 2014, Michael *et al.*, 2002, Jernberg *et al.*, 2010, Dethlefsen *et al.*, 2008).

Knecht *et al.* (2014) employed high throughput sequencing to establish the effect beta lactam antibiotics had on the gut microbiota and found a significant effect on microbial composition within the environment including a decrease in α-diversity but no overall community collapse. Notably long-term disruptions of this composition seem to be relatively trivial although both culture based and molecular techniques have revealed an increase in and a persistence of, antibiotic resistance genes, especially those of the beta-lactam, lincosamide and glycopeptide classes (Jernberg *et al.*, 2010). Based on results obtained in this chapter and those of other recent studies investigating TA modules and persistence to antibiotics (Black *et al.*, 1991, Shah *et al.*, 2006, Drlica *et al.*, 2008), it is likely that Type II TA modules and perhaps TA systems in general are involved in transient persistence when periods of antibiotic treatment are acute. Carriage of these modules and their influence on persister cell formation may also offer part of the explanation as to why the gut microbiome is also able to recover to a relatively pre-treatment composition.

It could be argued that these persister cells formed through TA expression, along with other methods such as biofilm formation (which again TA modules are known to play a direct role in, Wang & Wood 2011) and perhaps even an indirect role in induction of spore formation (although no evidence for this exists as of yet) may offer a plausible reason for the gut microbiota being able to avoid a complete collapse of diversity after an aggressive antimicrobial treatment period.

Alternatively this retention of diversity may be due to antibiotic specificity but this does not explain recovery after a broad spectrum antibiotic is utilised, such as penicillins and aminoglycosides. Interestingly, antibiotics are used often in parallel with others due to synergistic effects, such as Trimethoprim and sulfamethoxazole, which both effect the synthesis of folic acid (although more commonly used for treating UTIs, or to avoid the opportunity for a pathogen to become resistant to one), such as metronidazole and ciprofloxacin. Carriage of general stress response loci such as TA modules may enable a population (or community in the case of the gut) to survive the multiple transient stresses, which are designed specifically to circumvent traditional resistance mechanisms.

6.5.3 The p22-RelBE module, colonisation of other body sites and role in pathogenesis

Carriage of TA modules (often in multiple copies) has been consistently found, both chromosomally and plasmid based, for many significant UTI causing species including *E.coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumonia* and the Proteus genus (Yamaguchi *et al.*, 2011, Donegan & Cheung 2009, Mckenzie *et al.*, 2000, Schureck et al., 2014, Doublet *et al.*, 2010). Of particular interest with regards to this study however is the pARS3 plasmid carried by a multi-aminoglycoside resistant UPEC strain. Resistance was due to the *npmA* gene encoding a Methyltransferase (Wachino *et al.*, 2007). This plasmid was found to have a region of approximately 9.1kb, a composite transposon, with high homology to the pTRACA22 plasmid, including the RelBE TA module (Jones *et al.*, 2010). This begs the question, why this module is present on a clinically relevant, resistance carrying mobile genetic element? Firstly, and most simply it could purely be due to random insertion of the Tn into pARS3 and this plasmid then

being unable to lose the module due to the addictive nature of TA systems. It may also be possible that this module is stabilising more than just the module itself ensuring the pARS3 plasmid and the associated resistance is passed on to progeny regardless of selective pressure. It may also be a combination of this addictive characteristic and ability of the module to regulate global translation together as it has often been suggested that TA modules may be a "Swiss-army knife" stress response system serving multiple purposes during different phases of an organism's life cycle (Gerdes 2013).

The concept of TA modules having multiple roles within a bacterial population has recently become a favoured hypothesis among many in this field. However, to date, very little evidence exists to support this, with the exception of their ability to stabilise plasmids. It is generally accepted that TA are able to induce persister formation and increase resistance to a number of antimicrobial classes, including the p22-RelBE module to several beta-lactams (as seen in this work) but carriage of these modules, both in the core and mobile genome often in high numbers, by pathogens, may also suggest a secondary role in these organisms as well. So far, the possibility that TA modules could be involved in bacterial virulence has largely been neglected, which is surprising due to their strong link with pathogenicity (Leplae *et al.*, 2011, Georgiades & Raoult 2011).

One of the only studies done addressing this was by De la Cruz *et al.*, in 2013 which firstly identified 11 Type II TA modules within *Salmonella typhirmurium*, secondly found many of them conserved throughout pathogenic strains but lost from non-pathogenic counterparts and finally isolated one module specifically (SehAB, a higAB-like system) which allowed increased intracellular uptake and enabled continued survival in the lymphoid organs in mouse models. This was recently further supported by a study characterising the fitness

advantage Type I and Type II TA modules provide *S. typhirmurium* inside fibroblasts (Lobato-Marquez *et al.*, 2015). Although not examined it is also possible that the SehAB system may be able to increase persister formation due to the SehA toxin mode of action and self-regulatory mechanisms of the module being similar to that of HigAB locus. It may be that TA modules play a bigger role in pathogenesis and the increasing resistance of these organisms to antimicrobial therapeutic treatment than previously realised.

As briefly mentioned when discussing the antimicrobial fosfomycin, whilst the gut microbiota plays multiple important roles within human development and gut function, some of the organisms this encompasses are also able to cause infections when migration and colonisation to other sites of the human body occurs. *E.coli* is an excellent example of one such species. Commensal and pathogenic *E.coli* can be classified into four different phylogenetic groups, A, B1, B2 and D with groups A and B1 mainly consisting of commensal strains found in the large intestine and which do not usually carry known virulence factors (Clermont *et al.*, 2000, Rodriguez-Siek *et al*, 2005) however there have been reports of these also leading to extra-intestinal infections (Rijavec *et al.*, 2008).

Specifically focussing on urinary tract infections (UTI), *E.coli* is a major cause of UTIs in humans resulting in serious expense (Foxman & Brown 2003, Litwin *et al.*, 2005) and strains able to cause these infections have been shown to persist within the gut from a few days, up to many years as part of this community (Stecher & Hardt 2008, Nowrouzain *et al.*, 2006). In parallel the gut-origin hypothesis of UTIs is further supported with clinical isolates being consistently identified within the faecal flora of the host (Moreno *et al.*, 2008) and those identified often carrying virulence genes required for colonisation of the urinary tract (Zhang *et al.*, 2002). Similarly a study conducted in 2001 found *E.coli* strains harbouring the *papG*

genes (encoding for pili adhesion proteins) were one of the major gut flora of children diagnosed with urosepsis between the age of 1 and 24 months (Jantunen *et al.*, 2001).

As with colonisation of any environment, bacterial cells must first overcome the obstacles that environment presents. Within the urinary tract these obstacles consist of antimicrobial peptides which are either able to block attachment of the organism to the epithelium, or damage the microbe before it can secure a foothold. Examples of this include the Tamm-Horsfall glycoprotein, which blocks microbial adherence to the uro-epothelium by binding to Type I fimbriae (Zasloff 2013, Saeman *et al.*, 2005). An additional factor to mention is the limitation of iron within this environment and its requirement for growth and virulence. When pathogens enter the urinary tract they encounter a period of iron starvation where a transcriptional response to this occurs promoting expression of siderophores and iron transporters (Kadner 2005).

Any role that TA modules play within this colonisation, survival and virulence within the human UT, especially during periods of antimicrobial treatment, is purely speculative. It is possible to hypothesise that whilst TA systems may not only aid survival in the human gut (based on the findings of Chapter 5), but also when the bacterium is exposed to different environments such as the within UT or perhaps even a more general stress response. TA modules have already been significantly linked to stress response for nutritional deprivation, especially that of amino acid shortage but to a lesser extent carbon as well (Christensen et *al.*, 2001). As previously discussed in chapter 5, Christensen and Gerdes (2003) suggested that it was highly probable, based on work with relE and nutritional deprivation, that the relBE TA module plays some role in general stress responses in (at least) nutritionally limited situations.

6.6 Conclusions

- Through viability plate counts, live/dead fluorescence staining and confocal microscopy it has been identified that carriage and expression of the p22-RelBE TA module enabled an increased percentage of cells to survive, over a 5 hour period, treatment with inhibitory concentrations of the beta-lactam antibiotics ampicillin and cephalexin.

- Conversely, expression of the p22-RelBE module decreased survival of *E.coli* EPI300 to treatment with clarithromycin, tetracycline and chloramphenicol.

- Expression of the p22-RelBE module did not have any effect on survival of *E.coli* EPI300 during treatment with doripenem, mecillinam, colistin E, rifaxamin, gentamicin, ciprofloxacin, sulphamethoxazole, fosfomycin and trimethoprim.

- This implies p22-RelBE has a function as a stress response element with potential to transiently aid resistance to multiple beta-lactam treatments.

Chapter 7: Phage-microbe interaction and modulation of phage replication by the p22-RelBE TA module

7.1 Introduction

Bacteria are considered the most abundant organisms on the planet; however they are dwarfed in number by their viral parasites, bacteriophages at a ratio of ten to one and they are predicted to cause approximately 10^{25} infectious events every second (Suttle 2005, Wommack & Colwell 2000, Lima-Mendez *et al.*, 2007). With the occurrence of these lethal events being so high, and the constant turnover of biological material because of this, phage predation significantly effects nutrient recycling of ecosystems and even global climate (Suttle 2005, Whitman *et al.*, 1998, Fuhrman 1999). With numbers of phage particles and individual infectious events being so high, effective mechanisms of resistance to phage infection is a requirement for bacterial populations to survive. Outside of the ocean environment the number of predicted viral particles is unclear, however due to the obligate requirement for a host; it can be assumed that phage particle numbers likely correlate in some way to bacterial/archaeal cell numbers also within that environment (Chibani-Chennoufi *et al.*, 2004). Despite mammalian abundance on the planet the numbers of prokaryotes associated with them is several orders of magnitude lower than the open ocean and sediments, soil and terrestrial sub-surfaces (Clokie *et al.*, 2011). It is approximated that there are 4.8 $x10^{23}$ prokaryotes per 220g of colonic material predicted as the average faecal content per individual (Clokie *et al.*, 2011).

While traditional culture-dependent and molecular based techniques pioneered phage diversity and abundance studies, the modern use of metagenomic analysis has unlocked the total viral components of entire environments through collection and sequencing. Through this powerful, culture-

independent technique, major insights in the study of microbial viromes have been gleaned, for example in ecosystems associated with the human body such as the human gut (Minot *et al.*, 2011, Reyes *et al.*, 2010, Ogilvie *et al.*, 2013). Ogilvie & Jones defined a virome as "the total population of viruses [or virus like particles (VLPS)] associated with the underlying microbial community" (Ogilvie & Jones 2015) with specific focus on that of the human gut.

More traditional molecular approaches have been utilised with some success for phage quantification and prokaryotic phylogenetic placement using 16S rRNA genes as a marker. This is because there are no genes which are suitably conserved between phage taxonomic groups (Paul *et al.*, 2002). However within these groups, such as marine cyanophages, some markers have been utilised successfully, an example being the g20 gene encoding a viral capsid assembly protein (Zhong *et al.*, 2002). Furthermore primers used for this have also been used to successfully investigate these sequences in T4-type phage (Filee *et al.*, 2005). Other molecular approaches have also been employed to investigate diversity such as restriction fragment length polymorphisms (RFLP) or denaturing gradient gel electrophoresis (Casale *et al.*, 2011).

7.1.1 The human gut virome

Unlike several of the marine and terrestrial environments mentioned above, it is believed that VLPs in the human gut exist at similar levels to their prokaryotic hosts (~10^9/g of feces) based on microscopy counts (Kim *et al.*, 2011, Reyes *et al.*, 2010, Minot *et al.*, 2011, Ogilvie & Jones 2015). There is a degree of inter-individual variation seen within the human gut-microbiomes between hosts and this variation is larger in host-associated viromes (Minot *et al.*, 2011). It has also

been shown that viromes tend to be more similar when dietary intake was also similar (Minot *et al.,* 2011). It has been suggested this reflects 'dietary induced convergence' which has also been identified in their bacterial hosts (Wu *et al.*, 2011). The high variation seen between individuals has been linked firstly to variation in bacterial hosts (Huttenhower *et al.*, 2012) and secondly to the plasticity of phage genomes and their ability to form novel types (Minot *et al.*, 2013). Investigation into temporal variation of human gut viromes has suggested remarkable stability over time with >95% of viral genotypes persisting with minor relative abundance changes over a 1 year period and 80% of these being retained over 2.5 years (Minot *et al.*, 2011).

Perhaps the most notable feature of human gut viromes, compared to environmental communities, is dominance of a temperate lifestyle rather than the more commonly seen lytic tendencies and Red Queen dynamics which refers to the constant change in dominance within a community (Van Valen 1973, Reyes *et al.*, 2010). Through the use of several different methods including; integrase presence and abundance, nucleotide identity to sequenced bacterial genomes which is often indicative of prophage formation and similarity between proteins and prophages annotated in the ACLAME database (Leplae *et al.*, 2009), it was identified that a substantial portion of phage within human gut samples were temperate in nature (Minot *et al.*, 2011, Reyes *et al.*, 2010). Phage which induce lysogenic conversion can alter phenotypes which, in turn, affects bacterial host fitness and can significantly impact on the dynamics within this environment (Thingstad *et al.*, 2008, Minot *et al.*, 2011, Duerkop *et al.*, 2012).

7.1.2 Bacteriophage resistance systems

Throughout the history of bacterial and bacteriophage evolution, the ever-present viral-host interplay has led to the development of multiple phage-resistance mechanisms and counter-mechanisms (Labrie *et al.*, 2010). Specifically focussing on the bacterial perspective, some of the most commonly employed methods to avoid phage predation include actively preventing DNA injection through restriction-modification systems, the altering of cell surfaces to avoid phage adsorption, phage-specific immunity due to clustered regularly interspaced short palindromic repeats (CRISPRs) and abortive infection systems (Chopin *et al.*, 2005). These systems are summarised in Table 7.1.

7.1.3 Abortive infection systems and Type III TA modules

Bacteria carry a large array of heterologous proteins, which provide resistance through cellular suicide during phage infection. These abortive infection (Abi) systems altruistically kill the infected cell unlike other anti-phage systems detailed in Table 7.1 by targeting an essential stage in the phage lifecycle. This behaviour is presumed to occur to allow the remaining, non-infected population to escape phage infection (Samson *et al.*, 2013).

Table 7.1: A summary of phage resistance mechanisms identified to date.

Method of phage resistance	Subcategory	Description	Examples & Sources
Prevention of phage adsorption	Phage receptor blocking	Adaption of structure/three-dimensional confirmation of cell surface receptors.	*S. aureus* 'immunoglobulin G-binding protein A' is able to bind the Fc fragment of immunoglobulin G, reducing phage adsorption by masking the receptor (Foster 2005, Nordstrom & Forsgren 1974).
	Production of extracellular matrix	Extracellular polymers that provide a physical barrier between phage and their receptors.	Alginate (an exopolysaccharide) producing *Azotobacter spp.*, displayed an increased resistance to phage infection however phage which could recognise and degrade this using alginate lyase increased adoption (Sutherland 1995, Hanlon *et al.*, 2001, Hammad 1998).
	Competitive inhibition	Molecules naturally present in environment able to bind specifically to the phage receptor, reducing capacity for phage binding.	*E.coli* iron transporter FhuA is the receptor for both coliphages and the antimicrobial molecule microcin J25. J25 can outcompete phage binding (Destoumieux-Grazon *et al.*, 2005).
Prevention at DNA entry	Super-infection exclusion (Sie) systems	Inhibition of DNA entry into cell cytoplasm by changing the conformation of the injection site or preventing degradation of the cell wall or membrane.	Coliphage T4 encodes two sie systems, *imm* & *sp. imm* has two transmembrane domains localised in the cell membrane (Lu *et al.*, 1993). *sp* inhibits activity of T4 lysozyme thus preventing degradation of peptidoglycan (lu *et al.*, 1994, Moak & Molineux 2000).
Degradation of phage nucleic acid	Restriction modification (R-M) systems	Cleave incoming, foreign nucleic acid whilst methylating bacterial DNA to protect it from restriction.	*E.coli* CT596 is able to attack glycosylated DNA as it possesses a glucose modified restriction system, however some T4-like phage can disable this activity by injecting mature internal protein Inhibitor* along with the genome.(Pingoud *et al.*,

			2004, Pingoud 2004)
	CRISPR-Cas systems	Clustered regularly interspaced short palindromic repeats and associated genes. Usually 21-48bp direct repeats and 26-72 non-repetitive spacers.	Phage insensitive mutants of Streptococcus thermophiles shown to have gained new repeat-spacer at the 5' end of the repeat-spacer region. This is 100% identical to the proto-spacer within the phage genome (Mojica *et al.*, 2005, Mojica *et al.*, 2009, Brouns *et al.*, 2008)..
Abortive infection	-	Abortive infection system (Abi) is a catch-all term covering a range of disparate systems with phage infected cells undergoing cellular suicide to protect the larger population.	- λ-lysogenic *E.coli* Rex system (Snyder 1995) *lit* gene (Snyder 1995) *PrrC* anticodon nuclease (Snyder 1995) 20+ Lactococcal Abi systems (Chopin *et al.*, 2005)
	Toxin-Antitoxin systems	Generally two gene systems encoding a relatively stable proteinaceous toxin and more labile antitoxin, with expression of both being tightly controlled.	The Type III ToxIN system has been shown to abort phage infection (Fineran *et al.*, 2009) whilst the Type I Hok-Sok* system prevents T4 phage infection (Pecota & Wood 1996). Finally the Type II MazEF system has been shown to provide protection against Phage P1 (Hazan & Engelberg-Kulka 2004).

Typically these systems consist of a single protein or protein complex and are often found on module genetic elements such as prophages, plasmids enabling easy dissemination throughout populations and even communities.

Some of the most abundant Abi systems to be described have been found in the gram-positive species *Lactococcus lactis*, a species used extensively in the production of cheese (Madigan *et al.*, 2009) and thus resistance to phage infection an important phenotypical trait to obtain. These systems (or homologs of) have been identified in multiple genera spanning both Gram positive and negative species including *Shigella dysenteriae*, *Vibrio cholerae* and *E.coli* (Smith et al., 1969, Chowdhury et al., 1989, Snyder 1995). To date, at least 23 Abi systems have been identified within this species (Chopin *et al.*, 2005, Samson *et al.*, 2013a), designated alphabetically, however the exact mode of action of many of these is still unknown although some phage able to bypass these have been characterised. The Abi phenotype is usually mediated by a single host gene, although there are cases of Abi systems consisting of up to four. These systems have been shown to effect: DNA replication (such as AbiA, F, K, P and T) (Hill *et al.*, 1990, Garvey *et al.*, 1995, Emond *et al.*, 1997, Domingues *et al.*, 2004, Bouchard *et al.*, 2002), RNA transcription (as seen with AbiB, G and U) (Dai *et al.*, 2001, Cluzel *et al.*, 1991, O'Conner et al., 1999), limit production of capsid proteins (with the only example of this so far identified in AbiC) (Durmaz *et al.*, 1992) and packaging of phage DNA (Abi E, I and Q) (Emond *et al.*, 1998).

7.1.3.1 The Hok/Sok System

The first report of TA modules preventing/reducing phage infection was seen in 1996, while investigating the Type I Hok/Sok system (Pekota & Wood 1996). Investigations into the ability of a plasmid based Hok/Sok loci to convey phage resistance in *E.coli* displayed continued growth of an exponential phase culture with carriage of this module when infected/co-incubated with T4 (Pekota & Wood 1996). This was compared to an *E.coli* population lacking the TA module but not the pBR322-based plasmid. Additionally, efficiency of plating of T4 was decreased, as was plaque size and time required to produce mature phage went from 22 minutes to 30 minutes (Pekota & Wood 1996).

It is possible that resistance to T4 is seen because of its ability to disrupt transcription whilst other phage do not affect cell metabolism in the same way. Host transcription declines by 60% within 2 minutes of infection, gradually increasing up to 99% by the 13 minute mark (just before cell lysis)(McCorquodale 1975), this would also reduce the levels of antitoxin present, liberating the Hok protein which can then disrupt the cell membrane leading to death (Gerdes *et al.*, 1990a). This would seem plausible, as the half-life of Hok is approximately 20 minutes, much longer than the 30 seconds of Sok (Gerdes et al., 1990b). With cell death occurring so rapidly, it is likely that very few mature phage particles would be produced, effectively providing resistance to the rest of the population. Interestingly while the effect on Hok/Sok carriage was also examined on other phage such as T1, T5, T7 and λ, none of these had their ability to infect reduced, perhaps due to their inability to effect cellular transcription to a large enough extent, such as λ (Wagner *et al.*, 1980).

7.1.3.2 The ToxIN System

'Abi systems' is a catch-all term commonly used to encompass a wide range of disparate and often completely unrelated systems which eventually lead to cell death during phage infection. It is therefore unsurprising that some TA models, with their extensive dissemination in bacteria, have been associated with phage resistance and this phenotype. In fact, while some of the *Lactococcal* Abi systems are often used to describe generic abortive infection phage resistance mechanisms (Chopin *et al.*, 2005), others have even been shown to have high homology to TA systems, specifically those belonging to the Type III subgroup (Blower *et al.*, 2012). Furthermore, of the *Lactococcal* Abi systems identified, at least 6 of these are known to be bicistronic often with one of the components being toxic to the host cells upon overexpression (Bidnenko *et al.*, 2009, Gerdes *et al.*, 2013), a trait remarkably similar to that of conventional two-gene TA modules.

The ToxN toxin of the ToxIN TA module, first identified in *P.atrosepticum* was recently shown to have high homology with AbiQ from *L.lactis*. Interestingly the TA loci provided high-level resistance against various phages and was found to be functional across a multiple different bacterial genera (Fineran *et al.*, 2009, Blower *et al.*, 2009, Blower *et al.*, 2012). ToxN is cytotoxic through its action as an endoribonuclease whilst expression of ToxI, a repetitive untranslated RNA antitoxin, inhibits this toxicity (Fineran *et al.*, 2009, Blower *et al.*, 2009). As with many TA modules, the module regulates its own expression through a negative feedback mechanism with ToxN RNase activity cleaving toxI transcripts, releasing the small RNA antitoxins, inactivating itself (Blower *et al.*, 2011, Dy *et al.*, 2014). Although TA modules have previous (albeit rarely) been linked to various forms of phage resistance ToxIN is the first direct functional link between TA and Abi systems (Fineran *et al.*, 2009, Blower *et al.*, 2011).

Recently Blower *et al.* hypothesised a model for ToxIn mediated abortive infection with constitutive transcription of the two-gene system under normal conditions to ensure an excess of antitoxin transcript (Gerdes et al., 2013). With no phage present, inhibitory ToxI RNA pseudoknots are bound to the ToxN protein inactivating it (Figure 7.1a). When phage infection occurs the inhibition placed on ToxN by ToxI interaction is removed (although the mechanism controlling this remains unknown) leading to the rapid degradation of RNA, arresting cell growth and additionally replication of phage, but eventually leading to cellular death (Figure 7.1b).

7.1.3.3 The MazEF System

The *E.coli* MazEF chromosomal TA pair is the only Type II module to have been linked with phage resistance and programmed cell death (PCD) to date (Hazan & Engelberg-Kulka 2004). MazF encodes a stable toxin, which cleaves mRNA specifically at ACA-sequences whilst the MazE gene encodes its complimentary, labile and proteinaceous antitoxin (Zhang *et al.*, 2003). As has been previously shown with multiple TA modules, especially those belonging to the Type II subgroup, reduced metabolic activity and cell death can often be induced through presence of the host cells within stressful conditions (Gerdes *et al.*, 2013). This is achieved through inhibition of further expression or through over expression of both components whilst the antitoxin is quickly degraded. Phage infection is one such condition. In 2004 a study by Hazan & Engelberg-Kulka found evidence to suggest that the Type II MazEF system could inhibit the spread of prophage P1 when converted to its lytic form through thermo-induction (Hazan & Engelberg-Kulka 2004).

Unfortunately it appears no further investigation has been undertaken on Type II modules conveying phage resistance, however it is entirely possible that MazEF and other TA systems could also undertake this physiological function, especially considering the multiple confirmed functions (often of the same module) across multiple different environments. With this in mind investigation into the gut-associated p22-RelBE TA module was undertaken to explore whether this module could provide protection for *E.coli* when challenged with phage isolated from sewage and from the human gut microbiome.
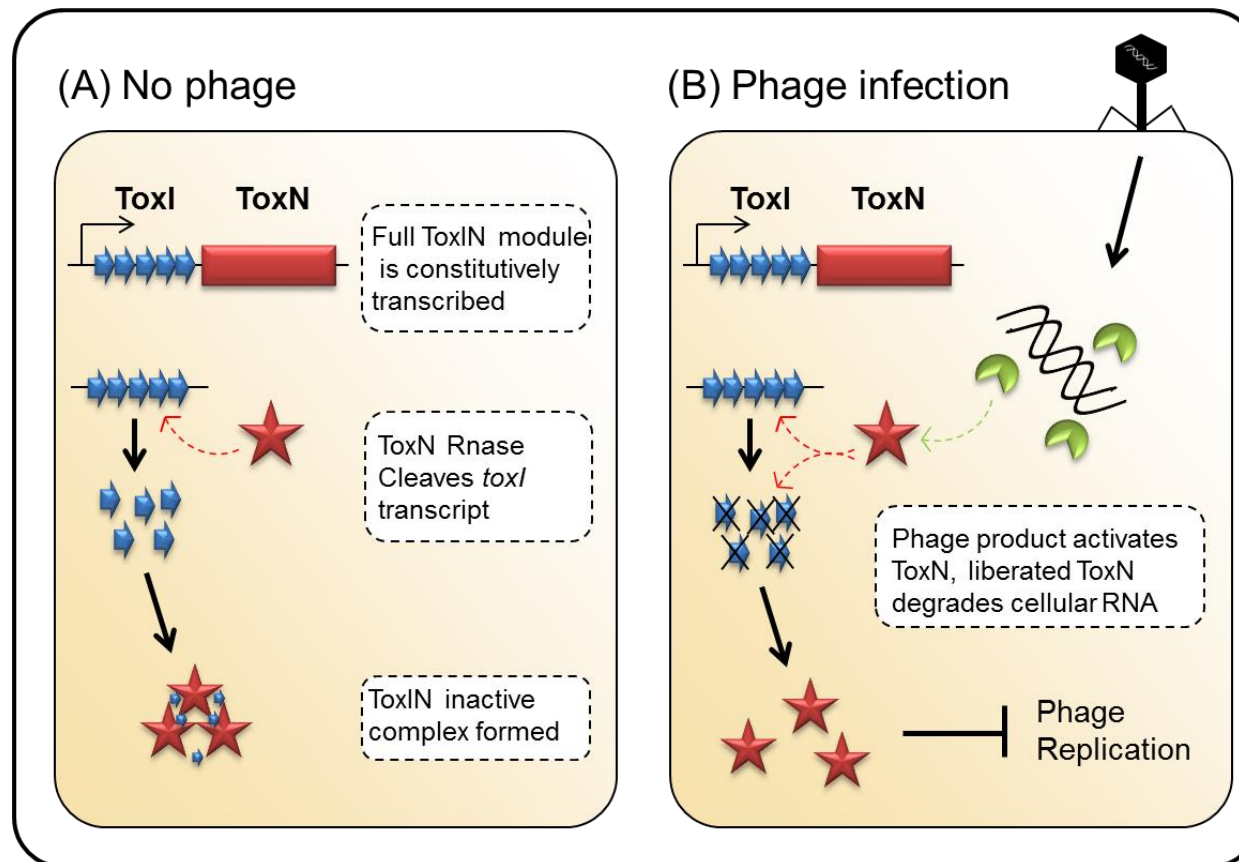
Figure 7.1: Hypothesised model for abortive infection by ToxIN TA module. **(A)** Demonstrates ToxIN activity under normal growth conditions whilst **(B)** Depicts activity of the module during phage infection. ToxI and ToxN are shown in blue and red respectively and red arrows represent ToxN RNase activity. ToxI is shown as multiple blue arrows to represent the tandem repeats of the gene. The hypothesised phage product which stimulates ToxN activity is shown as green (Adapted from Gerdes *et al.*, 2013).

7.2 Aims

The aims of the work presented in this chapter were to isolate bacteriophage capable of infecting *E.coli* EPI300 from a human faecal sample, characterise those phage isolated and identify the effect expression of the pTRACA22 RelBE TA module has on EPI300 when challenged with those gut-associated bacteriophage in parallel to those isolated from sewage.

7.3 Materials and methods

7.3.1 Isolation of bacteriophage infecting *E.coli* EPI300 from human faecal material

7.3.1.1 Faecal water preparation

A stool sample of approximately 15 grams in weight was collected from a 26 year-old, healthy, Caucasian male who had undergone no antibiotic treatment in the 6 months prior to collection. Ethical approval for this study was granted by the University of Brighton, School of Pharmacy and Biomolecular Sciences ethics committee. The stool sample was homogenised in 20 mL of phage buffer ($NaHPO_4$ anhydrous 7 g/L, KH_2PO_4 anhydrous 3 g/L, NaCl 5 g/L, 1 mMol $MgSO_4$, 1mMol $CaCl_2$) before being centrifuged for 10 minutes at 5000 xg for 15 minutes to remove bacteria and particulate matter. This was then filter-sterilised using a 0.22 µM Minisart filter (Fisher, UK) into a sterile 50 mL Falcon tube. This will be subsequently referred to as faecal water and was stored at 4 ^{o}C in and protected from light for no longer than 2 weeks.

7.3.1.2 Bacteriophage enrichment from faecal water

2 mL of a mid-log phase *E.coli* EPI300 (OD_{600} 0.5) culture was added to 100mL of Tryptone soya both (TSB, Oxoid UK) and incubated for 1 hour at 37 ^{o}C with shaking at 150 rpm. After 1 hour, 2 mL of filter sterilised faecal water was added and the culture incubated for a further 18 hours under the same conditions. Following this, the culture was centrifuged at 5000 xg for 15 minutes, the supernatant removed and filter-sterilised using a 0.22 µM Minisart filter in 20 mL, sterile, glass universals. The phage-enriched samples were also stored at 4 ^{o}C and protected from light for no longer than 2 weeks.

7.3.1.3 Bacteriophage isolation

Both the unenriched faecal water and enriched samples were used to isolate gut-associated bacteriophage using the double agar plating method. A culture of *E.coli* EPI300 was grown in TSB for 4 hours at 37 ^{o}C with shaking at 150 rpm. In Parallel, 5 mL soft overlay 0.4% (w/v) Tryptone soya Agar (TSA) was prepared using TSB and technical agar (Oxoid, UK) in 10 mL sterile bijou bottles, sterilised and allowed to cool to 45°C using a water bath. In addition regular TSA plates were prepared and dried for 1 hour using a laminar airflow cabinet while serial dilutions from neat to 10^{-5} were prepared of the faecal water and enriched samples using phage buffer. 0.1 mL of mid-log phase *E.coli* EPI300 was added to each bijou along with 0.1 mL of the serially diluted faecal water and enriched samples, mixed briefly by swirling before being poured uniformly, onto the pre-dried TSA base plates. Once set, all double agar plates were incubated for 18 hours at 37 ^{o}C along with control plates of no bacteria, no phage and no bacteria or phage.

7.3.2 Purification of bacteriophage isolates.

Finally after the presence of phage had been established through plaque production, individual isolates were purified. These were distinguished based on plaque size and morphology and several for each were picked using sterile Pasteur pipettes. Plaques were then suspended in 1 mL of phage buffer overnight in Eppendorfs at 4°C and protected from light. These were enriched against *E.coli* EPI300 again using the double agar plating method to ensure purity of each isolate. If necessary, isolates were stored for up to 1 week at 4°C and protected from light.

7.3.3 Production of high titre bacteriophage stocks

To generate high titre stocks of bacteriophage isolated from the human gut, phage previously isolated from sewage, T4 and λ, 1 mL of an 18 hour *E.coli* EPI300 culture was added to 100 mL of sterile TSB. This was incubated at 37 ^{o}C with shaking (150 rpm) for approximately 4 hours until an OD_{600} of 0.5 was reached. 1 mL of the previously isolated phage was added to the suspension, mixed gently and statically incubated for 15 minutes at 37 ^{o}C to allow phage to adsorb to the host cells. Shaking was then resumed and the culture incubated for 18 hours. 10 mL of chloroform was added to lyse the remaining cells/release mature phage particles and the culture incubated for a further 15 minutes. 5.84 g of NaCl was added to give a 1 Mol concentration and slowly dissolved on ice over a 1 hour period. The entire lysed culture was then centrifuged at 11,000 xg for 10 minutes at 4 ^{o}C. The method used was adapted from Yamamoto *et al.*, 1970.

The resulting supernatant was transferred to a sterile 250 mL conical flask and 10 g of polyethylene glycol (PEG) 8000 was added and dissolved at RT. When completely dissolved the conical flask was wrapped in foil to protect from light and incubated once again at 4 ^{o}C for 18 hours. Once complete, the phage preparation was transferred to a 150 mL chloroform-resistant and sterile centrifuge tube and centrifuged at 11,000 xg for 10 minutes also at 4 ^{o}C. The supernatant was subsequently discarded and the pellet resuspended in 2 mL of phage buffer and transferred to a 50 mL chloroform resistant Falcon tube (Alpha laboratories, UK). 2 mL of chloroform was added, and the tube mixed vigorously by vortex for 30 seconds. This was separated into layers by centrifugation at 3000 xg for 15 minutes at 4 ^{o}C. The top phage buffer layer containing the high titre phage stock was carefully drawn off and transferred to a sterile bijou bottle. This was then wrapped in foil to protect from light and stored at 4 ^{o}C.

7.3.4 Bacteriohage storage

All phage used were kept at both $4^{o}C$ and $-80^{o}C$ for long-term storage. For storage at $4^{o}C$, the phage stock was transferred to a sterile glass universal tube, sealed with parafilm and wrapped in foil to protect them from light. For storage at $-80^{o}C$ stocks were transferred to a sterile cryovial and sterile glycerol added to 20% (v/v) final concentration. This was then sealed with parafilm and wrapped in foil to protect from light before being frozen at $-20^{o}C$ for 24 hours. Finally this was transferred to $-80^{o}C$.

7.3.5 Transmission electron microscopy

The structure of individual bacteriophage isolates were examined using a Zeiss SIGMA field emission gun scanning transmission electron microscope (FEG-STEM) at 20 kV of accelerating voltage a 20 μM aperture and 2.7 mM working distance. 24 hours before visualisation, purified phage particles (10^{10} PFU/mL) were immobilized on 200-mesh Formvar/carbon filmed copper electron microscope grids (Agar Scientific, UK). A single drop was placed onto the grid, left for one minute and then drawn off using sterile filter paper. This was repeated three times followed by a single drop of 2% (w/v) phosphotungstic acid (pH of 7.4) for 1 minute. This was subsequently drawn off and the grid left to air dry for 5 minutes before being transferred to a sterile petri dish for storage. The entire process was conducted at RT.

7.3.6 Effect of p22-RelBE TA gene expression on phage replication

To investigate the ability of the p22-RelBE module to provide resistance to phage infection, a double agar plating assay was designed which normalised results by Cfu/mL. This was to ensure that any reduction in plaque number was not due to a drop in host cell number because of p22-RelBE module carriage and/or expression. Furthermore live/dead cell counts were conducted as with survival assays in previous chapters (Section 5.3.10).

1 x 100 mL volume of LB containing 0.2% (w/v) glucose was inoculated with an overnight culture of *E.coli* EPI300 harbouring p22-RelBE constructs and grown until an OD_{600} of 0.3. These were then pelleted by centrifugation at 5000 xg for 5 minutes, washed 3 times in sterile PBS before being resuspended in fresh, pre-warmed LB containing the required supplement for expression of p22-RelBE genes (100 mMol rhamnose or 2% (w/v) arabinose). Cultures were further incubated until the WT reached an OD_{600} of 0.5. 100 µL samples of these were then added to molten (45°C) overlay agar along with 100 µL of a 1x 10^{8} Pfu/mL stock of the phage of interest. The overlay was then mixed gently before being poured over a pre-dried agar plate. All plates were then incubated at 37°C for 18 hours. A further 2 x 1 mL samples were also taken, pelleted by centrifugation at 5000 xg for 5 minutes, washed 3 times in PBS before being resuspended in fresh PBS. One of these samples was then serially diluted from neat to 10^{-10} before being incubated for 18 hours at 37°C. Viable counts were performed as in Section 4.3.9 to identify the Cfu/mL of each culture. The second sample taken was stained using LIVE/DEAD® BacLight Bacterial Viability Kit (as in Section 4.3.10) to calculate the percentage of live cells. All agar and overlay agar used within this study contained the inducer for the construct being used. If the construct was a control 0.2% (w/v) glucose was added instead. The data was presented as

nPFU/mL (normalised by viable cells PFU/mL) with percentage live data used to confirm constructs were not causing cell death.

As a further control to ensure the sugars used to induce and repress expression of the p22-relBE module did not affect the phage lifecycle or infection process, *E.coli* EPI300 WT was grown in LB containing 0.2% (w/v) glucose, 2% (w/v) arabinose and 100 mMol rhamnose until an OD_{600} of 0.5. 100 µL of $1x10^8$ phage was added to soft overlay agar along with 100 µL of the cell suspension and a double overlay plating assay was undertaken with agar also containing the sugars at the same concentrations and Pfu/mL identified.

7.3.7 Characterisation of phage replication

7.3.7.1 For all phage isolated

To assess the characteristics of a single replication cycle for phage isolated, one-step growth curves were undertaken. A culture of *E.coli* EPI300 was grown in LB over 18 hours at 37 ^{o}C with shaking at 150 rpm. This was then used to inoculate 2 x 100 mL of fresh LB and allowed to reach an OD_{600} of ~1.0 (~$1.0x10^9$ Cfu/mL) at 37°C, also with shaking. One of these cultures would be used to create a bacterial lawn in the double agar plating method used. 1 mL of the other 100 mL culture this was removed and 100 µL of 10^{11} PFU/mL was added to give a multiplicity of infection of 10:1 (phage:cells). This was incubated for 1 minute at 37°C without shaking. The 1 mL infected culture was briefly centrifuged at 17,000 xg for 1 minute to pellet the cells, the supernatant discarded and resuspended in 1 mL of fresh LB. This was then returned to the remaining 99 mL uninfected culture and the initial 0 minute time-time point sample taken.

1 mL samples were taken every 4 minutes. 500 µL of each sample was lysed by the addition of 100 µL chloroform, centrifuged at 17,000 xg for 1 minute and the supernatant carefully removed to ensure no chloroform was also drawn off. This was to release and calculate total phage. The remaining 500 µL sample was used to calculate free phage only. Both 500 µL samples were then serially diluted and plated using the double agar plating methodology described in Section 7.3.1.3 with the exception of LB agar used in place of TSA. Data obtained was then used to identify the length of the latent period, total length of the replication cycle and burst size. Burst size was calculated as final total phage titre/initial total phage titre.

7.3.7.2 Phage presenting reduced PFU with EPI300 expression of p22-RelBE genes

When phage exhibited lower PFU/CFU per mL due to expression of p22-RelBE TA genes (Section 7.3.6), further one-step growth curves were conducted following the methodology in Section 7.3.7.1. In addition to the WT, EPI300 harbouring each of the p22-RelBE plasmids and constructs were also used with and without expression of genes. When expression of the RelBE genes were required 100 mMol rhamnose or 2% (w/v) arabinose was added to the remaining 99 mL of culture before that phage-infected 1 mL cell suspension was returned to the total culture.

7.4 Results

7.4.1 Bacteriophage acquired and isolated

Bacteriophage isolated from faecal samples along with others previously isolated from sewage and well known reference phage that were utilised within this study are tabulated in Table 7.2. This includes a description of the phage, notable plaque morphology and the source.

7.4.2 Morphology of gut-associated bacteriophage

To further characterise the bacteriophage isolated, scanning transmission electron microscopy (STEM) was undertaken for phage A1, B1, B2, BM13 and BM15 (Table 7.2). Clear images were obtained at magnifications of between 50000x to 1000000x for all phage except A1. The morphology of these phage can be seen in Figure 7.2. Phage B1, B2 and BM13 appear to be myoviridae whilst micrographs of BM15 show a siphoviridae-like form. In size, the largest was B1 at 120 nm in length while both gut-associated phage were approximately 80 nm in length. All phage examined appear to have an icosahedral capsid, with BM13 appearing to have a collar.

Table 7.2: A summary of phage isolated and used within this study and subsequent experiments.

Name	Description	Source	Reference(s)
T4	Double stranded genome ~169 kbp in length. Consists of a icosahedral head, hollow tail and fibres to recognise and aid host attachment. Plaque size ~ 4mm in diameter. Smooth edged.	Thought to have originated from either sewage or faecal matter	Demerec & Fano 1945, Delbruck 1946, Miller *et al.*, 2003. Yu & Mazushima 1982.
Coliphage λ	Double stranded genome ~48 kbp in length. Also Myoviridae in structure. Under certain conditions, lysogenic lifecycle can be followed. Plaque size ~3 mm in diameter. Smooth edged.	*E.coli* pure cultures treated with UV light	Lederberg & Lederberg 1951, Kobiler 2007.
A1	Plaque size ~1.5 mm in diameter. Smooth edged.	Sewage water	Used with permission from Mrs Cinzia Dedi.*
B1	Plaque size ~1 mm in diameter. Smooth edged.	Sewage water	Used with permission from Mrs Cinzia Dedi.*
B2	Plaque size ~1 mm in diameter. Smooth edged.	Sewage water	Used with permission from Mrs Cinzia Dedi.*
BM13	Isolated from the human gut microbiome of a healthy adult male. Plaque size ~0.5-1 mm in diameter. Smooth edged and clear.	Faecal sample	This study
BM15	Isolated from the human gut microbiome of a healthy adult male. Plaque size ~0.5-1 mm in diameter. Smooth edged with a very faint and thin halo.	Faecal sample	This study

*School of Pharmacy and Bimolecular Sciences, University of Brighton.
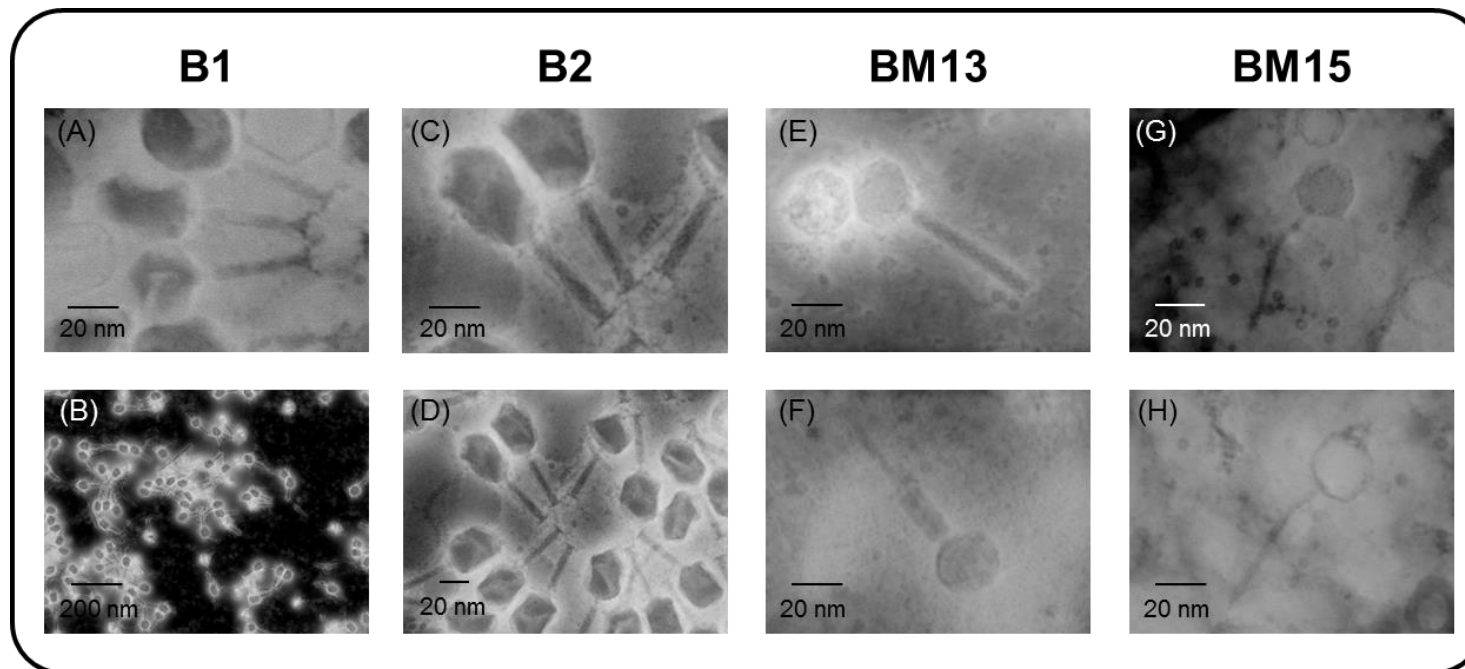
Figure 7.2: STEM micrographs showing the morphology of two previously isolated phage from sewage samples and two phage isolated from the human gut. (**A**) and (**B**) Morphology of phage B1 at two different magnifications. It is Myoviridae-like in structure and approximately 120 nm in length. (**C**) and (**D**) Phage B2, also myoviridae shaped. B2 is approximately 100 nm in length and appears to have a slightly shorter, granulated tail with a thick plate at the base of this. Thin tail fibres are also faintly visible. (**E**) Phage BM13 morphology. Also with a myoviridae structure, BM13 appears to have a longer and thicker tail with no visible tail fibres approximately 50 nm in length. The head structure is slightly smaller than the previous two at ~30 nm diameter. (**F**) Also BM13 at the same magnification however the unsheathed tail is visible. (G) and (H) Phage BM15 morphology. BM15 is approximately 80 nm in length and siphoviridae in form. The second micrograph shows a phage-like particle lacking the genomic content of the head component.

7.4.3 Bacteriophage reaction to the presence of p22-RelBE-construct inducers and repressors

To assess the effect glucose, arabinose and rhamnose had on the ability to infect *E.coli* EPI300 for the 7 phage examined within this chapter, double agar plating assays were carried out for all 7 phage using *E.coli* EPI300 WT. Following plating in agar with no supplement, 0.2% (w/v) glucose, 2% (w/v) arabinose and 100 mMol rhamnose no significant differences in Pfu/mL could be seen ($P \geq 0.05$ using one way ANOVA for each phage between supplements). See Figure 7.3. This suggests that none of the sugars interfere with phage infection for any of these phage or the production of mature phage particles that are able to replicate. These sugars could then be used to repress/induce expression of the p22-RelBE module and the individual toxin and antitoxin component in phage resistance assays.

7.4.4 Effect of p22-RelBE carriage and gene expression on EPI300 susceptibility to bacteriophage

To assess the potential for the p22-RelBE TA module to confer resistance to replication of phage for *E.coli* EPI300, a plaque assay was designed using the double agar plating method for calculate the Pfu/mL which was normalised by viable cells (nPFU/mL). Furthermore the percentage of live cells was examined by confocal microscopy and fluorescent staining to ensure constructs were not causing cell death. Results for these are shown in Figures 7.4.
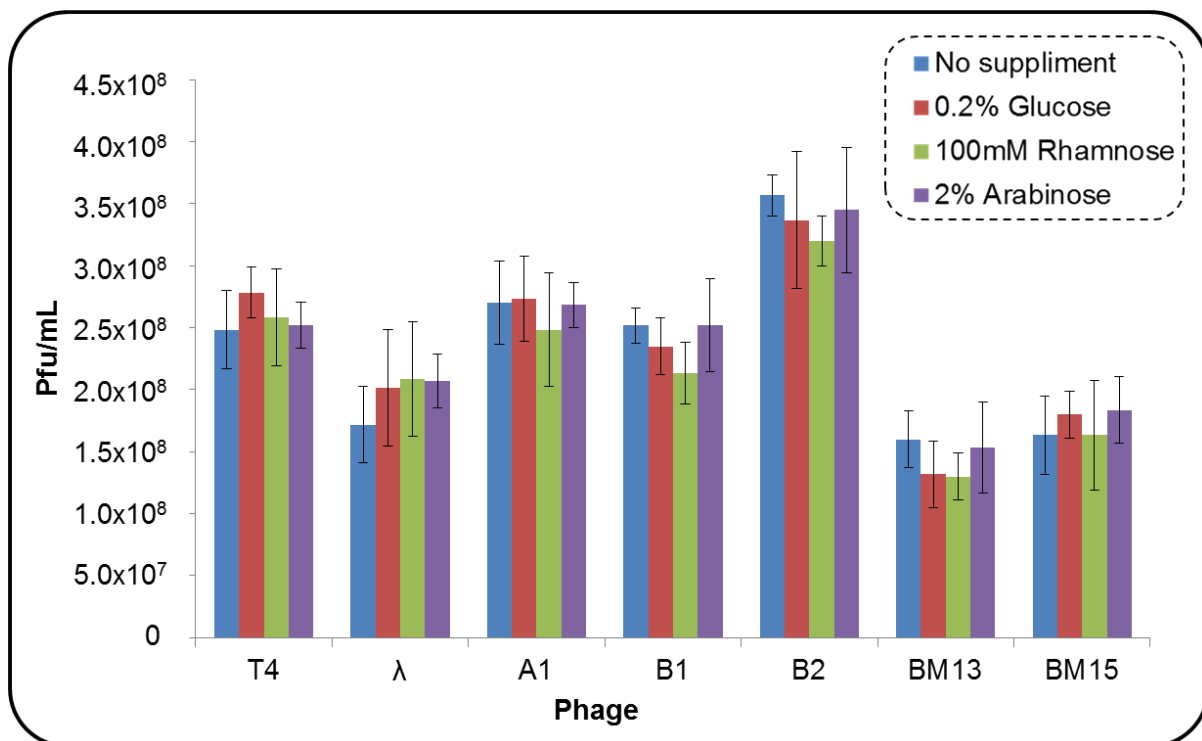
Figure 7.3: Plaque forming units per mL of 7 phage infecting _E.coli_ EPI300 WT grown in the presence of sugar supplements required to induce/repress the p22-RelBE TA module. _E.coli_ EPI300 WT was grown in LB containing 0.2% (w/v) glucose, 2% (w/v) arabinose and 100mM rhamnose until an OD600 of 0.5 (1.8×10^8 CFU/mL). 100 µL of 1×10^9 phage (MOI 1:1) was added to soft overlay agar along with 100 µL of the cell suspension and a double overlay plating assay was undertaken with agar also containing the sugars at the same concentrations and Pfu/mL identified. Results shown are the mean of three independent replicates and error bars represent standard error. There were no significant differences in PFU/mL for all 7 phage examined in the presence of glucose, rhamnose and arabinose when compared negative control (no supplement).
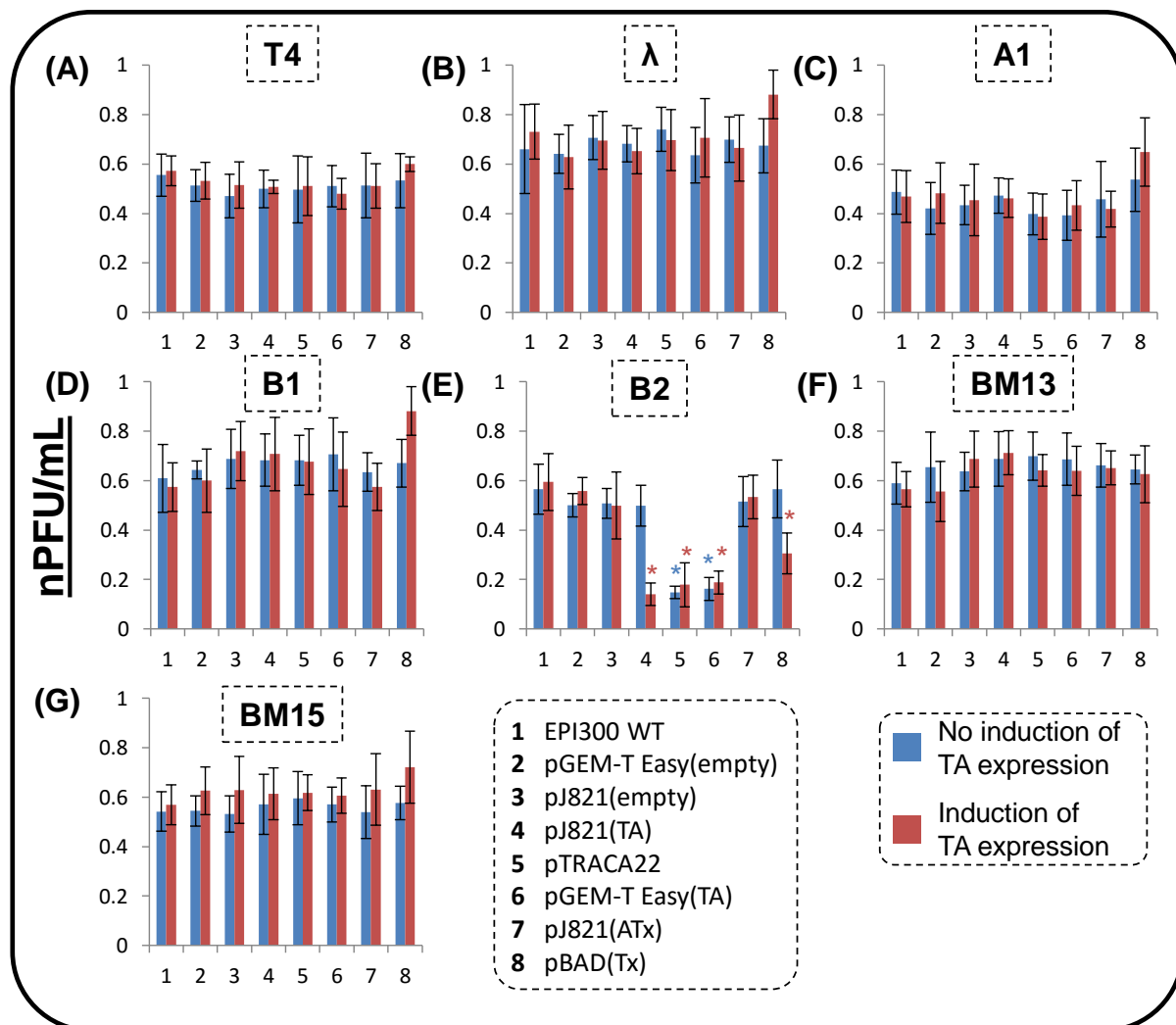
Figure 7.4: Phage resistance assay of *E.coli* EPI300 expressing p22-RelBE plasmid constructs during infection by multiple phage of interest. Cells were grown to an OD_{600} of 0.3 before having expression of p22-RelBE (and individual components) induced. These were grown until OD_{600} of 0.5 then plated in the presence of phage T4 and Pfu/mL calculated. Data was then normalised by viable cells (CFU/mL) to account for effects of TA expression on growth of *E.coli* (nPFU/mL). (A) Phage T4, (B) Phage λ, (C) Phage A1, (D) Phage B1, (E) Phage B2, (F) Phage BM13 and (G) Phage BM15.

All experiments were undertaken in triplicate and results shown are the mean ±SEM of three independent experiments.

* = A significant difference in Pfu/mL (P<0.05) when compared to the WT (EPI300 WT).

In comparison to *E.coli* EPI300 WT and EPI300 harbouring the control constructs (pGEM-T(empty), pJ821(empty)), carriage and expression of full TA module under control of the natural promoter (pTRACA22, PGEM-T Easy(TA)), showed a significant decrease in nPFU/mL for phage B2 (P<0.05, Figure 7.4E). This was also seen when expression of the full module (pJ821(TA)) and individual RelE toxin (pBAD(Tx)) was induced (P<0.05, Figure 7.4E). This was not seen for any of the other phage investigated in this study. This decrease appeared to be larger for induced expression of the module rather than expression from the natural promoter however the difference was not significant. The percentage of live cells for these cultures seemed to increase slightly, but again, it was not found to be significant when compared to control-constructs cultures. In addition live/dead fluorescence staining identified a decrease in percentage live cells for all *E.coli* EPI300 cultures expressing the pBAD(Tx) toxin construct prior to incubation with phage (P<0.05). No other significant decreases in percentage live cells were seen during expression of p22-RelBE constructs.

7.4.5 Bacteriophage replication characterisation

To elucidate the process of infection for each phage to be examined later one-step growth curves were employed allowing the latent period, burst size, time for one replication cycle to be completed, total Pfu and free Pfu to be determined. Results can be seen in Figure 7.5. Values for latent period, total length of the replication cycle and burst size can be seen in Table 7.3. Of the seven phage investigated, BM15 had the longest replication cycle, taking 47.5 minutes to complete. In comparison the shortest cycle was that of phage B2, which took approximately 32.5 minutes to complete one cycle of replication. Phage A1 had the highest burst size yield with ~98 phage-like particles being released per infected cell, while

BM15 had the smallest of approximately 8. Very few similarities between phage investigated can be seen with the exception of both gut-derived phage having a burst period of 20 minutes with yield and latent phases were being relatively similar also. No significant differences were seen with the addition of 0.2% (w/v) glucose, 100mM rhamnose and 2% (w/v) arabinose (data not shown). Additionally for Phage B2, which showed reduced replication with expression of the p22-RelBE module in *E.coli* EPI300 (Section 7.4.4), one-step growth curves were also undertaken for EPI300 harbouring each of the p22-RelBE plasmids/constructs.

Replication of phage B2 within EPI300 harbouring pTRACA22 and pGEM-T Easy(TA) was significantly prolonged with an increased latent phase ($P \geq 0.05$). When expression of the full TA module (pJ821(TA)) and RelE toxin (pBAD(Tx)) was induced this increase in length of the latent phase was also increased ($P \geq 0.05$). Figure 7.6 shows this. However, burst size did not appear to be effected significantly regardless of expression ($P < 0.05$). All other constructs/plasmids did not influence replication of Phage B2 when compared to EPI300 WT (Data not shown). Table 7.4 shows values for latent periods, total length of the replication cycle and burst sizes with expression of p22-RelBE or individual components.
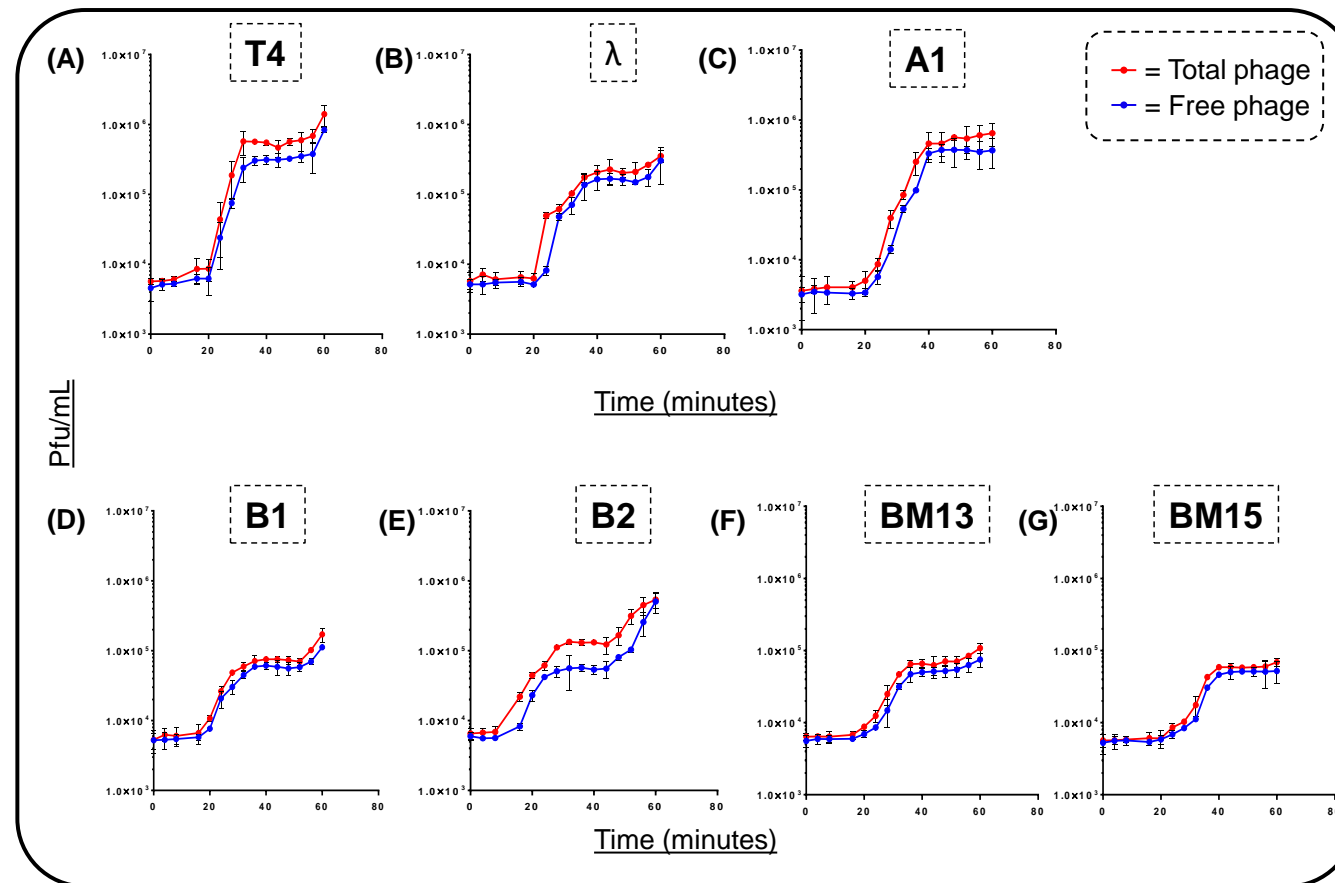
Figure 7.5: One-step growth curves of the 7 phage used within this study. To elucidate characteristics of the phage replication cycle for the 7 phage used in this study. pfu/mL was measured in 4 minute intervals for a 60 minute period. Error bars represent standard error and data points represent the mean of three independent repeats. Due to the log scale used, the X axis begins from 1.0×10^3.

Table 7.3: Characteristics of the replication cycle for 7 bacteriophages of interest.

Phage	Replication cycle (mins)	Latent period (mins)	Burst size (Final total PFU /initial PFU)
T4	35	20	38.503
Coliphage λ	45	20	32.016
A1	40	20	97.541
B1	40	16	10.578
B2	32	20	9.940
BM13	35	15	8.420
BM15	47	27	8.246

Burst size was calculated by dividing the final phage titre by the initial phage titre after a single replication cycle had completed for each phage.
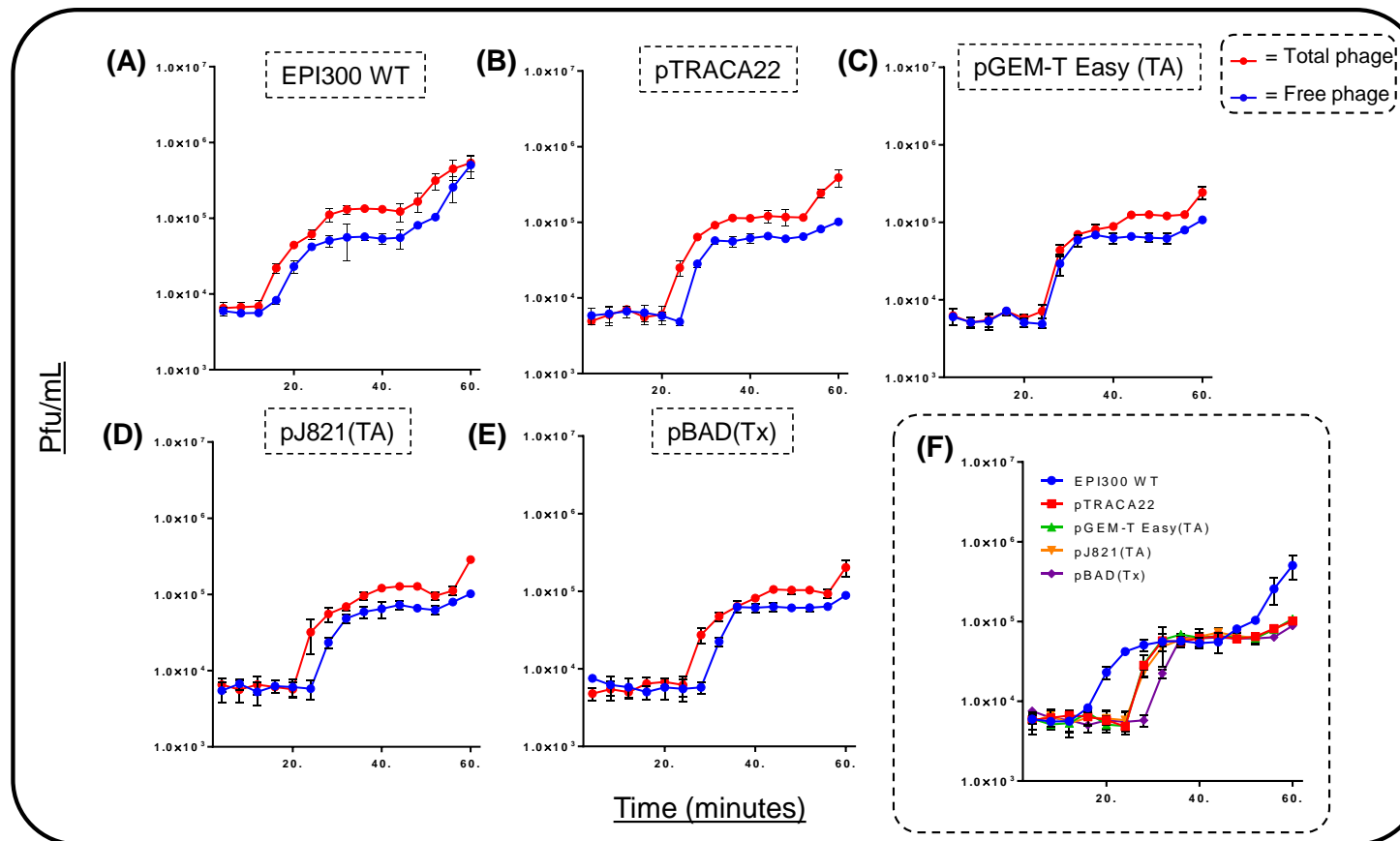
Figure 7.6: One step growth curves of phage B2 with EPI300 expressing pTRACA22-RelBE TA module. PFU/mL was measured in 4 minute intervals for a 60 minute period with expression of the p22-RelBE module and individual components. (A) EPI300 WT control, (B) EPI300 + pTRACA22, (C) EPI300 + pGEM-T Easy(TA), (D) EPI300 + pJ821(TA), (E) EPI300 + pBAD(Tx). (F) B2 free phage (PFU/mL) for EPI300 WT and EPI300 expressing the RelE toxin. Error bars represent standard error and data points represent the mean of three independent repeats. Due to the log scale used, the X axis begins from 1.0 x10^3.

Table 7.4: Characteristics of the replication cycle for phage B2 Whilst EPI300 expressed the p22-RelBE TA module

P22-RelBE construct	Replication cycle (mins)	Latent period (mins)	Burst size (Final total PFU /initial PFU)
EPI300 WT	32	20	9.940
pGEM-T Easy(empty)	45	20	10.014
pJ821(empty)	40	20	9.936
pJ821(TA)	42	24	11.14
pTRACA22	40	24	10.221
pGEM-T Easy(TA)	40	24	9.671
pJ821(ATx)	32	20	10.533
pBAD(Tx)	44	28	11.098

Burst size was calculated by dividing the final phage titre by the initial phage titre after a single replication cycle had completed for each phage.

7.5 Discussion

7.5.1 Phage morphology and replication cycle characteristics

Phage utilised within this study were chosen for a number of reasons. T4 and λ were chosen because either (i) resistance to the phage has been shown in other TA modules or (ii) they are well characterised and understood (Pekota & Wood 1996, Miller *et al.*, 2003, Oppenheim *et al.*, 2005). *E.coli* phage isolated from sewage (A1, B1 and B2) were also included as often their bacterial hosts are derived from human faecal matter and are therefore relevant to the human gut microbiome (Muniesa & Jofre 1998, Chibani-Chennoufi *et al.*, 2004). Finally the inclusion phage isolated directly from human faeces and thus present within the human gut microbiome was required, as this would provide the greatest insight into how a gut-associated TA module, which is highly abundant within this environment, may aid resistance to bacteriophage infection within that habitat.

Analysis of replication and phage yield was undertaken using One-step growth assays and results varied between phage as might have been expected. For those specifically gut associated the entire replication cycle ranged from 35 minutes to 47 minutes with similar burst phage lengths but almost 12 minutes difference in latent period length. To date, very few studies have investigated the potential impact on phage fitness the length of the latent period can have. It has been shown several times that lytic-defective cells can continue to accumulate progeny for much longer than normal lysis would occur suggesting lysis is not prompted by a lack of resources within the infected host (Josslin 1970, Reader & Siminovitch 1971). Arguably it could be beneficial to increase this phase leading to more progeny being released per infected cell. However results from studies undertaken within this chapter, which saw an extended latent phase for phage B2, yet a drop in PFU would suggest this is not the case for p22-RelBE in the context

of phage B2. A study in 2006 suggested two external factors may influence the length of time it takes for lysis to occur: host fitness and host density (Wang 2006). With the density of bacterial cells within the distal colon, it is possible to conclude that this would not be a significant influence on the latent phage for those phage isolated from that environment. However *Proteobacteria* are much less abundant than that of the *Firmicutes* and *Bacteriodetes* (Backhed *et al.*, 2005, Shin *et al.*, 2015). Therefore when exploring the role of p22-RelBE in phage resistance it must always be considered that its effect when expressed in *Enterobacteria*, may well be different to expression of the module when in its expected originator phylum, the *Firmicutes* (Jones *et al.*, 2010).

Of the four phage examined under STEM, a range of morphologies were seen with differences in size, tails, and tail fibres clearly visible. Notably all phage examined appeared to have the extremely common icosahedral-like capsid structure with slight variations in size. This has been repeatedly argued to be so common because it allows for the lowest-energy configuration of particles interacting whilst still retaining stability (Bruinsma *et al.*, 2003). It could be argued that the myoviridae structure seen for phage from the human gut may allude to a functional purpose within this environment. Perhaps this structure is preferred within the human gut microbiome due the stress exhibited on phage varying stress both before and during arrival in that environment? Myoviridae have been shown to often have large genomes which could encode multiple mechanisms for survival however the identification of both gut related phage as myoviridae may be explained more simply by it being a common phage type for Proteobacteria in general (Comeau *et al.*, 2012, Ackermann 2006).

All phage examined also had tails, which would be responsible host recognition, initiation of the infection process, act as a conduit for genomic DNA

and also the termination process where the tail can act as a binding interface for head attachment (Davidson *et al.*, 2012, Leiman & Shneider 2012). Interestingly in myoviridae, a retractable tail sheath is also present, as can be seen in phage BM13 (Figure 7.3F). The apparent prevalence of tailed phage, in the human gut microbiome and also generally (Drulis-Kawa *et al.*, 2012), regardless of the complexity and metabolic cost of building these structures suggests that it is able to provide a significant advantage likely for injection of genomic material through the cell wall and double membranes.

7.5.2 RelBE TA module and resistance to phage B2

Results from this chapter have suggested that *E.coli* expressing the p22-RelBE module, under both natural and inducible promoters, has an increased resistance to phage B2, which was initially isolated from sewage. While the Type II RelBE module has been shown to undertake several important cellular functions including tolerance to multiple different stresses until now, phage predation has not been one of them.

A possible mechanism for phage resistance provided by the expression of p22-RelBE is the global inhibition of translation triggered by free RelE toxin. During infection by lytic phage, the host metabolic machinery and intracellular resources are used in order to generate large amounts of components for mature phage particles (Clokie *et al.*, 2011). Furthermore host DNA and RNA synthesis ceases and transcription of phage-specific genes is prioritised (Madigan *et al.*, 2009). As showed by Christensen *et al.*, (2001) any global reduction on bacterial cell transcription would also effect TA gene expression. This would lead to a depletion of RelB antitoxin both actively (by Lon proteases) and passively (through a lack of

continued synthesis) and an abundance of free RelE toxin, enabling RelE to interact with host transcriptional machinery. Furthermore, the mechanism of RelE when bound to the ribosome would actively destroy phage mRNA ensuring mature phage particles could not be assembled as the infecting phage is dependent on its own mRNA being translated and the ribosomal machinery being functional, mature phage particles cannot be formed, terminating replication.

Although RelE does not specifically induce cell death (i.e. act like an abortive infection system) the continued lack of cellular metabolism may eventually lead to another factor doing so. Regardless, bacteriophage replication would be aborted (See Figure 7.7 for a summary of this). Whilst this would seem the most logical method of providing phage resistance by the RelBE TA module, it would be expected that this was a global reaction to phage infection utilising a lytic lifecycle. In fact what was seen based on experimental data generated in this chapter was lower Pfu/Cfu for a single phage (B2) rather than all phage as might be expected from the model described above. This may mean that p22-RelBE module (probably the RelE component) is affecting some other stage in phage replication or effecting translation more specifically that hypothesised above. Alternatively phage B2 may/may not encode unknown factors that other phage examined in this study do/do not. As an example it may be possible that other phage may affect TA expression providing a level of protection to the host cell by neutralising the RelE toxin.
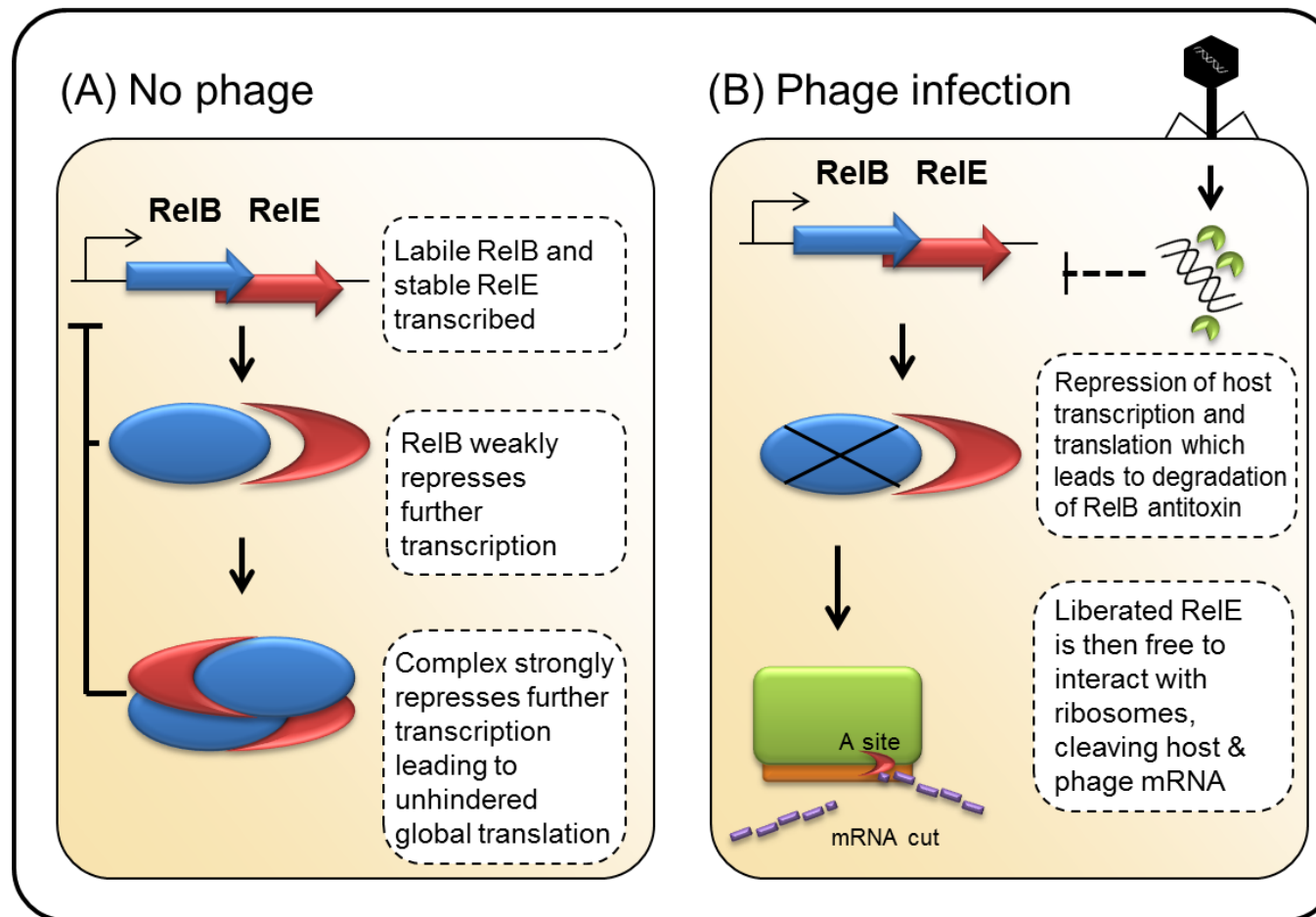
Figure 7.7: Hypothesised model for phage resistance mechanism by the p22-RelBE TA module. (A) Demonstrates natural RelBE activity under normal growth conditions whilst (B) depicts activity of the module during phage infection. RelB and RelE are shown in blue and red respectively. The green and orange rectangles represent the large and small rRNA subunits and cleaved signified by the broken purple chain.

Previous studies of phage resistance involving TA modules have seen varying levels of generalised resistance depending on the Type of TA module under investigation. The Type III ToxIN system, perhaps the most well understood TA module in terms of phage resistance, has been shown to provide protection against a wide range of phage in multiple enteric bacterial backgrounds (Fineran *et al.*, 2009). Of 25 phages known to infect *Erwinia carotovora* Subsp. *atroseptica*, 13 were found to be sensitive to ToxIN expression through simple efficiency of plaquing assays (plaque forming ability when related to plating without the TA module present). Subsequently when transferred to *E.coli* DH5α, 4 previously identified coliphage (including T4 and λ) were shown to be insensitive; however of 10 newly isolated coliphage, 50% were sensitive to ToxIN expression. Finally when expressed in *Serratia marcescenes* DB11 3 out of 11 phages were also sensitive but also had reduced plaque sizes (Fineran *et al.*, 2009, Blower *et al.*, 2012). This indicates that TA modules (at least those belonging to the Type III category) are able to act as fairly generic anti-phage systems across multiple enteric genera. Whether or not this can be expanded to TA modules as a whole is still unclear, as little investigation into this area has been reported.

As mentioned previously, of Type II TA systems, only the MazEF system has been reported to have anti-phage activity and like p22-RelBE in this study, it appears to be much more specific than the ToxIN Type III module. As the only other study of Type II modules and phage resistance it is impossible to judge whether the MazEF programed cell death system is able to provide phage resistance to *E.coli* populations for multiple phage as only P1 was investigated (Hazan & Pecota *et al.*, 2004). Interestingly the *E.coli* phage P1 carries a TA module, PhD/Doc, which is also a Type II system and is used by the phage to ensure replication and stability of its genome within bacterial cells when temperate

(Tyndall *et al.*, 1997, Lehnherr *et al.*, 1993). While no data has been shown to date, it can be hypothesised that this module favours a lysogenic lifestyle, ensuring population stability within the environment of its host bacterium. It may be that this module favours lysogenic lifestyle and kills cell populations where phage attempt lytic behaviour as this would influence dissemination of the module. This kind of inter-mobile genetic element competition can be expanded to include others also. Furthermore carriage of TA components on phage may reduce phage infection due to the mechanics of inter-element competition.

7.6 Conclusions

- The presence of glucose, rhamnose and arabinose did not affect the PFU forming ability of any phage used within these studies.

- Expression of the p22-RelBE module, specifically the RelE toxin, in *E.coli* EPI300 was shown to reduce PFU for the sewage isolated phage B2.

- Replication of phage B2 was slowed by expression of the p22-RelBE module in *E.coli* EPI300, increasing the length of the latent phage; however this did not affect the burst size.

Chapter 8: Host-microbe interactions with *E.coli* EPI300 expression of the p22-RelBE module

8.1 Introduction

8.1.1 Effect of TA modules on eukaryotic cells

Bacterial TA module toxins severely restrict prokaryotic cell growth through a number of different mechanisms (these are listed in Table 1.1). Perhaps the most fully understood of these modules is the Type II *E.coli* RelBE. The relE toxin functions as a global inhibitor of translation by binding to the ribosomal A-site inducing a conformational change and promoting cleavage of mRNA (Neubauer *et al.*, 2009). Whilst Eukaryotic ribosomes are different in some respects to the prokaryotic counterparts; being larger, having different initiating amino acids, employing different elongation factors and utilising only one termination factor compared to the prokaryotic two (Berg *et al.*, 2002), the mechanism of translation is relatively conserved between both kingdoms. The similarities between the process of translation and conservation of the A-site within the ribosome enable the relE toxin to interact with the eukaryotic ribosome and inhibit translation in the same way.

While no relBE TA modules have been found encoded within eukaryotic genomes, artificial extra-chromosomal expression has been attempted to examine if the relE toxin is able to inhibit translation within a eukaryotic background. This possibility was first explored in *Saccharomyces cerevisiae* as a potential mechanism of controlling cell proliferation in industrial fermentation processes and as a safety measure against release of genetically modified microorganisms into environments for remediation purposes (Kristoffersen *et al.*, 2000). In this study, relE was shown to be highly toxic to yeast, whilst co-expression of the relB

antitoxin partially neutralised these effects. However, no exploration into the mechanism by which the relE peptide is toxic was undertaken.

Following on from these findings, a relE toxin was artificially expressed the same RelBE module within the human TREx-U2OS osteosarcoma cell line under the control of an inducible promoter (Yamamoto *et al.*, 2002). Expression of relE by this cell line retarded growth and specifically resulted in cell death by apoptosis (Yamamoto *et al.*, 2002). It was hypothesised that this was through the same mechanism as in prokaryotes by direct interaction with ribosome and a number other ribosome-inactivating proteins have been previously identified which are active across both kingdoms (Endo *et al.*, 1998, Suh *et al.*, 1998).

It has since been confirmed that the action of relE when expressed by eukaryotic cells is through cleavage of mRNA on ribosomes at the universally conserved A-site in ribosomal-translation initiation complexes (Andreev *et al.*, 2008). Jones (2010) hypothesised that the pTRACA22-RelBE module might behave as a control for epithelial cell proliferation within the human gut especially with the apparent prevalence of the module within the gut microbiome (Jones 2010). Notably all observations made to date have used artificial expression systems and it is not yet known if microbial cells expressing the relE toxin will reproduce the effects.

8.1.2 Human gut epithelial lining

The mucosal surfaces of the gastro-intestinal tract (GI tract) act as a barrier against pathogenic organisms and as an attachment site for commensal species (Hao & Lee 2004). The epithelium of both these sections of the GI tract consists of a single layer of polarised cells with the apical surface facing outwards towards the

lumen and basolateral surface surrounded with the submucosa harbouring nerve endings, smooth muscle and lymphatic/blood vessels for the transport of absorbed nutrients (Martini & Nath 2009). As shown in Figure 8.1, the human gut is organised into villi, which are undulating projections protruding from the epithelial lining and between these, the crypts. At the base of crypts reside pluripotent intestinal epithelial stem cells, which give rise to transit-amplifying populations of cells that undergo rapid proliferation and subsequent differentiation. With the exception of Paneth cells (described below), the terminally differentiated epithelial cells migrate up the crypt until they are eventually lost through apoptosis (Barker *et al.*, 2014).

Enterocytes are the most numerous of cells in the gut epithelium interacting with the luminal content and their microvilli providing a large surface area for nutritional absorption (Abreu 2010). It is estimated that the corrugated structure of the microvilli and villi provide approximately 260-300m^2 of surface area in an adult human (Helander & Fandriks 2014). Goblet cells are less numerous but vital due to the secretion of mucus which forms an interface between the epithelium and environment whilst paneth cells produce antimicrobial proteins to promote the exclusion of some microorganisms from the epithelial surface (Umar 2010). Enteroendodocrine cells are also present within the epithelium, releasing hormones involved in digestion and maintain a link between the central and enteric neuroendocrine systems (Peterson & Artis 2014). With the importance of the epithelial barrier to gut health and immunity, microfold cells and macrophages are also present within this layer however the latter being more commonly in the submucosa (Peterson & Artis 2014). These mediate transport of antigens and bacteria across the barrier to dendritic cells and also sample the luminal content (Kucharzik 2000).
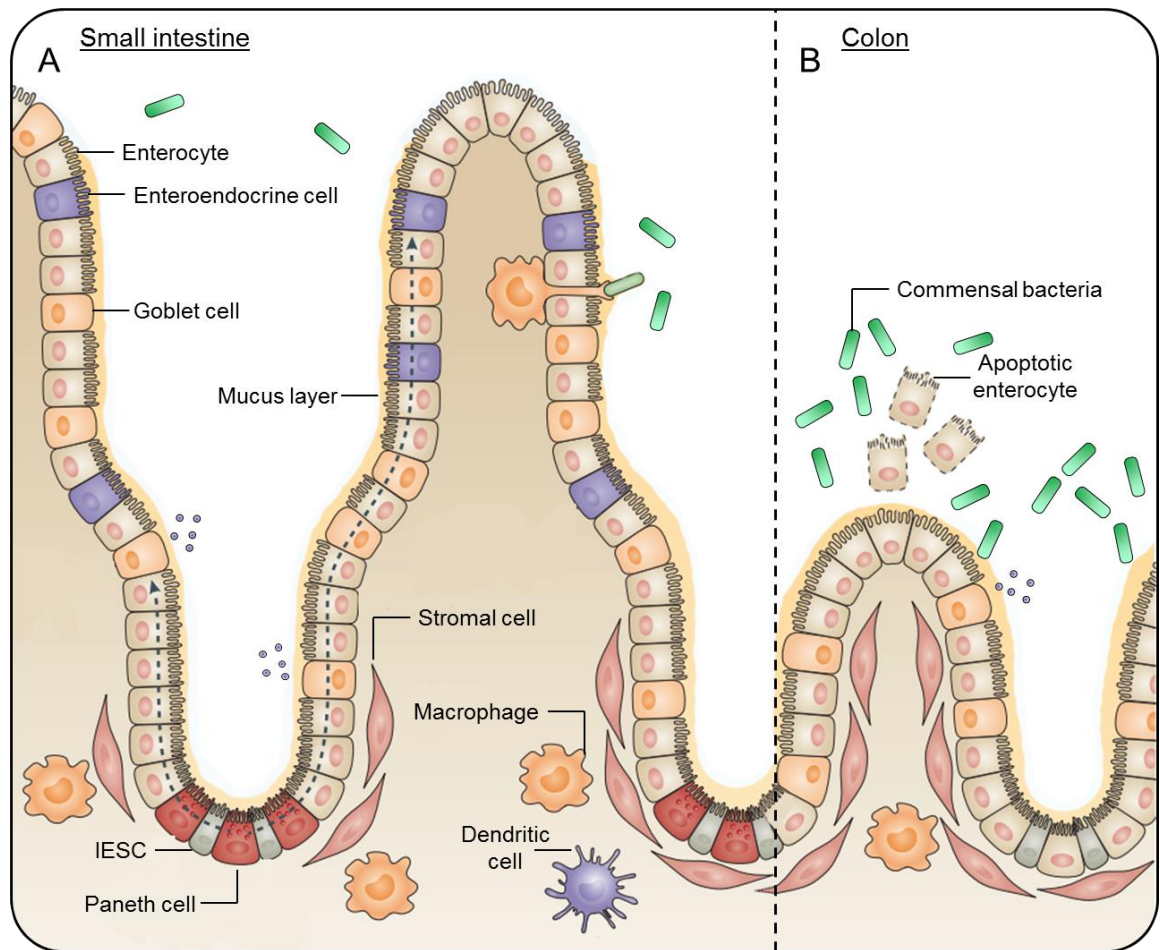
Figure 8.1: Diagram of the human small intestinal and colonic cell structure. Adapted from Peterson & Artis 2014.

The structure of both the (**A**) small intestine and (**B**) colon are depicted. Pluripotent Intestinal stem cells ensure constant renewal of the epithelial layer with differentiated cells migrating away from the crypts. Enterocytes enable absorption of nutrients whilst goblet cells secrete a protective mucus layer, which also behaves as an interface for cell-lumen interaction. Enteroendocrine cells release intestinal hormones related to digestive processors and secretion of antimicrobial peptides is accomplished by the paneth cell population. Microfold cells and macrophages sample and mount responses to antigens present within the luminal content.

8.1.3 Microbial-induced cell death of the gut epithelium

The cells of the gut epithelium separate the gut microbiota and submucosal structures (known as the lamina propria) physically through cells linked by tight junctions and by the secretion of proinflammatory cytokines and reactive oxygen species (Yu *et al.*, 2012). In parallel the commensal bacteria inhabiting the gut are involved in various physiological and developmental functions whilst dysbiosis of this community often leads to pathology (Backhed *et al.*, 2005, Ley *et al.*, 2006). A key function of gut-commensal microbes is the intestinal epithelial cell turnover rates. This function is highly important as evasion of apoptotic events, especially at the peak of the villi or 'extrusion zone' is known to be a factor in determining early stages of colorectal carcinogenesis (Renehan *et al.*, 2002, Oumouna-Benachour *et al.*, 2007). Alternatively, increased epithelial apoptosis without sufficient proliferation would result in barrier damage leading to possible submucosal tissue invasion. Precise control of this process is therefore necessary for both the higher host and the community inhabiting the gut environment to retain the mutualistic relationship they share.

Evidence for the role of the gut community in epithelial turnover has been rapidly increasing, often employing comparisons between germ-free and conventionally raised animals. In germ-free piglets, aberrant intestinal morphology was shown with elongated villi and shallower crypts than conventional counterparts, whilst oral inoculation of commensal bacteria obtained from faeces was shown to stimulate epithelial apoptosis (Willing & Van Kessel 2007, Danielsen *et al.*, 2007). *In vitro* studies have also identified pro-apoptotic effects on human intestinal epithelial cell lines and colon explants when in the presence of non-pathogenic *E.coli* and commensal Lipopolysaccharides (LPS, Yu *et al.*, 2005, Jarry *et al.*, 2008, Yu *et al.*, 2006). Precise control of epithelial turnover and the factors

affecting it still require more investigation, especially from the position of the gut prokaryotic community, which clearly impacts this process. The abundance of some Type II TA modules specifically within the human gut and the induction of apoptosis (not necrosis) in human cell lines by the RelE toxin may offer one possible mechanism by which the microbiota help modulate the epithelial lining of the human gut.

8.1.4 *In vitro* intestinal-epithelial cell culture

There are generally considered to be two approaches to the generation of *in vitro* gut epithelial models; the use of freshly harvested primary cells or the use of 'immortalised' and abnormal tumour cells which have similar physiological characteristics to the original donor cells (Castellanos-Gionzales *et al.*, 2013). The former of these two methods is considered more representative of enterocytes *in vivo* however these tend to be short lived and undergo apoptosis soon after culture begins. Alternatively, the latter, although not resembling the required cell type as closely (with mutations modifying cell cycle regulation and senescence) offer a stable source of cells which can proliferate indefinitely when exposed to optimal culture conditions (Meunier *et al.*, 1995). The Caco-2 enterocyte-like and HT-29 goblet-like cells lines have both been shown to have similar protein profiles to primary cell counterparts however not identical (Lenaerts *et al.*, 2007).

Regardless, the human intestinal Caco-2 cell line is still one of the most extensively studied and characterised and has been used widely in studies of the intestinal barrier, host-microbe interactions and drug absorption (Lee & Salminen 2009, Cencic & Langerholc 2010). The cell line was originally derived from a tumour and differentiates spontaneously depending on cell density (Fogh *et al.*, 1977, Pinto *et al.*, 1983). When seeded on impermeable substrates such as plastic

or glass, cells form well-polarized monolayers with tight junctions and developed apical microvilli and brush border (Chantret *et al.*, 1988, Pignata *et al.*, 1994). Before confluency is reached Caco-2 cells maintain a high level of homogeny, suggesting they represent neoplastic equivalents of the crypt-pluripotent precursors to enterocytes (Neutra & Louvard 1989, Lee *et al.*, 2009, Uchida *et al.*, 2009).

More recently, a study investigating the disruption of the well characterised probiotic, *E.coli* Nissle 1917's K5 capsule biosynthesis employed a co-culture methodology with the Caco-2 cell line (Nzakizwanayo *et al.*, 2015). This co-culture method was employed to examine apoptotic and cytotoxic effects of Nissle mutants when in culture with Caco-2 cells and offers a good model for the investigation of the pTRACA22-RelBE module expression in *E.coli EPI300* on Caco-2, and subsequently host epithelial health.

8.2 Aims

The aim of the work presented in this chapter was to investigate the effect of pTRACA22-RelBE expression in *E.coli* EPI300 on the human intestinal Caco-2 cell line using an *in vitro* co-culture model to identify any apoptotic or cytotoxic effects expression of the module may have.

8.3 Material and methods

8.3.1 Caco-2 cell line and culture conditions

Cells were used between passage numbers 42-77. Cells were seeded in T75 (75 cm^2) flasks and grown at 37°C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium – high glucose (DMEM, 4.5 g/L glucose, PAA Laboratories, UK) supplemented with 10% fetal bovine serum (FBS, PAA Laboratories, UK) and 1x non-essential amino acids (NEAA, PAA Laboratories, UK). 1x non-essential amino acids consisted of 7.5 mg/L glycine, 8.9 mg/L L-Alanine, 13.2 mg/L L-Asparagine, 13.3 mg/L L-Aspartic acid, 14.7 mg/L L-Glutamic acid, 11.5 mg/L L-Proline and 10.5 mg/L L-Serine. For the purposes of this study this complete media will be referred to as "CDMEM". Cell culture medium which is serum and glucose free will be referred to as "SGFM" All flasks, plates and other reagents were obtained from Fisher Scientific (UK) unless otherwise stated and all media used was kept at 4°C until required where it was then pre-warmed in a 37°C for 30 minutes.

Upon reaching ~80% confluence within a T75 flask, the media was removed and the monolayer washed with sterile PBS. Cells were then detached from the flask by the addition of 5mL of 0.05% w/v trypsin in 0.5mM EDTA and incubated for 7 minutes at 37°C in the same humid environment as they were grown. After incubation trypsin present in the flask was neutralised through the addition of 10mL of cell culture media excluding the additional NEAA. The cell suspension was transferred to a 20 mL sterile universal tube then centrifuged at 500 g for 5 minutes to pellet the cells. After discarding the supernatant, the pellet was re-suspended in 1 mL of CDMEM by gentle pipetting 100 times with a 1 mL and 200 µL pipette each to ensure complete re-suspension. Caco-2 cells were seeded into

new flasks at a cell density of $0.5 \times 10^{5\text{-mL}}$ which required approximately 4 days to form a 80% confluent monolayer with growth media changed every 2 days.

8.3.2 Storage and thawing of cells

To store cells long-term, cryogenic preservation in a dry nitrogen store (-164°C) was employed. Caco-2 cells were grown and pelleted as previously described (Section 8.2.1). The resulting pellet was resuspended in 3 mL of CDMEM containing 5% (v/v) DMSO. 1mL aliquots of cell suspension were transferred to 1.5 mL sterile cryo-vials and these were stored in a polystyrene vessel at -80°C for ~48 hours before being transferred to a dry nitrogen container until required.

When needed, cells were thawed by removal of the cryo-vials from the storage facility and immediately placed in a 37°C water bath. The 1 mL thawed suspension was then transferred to a 50 mL tube containing 19 mL of CDMEM, gently mixed by pipetting and the entire contents finally transferred to a sterile T75 flask to be incubated as in Section 8.2.1.

8.3.3 Cell number quantification

To determine cell-seeding densities before passaging, a haemocytometer count was used. A 1/10 dilution of re-suspended, freshly trypsinised cells was done using sterile PBS in a 1.5 mL Eppendorf tube and counted using a Neubauer haemocytometer. The haemocytometer and its cover slide were carefully cleaned using 100% ethanol, with each chamber then loaded with 10µL of cell suspension. Cells within the defined square counting zones were manually counted using a microscope at 100x magnification with at least 8 counting squares used per sample to calculate the cell concentration using the following formula:

$$\text{Total cells/mL} = \frac{\text{Total cells counted } \times \text{ dilution factor } \times \text{ } 10^4 \text{ cells/mL}}{\text{Number of squares}}$$

8.3.4 Generation of bacterial culture supernatants and total protein extract

EPI300 cultures containing the required plasmid construct for expression were grown in 100 mL of LB broth supplemented with 0.1% (w/v) glucose at 37^oC with shaking to an OD_{600} of ~0.2 (4×10^6 CFU/mL), pelleted, washed 3 times in sterile PBS and re-suspended in SGFM. This media was also supplemented with the required inducer (Sections 5.3.5 and 5.3.6) at optimum concentrations as decided in Chapter 5 and grown at 37^oC with shaking and expression of the TA module/individual components until an OD_{600} of ~1.0 (4.6×10^7 CFU/mL).

For harvesting the supernatant only, cells were transferred to sterile centrifuge tubes and spun down at 3500 xg for 10 minutes. The supernatants were then carefully drawn off leaving the ~5 mL of supernatant closest to the pellet to avoid disturbing it, pH adjusted to 7.2 and filtered through a 0.44 µm Millipore filter to ensure sterility. Sterility for both the supernatant and crude culture lysates was confirmed through CFU counts on LB agar (data not shown). Finally, sterile supernatants were diluted at a 1:1 ratio in fresh SGFM.

For generation of the total protein extract 30x 1 mL of overnight culture for each construct to be used were subjected to 3 rounds of freeze/thaw cycles, freezing at -80^oC for 20 minutes and thawing at 30^oC for 2 minutes. Pellets were re-suspended in 400 µL of celLytic B Bacterial Cell Lysis Extraction Reagent (Sigma, UK) and 8 µL of 1x Bacterial cell Protease Inhibitor Cocktail (Sigma, UK) added with 0.2 mg/mL lysozyme (Sigma, UK) and 4 units of DNAse I. Cell suspensions were subsequently incubated at RT for 30 minutes and supernatant

collected by centrifugation at 16 000 xg for 10 minutes. Soluble protein fractions were stored at -80°C until required. Proteins were precipitated with 4 volumes of ice-cold acetone and finally resuspended in nuclease free water or SGFM.

8.3.5 Co-culture model optimisation by trypan blue exclusion assay

The effect on Caco-2 cell population viability of the p22-RelBE module was investigated using monolayers of Caco-2 cells after incubation with p22-RelBE expressing EPI300, culture supernatant or crude lysate of a culture expressing the module. Optimisation of this model was achieved by determining the effect of the bacterial culture density and exposure duration using the trypan blue exclusion method (Freshney 2002).

 Cells were grown in CDMEM in 6-well plates at 37°C and 5% CO_2 until 1x10^6 cells/well (80% confluence), whilst bacterial cultures to be used were grown in SGFM and 1x NEAA and harvested at mid log phase (~1.1x10^7 CFU/mL), washed 3 times in PBS and resuspended in DMEM at 5x10^7 CFU/mL. If cultures were to be used as controls 0.1% (w/v) glucose was added to growth media to ensure repression of constructs. Monolayers of Caco-2 cells were washed gently by rinsing with 2 mL of PBS before adding 2 mL of EPI300 bacterial suspensions (corresponding to MOIs ranging from 0.1:1 to 100:1), culture supernatants and crude culture lystates (generated from the neat 1.1x10^7 CFU/mL mid log phase cultures and diluted at a ratio of 1:1. The plates were incubated for 2, 4 and 6 hours to allow cells to interact. 2 wells on each plate were left as untreated monolayers with only Caco-2 cells and sterile SGFM. When each time point was reached the media, bacterial suspensions and detached Caco-2 cells were removed by gentle aspiration and monolayers washed once in PBS and tripsinised (as in section 8.2.1).

Freshly trypsinised cells were diluted in sterile PBS. A 50 µL aliquot of mixed cells was transferred to a 1.5 mL Eppendorf tube and mixed with an equal volume of trypan blue solution at a concentration of 0.25% (w/v) in sterile PBS. The stained suspension was loaded onto a haemocytometer and cells were observed under a microscope. Two counts were taken per square zone, the total number of cells and the number of stained, blue cells (signifying they were dead). The viability of cells in treated samples was expressed as a percentage of viable untreated cells using the following formula:

$$\textbf{Total cells/mL} = (\text{viable cells in treated sample} \ / \ \text{viable cells in untreated control}) \ x \ 100$$

Optimal conditions for this model were determined as the bacterial cell densities or concentration of supernatants/lysates and co-incubation durations producing ≥95% viability of the Caco-2 cells.

8.3.6 Hoescht propidium iodide staining of Caco-2 cells in co-culture with *E.coli* EPI300, total protein extracts or culture supernatants with expression of the p22-RelBE TA module

To determine cell health status (death and viability) of Caco-2 cells in co culture with EPI300 cultures expressing the p22-RelBE TA full module/individual toxin or antitoxin the Hoechst 33342/Propidium iodide (HPI) double fluorescence staining technique was used as described by Dive *et al.*, (1992). Hoechst 33342 (H33342) is a blue-fluorescent dye that binds to DNA of eukaryotic cells, fluoresencing more brightly when bound to the condensed chromatin of apoptotic cells when compared to chromatin of healthy cells. Propidium iodide (PI) also binds to DNA however is membrane-impermeant and is generally excluded from viable cells

indicating those that are necrotic (Belloc et al., 1994, Elstein & Zucker 1994, Darzynkiewicz *et al.*, 1997).

For the assay, Monolayers of Caco-2 cells and EPI300 inocula were prepared as described in Section 8.2.4, with the exception of being grown to approximately 60% confluency and HPI staining solution was prepared in SGFM containing 5 mg/mL H33342 (Thermofisher, UK) and 1 mg/mL PI (Sigma Aldrich, UK). Momolayers of Caco-2 cells were exposed to bacterial suspensions at a range of MOIs (5:1 to 0.1:1) and plates incubated for 4 hours. Untreated cells containing SGFM were used as experimental controls.

After incubation, media was gently removed from the 6 well plates by aspiration and monolayers were washed once with PBS before the careful addition of 250 µL of HPI staining solution to avoid damaging the monolayer. All plates were immediately covered in foil and incubated in the dark at room temperature for 5 minutes. Stained cells were then visualised using the Zeiss AxioVert 25 inverted fluorescent microscope with attached camera.

8.3.7 Caspase activity (apoptotic) analysis of Caco-2 cells in co-culture with *E.coli* EPI300, total protein extracts or culture supernatants with expression of the p22-RelBE TA module

The activity of caspase 3/7 was measured in Caco-2 cells treated with the total protein extraction, culture supernatants and live cultures of *E.coli* EPI300 expressing the p22-RelBE TA module or individual components of this using the Capase-Glo® 3/7 Assay. Programmed cell death, or apoptosis, is a vital process by which multicellular organisms ensure regulation of cell numbers and removal of damaged cells (Kanduc *et al.*, 2002). A series of cysteine-aspartic acid specific proteases are activated during the early stages of apoptosis and these caspases

are synthesised as dormant precursors that can be stimulated by cytotoxic agents and cytokines leading to packaging of cellular contents into vesicles, dispersion of these and eventual cell death (Gnesutta & Minden 2003).

Human caspases are classified based on function and structure. To date, two major groups have been described; firstly, monomeric initiator caspases (-8. -9 and -10) and secondly dimeric effector caspases (-3, -6 and -7, Pop & Salvesen 2009). After receiving pro-apoptotic signals, Initiator caspases activate effector caspases, which, following activation, target multiple structural proteins, triggering a cascade of biochemical and morphological changes. These include cellular shrinking, convolution and internucleosome cleavage, which eventually leads to cell death (McStay et $al.$, 2008).

Caco-2 cells were seeded in 96-well plates at 5000 cells/well in 100 μL CDMEM and grown at $37^{o}C$ in 5% CO_2 for 60 hours to reach ~70% confluency (~14000 cells/well). After 66 hours media was replaced with SGFM and incubated as above for a further 6 hours. After 72 hours the media was then removed from all wells and were washed once with PBS followed by exposure to the experimental conditions in SGFM in 100 μL per well. Plates were incubated for 6 hours at $37^{o}C$ and 5% CO_2. After 4 hours of co-incubation, the media containing the bacterial suspensions was removed and replaced with SGFM containing 200 μg/mL gentamicin to completely stop bacterial growth.

Experimental conditions were culture supernatant, total protein extracts and bacterial suspensions which were prepared as in Section 8.2.4 above with bacterial suspensions added at a MOI of 5:1 (EPI300:Caco-2). Control plates were also included for each plates which were untreated cells (Caco-2 cells in media only), bacterial cells ($E.coli$ EPI300 in media only), media only (media without Caco-2 or $E.coli$ EPI300) and Caco-2 cells treated with serum and glucose free

media containing 0.1mM camptothecin (Sigma, UK) as a positive control for apoptosis. Camptothecin, an extract of the Chinese tree *Camptotheca acuminata*, is a potent inhibitor of topoisomerase I, a molecule required for DNA synthesis. Camptothecin has been shown to induce apoptosis in a dose-dependent manner In vitro and is routinely used as a general method for inducing apoptosis (Traganos *et al.*, 1996).

After exposure to the experimental conditions for 4 hours and without removal of media, 100 µL of the Capase-Glo$^®$ 3/7 reagent was added to each well, including controls. The contents of each well were mixed gently using a microplate shaker at 300 rpm for 2 minutes then incubated in the dark for 1 hour at RT. Luminescence proportional to the caspase 3/7 acitvity was measured as relative light units with a Synergy Multi-Mode Plate reader (BioTek, UK) using BioTek Gen5.20 software.

8.3.8 Cytotoxicity analysis Caco-2 cells in co-culture with *E.coli* EPI300, total protein extracts or culture supernatants with expression of the p22-RelBE TA module

The release of lactate dehydrogenase (LDH) was quantitatively measured in Caco-2 cells treated with the total protein extraction, culture supernatants and live cultures of *E.coli* EPI300 expressing the p22-RelBE TA module or individual components of this using the CytoTox 96$^®$ Non-Radioactive Cytotoxicity Assay (Promega, UK). Necrosis is a type of cell death that is morphologically characterised by the swelling rupture of intracellular organelles, leading to the eventual break down of the plasma membrane and leakage of intracellular contents (Chan *et al.*, 2013). LDH is a stable cytosolic enzyme that is released upon cell lysis, enabling detection of necrosis and allowing quantification of the

process as well. The released LDH can then be measured in culture supernatants by coupling an enzymatic assay which results in the conversion of tetrazolium salt (INT) into a red formazan product. The amount of colour formed is proportional to the number of lysed (necrotic) cells.

The CytoTox 96® Non-Radioactive Cytotoxicity assay was performed as detailed in Section 3.3.7. After treatment, 50 µL of supernatant from each well was collected and transferred into a fresh 96 well plate and 50 µL of the CytoTox 96® substrate was added. Plates were incubated in the dark at RT for 30 minutes before the addition of 50µL of the provided stop solution. To generate the maxium LDH release possible, 10x lysis solution was added to lyse all Caco-2 cells 45 minutes before addition of the CytoTox 96® substrate. Absorbance was measured at 490 nm (OD_{490}) and the percentage cytotoxicity was calculated using the following equation:

$$\frac{\text{Treated cell LDH } (OD_{490})}{\text{Maximum LDH release } (OD_{490})} \times 100$$

For wells containing bacterial suspensions, before OD readings were entered into the equation, the OD_{490} values of the bacteria-only control wells were subtracted.

8.4 Results

8.4.1 Optimisation of a co-culture model of *E.coli* EPI300 and the Caco-2 cell line

For use in subsequent experiments, the effect of complete, serum-free medium (SFM) and serum and glucose-free medium (SGFM) on the growth of EPI300 was evaluated. Serum and glucose free options were included as serum is known to produce false positives in the Capase-Glo[®] 3/7 and CytoTox 96[®] Non-Radioactive Cytotoxicity Assay whilst glucose acts as a metabolic repressor of TA modules harboured on some of plasmid constructs used. Results are shown in Figure 8.2. All 3 variations of the cell culture medium supported growth similarly up until 6 hours. After this time point, EPI300 growing in SGFM (8 and 12 hours) and SFM (12 hours) was significantly less than the complete medium ($P < 0.05$).

To optimise the co-culture model of EPI300 and Caco-2 to be used in later experiments, cell viability of Caco-2 was investigated in the presence of bacterial cells at different densities over 2, 4 and 6 hours. Data is shown in Figure 8.3. Caco-2 viability was found not to be significantly affected by co culture with EPI300 at an MOI equal to or less than 5:1 (EPI300:Caco-2) at 2 and 4 hours. An MOI of 25:1 or above was shown to significantly reduce cell viability of Caco-2 at 2, 4 and 6 hours of incubation whilst 6-hour co-incubation was detrimental even at an MOI of 0.1:1. It was determined that optimal conditions for the EPI300-Caco-2 co-culture module were an MOI of 10:1 and incubation duration of 4 hours or less.
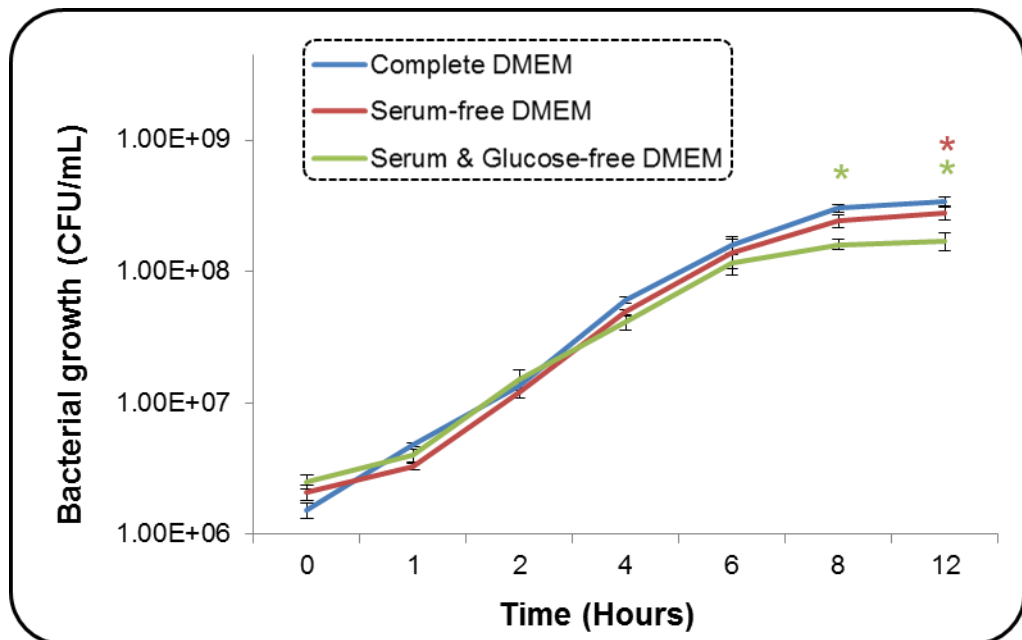
Figure 8.2: Growth of *E.coli* EPI300 WT in CDMEM, SFM and SGFM. Cultures were grown without agitation at 37°C in 5% CO_2 and growth monitored using whole plate viable counts. Results shown are the mean of three independent experiments and error bars represent standard error of the mean. * = P value <0.05.
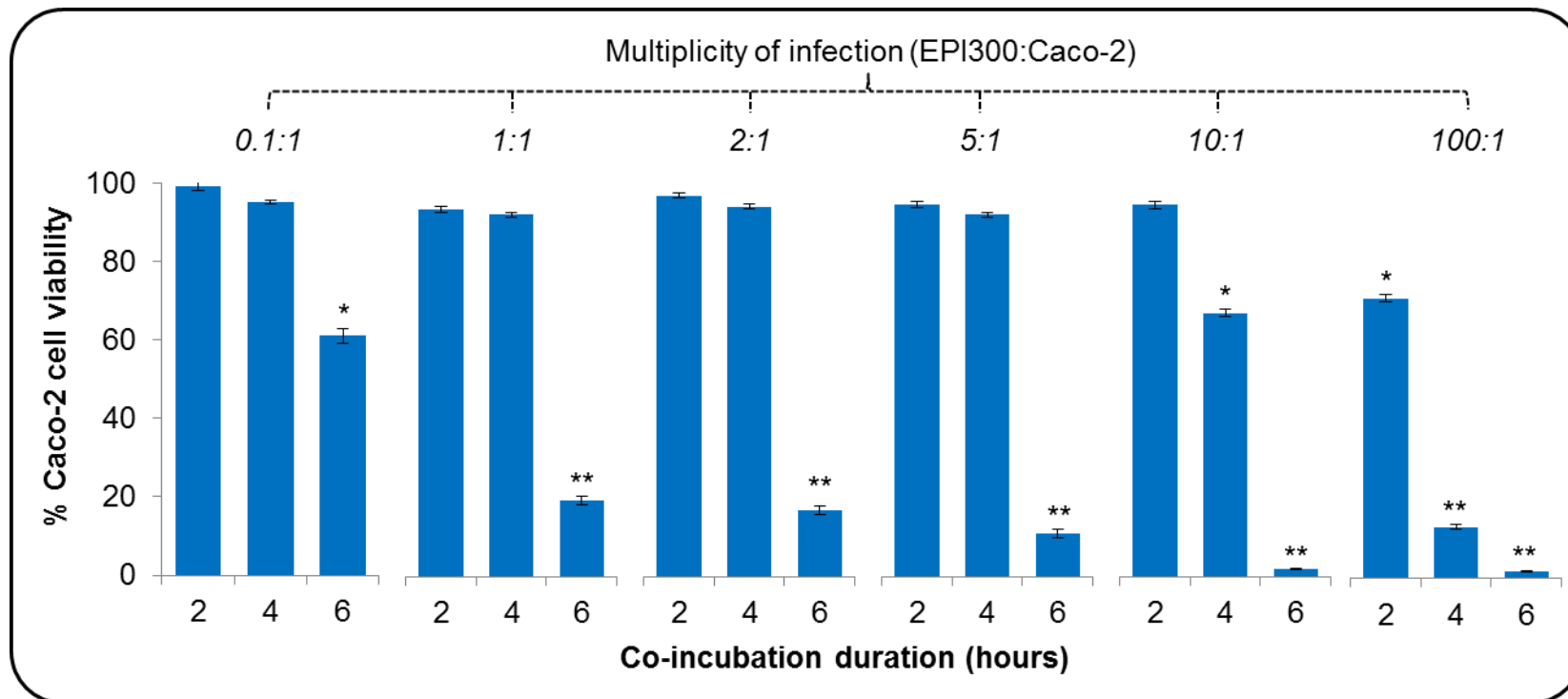
Figure 8.3: Assessment of Caco-2 viability in co-culture with *E.coli* EPI300. Monolayers of Caco-2 cells grown to approximately 75% confluency (1x 10^6 cells/well) were co incubated with mid-log phase culture of *E.coli* EPI300 WT at a range of multiplicities of infection (MOI) and 3 different incubation durations. Co-culture was done at 37^oC and in 5% CO_2. Cell viability was determined using a trypan blue exclusion assay and the percentage viability of Caco-2 cells was calculated using control wells containing media only as a comparison. Data represented is the mean of three independent replicates and error bars represent standard error of the mean. * = P value <0.05, ** = P value <0.001.

8.4.2 HPI staining of Caco-2 cells treated with EPI300 expressing the p22-RelBE TA module

To investigate the impact of p22-RelBE expression by EPI300 on Caco-2 cell health HPI staining was undertaken. Applying the HPI stain to Caco-2 cells with bacterial cultures at an MOI of 5:1 after a 4-hour of co-incubation time showed no detrimental effect on the epithelial cell monolayer. Incubation with bacterial suspensions expressing the TA module and antitoxin only, displayed no noticeable qualitative differences in Caco-2 viability when compared to the untreated and WT controls (Figure 8.4). Expression of the p22-RelE toxin appeared to reduce the number of apoptotic cells (bright blue in colour) when assessed subjectively. These results indicated that expression of the p22-RelE toxin may affect levels of apoptosis in Caco-2 cells and so further quantitative investigation was undertaken.

Figure 8.4: HPI staining of Caco-2 cells treated with *E.coli* EPI300 expressing the p22-RelBE TA module and individual components. Caco-2 monolayers were grown to approximately 60% confluency in 6-well plates and co-cultured with mid log phase *E.coli* EPI300 expressing the p22-relBE complete module, toxin and antitoxin at an MOI of 5:1 for 4 hours at 37°C and 5% CO_2. Untreated Caco-2 cells were used as the experimental control. HPI stained cells were visualised using a Zeiss Axiovert 25 inverted fluorescent microscope at 5x magnification. Images are representative of 3 independent experiments.

8.4.3 Analysis of apoptosis and cytotoxicity in Caco-2 cells when treated with supernatant from EPI300 cultures expressing the p22-RelBE TA module

Induction of apoptosis in Caco-2 cells when treated with supernatant from EPI300 cultures, expressing the p22-RelBE TA module, was quantified measuring caspase-3/7 activity. As displayed in figure 8.5A, caspase-3/7 activity was not significantly increased by treatment with supernatant. The cytotoxic effects of the p22-RelBE TA module were also investigated by quantifying LDH release. Exposure to supernatants from cells harbouring the TA module or derivative constructs showed no impact on LDH release regardless of gene expression (Figure 8.5B).

In concurrence with results from HPI staining (section 8.3.2) co-culture with EPI300 expressing the complete TA module under the control of an inducible and natural promotor did not significantly alter caspase-3/7 activity in Caco-2 cells. This was also the case for Caco-2 cells co-cultured with EPI300 expressing the p22-RelB antitoxin. Although expression of the p22-RelE toxin appeared to exhibit an increased level of apoptosis when using HPI staining, this was not supported by quantitative analysis undertaken here. Overall there were no significant differences in caspase activity between the control wells (untreated Caco-2, EPI300 WT and EPI300 carrying empty plasmid constructs), EPI300 harbouring the TA module constructs but with no expression and EPI expressing the TA module and the individual toxin/antitoxin. Caspase-3/7 assay results for Caco-2 and EPI300 co-culture can be seen in figure 8.6.

Treatment of Caco-2 cells with total protein extracts from EPI300 cultures with and without the expression of the TA module significantly increased caspase activities when compared to the untreated Caco-2 control wells (Figure 8.7).
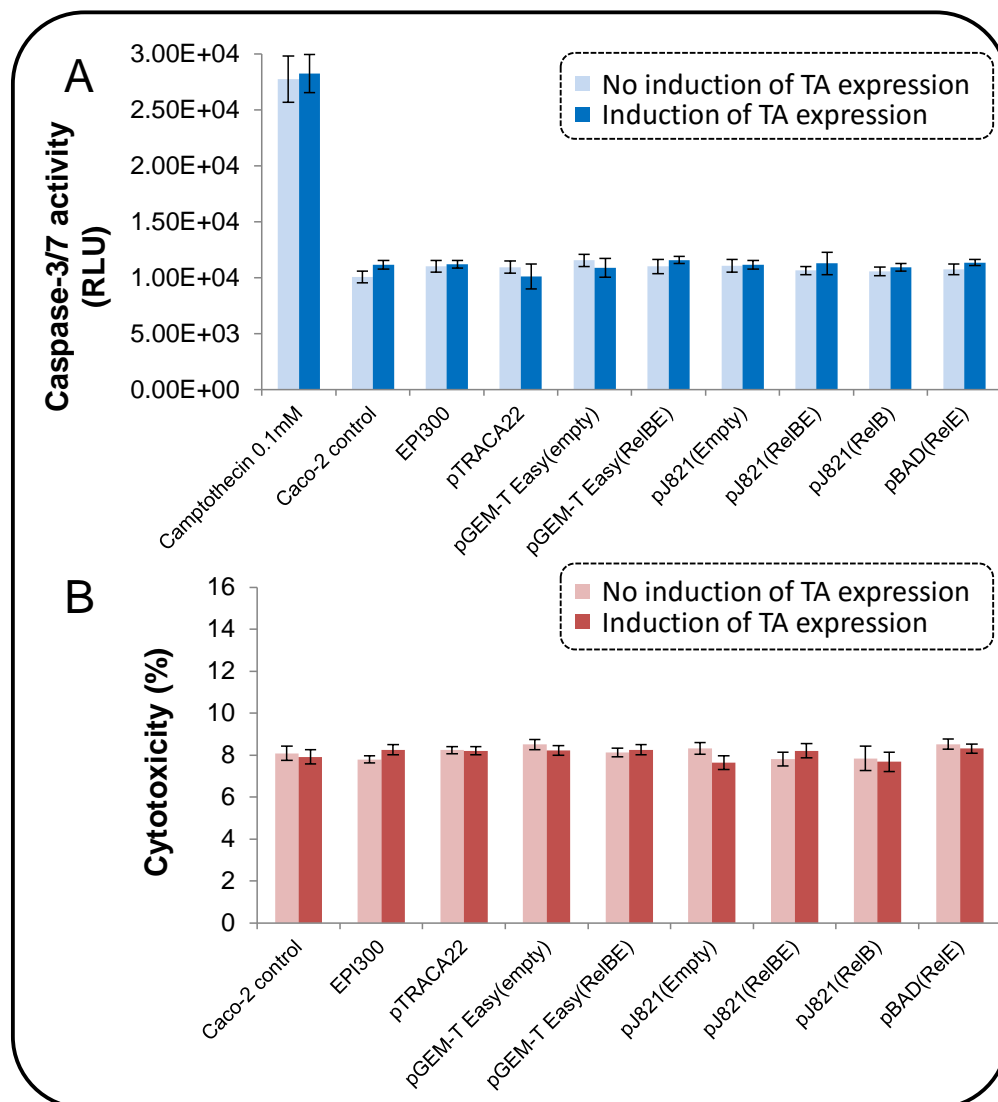
Figure 8.5: Analysis of Caco-2 caspase activity and LDH release when treated with bacterial culture supernatant of *E.coli* EPI300 expressing the p22-RelBE TA module and individual components. Caco-2 cells were grown in 96 well plates until 70% confluent (~14000 cells/well) and then treated with bacterial culture supernatants. Plates were incubated for 6 hours at 37°C and 5% CO_2. Bacterial supernatants were prepared by growing in SGFM overnight, collection by centrifugation and the pH adjusted to 7.2 before filter sterilising and diluting in SGFM.

(**A**) Capase-Glo® 3/7 Assay was performed measuring luminescence proportional to caspase activity and recorded using a Synergy Multi-Mode Plate reader as relative light units (RLU). Data is expressed as the mean of three independent replicates and error bars represent standard error of the mean.

(**B**) The CytoTox 96® Non-Radioactive Cytotoxicity assay was performed and absorbance was measured at 490 nm (OD490). The percentage cytotoxicity was calculated as LDH released in treated cells/maximum LDH release x 100. Data is expressed as the mean of three independent replicates and error bars represent standard error of the mean.

8.4.4 Analysis of apoptosis and cytotoxicity in Caco-2 cells when treated with total protein extracts from EPI300 cultures expressing the p22-RelBE TA module

Induction of apoptosis in Caco-2 cells when treated with total protein extracts from EPI300 cultures expressing the p22-RelBE TA module was quantified measuring caspase-3/7 activity. As displayed in figure 8.6A, caspase-3/7 activity was significantly increased by treatment with total protein extracts for all constructs regardless of TA expression (P<0.05). In addition the use of total protein extracts also showed increased LDH release (P<0.05, Figure 8.6B). The increase in both caspase-3/7 activity and LDH release was comparable between protein extracts from cultures with no induction of gene expression and cultures in which expression of the RelBE module or component genes had been enhanced prior to protein extraction.

8.4.5 Analysis of apoptosis and cytotoxicity in Caco-2 cells when co-cultured with EPI300 expressing the p22-RelBE TA module

Induction of apoptosis in Caco-2 cells when co-cultured with EPI300 expressing the p22-RelBE TA module at an MOI of 5:1 was quantified measuring caspase-3/7 activity. As displayed in figure 8.7A, caspase-3/7 activity was not significantly increased by co-culture with EPI300 regardless of expression of the TA module or individual genes. The cytotoxic effects of the p22-RelBE TA module were also investigated by quantifying LDH release. Co-culture of Caco-2 cells with EPI300 expressing TA module showed no impact on LDH release (Figure 8.7B).
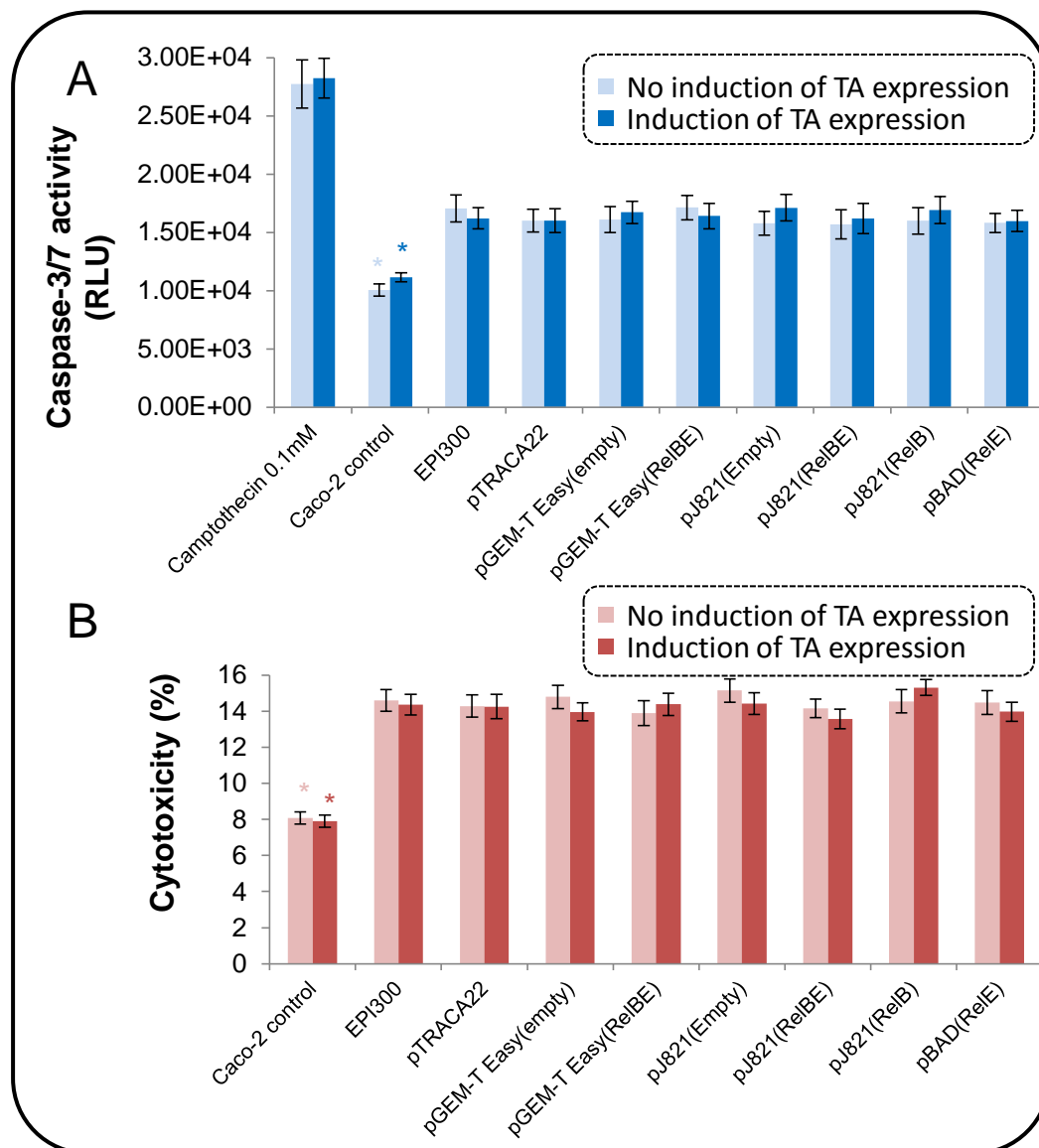
Figure 8.6: Analysis of Caco-2 caspase activity and LDH release when treated with total protein extractions from *E.coli* EPI300 cultures expressing the p22-RelBE TA module and individual components. Caco-2 cells were grown in 96 well plates until 70% confluent (~14000 cells/well) and then treated with total protein extracts. Extracts were prepared by 3 freeze-thaw cycles before incubation for 30 minutes in CelLytic B Bacterial lysis solution containing 0.2 mg/mL lysozyme, 4 units of DNAse I and 1x protease inhibitor cocktail. The supernatant was then collected by centrifugation and protein precipitated in ice-cold aceteone before resuspension in SGFM. Plates were incubated for 6 hours at $37^{\circ}C$ and 5% CO_2.

(**A**) Capase-Glo® 3/7 Assay was performed measuring luminescence proportional to caspase activity and recorded using a Synergy Multi-Mode Plate reader as relative light units (RLU). Data is expressed as the mean of three independent replicates and error bars represent standard error of the mean.

(**B**) The CytoTox 96® Non-Radioactive Cytotoxicity assay was performed and absorbance was measured at 490 nm (OD490). The percentage cytotoxicity was calculated as LDH released in treated cells/maximum LDH release x 100. Data is expressed as the mean of three independent replicates and error bars represent standard error of the mean.

* = P value <0.05.

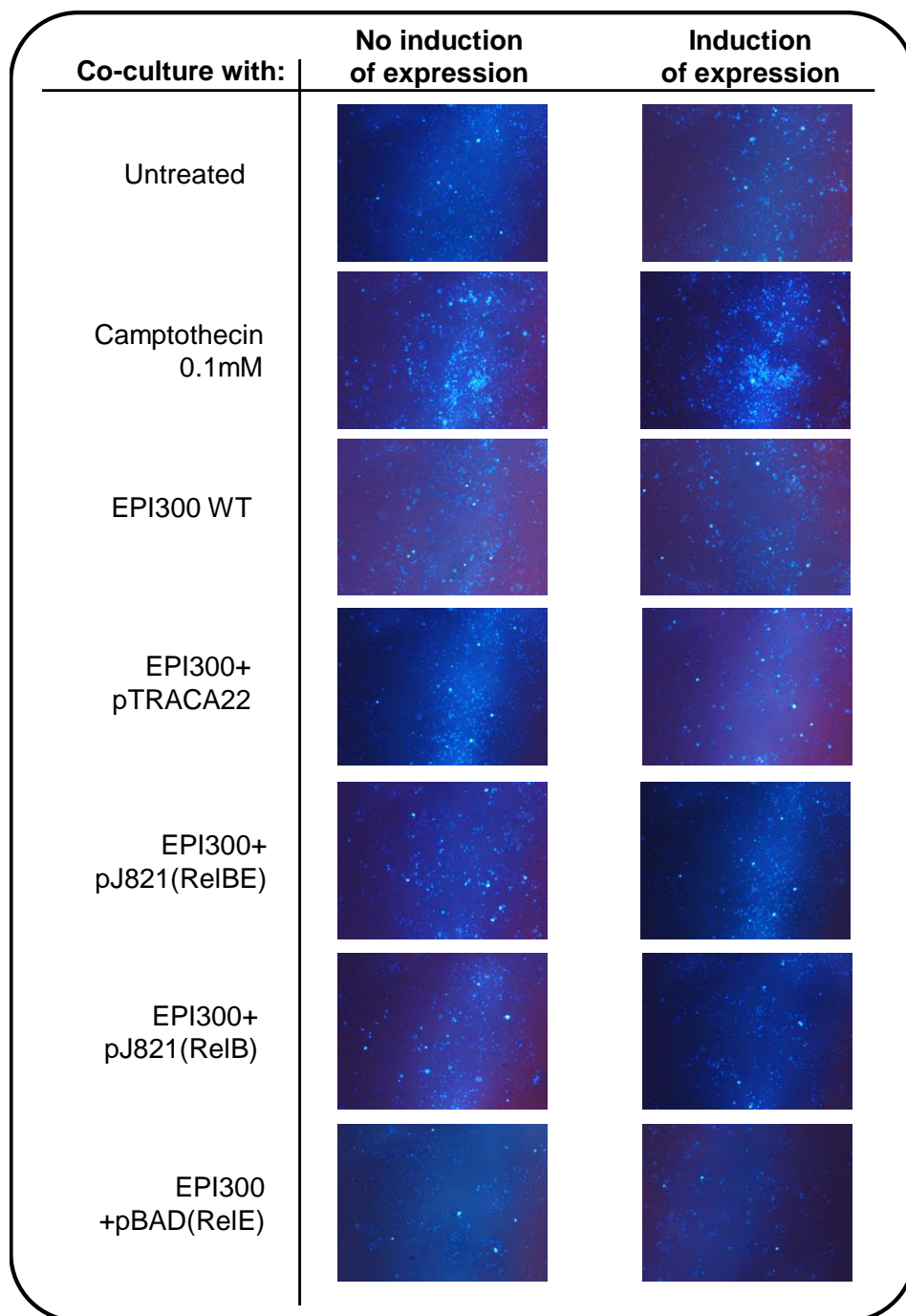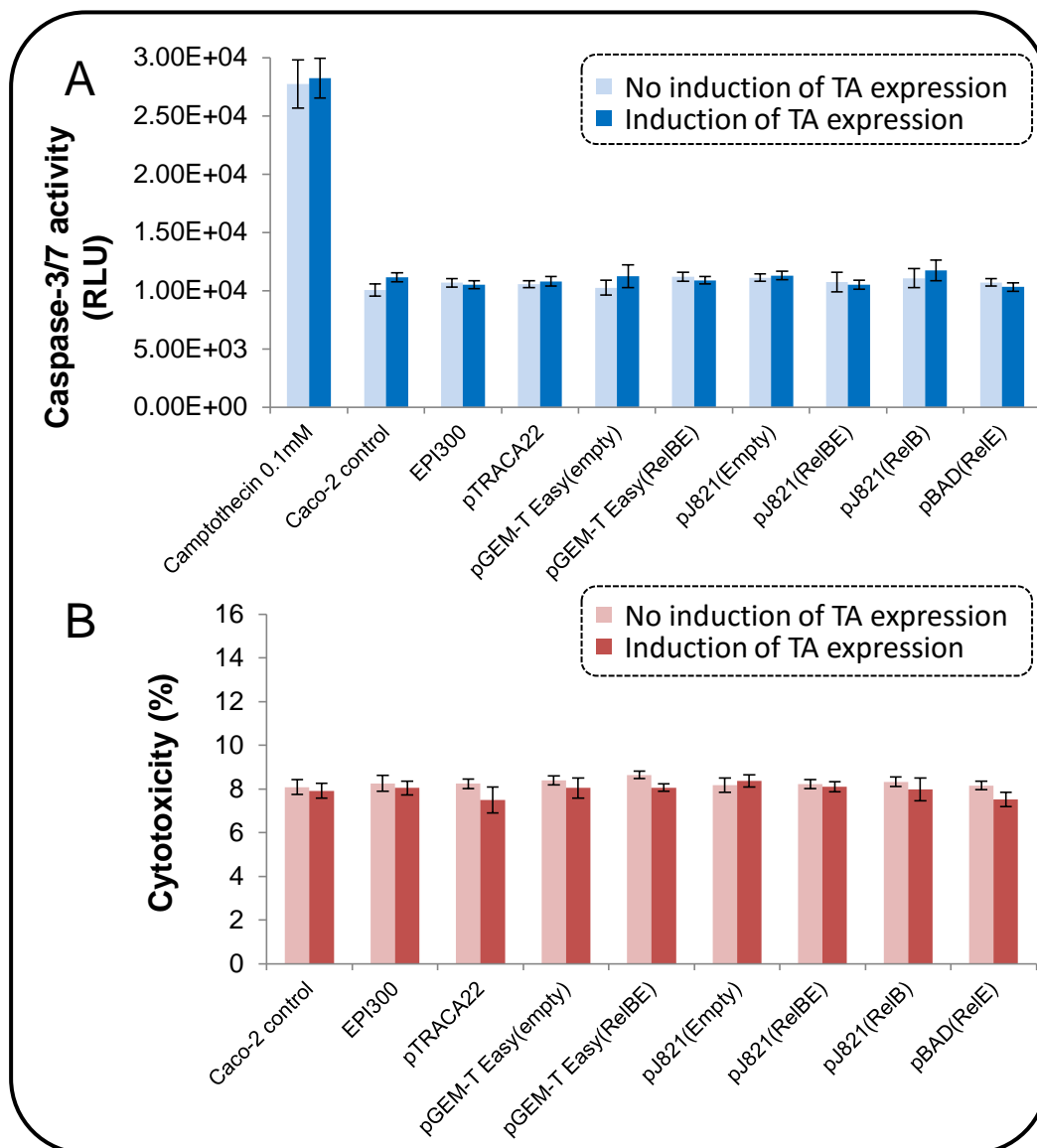**Figure 8.7: Analysis of Caco-2 caspase activity and LDH release when co-cultured with *E.coli* EPI300 expressing the p22-RelBE TA module and individual components.** Caco-2 cells were grown in 96 well plates until 70% confluent (~14000 cells/well) and then treated with bacterial suspensions at an MOI of 5:1 prepared in SGFM. Plates were incubated for 6 hours at 37°C and 5% CO_2 with media removed and replaced with SGFM supplemented with 200 µg/mL gentamicin after 4 hours.

(**A**) Capase-Glo® 3/7 Assay was performed measuring luminescence proportional to caspase activity and recorded using a Synergy Multi-Mode Plate reader as relative light units (RLU). Data is expressed as the mean of three independent replicates and error bars represent standard error of the mean.

(**B**) The CytoTox 96® Non-Radioactive Cytotoxicity assay was performed and absorbance was measured at 490 nm (OD490). The percentage cytotoxicity was calculated as LDH released in treated cells/maximum LDH release x 100. Data is expressed as the mean of three independent replicates and error bars represent standard error of the mean.

8.5 Discussion

8.5.1 The co-culture model: An effective tool for investigating the effect of bacterial TA expression on human cells

The human GI tract is a unique environment in which intestinal epithelial cells and non-pathogenic bacteria co-exist with relative stability (Lozupone *et al.*, 2012). However because of the complexity and diversity of the system, generation of *in vitro* models of study can be difficult. In terms of host-microbial interactions, the culture of human epithelial cells and bacterial cells together, at least to a certain extent, offers a useful tool to examine these interactions. Many co-culture models between human GI tract epithelial cells and bacteria have been designed, with most intended for investigation of probiotic colonisation (Alemka *et al.*, 2010, Hardy *et al.*, 2013, Trapecar *et al.*, 2014), stimulation of the immune system and cell differentiation (Vinderola *et al.*, 2005, Fink & Frokiaer 2008) and host-pathogen interactions. They are also often employed as a precursor for animal *in vivo* models (Fritz *et al.*, 2013).

Co-culture optimisation between the Caco-2 and *E.coli* EPI300 undertaken in this study identified densities of bacterial cells and the durations that these could be co-incubated to allow optimal growth of the cell line and EPI300 without affecting cell viability. In this study 4 hours was found to be optimal however other studies using different strains (including *E.coli* K-12, Nissle and BW25113) have used variations from 60 minutes to 48 hours (He *et al.*, 2013, Hafez *et al.*, 2010 Kim *et al.*, 2009). To consider length of co-incubation, flow rate of the culture vessel also needs to be considered. Experimental conditions within this chapter, as well as several other recently published studies, were done under static conditions (Kim *et al.*, 2009, Nzakizwanayo *et al.*, 2015); however this lack of

movement in growth medium may lead to bacterial overgrowth effecting pH and waste product accumulation.

Bacterial metabolism in growth media to be used during co-culture experiments was examined as expression and translation of the p22-RelBE peptide components was an important factor in the experimental design. The complete DMEM media used for general Caco-2 growth and maintenance contained 10% foetal bovine serum, which has been found on multiple occasions to have bactericidal effects on multiple species including *E.coli* (Blum *et al.*, 1995, Holm *et al.*, 2004, Shibayama *et al.*, 2006). With this said, serum did not appear to be detrimental *E.coli* EPI300 growth in DMEM. Additionally, the removal of glucose from this media did not affect growth up until the 8-hour time point which was necessary for later co culture experiments to be undertaken as glucose behaves as a metabolic repressor for p22-RelBE expression of several constructs designed. This may be a result of EPI300 utilising other carbon sources present in the media such as the additional non-essential amino acid supplements.

8.5.2 The effect of bacterially expressed p22-RelBE on human epithelial cells
Using caspase-3/7 activity as a quantitative marker of apoptosis, it was observed that the p22-RelBE module or individual gene products did not influence the incidence of apoptosis in Caco-2 cell populations. Furthermore no increase in LDH release, a marker of cellular cytotoxicity, was found either. It can be hypothesised that this was the case because i) the RelE toxin does not affect the process of translations within Caco-2 cells, ii) the RelE toxin cannot be secreted by EPI300, iii) Caco-2 cells cannot uptake the RelE protein iv) a combination of the previous. The likelihood that the p22-RelE toxin does not interact with the eukaryotic ribosome is low, firstly because of significant evidence existing showing induction

of cell apoptosis by other RelE proteins and secondly due to the very high percentage identity of the p22-RelE module to a well characterised homologue of the relE toxin in *Blautia hydrogenotrophica* (Jones *et al.*, 2010 - supplementary table 1).

It seems likely that the p22-RelE toxin cannot be secreted from EPI300 and is therefore not available to interact with the Caco-2 monolayer. Bacteria have evolved a wide range of secretion systems for small molecules including proteins and peptides, which have key roles in processes such as adhesion, pathogenicity, adaption and survival (Costa *et al.*, 2015). These secreted substrates can remain associated to the bacterial outer membrane, be released into the extra-cellular environment or injected into a target cell (Kubori *et al.*, 1998, Gerlach & Hensel 2007). In Gram negative organisms, the cellular structures facilitate these processes (pili and curli) can span both the inner and outer membrane or the outer membrane alone (Lycklama-a-Nijehot & Driessen 2011). To date seven secretion systems have been classified, Types I – VII, with Type V including machinery involved with assembling pili and curli, whilst Type VII have only been observed in mycobacteria (Costa *et al.*, 2015). Type I-IV secretion systems are known to be involved in toxin secretion in Gram negative bacteria along with several other mechanisms including the Sec pathway and specific toxin secretion systems, such as for the tetanus toxin (Lee & Schneewind 2001). However; to date no link to the secretion of Type II TA module toxins or evidence that they are even substrates for these systems has been identified.

Molecules which are to be secreted across the bacterial envelope, can either be directly transported from the cytoplasm to the extracellular space, or first translocated to the periplasmic space by inner-membrane spanning transporters Rego *et al.*, 2010). In *E.coli*, proteins which are to be exported out of the cell follow

the Sec-dependent protein export pathway and secretion is achieved by remaining partially unfolded (Peterson *et al.*, 2006). Additionally, to-be-exported proteins (often referred to as preproteins) are synthesised with a cleavable amino acid signal sequence that functions both to slow folding of the preprotein and aids recognition for export factors (Sjostrom *et al.*, 1987, Shiomi *et al.*, 2006). Furthermore the use of Phobius; an online signal peptide predictor (http://phobius.sbc.su.se/) did not identify signal peptides in the amino acid sequences of the toxin or the antitoxin. Based on results obtained in this chapter, it seems more probable that if there is any involvement of the p22-RelBE system in epithelial cell proliferation regulation, it would be achieved through bacterial cell lysis by a portion of the population. This kind of behaviour has been seen in populations expressing TA modules generally as a method of relieving nutritional stress on the community. One of the most understood examples of this are by the MazEF TA system during stressful/unfavourable growth conditions (Hazan *et al.*, 2004), which is considered as a possible method of programmed cell death in prokaryotic populations (Erental *et al.*, 2012, Bayles 2014). RelBE modules have not been documented to cause rapid cell lysis with expression of this module and this tends to be a much slower process (Christensen *et al.*, 2003). However, in the context of the gut environment, slower lysis and release of RelE toxin may be a possibility due to the low flow rate.

If bacterial cell lysis is considered a viable method for p22-RelBE to interact with host epithelial cells, it is imperative to consider the effect of all other cellular contents being released in parallel. Results indicated that co-incubation of Caco-2 cell monolayers with EPI300 total protein extracts did significantly increase apoptosis and cytotoxicity however this was independent of p22-RelBE previous expression by bacterial cultures used to generate the extracts used. This suggests

that certain proteins within these extracts were detrimental to caco-2 viability but whether or not the components of the RelBE module were involved is unclear. The purification of toxin-antitoxin module proteins would allow for the enable examination of the p22-RelE and p22-relB antitoxin effects on Caco-2 viability without other proteinaceous components also interacting.

The concept of using TA module toxins in biotechnology or as targets of antimicrobial development has been gaining momentum and this in turn has led to methodologies for efficiently purifying multiple TA systems (Lioy *et al.*, 2010, Zhu *et al.*, 2009, Nariya & Inouye 2008). Production of these purified proteins is problematic as overproduction of the toxin is lethal not only to bacterial cells, but for some eukaryotic cells as well. Furthermore, over expression of the antitoxin often leads to degradation of the antitoxin due to the inherently unstructured nature of these molecules (Rathore & Gautam 2014). Most recently, Sterckx *et al.*, (2015) have developed a methodology for this which has allowed the isolation of active toxin and antitoxin for the Phd/Doc, MazEF, ParAE, and HigAB modules from multiple species including *E. coli* and *V. cholerae* (Sterckx *et al.*, 2015).

Finally, although RelE toxins have been shown to be active within eukaryotic cells, this does not necessarily translate to a role in host-microbe interaction. Excluding other roles of TA modules within the microbiome, whether these systems (as a whole rather than just the RelBE module) have host-microbe functionality remains to be seen and much more investigation is needed.

Findings from studies undertaken in this chapter suggest that the pTRACA22-RelBE module when carried and expressed by *E.coli* EPI300 does not significantly affect levels of apoptosis and cytotoxicity in the Caco-2 gut epithelial cell line. This is probably because the toxin, which for this study could be considered the functional portion of the module, was unable to reach the intended target of the

eukaryotic ribosome. Whether this is due to i) no secretion of the toxin from bacterial cells or ii) a lack of uptake by the human cell line is still unclear, but both factors likely influence this. To further investigate the effect of p22-RelBE bacterial expression on Caco-2 cells purification of the protein components may be a possible route; however how representative this may be of an *In vivo* situation is debatable.

8.6 Conclusions

- Multiplicities of infection higher than 5:1 in a Caco2-*E.coli* EPI300 co culture module as well as duration for longer than 4 hours are detrimental to Caco-2 cell viability.

- Expression of the pTRACA22 RelBE module, individual antitoxin and toxin by *E.coli* EPI300 when in co culture with Caco-2 cells did not increase apoptosis or cytotoxicity.

Chapter 9: General discussion

TA modules have been linked both directly and indirectly with a number of possible physiological roles within bacterial populations and communities, often dependent on the host background, genomic location of the module and pressures exerted by the external environment (Gerdes 2013). With such varied functions and the apparent ubiquity of TA modules within prokaryotes, the distribution and cellular functions of TA systems in the microbial community colonising the human gut is of interest from both a medical and developmental perspective. However, little is known regarding TA abundance, the role they undertake or how expression of these modules can affect the interaction between the gut community and the higher host within this environment. The two overarching hypotheses of studies undertaken within this thesis were firstly, that Type II TA systems were highly prevalent within the human gut microbiome and secondly, that expression of the gut-abundant p22-RelBE Type II TA module could directly impact bacterial survival when challenged by stress associated with the human gut environment.

9.1 Distribution and carriage of TA modules alter functional neighbourhoods surrounding them

The application of comparative genomic and metagenomic analysis provided a powerful tool with which to explore Type II TA distribution and abundance of both genetic units and discrete environments. Additionally this enabled investigation of gene neighbourhood composition surrounding putative modules identified through this method.

The results obtained during gene neighbourhood studies indicated that approximately 40% of assignable open reading frames (ORFs) appeared to be

viral in origin (33% associated with prophage and 7% to viruses), compared to 60% plasmid based. In comparison, abundance data on the prevalence of Type II TA modules within genomes found only a small proportion carried on plasmids and fewer still on phage genomes with the majority, especially for those most abundant within the human gut environment, present on chromosomes. These differences may be explained by the differences between the queries searched. The abundance study undertaken specifically identified hits matching TA module queries alone, whilst the gene neighbourhood study searched databases with predicted ORFs in sequence data surrounding putative TA module genes. While the original putative TA hits may be primarily located on chromosomes, the segment of DNA harbouring TA systems within the chromosome sequence may have plasmid or bacteriophage origins. This seems highly plausible due to the addictiveness of these modules and the repeated finding of TA systems being within or closely related to mobile genetic elements on bacterial chromosomes (Pandes & Gerdes 2005, Van Melderen & De Bast 2009).

The notable presence of predicted MGEs surrounding putative TA genes (approximately 10% greater than randomly generated sequence ORFs and 30% greater than ORFs surrounding RpoB housekeeping genes) may suggest a role in dissemination of virulence factors, resistance determinants and other bacterial traits. Predicted determinants for beta lactam resistance were highly abundant in TA-associated regions of interest, demonstrating stabilisation of these factors within chromosomes at the very least but may also indicate MGEs currently residing within bacterial chromosomes, but able to move via horizontal transfer may be stabilised by Type II TA modules within the human gut environment. The presence of resistance determinants within MGEs harbouring putative TA genes has also been shown and mapped in four completely sequenced *Pseudomonas*

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aeruginosa genomes, supporting this hypothesis that TA systems may play a role in the stabilisation of self-transmissible integrative elements along with useful auxiliary traits (Bi *et al.*, 2012). Identifying MGEs on regions that appear to have both a toxin and antitoxin (both separated and in the more conventional overlapping confirmation) may help further understand whether an entire module, or just one component is needed for stabilisation.

9.2 Reduction of phage predation by expression of the p22-RelBE TA module

In addition to *In Silico* findings that TA systems appear to have an association with prophage elements rather than viral, and the distinct lack of Type II TA modules associated with phage genomes, it was identified that expression of the p22-RelBE TA module toxin could reduce PFU counts significantly when infected with bacteriophage B2. Primarily this ascertains that the p22-RelBE module can behave as an anti-phage mechanism with *E.coli* EPI300, particularly as both the individual toxin and antitoxin component returned no hits to any of the phage genomes or metagenomic fragments searched. The presence of putative TA genes in the proximity of prophage elements however suggests they may still be harboured within phage and it may be that differences in phage lifestyle might dictate which phage carry these modules.

With this in mind, it is worth considering the requirements and pressure placed on the human gut microbiome by the higher host. Emerging evidence from virome studies has indicated that the gut microbiome is dominated by temperate phage (Reyes *et al.* 2010; Minot *et al.* 2011; Stern *et al.* 2012; Ogilvie *et al.* 2013). If the overall functional stability of the human gut microbial community is a prerequisite for the close relationship between the higher host and microbiome

(which would seem logical due to the close co-evolution of that community with the host), it can be hypothesised that host level selective pressure will push for a community that is stable, with a consistent functional output in terms of emergent properties that benefit the higher host. Phage can severely disrupt ecosystems, as is clear from their use in phage therapy (Letarov *et al.* 2010), and with regards to the human gut habitat this would be detrimental to the host. There has also been preliminary evidence showing an increase of phage in CD sufferers compared to healthy patients (Wagner *et al.* 2013), suggesting a potential involvement in the disease. Furthermore it may be that phage attack may initiate disease, triggering community de-stabilisation and the dysbiosis characteristic seen in CD. Could the microbiomes of some individuals be more susceptible or resistant to this disruption and, if this is the case, how is this resistance achieved? The finding that PhD/Doc protects the loss of lysogenic P1 whilst other modules, such as ToxIN, MazEF and now p22-RelBE suggests TA modules may offer at least a partial solution to this question. In parallel to this carriage of TA systems within temperate phage may also be a mechanism by which they can outcompete rival phage for hosts, allowing retention of the prophage and potentially providing protection from incoming lytic phage.

9.3 The p22-RelBE TA module as a mechanism for stress tolerance

Perhaps the clearest outcome of studies undertaken is that the p22-RelBE module is able to provide (at least transiently) a mechanism by which *E.coli* EPI300 can tolerate stress associated with the human gut environment. *E.coli* EPI300 expressing the p22-RelBE module showed increased persistence when nutritionally challenged by a lack of nitrogen and carbon, when treated with normally inhibitory doses of certain beta lactam antimicrobial compounds and

infected by bacteriophage B2. Although Type II TA systems have been previously shown to undertake many of these functions, such as persistence when starved of nitrogen by a RelBE module (Christensen *et al.*, 2001), reduced spread of prophage P1 by expression of MazEF (Hazan & Engelberg-Kulka 2004) and increased killing by both ciprofloxacin and ampicillin when natural TA systems in *E.coli* were deleted (Maisonneuve *et al.*, 2011), this is the first time showing that one individual module (p22-RelBE) can offer some level of protection against multiple stresses impacting one environment.

What effect expression of such a module might have on colonisation of the human gut remains to be explored, but it seems plausible that carriage of genes enabling survival in low nutritional conditions, such as outside the gut environment, prior to colonisation and during feed-famine cycles within the gut would be highly beneficial. Furthermore a mechanism to avoid some level of phage predation once within the gut would offer a fitness advantage over other competing organisms, especially during initial colonisation of new-borns. Finally, systems that can provide transient resistance to antimicrobial stress would allow persistence and recovery of a community after periods of antibiotic therapy to treat infections, allowing normal gut function to be maintained. Notably other TA systems also appear to be linked with general stress resistance and colonisation of niche environments, such as the Type II YefM-YoeB module and the role it plays in colonisation of uropathogenic *E.coli* (Norton & Mulvey 2012). To this end a key next step in understanding the role of TA systems within the gut would be the colonisation of animal models by commensal organisms expressing the p22-RelBE module and challenging these organisms *in vivo*.

It is also worth considering that while these modules provide some level of population-level protection to stressors, many organisms often contain multiple

different TA systems within their genome (Pandey & Gerdes 2005). It may be that the carriage and expression of multiple systems may lead to a cumulative effect where persistence is increased up to a point dependent on TA numbers. This has been documented previously where progressive deletion of multiple TA systems led to proportional reduction in *E.coli* tolerance to both ciprofloxacin and ampicillin treatments (Maisonneuve *et al.*, 2011). Alternatively it may be that TA systems are less global in there stress response, but instead function together, each providing avoidance mechanisms to different stresses, but leading to increased tolerance of many stresses as a whole.

Approaching the persistence provided by TA systems from an alternative angle, TA systems present useful targets for the development of new antibacterial therapies and may be achieved through inhibition of the antitoxin before it can neutralise the toxin component. One notable finding from studies within this thesis was that the increased persistence to stress was transiently present before returning to WT values. Prolonged exposure to TA toxins (even if bacteriostatic) would eventually lead to cell death. Several TA systems which utilise regulators could be useful starting points as regulation of the module is often achieved through regulator-encoded ligands which can bind to the antitoxin component and effect feedback loops responsible for TA regulation (Trovatti *et al.*, 2008, Collin *et al.*, 2011, Barbosa 2012).

9.4 Future work

The following should be investigated in future studies:

- Further exploration of ACLAME hits, focussing primarily on the five most gut abundant toxins, antitoxins and regulators to identify if sequences surrounding these harbour phage-related ORFs.

- Expansion and further analysis of CRC gut metagenomes for use in RT-PCR studies to investigate differences in TA copy number between healthy and sufferer samples. This may strengthen any link identified in this study between TA carriage and alterations in the gut community of CRC sufferers.

- Cloning of other gut-abundant Type II TA module components and utilising methodologies presented within this thesis to gauge the affect they have on bacterial stress tolerance. This would provide further evidence of a general role for TA modules within the human gut microbiome.

- Investigate the potential for several of the most-gut abundant TA module antitoxins to neutralise multiple toxins, especially those found abundantly with the gut. This may help establish whether Xre-related antitoxins identified have multiple toxin counterparts with the gut environment.

- Use of a Gram-positive bacterial background, such as *Lactococci,* for further investigation into the ability of p22-RelBE to increase tolerance to gut-related stress, as the module appears to have originated from the Firmicutes.

- Examine the effect carriage and expression of gut-abundant TA modules has on inter-plasmid and inter-phage competition within a bacterial cell population. Preliminary insight may be achieved through simple plasmid loss experiments and plaque-recovery assays.

Chapter 10: References

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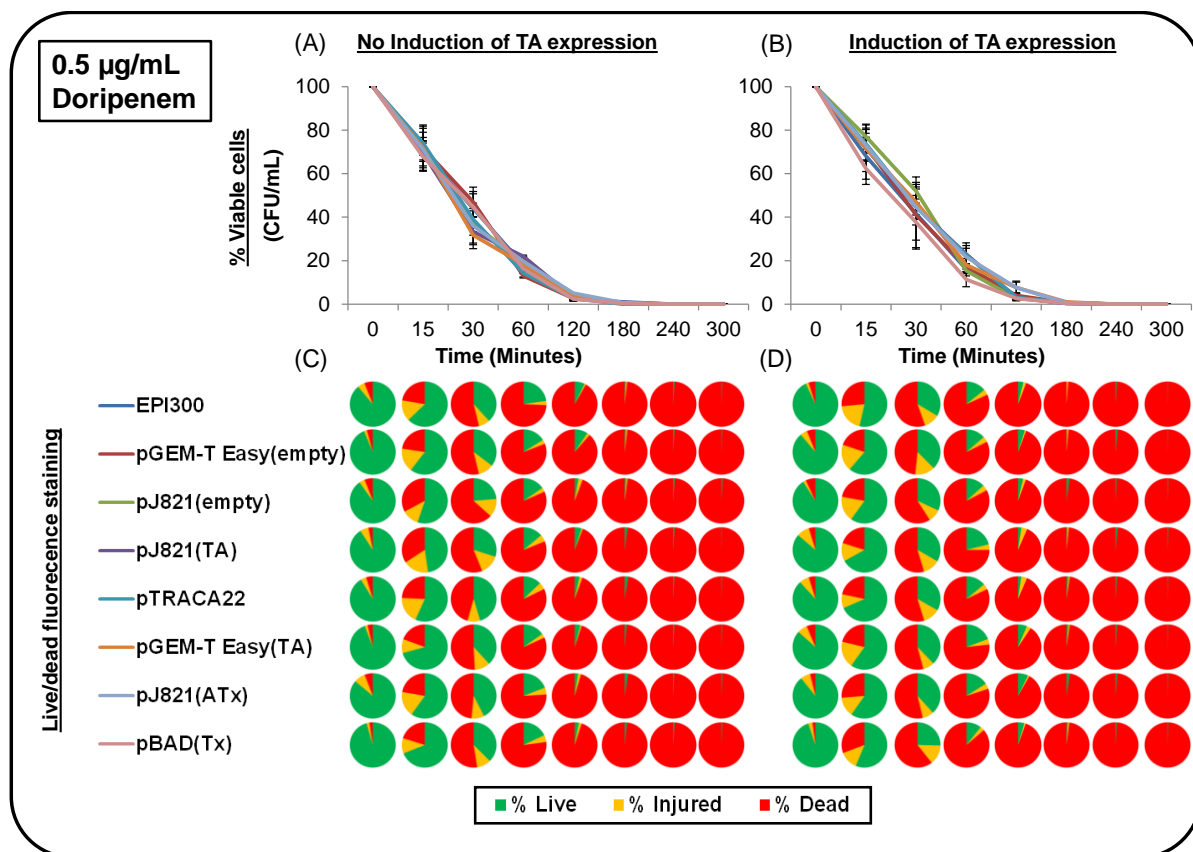
Figure 1: The effect of the p22-RelBE TA system on E.coli EPI300 survival during treatment with 0.5 µg/mL doripenem. Cell suspensions in LB broth supplemented with 0.5 µg/mL doripenem were incubated for 5 hours and used for both viable plate counts and live/dead fluorescence staining. 0.2% (w/v) glucose was added at time point 0 to cultures in which cells were not induced. For those induced, 100mM rhamnose or 2% (w/v) arabinose was added. A & B) graphs refer to percentage viable cells (CFU/mL), whilst C & D) pie charts represent percentage live/dead/injured. All experiments were undertaken in triplicate and results shown are the mean ± SEM of three independent experiments.
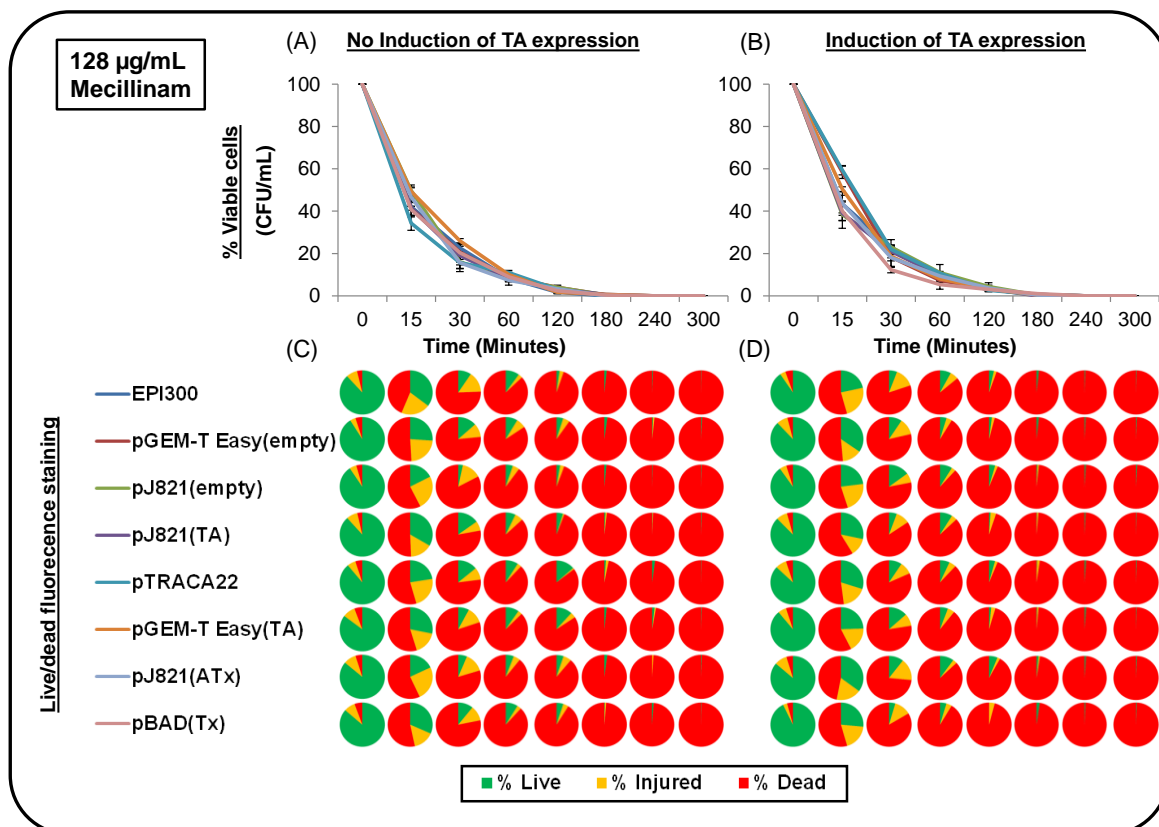
Figure 2: The effect of the p22-RelBE TA system on E.coli EPI300 survival during treatment with 128 µg/mL mecillinam. Cell suspensions in LB broth supplemented with 128 µg/mL mecillinam were incubated for 5 hours and used for both viable plate counts and live/dead fluorescence staining. 0.2% (w/v) glucose was added at time point 0 to cultures in which cells were not induced. For those induced, 100mM rhamnose or 2% (w/v) arabinose was added. A & B) graphs refer to percentage viable cells (CFU/mL), whilst C & D) pie charts represent percentage live/dead/injured. All experiments were undertaken in triplicate and results shown are the mean ± SEM of three independent experiments.
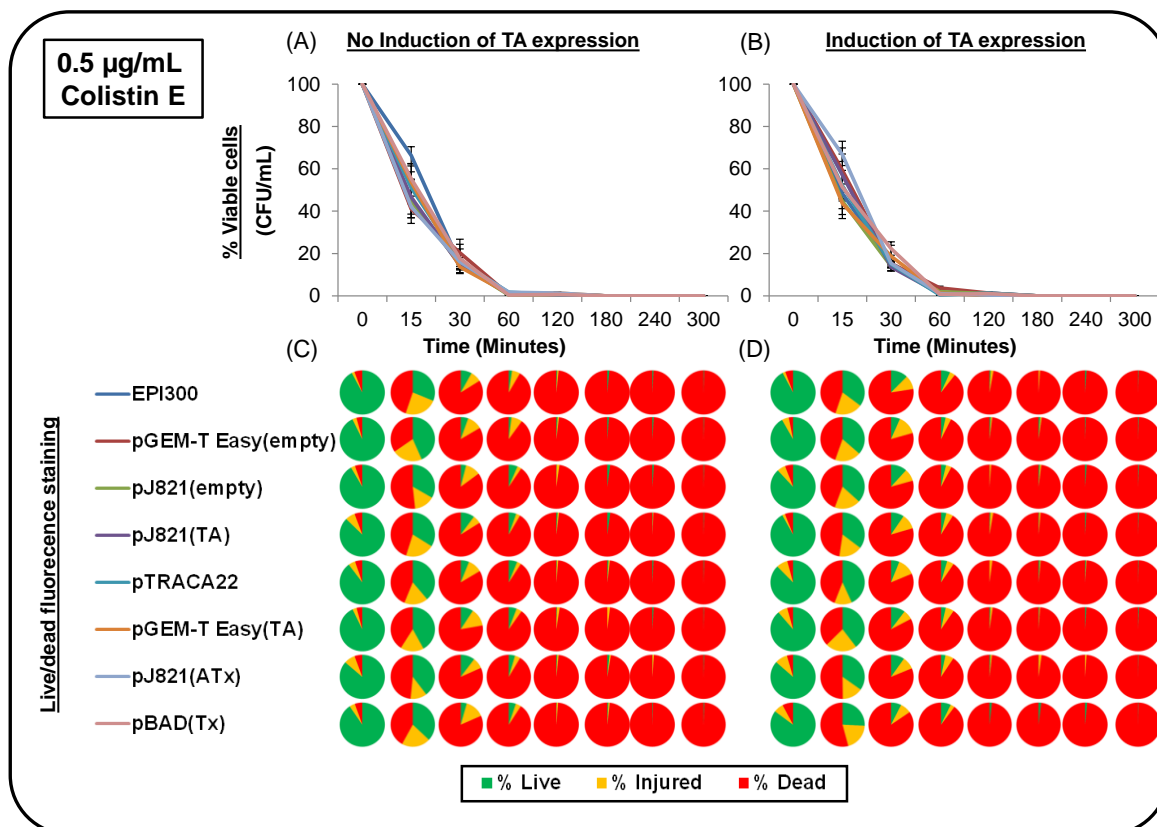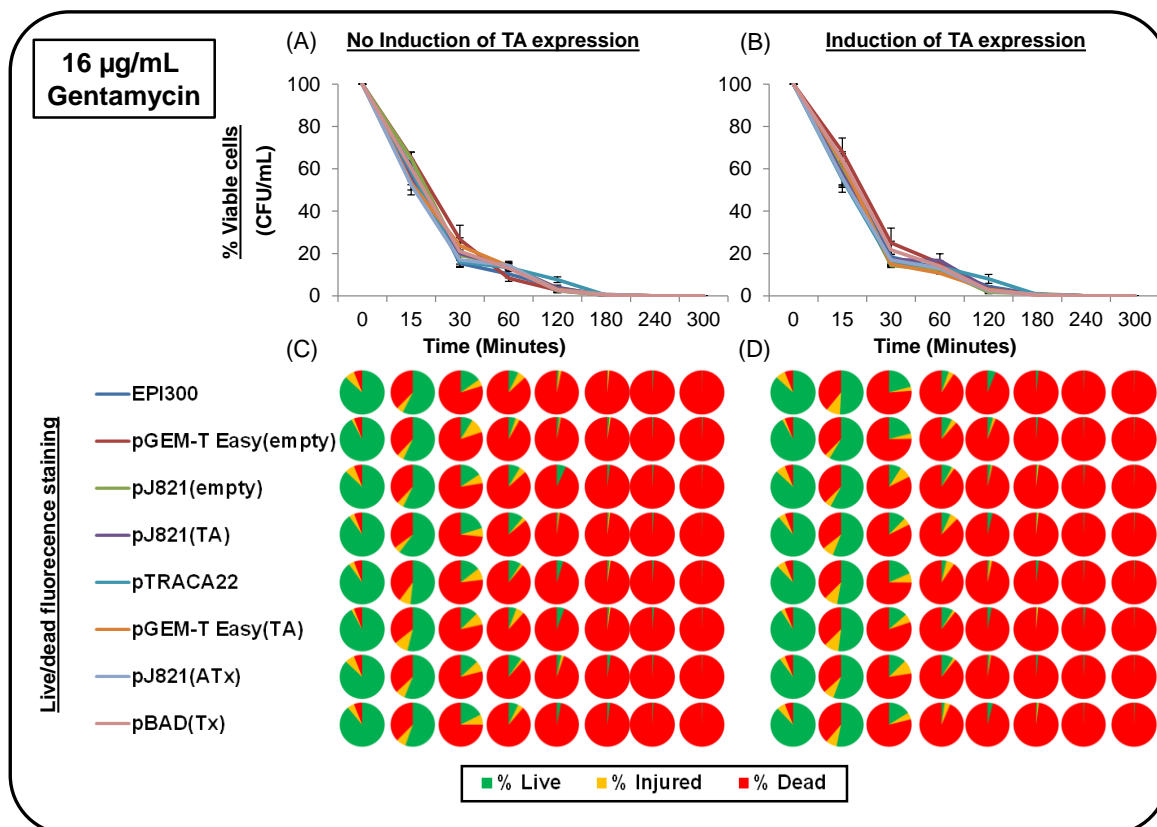
Figure 3: The effect of the p22-RelBE TA system on E.coli EPI300 survival during treatment with 0.5 μg/mL colistin. Cell suspensions in LB broth supplemented with 0.5 μg/mL colistin were incubated for 5 hours and used for both viable plate counts and live/dead fluorescence staining. 0.2% (w/v) glucose was added at time point 0 to cultures in which cells were not induced. For those induced, 100mM rhamnose or 2% (w/v) arabinose was added. A & B) graphs refer to percentage viable cells (CFU/mL), whilst C & D) pie charts represent percentage live/dead/injured. All experiments were undertaken in triplicate and results shown are the mean ± SEM of three independent experiments.
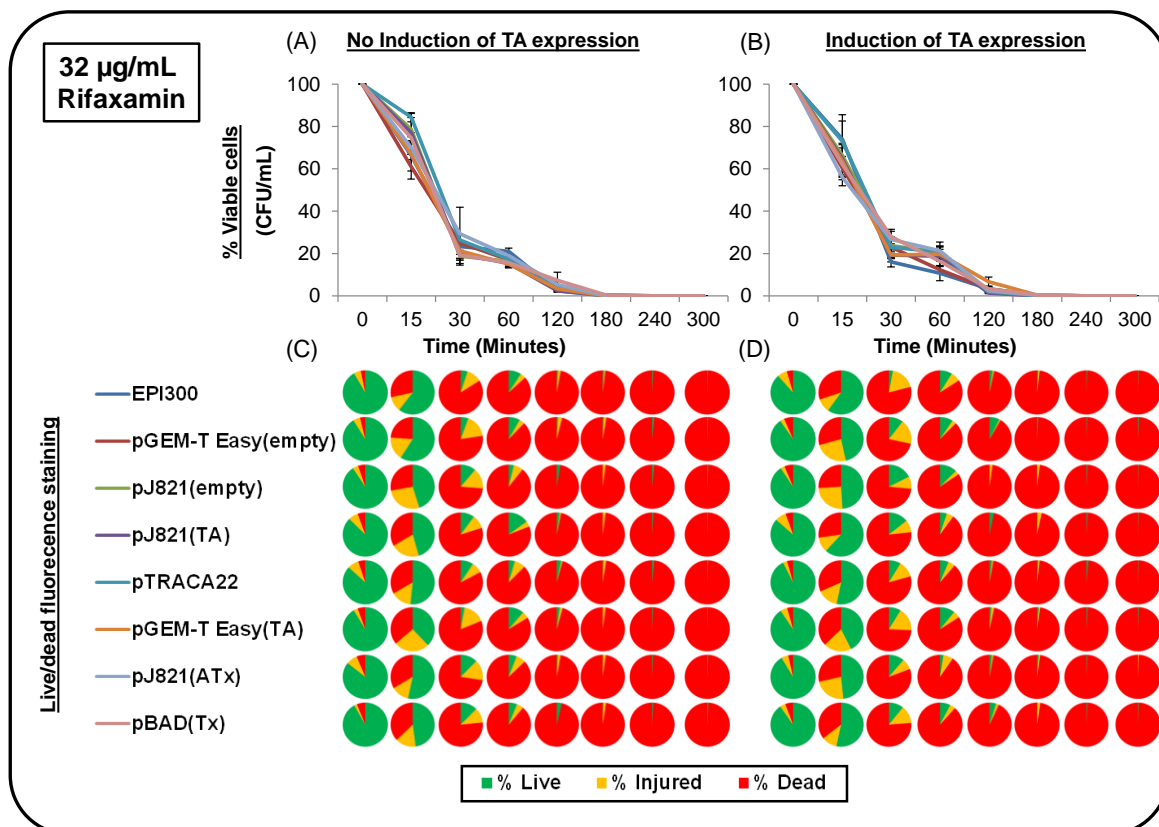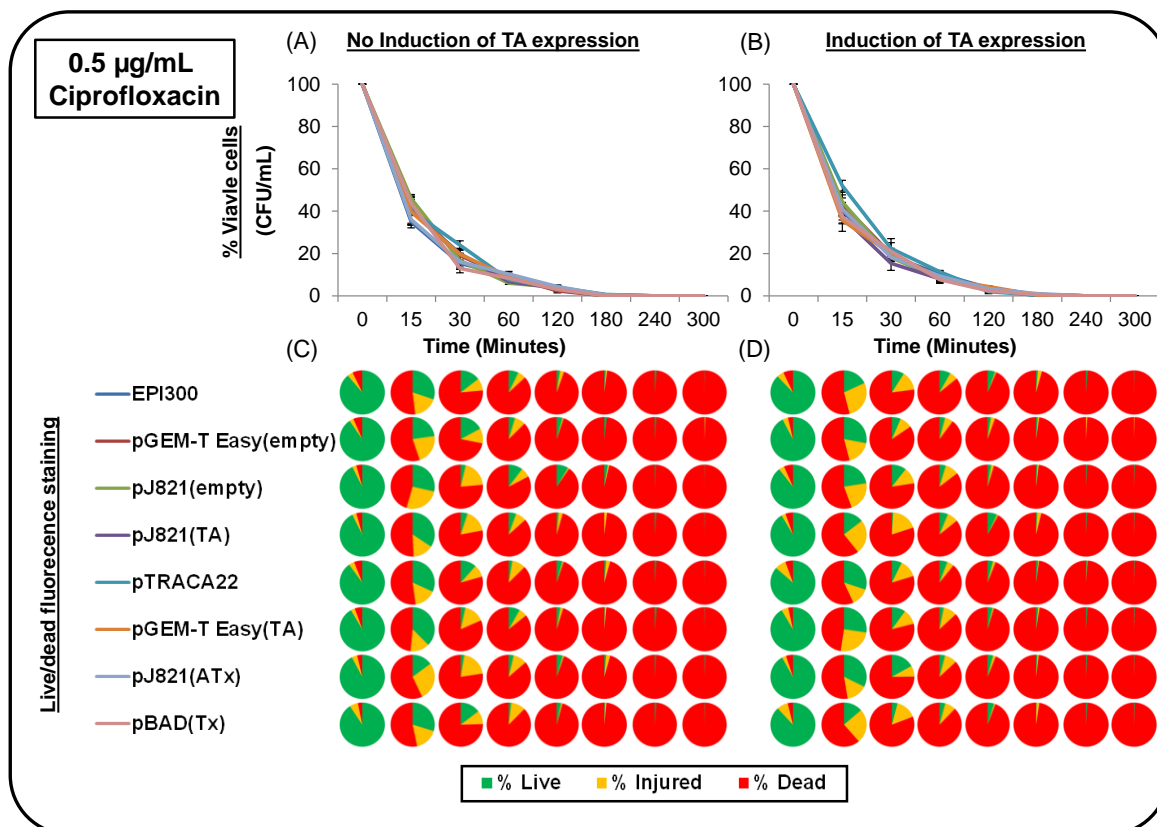
Figure 4: The effect of the p22-RelBE TA system on E.coli EPI300 survival during treatment with 32 µg/mL Rifaxamin. Cell suspensions in LB broth supplemented with 32 µg/mL Rifaxamin were incubated for 5 hours and used for both viable plate counts and live/dead fluorescence staining. 0.2% (w/v) glucose was added at time point 0 to cultures in which cells were not induced. For those induced, 100mM rhamnose or 2% (w/v) arabinose was added. A & B) graphs refer to percentage viable cells (CFU/mL), whilst C & D) pie charts represent percentage live/dead/injured. All experiments were undertaken in triplicate and results shown are the mean ± SEM of three independent experiments.
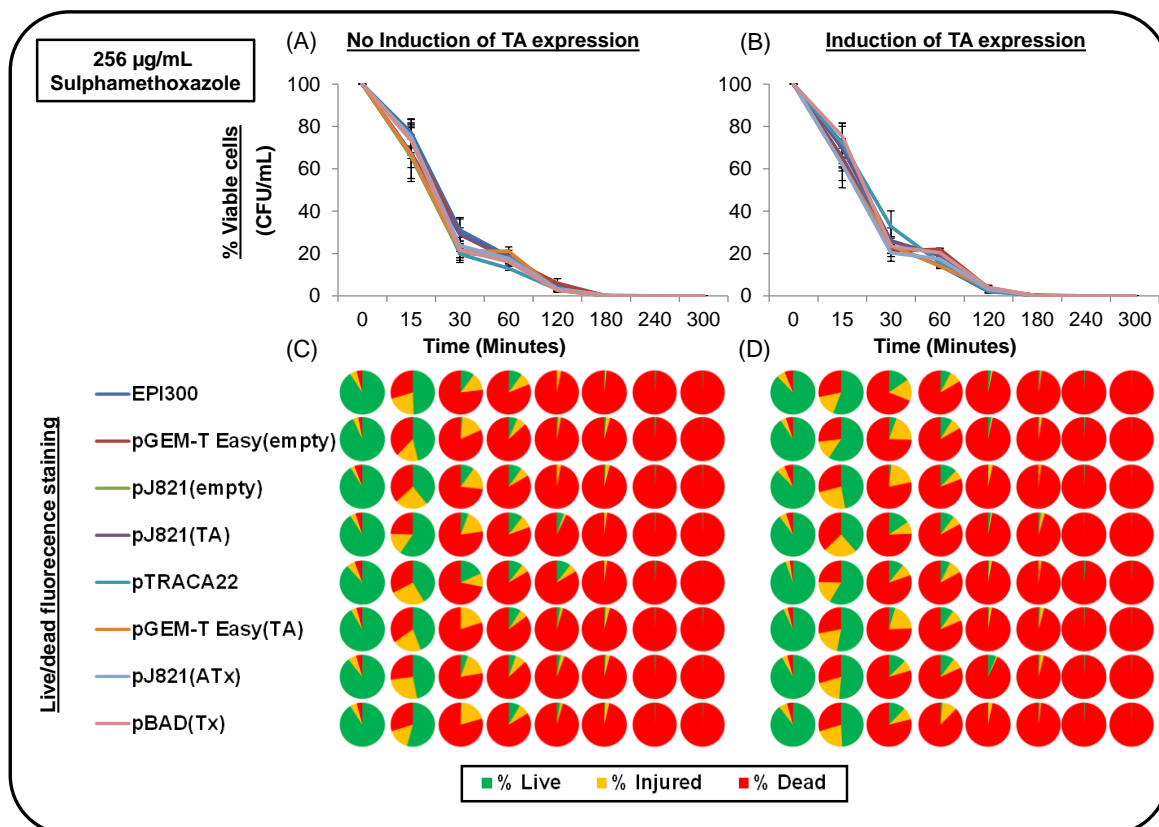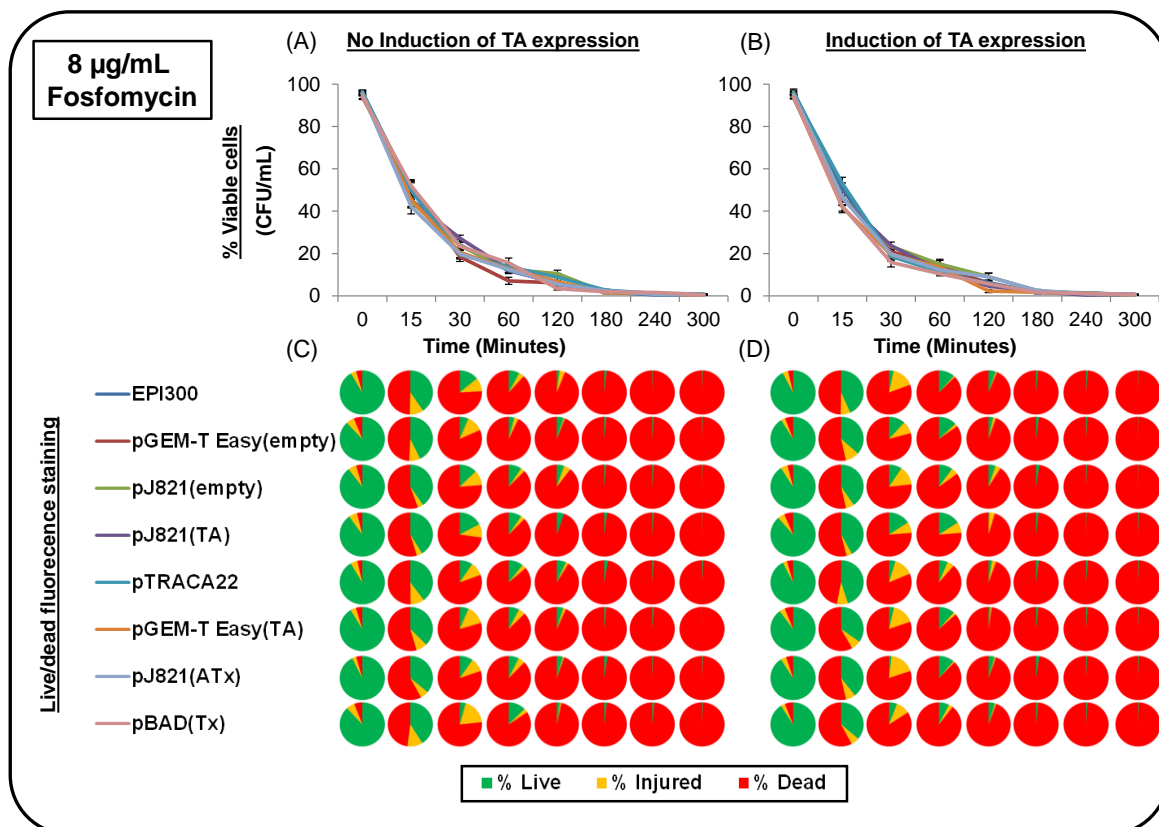
Figure 5: The effect of the p22-RelBE TA system on E.coli EPI300 survival during treatment with 16 μg/mL gentamicin. Cell suspensions in LB broth supplemented with 16 μg/mL gentamicin were incubated for 5 hours and used for both viable plate counts and live/dead fluorescence staining. 0.2% (w/v) glucose was added at time point 0 to cultures in which cells were not induced. For those induced, 100mM rhamnose or 2% (w/v) arabinose was added. A & B) graphs refer to percentage viable cells (CFU/mL), whilst C & D) pie charts represent percentage live/dead/injured. All experiments were undertaken in triplicate and results shown are the mean ± SEM of three independent experiments.
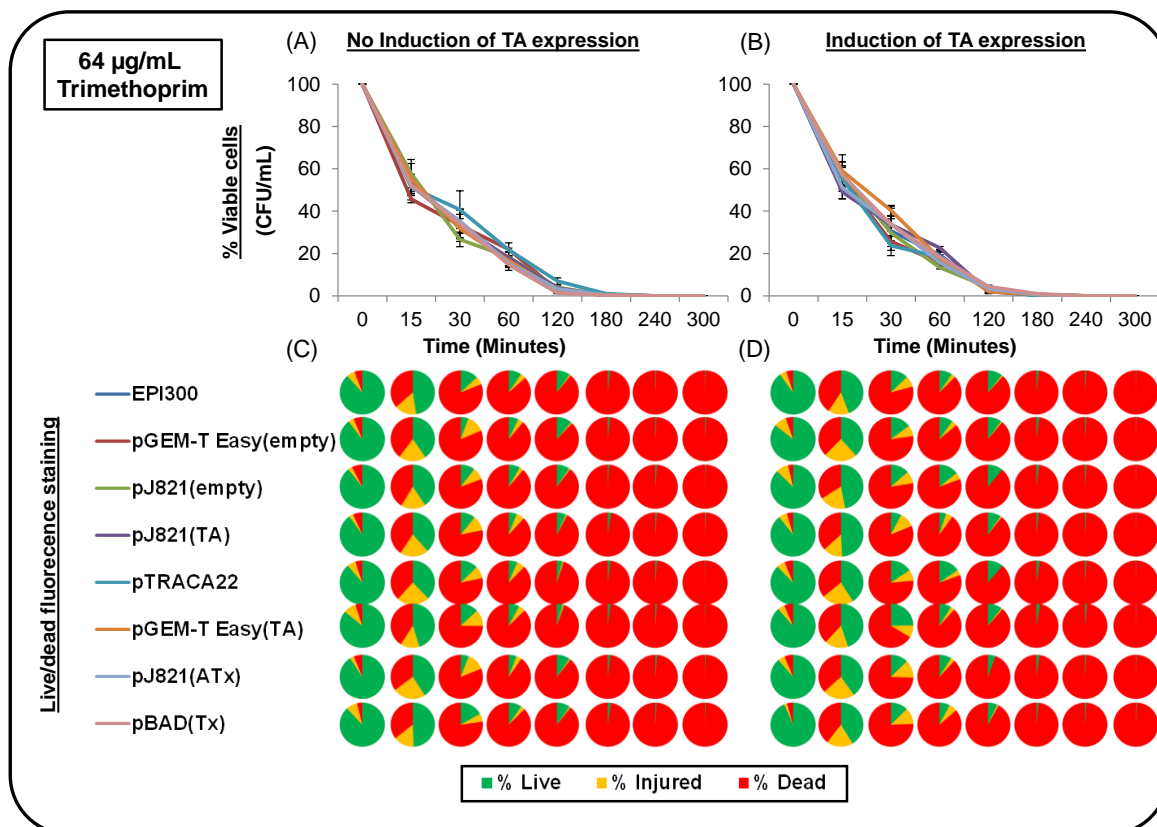
Figure 6: The effect of the p22-RelBE TA system on E.coli EPI300 survival during treatment with 0.5 μg/mL ciprofloxacin. Cell suspensions in LB broth supplemented with 0.5 μg/mL ciprofloxacin were incubated for 5 hours and used for both viable plate counts and live/dead fluorescence staining. 0.2% (w/v) glucose was added at time point 0 to cultures in which cells were not induced. For those induced, 100mM rhamnose or 2% (w/v) arabinose was added. A & B) graphs refer to percentage viable cells (CFU/mL), whilst C & D) pie charts represent percentage live/dead/injured. All experiments were undertaken in triplicate and results shown are the mean ± SEM of three independent experiments.

Figure 7: The effect of the p22-RelBE TA system on E.coli EPI300 survival during treatment with 256 µg/mL sulphamethoxazole. Cell suspensions in LB broth supplemented with 256 µg/mL sulphamethoxazole were incubated for 5 hours and used for both viable plate counts and live/dead fluorescence staining. 0.2% (w/v) glucose was added at time point 0 to cultures in which cells were not induced. For those induced, 100mM rhamnose or 2% (w/v) arabinose was added. A & B) graphs refer to percentage viable cells (CFU/mL), whilst C & D) pie charts represent percentage live/dead/injured. All experiments were undertaken in triplicate and results shown are the mean ± SEM of three independent experiments.

Figure 8: The effect of the p22-RelBE TA system on E.coli EPI300 survival during treatment with 8 µg/mL fosfomycin. Cell suspensions in LB broth supplemented with 8 µg/mL fosfomycin were incubated for 5 hours and used for both viable plate counts and live/dead fluorescence staining. 0.2% (w/v) glucose was added at time point 0 to cultures in which cells were not induced. For those induced, 100mM rhamnose or 2% (w/v) arabinose was added. A & B) graphs refer to percentage viable cells (CFU/mL), whilst C & D) pie charts represent percentage live/dead/injured. All experiments were undertaken in triplicate and results shown are the mean ± SEM of three independent experiments.

Figure 9: The effect of the p22-RelBE TA system on E.coli EPI300 survival during treatment with 64 µg/mL trimethoprim. Cell suspensions in LB broth supplemented with 64 µg/mL trimethoprim were incubated for 5 hours and used for both viable plate counts and live/dead fluorescence staining. 0.2% (w/v) glucose was added at time point 0 to cultures in which cells were not induced. For those induced, 100mM rhamnose or 2% (w/v) arabinose was added. A & B) graphs refer to percentage viable cells (CFU/mL), whilst C & D) pie charts represent percentage live/dead/injured. All experiments were undertaken in triplicate and results shown are the mean ± SEM of three independent experiments.