Investigating the molecular mechanisms of colicin import into *Escherichia coli* cells

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DECLARATION

I do hereby declare that this thesis is my own work and has not been submitted elsewhere. All work contained within this report is my own unless otherwise acknowledged.

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Supervisor

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To my father Sfouq Shwaish Ben Mnikher (RIP), you are always with me.

Abbreviations

2YT	2 x yeast tryptone
Å	Angstrom
APS	Ammonium persulfate
bp	DNA base pairs
BRP	Bacteriocin Release Protein
cAMP	Cyclic adenosine monophosphate
CD	Circular Dichroism
C domain	Cytotoxic domain
ColA	Colicin A
ColB	Colicin B
ColD	Colicin D
ColE1	Colicin E1
ColE3	Colicin E3
ColE2	Colicin E2
ColE4	Colicin E4
ColE5	Colicin E5
ColE7	Colicin E7
ColE8	Colicin E8

ColE9	Colicin E9
ColK	Colicin K
ColN	Colicin N
ColM	Colicin M
CRP	cAMP receptor protein
CM	cytoplasmic membrane
Da	Dalton
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
g3p	gene 3 protein (bacteriophage minor coat protein)
IM	Inner membrane
Im7	Immunity protein for colicin E7
Im8	Immunity protein for colicin E8
Im9	Immunity protein for ColE9
IPTG	Isopropyl-β-D-thiogalactopyranoside
Kb	DNA kilobase pairs
K_d	Dissociation constant

kDa	kilo Dalton
l	Litre
LB	Luria-Bertani broth
LPS	Lipopolysaccharide
LPP	Lipoprotein
mM	Millimolar
mRNA	Messenger ribonucleic acid
μM	Micromolar
nM	Nanomolar
mpc	molecules per cell
NDR	Natively disordered region
NMR	Nuclear magnetic resonance
OD	Optical density
OM	Outer membrane
OMPLA	Outer membrane phospholipase A
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
Pal	Peptidoglycan associated lipoprotein
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PG	peptidoglycan
Pmf	proton motif force
Ppm	part per million
R domain	Receptor-binding domain
RFU	Relative fluorescence units
RLU	Relative luminescence units
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SDM	Site-directed mutagenesis
SDS	Sodium dodecyl sulphate
SPR	Surface Plasmon Resonance
tRNA	Transfer ribonucleic acid
T domain	Translocation domain
Ta	anealing temperature
Tm	Melting temperature
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol
V_e	Elution volume
V_o	Void volume

List of Conference / Oral and poster presentations

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Fadilah Aleanizy, Mireille Vankemmelbeke, Christopher Penfold and Richard James (2010). Periplasmic expression of the translocation domain of ColAconfers protection of *E. coli* cells against killing by ColE9 presented as a poster presentation at CBS Researchers Symposium, 22rd June 2011, Centre for Biomolecular Sciences, University of Nottingham, UK

Abstract

Colicins are a family of bacterial toxins, which kill *Escherichia coli* cells and other closely related species. Their mode of action requires binding to an outer membrane receptor, translocation across the cell envelope leading to cytotoxicity at specific targets. The mechanism of colicin cytotoxicity includes a non-specific endonuclease activity or depolarizing of the cytoplasmic membrane by pore-forming activity. The cytotoxic activity can be inhibited by the high affinity binding of an immunity protein. For Group A colicins, translocation requires interaction between the N-terminal domain of the colicin and a series of membrane bound and periplasmic proteins called the Tol system (TolB, TolR, TolA, TolQ and Pal). In order to allow cytotoxicity, the immunity protein of enzymatic colicins must be lost after binding of the colicin to a target cell and the cytotoxic domain has to be translocated through both the outer and inner membranes.

This work has studied colicin translocation by using a periplasmic protection assay combined with an *in vivo lux*-reporter assay and a potassium release assay. Expressing the translocation domain of colicin A in the periplasm and challenging the cells with external colicins showed that the translocation of group A colicins is inhibited as it requires an interaction with the Tol system. Surprisingly, the TolA protein was found to play the major role during the translocation of both ColA and the endonuclease colicin E9 even though the latter colicin has no direct interaction with the TolA protein. This study also suggests that the interaction with TolB is important for both colicins A and E9. Moreover, a series of ColA constructs with a truncated T domain were made by site directed mutagenesis to define the important residues of the TolA and TolB boxes for their

interaction with Tol proteins. The results showed that tyrosine 58 residue of the TolA box of colicin A is essential for TolA binding, whilst the glutamate 18 residue of the TolB box of ColA did not show an effect on TolB binding.

Colicin-producing cells are protected by the co-expressed immunity protein which prevents killing of the producing cells. The immunity protein must be released from the cytotoxic domain of endonuclease E colicins prior to reaching the *E. coli* cytoplasm to degrade DNA. Little is known about the mechanism of release of immunity protein from E colicin cytotoxic domains and the role of Tol proteins in the release of immunity protein. Finally in this work an attempt was made to develop a sensitive, real-time assay to study the mechanism by which immunity protein is released from colicin E9 and to study the physiological role of the Tol system in the release process. Further developments are required for improving this assay which would be of great value.

CHAPTER ONE

1 General Introduction

1.1 Colicin overview

Colicins are a family of bacterial antibiotic proteins that are produced by and active against *Escherichia coli* and closely related bacterial species to give a competitive advantage against competitors that occupy the same ecological niche. Colicins were first discovered in 1925 by Gratia, a Belgian scientist, who isolated an *E. coli* V culture which appeared to be lethal for *E. coli* Φ (reviewed in (Cascales, Buchanan et al. 2007). Subsequently, many types of colicins were found to be produced by numerous strains of the Enterobacteriaceae family including *Escherichia, Enterobacter, Salmonella, Shigella* and *Proteus* species. The activity of colicin was shown to be dependent upon binding to a specific receptor at the surface of the susceptible strains (Cascales, Buchanan et al. 2007).

During the 1950s it was shown that colicin production was induced by DNA damaging agents (Ozeki, Stocker et al. 1959). Furthermore, producing strains were found to be immunized against killing by the colicin due to the presence of an immunity protein. The absence of this protein rendered the cells sensitive causing subsequent death as a result of a single hit killing mechanism (Cascales, Buchanan et al. 2007).

Further studies have revealed different modes of action, characterized by a narrow target range, of various colicins including; inhibition of macromolecular synthesis; non-specific break down of cellular DNA; degradation of 16S rRNA or specific tRNA eventually leading to the inhibition of protein synthesis; and inactivation of Lipopolysaccharide (LPS) and peptidoglycan (murein) synthesis (Oudega, Klaasen-Boor et al. 1975; Schaller and Nomura 1976; Harkness and Braun 1989; Ogawa, Tomita et al. 1999;

James, Penfold et al. 2002). Colicins have been shown to have an important role in microbial biodiversity and maintaining a stable coexistence of bacterial populations (Gratia 1925; Gratia 1946). Colicinogenic strains are widely distributed in nature and particularly abundant in the mammalian colon (Kleanthous 2010).

1.2 Colicin classifications

Over 20 different colicins (all carrying the prefix 'Col') have been identified and extensively studied (Table 1-1). Colicins are extremely diverse; they can utilize one of several different outer-membrane receptors, one of two different translocation systems, and are lethal by one of four specific cytotoxic activities. Defining the machinery by which colicins gain entry to the cells allowed the first classification of these colicins into two main groups (group A and B) based on cross-resistance studies (Davies and Reeves 1975a; Davies and Reeves 1975b). Group A colicins are inactive against strains containing mutations in the *tolA* gene, whilst group B colicins are inactive against bacteria carrying mutations in the *tonB* gene. Colicins were then sub-grouped based on their cytotoxic activity.

Table 1-1: Colicin classification (adapted from Kleanthous, C; 2010)

Colicin	OM receptor	Translocation proteins	Cytotoxic activity
Group A			
ColA	BtuB	OmpF, TolABQR	Pore forming
ColE2, E7, E8, and E9	BtuB	OmpF, TolABQR	DNase
ColE3, E4 and E6	BtuB	OmpF, TolABQR	RNase targeting 16S ribosomal RNA
ColE1	BtuB	TolCAQ	Pore forming
ColE5	BtuB	OmpF, TolABQR	RNase targeting tRNA
ColN	OmpF	OmpF, TolAQR	Pore forming
ColK	Tsx	OmpF, TolABQR	Pore forming
Col4	OmpW	OmpF, TolABQR	Pore forming
ColU	OmpA	OmpF, LPS, TolABQR	Pore-forming
Col28b	OmpA	OmpF, LPS, TolABQR	Pore-forming
CloDF13	IutA	TolAQR	16s rRNase
Group B			
Col5-Col10	Tsx	TolC, TonB, ExbBD	Pore forming
ColIa and Ib	Cir	Cir, TonB, ExbBD	Pore forming
ColB	FepA	TonB, ExbBD	Pore forming
ColD	FepA	TonB, ExbBD	RNase targeting tRNA
ColM	FhuA	TonB, ExbBD	Inhibition of PG synthesis

1.3 Colicin Expression, Regulation and Release

1.3.1 Col Plasmids

All colicinogenic *E. coli* strains contain a colicin producing plasmid, named pCol, these plasmids are classified into two groups, where the fundamental difference lies in the molecular weight of the plasmid DNA. Type I are small (6-10 kb), high copy number and mainly encode group A colicins. Type II are large (40 kb), conjugative plasmids mainly encoding group B colicins (Hardy, Meynell et al. 1973; Riley and Gordon 1992).

The genetic organization of colicin operons is summarized in the (Figure 1-1). Typically colicin operons contain three genes. The first one, named *cxa*, is the colicin structural gene that encodes a specific colicin protein, where x is the specific colicin activity (for example *cna* encodes ColN). The second gene is a specific immunity gene (*cxi*), encoding the immunity protein, which protects producing cells against the lethal activity of the colicin (Chak and James 1986; Kuhar 1999). The last gene is the lysis gene (*cel*), which is also called the Bacteriocin Release Protein (BRP), encoding the lysis protein, that facilitates colicin release from the producing cell (van der Wal, Luirink et al. 1995). The exceptions to this are colicins Ia, Ib, B and M which lack a lysis gene (Pugsley 1983).

The detailed arrangement of a colicin operon depends on the killing mechanism of the encoded colicin. For pore-forming colicins, the structural and lysis genes are separated by a large polycistronic region of approximately 550 bp, in which the gene encoding the immunity protein is located on the opposite strand, resulting in it being transcribed in the opposite direction to the

colicin and lysis genes (Lloubes, Baly et al. 1986). A transcriptional terminator (T1) is located immediately after the colicin structural and immunity gene (Figure 1-1). For nuclease colicins, however with the structural, immunity and lysis genes are present on the same DNA strand and are transcribed in the same direction and regulated by a common SOS promoter. The lysis gene is located a few base pairs downstream of the immunity gene, and the transcriptional terminator (T1) is located just after the immunity gene (Mark, Lawrence et al. 1984; Chak and James 1985; Carvard and Oudega 1992; James, Kleanthous et al. 1996). Despite the common organization of these three closely linked genes, some differences in the arrangement of colicin operons have been observed. For instance, the enzymatic ColE3 and E9 operons enclose an additional immunity gene for ColE8 and E5, respectively (Chak and James 1984; Cooper and James 1984; Lau, Rowsome et al. 1984; Chak and James 1986; Curtis, James et al. 1989).

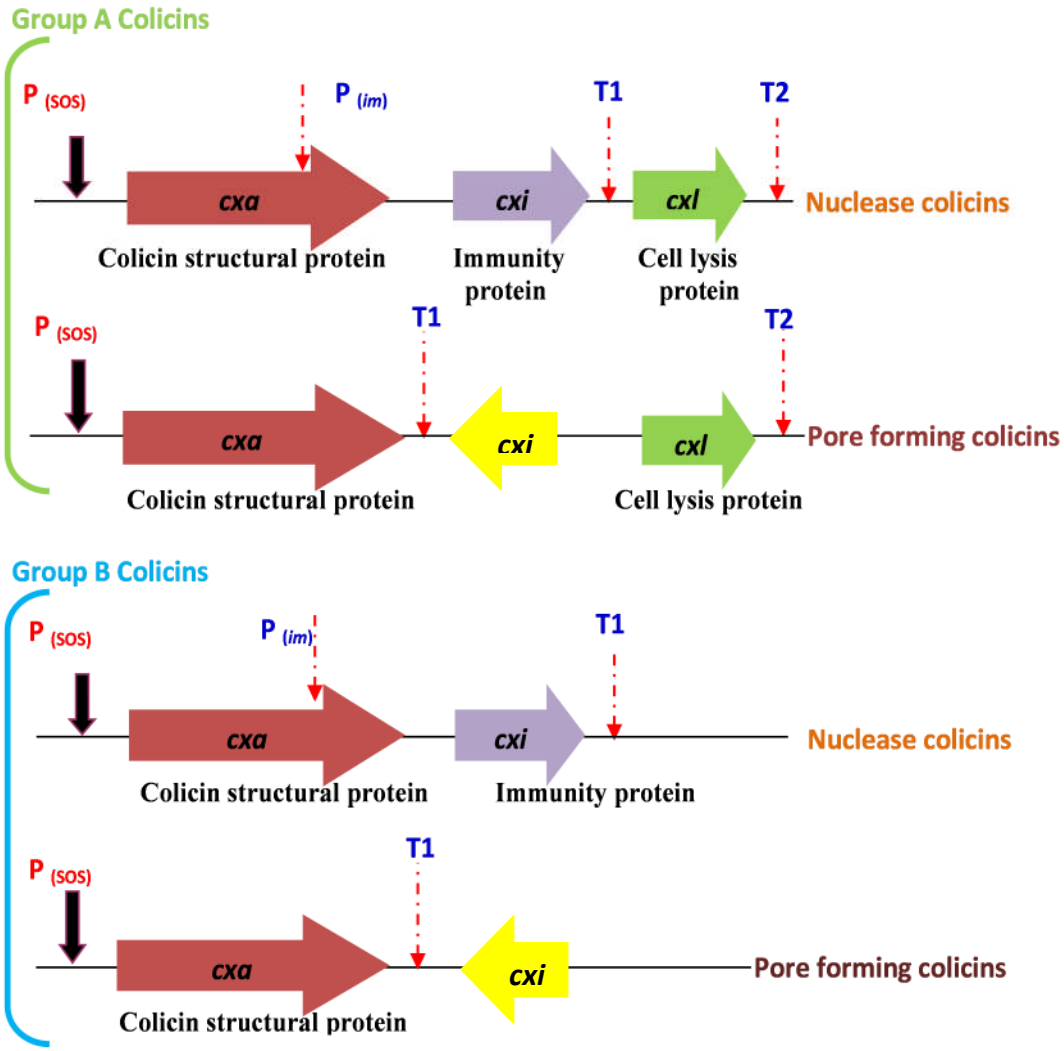


Figure 1-1: Colicin operon organization [adapted from (Cascales, Buchanan et al. 2007)]. The promoter of the colicin operon is indicated as P (SOS). The constitutive promoter of the immunity gene is indicated as P(im). The genes encoding the colicin structural protein (*cxa*), immunity protein (*cxi*) and lysis protein (*cxl*) are shown as coloured arrows. The direction of transcription is shown by the arrowheads on the relevant genes. The immunity gene for nuclease colicins is located between the structural and lysis genes on the same positive strand. For pore-forming colicins; the immunity gene is transcribed in the opposite direction (yellow arrows). The points labelled as T1 and T2 indicate the position of the transcription terminator sites.

1.3.2 SOS Response

E. coli displays a complex phenomenon that induces a number of genes in response to DNA damage, termed the SOS response that is controlled by the LexA protein (Witkin 1976; Ebina, Takahara et al. 1983). LexA, is the repressor of SOS inducible promoters that induces substantial DNA bending upon interaction with the operator of the SOS inducible genes such as colicin gene (Lloubes, Granger-Schnarr et al. 1988). Colicin SOS promoters comprise two LexA binding sites. Footprinting experiments showed that LexA binds tightly (apparent K_d = 0.4 nM) to a sequence (40 bp) containing two SOS boxes overlapping by one or two bases, in the case of *caa* (ColA) and *cea* (ColE) (Ebina and Nakazawa 1983; Lazdunski 1988; Lu and Chak 1996). Conversely, colicins DF13 and Ib contain only one SOS box (van den Elzen, Maat et al. 1982; Gillor, Vriezen et al. 2008). In the absence of DNA damage a dimer of the LexA protein is bound to each of the two SOS boxes, located just downstream of the pribnow box of the colicin promoter (Lloubes, Baly et al. 1986). This binding of LexA to the SOS boxes results in an inhibition of the transcription of colicin operon genes (Ebina, Kishi et al. 1982). DNA damage activates the proteolytic activity of the RecA protein which induces the cleavage of the LexA repressor (Little, Edmiston et al. 1980), therefore allowing colicin transcription and associated co-expression of the *kil* gene in spite of the presence of the transcription terminators (Suit and Luria 1988). Repair of the DNA damage restores the repression of the colicin promoter (Salles, Weisemann et al. 1987). Expression of the gene encoding ColE1 (*ceaA*) takes place 20-30 minutes after induction of the SOS response (Sanger, Nicklen et al. 1977). The observed lag time is not seen for other SOS response

genes and could be due to (1) a combination of the two SOS boxes found upstream of the start codon of the E colicin structural genes, allowing a cooperative binding of LexA to this regulatory region or, (2) the requirement for accumulation of an SOS-controlled factor in the cell before *cea* expression takes place (Sanger, Nicklen et al. 1977). This considerable delay could allow the cells to repair low levels of DNA damage without being killed by lethal expression of the *kil* gene.

1.3.3 Colicin Expression and Release

Under normal conditions, a basal level of colicin is always found in colicinogenic cell cultures in the absence of any external inducing agents (Durkacz, Kennedy et al. 1974; Mulec, Podlesek et al. 2003). Presumably, this is due to the leaky expression of the colicin structural gene (Durkacz, Kennedy et al. 1974; Mulec, Podlesek et al. 2003). Colicin transcription is primarily controlled by a strong SOS promoter located proximal to the colicin structural gene *cxa*. In times of stress, such as nutrient depletion and overcrowding, a small fraction of colicinogenic bacteria produce a substantial level of colicin through activation of the SOS response. Apart from the SOS response, an additional regulatory system that regulates the expression of the *cea* and *kil* genes is catabolite repression, which is dependent on cAMP and the cAMP binding protein (CRP) (Ebina and Nakazawa 1983). Transcription of the ColE1 gene *ceaA* is stimulated by the presence of cAMP and CRP where there are two binding sites near the *cea* promoter, only one of which has been shown to be involved in the control of *cea* (Shirabe, Ebina et al. 1985; Salles, Weisemann et al. 1987). Expression of *ceaA* post DNA damage is delayed by catabolite repression, suggesting a potential role for the cAMP-CRP complex,

which is thought to be involved in removing the tightly bound LexA from the *ceaA* regulatory region, thus improving the accessibility of RNA polymerase to the promoter (Salles, Weisemann et al. 1987). Figure 1-2 represents a simple model for protein export from the colicinogenic cells and import into the target susceptible cells, respectively (James, Kleanthous et al. 1996).

Within the DNA sequence at the 3' end of the colicin structural gene two constitutive promotors are responsible for expression of the immunity protein (*cei*) (Soong, Hsieh et al. 1994). In the case of ColE7, under normal conditions, cells are protected against exogenous ColE7 by the amount of ImE7 present, found to be 20 times greater than the amount of ColE7 (Hsieh, Ko et al. 1997). This is also true for the nuclease E type colicins (Chak and James 1985). SOS-induced transcription of the ColE7 operon produces one mRNA transcript for the three genes, in addition to constitutive expression of the ImE7. It has been proposed that ImE7, in a dimeric form, is involved in the cleavage of the colicin operon mRNA (Hsieh, Ko et al. 1997). The putative active site was suggested to be located at the interface of two immunity protein molecules. The site of mRNA cleavage is within the Im7 gene and is thought to uncouple translational expression. However, if the SOS response is activated for more than 100 minutes as is seen with ColE7 (Lu and Chak 1996), the ImE7 dimer is unable to completely degrade the mRNA transcribed, resulting in expression of ColE7and BRP (Hsieh, Ko et al. 1997).

For several reasons, the release of colicins into the culture medium of producing bacteria is unlike any other extra-cytoplasmic proteins that are released by Gram-negative bacteria. Colicins lack the N-terminal or internal signal sequence that usually directs extracellular proteins across the

cytoplasmic membrane to the terminal division of the general secretory pathway and does not use the other protein secretory pathways (Gilson, Mahanty et al. 1990). In contrast, their release involves only a single gene product, the colicin lysis protein (BRP) (Carvard and Oudega 1992). They are also exported late after synthesis and are non-specifically released during a quasi-lysis state (a true lysis state involves degradation of peptidoglycan) (Suit and Luria 1988). Quasi lysis provides a good reporter for the functionality of lysis proteins. Both colicin release and quasi lysis occur simultaneously. The quasi lysis state is characterised by a reduction in culture turbidity, which is caused by the activity of the lysis protein and its stable signal peptide (van der Wal, Luirink et al. 1995).

BRPs are synthesised as precursor polypeptides of around 4.5 kDa and all lysis protein precursors share sequence similarity (Riley 1993; van der Wal, Luirink et al. 1995), comprising a signal sequence for entry into the periplasm; this contains a Leu-X-Y-Cys sequence around the signal peptide cleavage site, where X is Ala, Val or Ser and Y is Ala or Gly (Carvard and Oudega 1992). Acylation of the cysteine residue takes place before the precursor protein is processed by signal peptidase II, generating a stable signal peptide and the BRP of approximately 30 amino acids. Modification of the amino group of the acylated cysteine residue of the BRP then occurs via a fatty acid residue (Cavard, Baty et al. 1987; Cavard 1992). Localization of the mature lipoproteins predominantly takes place in the outer membrane, except in the case of ColN, where the BRP is localised only in the outer membrane (Pugsley 1988)

BRPs not only possess similar amino acid sequences but are additionally functionally interchangeable. This is demonstrated by the secretion of colicin E2 which requires simultaneous expression of the colicin E1, E3, E7, A or D gene clusters (Pugsley and Schwartz 1983) and suggests a shared mode of action for the different BRPs. Therefore, although most of the work on BRPs has concentrated on those from colicin E1, A and a bacteriocin from *Klebsiella pneumoniae*, cloacin DF13 (which is closely related to colicin E3), it is reasonable to assume that the BRPs of other colicins will function similarly. BRPs have been shown to be involved in the activation of outer membrane phospholipase A by dimerisation (Pugsley and Schwartz 1984; Cavard, Baty et al. 1987; Dekker, Tommassen et al. 1999). Investigations into the action of the phospholipase by measuring changes in phospholipid content have revealed the major variations were a decrease in phosphatidylethanolamine (PE) and an increase in lysophosphatidylethanolamine (lysoPE) and free fatty acid content (Pugsley and Schwartz 1984; Cavard, Baty et al. 1987). Bacterial cells possessing mutations in the *pldAI* gene, encoding phospholipase A, were considerably less proficient at release of both colicins E1 and A, and also showed no quasi-lysis (Pugsley and Schwartz 1984; Cavard, Baty et al. 1987). Therefore, both BRP and phospholipase A seem to be required for efficient colicin release. However, it has been observed with *pldA1* mutants that BRP is degraded by the DegP protease causing a reduction in the BRP level (Cavard, Lazdunski et al. 1989), hence levels were insufficient to enable colicin release and lysis. Ultimately, this means that the lack of colicin released in these mutants is an artefact. Independently of phospholipase A, ColA has also been

shown to affect the integrity of the outer membrane (Howard, Cavard et al. 1991).

Before release, the enzymatic colicins form a heterodimeric complex with its cognate immunity protein (9.5 kDa) to neutralise its activity (Figure 1-2). Heterodimeric complexes released from colicin producing cells then make their journey into sensitive cells by first binding to specific outer membrane receptors. The immunity protein of enzymatic colicins must be lost from the colicin to initialise killing and the precise details of how this occurs are still under investigation (Cascales, Buchanan et al. 2007). A recent investigation into the ColE7 lysis protein using specific point mutations that were introduced into the lipobox (Wu and Tokunaga 1986; Oudega 2003) or sorting signal sequence of the lysE7 gene resulted in the production of various forms of lysis protein (Chen, Yang et al. 2011). The results showed that cells with wild-type mature LysE7 protein demonstrated a higher efficiency of ColE7 translocation across the inner membrane into the periplasm than those with premature LysE7 protein (Chen, Yang et al. 2011). Additionally, the mature LysE7 protein showed a significant increase in the degree of permeability of the inner membrane as compared to the unmodified LysE7 precursor. These results suggest that the efficiency of colicin movement into the periplasm is associated with the increase in inner membrane permeability induced by the LysE7 protein. Thus, it was proposed that the mature LysE7 protein has two critical roles: firstly in facilitating the translocation of ColE7 across the inner membrane into the periplasm, and secondly in activating the outer membrane phospholipase A (OMPLA) to permit colicin release from the cell (Chen, Yang et al. 2011).

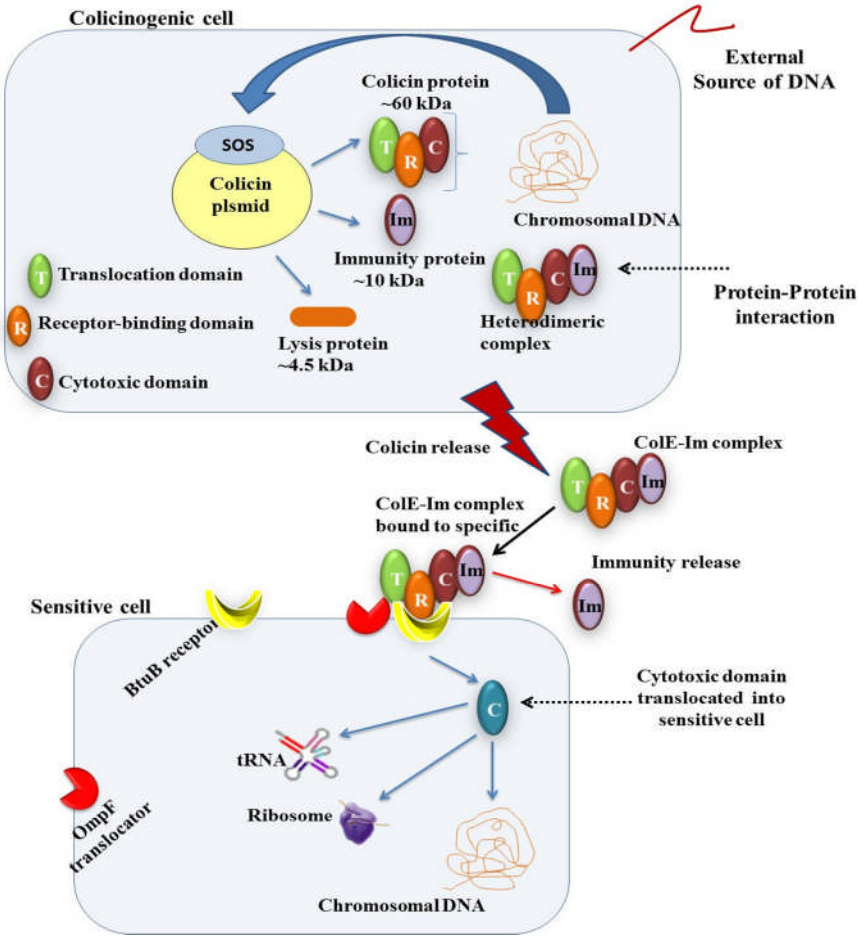


Figure 1-2: Schematic diagram illustrating the expression and release of a nuclease colicin from a producing *E. coli* cell (James, Kleanthous et al. 1996; Cascales, Buchanan et al. 2007). The colicin-immunity protein complex is exported from the producing cell where it first binds to a specific receptor (BtuB) on the target sensitive cell before translocating the nuclease (DNase or RNase) domain into the target sensitive cell where colicin activity either cleaves DNA non-specifically, or RNA (tRNA or rRNA) (James, Kleanthous et al. 1996).

1.4 Colicin Structure and Function

The structural organization of nuclease E colicins is identical and can be divided into three domains on the basis of function: an N-terminal translocation (**T**) domain, a receptor-binding (**R**) domain and a C-terminal cytotoxic (**C**) domain (Figure 1-3). After binding to its cognate immunity protein, which protects the producing cell from the colicin's cytotoxic activity, the colicin is released from the producing cell (James, Kleanthous et al. 1996).

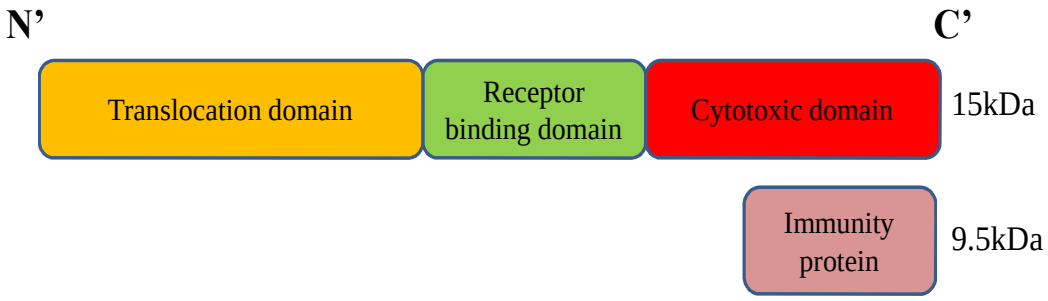


Figure 1-3: General Schematic diagram showing the functional domains of a nuclease E colicin protein. The Immunity protein binds to IBR (immunity binding region) of the 15 kDa C domain of enzymatic E colicins on synthesis (James, Kleanthous et al. 1996).

The structures of the group A endonuclease ColE3 (Soelaiman, Jakes et al. 2001), ColN (Vetter, Parker et al. 1998) and ColM (Zeth, Romer et al. 2008); and the group B pore-forming colicin Ia (Wiener, Freymann et al. 1997) and colicin B (Hilsenbeck, Park et al. 2004) have been determined (Figure 1-4 and Figure 1-5). Generally, all the published structures agree with the three domain organisation , although a fourth domain between the translocation domain and the receptor binding domain has been reported for ColE1 and colicin 10, which is involved in binding to TolC (Pilsl and Braun 1995). The pore-forming colicins differ in that, unlike the nuclease colicins, their immunity proteins do not bind to the colicin and form a complex on synthesis.

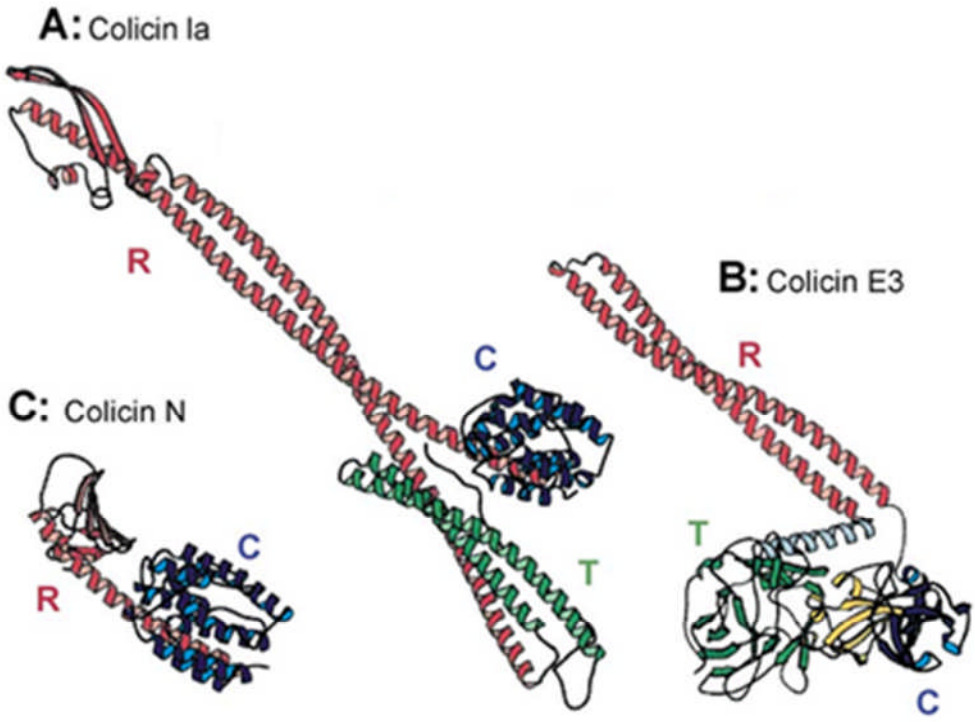


Figure 1-4: Crystal structures of colicins Ia, E3 and N (Zakharov and Cramer 2002). The structures of colicin Ia and E3 proteins were determined by X-ray crystallography at 3.0Å resolution, and ColN was resolved at 3.1Å resolution. The receptor binding domains of colicins Ia and E3 are formed by long helical hairpins, on the top of which sit the translocation domains and cytotoxic domains. The first 66 residues of ColN, the first 83 residues of ColE3 and the first 22 residues of colicin Ia are missing in the crystal structures. The yellow shaded region in the structure of ColE3 corresponds to its immunity protein.

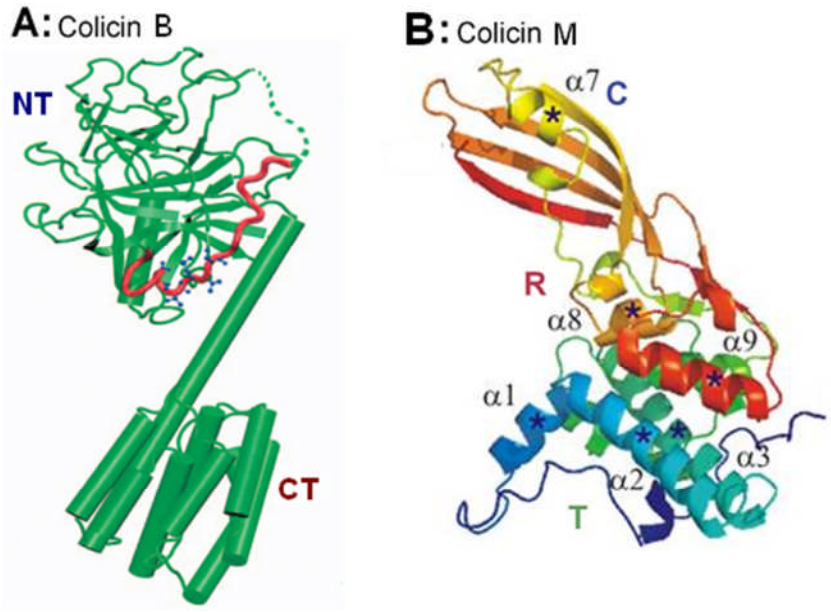


Figure 1-5: The crystal structure determined by X-ray crystallography of ColB (Hilsenbeck, Park et al. 2004) and ColM (Zeth, Romer et al. 2008). The amino terminal (NT) and carboxy terminal (CT) regions of ColB and the T, R and C domain of ColM are indicated. For colicin B no electron density was detected for residues 1-10 and 29-43 (dotted line). In contrast to the full-length structures of other colicins such as ColM, which have clearly delineated cytotoxic, translocation and receptor-binding domains, ColB is dumbbell shaped with the intertwined receptor-binding and translocation domains forming one end of the dumbbell (top half) and the pore-forming domain forming the other end (bottom half). The two ends of the dumbbell are held together by one long 74 Å α-helix.

ColE3 and Ia showed characteristic hairpin-like structures. A central R domain that includes the bend of the hairpin-like structure mediates binding to the outer membrane receptor (Parker, Pattus et al. 1989). All E group colicins bind to the product of the chromosomal btuB gene, which is responsible for vitamin B12 transport in *Escherichia coli* (James, Kleanthous et al. 1996). These have been subdivided into nine types (ColE1-E9), and fall into one of the three cytotoxic classes: membrane-depolarizing (or pore-forming) agents such as Col E1, DNases such as colicins E2, E7, E8 and E9, and RNases such as colicins E3, E4, E5 and E6 (James, Kleanthous et al. 1996). The only complete crystal structure that has been solved among the E colicins is one for ColE3 (Soelaiman, Jakes et al. 2001). Although this colicin is an RNase, a high level of amino-acid sequence homology is shared between the T & R domains of most other nuclease E colicins. Therefore ColE3 may provide a model for the T and R domains of ColE9, a DNase type E colicin (James, Penfold et al. 2002). Three dimensional (3D) crystal structures of the soluble DNase domains of ColE7 and E9 bound to their cognate immunity protein Im7 and Im9, respectively (Kleanthous, Kuhlmann et al. 1999; Ko, Liao et al. 1999) has been resolved.

Elucidation of the crystal structure of a complex between TolB and a 107-residue peptide ($TA_{1–107}$) of the translocation domain of colicin A, together with site-directed mutagenesis, identified the TolB box as a 12-residue peptide that bound to β-propeller canyon of TolB (Zhang, Li et al. 2010). Comparison of this structure with the published co-crystal structure of the ColE9 TolB box–TolB complex (Loftus, Walker et al. 2006) indicated differences in the intermolecular binding patterns of both colicins with TolB (Figure 1-6). This

study also reported differences in the binding affinities and recruitment mechanisms of these two colicins for TolB and allowed a model to be proposed for the translocation of these two colicins in *E. coli* cells.

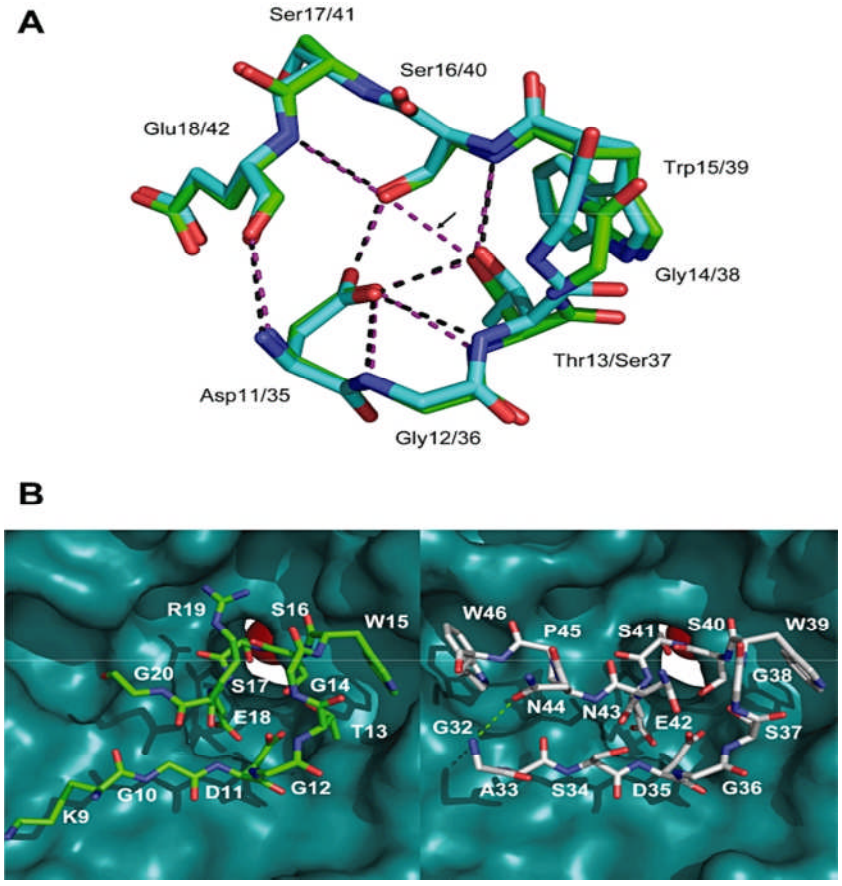


Figure 1-6: Comparison of $TA_{1–107}$–TolB with TE9pep32–47–TolB (PDB entry 2IVZ) (Zhang, Li et al. 2010). (A) Ball and stick comparison of the stereochemistry of the core region of the TolB box of ColA (blue) with ColE9 (green). (B) Ball and stick representation of residues 9–20 of $TA_{1–107}$ bound to the central canyon of the TolB β-propeller (left) in comparison with the TE9pep32–47 bound to TolB (right).

1.4.1 Receptor binding domain

The R domain of all colicins is located within the central region of the protein and separates the T and C domains by a long coiled-coil. The structure of ColE3 (Figure 1-4) shows that the central R domain adopts a 100 Å long helical-hairpin located between residues 316-450 (Soelaiman, Jakes et al. 2001). The R domain of colicin Ia as shown in Figure 1-4 comprises 104 residues, located between residues 282-385 of the colicin. It forms a double stranded β-hairpin fold around the first helix of the C-domain which facilitates the display of high density charged amino acids near its terminal loop (Wiener, Freymann et al. 1997). To assist structure-function analysis of the R domain of ColE9, a number of receptor-binding polypeptides have been characterised. Using random mutagenesis, screening of single point mutations identified A395D and A398P to be biologically inactive, the loss of cytotoxicity of which was due to the inability of the mutant toxins to bind BtuB. Mutations in residues 395 and 398 are thought to have caused conformational changes (Yue, Grizot et al. 2003) . Analysis of the 3D structure of ColE3 provides an explanation for the role of these two residues in receptor binding (Soelaiman, Jakes et al. 2001).

Using an *in vivo* biological protection assay and a novel *in vitro* fluorescent competition assay, the minimum R domain of ColE9 that retains receptor binding activity was identified to consist of residues 343-418 of the 582 amino acid protein (Penfold, Garinot-Schneider et al. 2000). The minimal R domain region is highly conserved amongst the enzymatic E colicins: 68 out of the 76 amino acids are identical when the sequences of E2, E3, E6, E7, E8 and E9 have been aligned (James, Penfold et al. 2002); while all 76 residues are

identical for ColE3 and E9 (Soelaiman, Jakes et al. 2001). Although its role remains unknown, for ColE9, it was speculated that a 30 residues (termed linker region) were involved in bridging the periplasm space to allow transport of the cytotoxic domain across the cytoplasmic membrane of *E. coli* cells (James, Penfold et al. 2002). However, unless this occurs at the adhesion site of OM and CM, this 30-residue α-helix would not be sufficient enough to span the 150 Å wide periplasmic space (Dorit and Riley 2002). Hence, the reason why this region is so highly conserved is intriguing; the α-helical stretch could presumably be maintained by a different sequence of amino acids.

1.4.2 Translocation domain

All the information required for translocation of colicins is found in their T domains. The composition of the ColE3 T-domain was shown by structural analysis to adopt a jellyroll structure of three β sheets flanked by two helical stretches. The first 83 residues of the T-domain were suggested to be disordered and unstructured due to the lack of electron density within this region (Soelaiman, Jakes et al. 2001); this has also been demonstrated by NMR studies, (Collins, Whittaker et al. 2002). Moreover, this region has been proposed to be involved in the interaction of the colicin with TolA (James, Penfold et al. 2002). The lack of secondary structure observed at the N-terminus of ColE9 within the first 79 residues is due to the presence of 34 glycine residues. In contrast, the T domain of the B group colicin Ia was shown by its crystal structure to contain three α helices (80 Å long) organized as an antiparallel helical sheet (Wiener, Freymann et al. 1997). The crystal structure of ColM revealed that the N terminal region shows a high degree of flexibility

and lack of secondary structure (Zeth, Romer et al. 2008); this finding is consistent with that of the crystal structure of ColN (Vetter, Parker et al. 1998).

1.4.3 The cytotoxic domain

1.4.3.1 Pore-formers

The X-ray structures of the pore-forming domains of ColA (Parker, Pattus et al. 1989), E1 (Elkins, Bunker et al. 1997), N (Vetter, Parker et al. 1998), B (Hilsenbeck, Park et al. 2004) and Ia (Wiener, Freymann et al. 1997) show that their pore-forming domains all consist of a bundle of eight amphipathic helices burying two hydrophobic helices. The average length of the helices is 13 residues, but the approximate requirement to span the membrane bilayer is 17-20 residues (Elkins, Bunker et al. 1997; Zakharov and Cramer 2002). However, ColE1 possesses a 25 % increased α-helical content for pore formation, which may result in an adequate extension to permit the helices to span the membrane (Elkins, Bunker et al. 1997; Zakharov, Lindeberg et al. 1998). The two longest helices found in pore-forming colicins similar to that of diphtheria toxin (the hydrophobic helices, 8 and 9) form a hydrophobic helical hairpin at the core of the domain, which is expected to form a membrane anchor after the initial interaction of colicin with the membrane bilayer, this mechanism of interaction could be comparable for pore-forming colicins (Zakharov and Cramer 2002).

The cytotoxicity of pore-forming colicins is a result of the formation of ion-permeable channels in the inner membrane of the susceptible cell, facilitated by the C-terminal domain of the colicin (Dankert, Uratani et al. 1982). The intriguing model suggesting that ColA channel domain activity that occurs at low pH might be possible through an unfolded molten globule intermediate

wherein the α-helix content is preserved (van der Goot, Gonzalez-Manas et al. 1991) this is not supported by the data for ColE1. For the ColE1 soluble channel domain, P190, the active low pH state is not accompanied by loss of tertiary structure as monitored by near-UV CD (Lakey, Duche et al. 1993). The loss of tertiary structure and conservation of secondary structure are required determinants of the molten globule state, but not sufficient (Zakharov and Cramer 2002).

It was anticipated that the initial interaction of the cytotoxic domain with the membrane was electrostatic, as pore-forming colicins require an acidic pH for binding to artificial membrane vesicles (Davidson, Brunden et al. 1985; Zakharov and Cramer 2002). Orientation of the pore-forming domain to the membrane surface is predicted to take place by a group of positive charges on the surface of the domain (Elkins, Bunker et al. 1997). Once the cytotoxic domain has bound to the membrane, it then unfolds, possibly via a molten globule intermediate, permitting extension of the helices and insertion into the membrane (van der Goot, Gonzalez-Manas et al. 1991; Cramer, Heymann et al. 1995; Zakharov and Cramer 2002). The requirement for partial unfolding of the pore-forming domain, as it interchanges from the aqueous to the membrane phase, has been inferred from the onset of channel activity and the decrease in structure, as monitored by CD, as a function of pH (Cramer, Heymann et al. 1995).

1.4.3.2 Endonucleases

The DNase domain of colicins E2, E7, E8 and E9 cleaves cellular DNA in *E. coli* sensitive cells. These four E colicins share high sequence identity in their

receptor binding and translocation domains in addition to 80 % homology in their DNase domains. Structures of the DNase domains of ColE7 and E9 bound to their cognate immunity proteins have been determined (Kleanthous, Kuhlmann et al. 1999; Ko, Liao et al. 1999).

X-ray crystallography and activity assays have localized the DNase domain of ColE9 to residues 449-582 (Pommer, Wallis et al. 1998; Kleanthous, Kuhlmann et al. 1999). The DNase activity of ColE9 is placed in the 134 residues of the cytotoxic domain and involves a metal ion.

The crystal structure of the E9 DNase-Im9 complex at 2.05 Å resolution displays the DNase domain to consist of a central core of β-sheet enclosed by α-helices (Kleanthous, Kuhlmann et al. 1999). The 32 amino acid residues at the C-terminus show sequence identity to the HNH family of homing endonucleases (Shub, Goodrich-Blair et al. 1994). The HNH motif resembles a distorted zinc finger and forms the core of the DNase active site (Hannan, Whittaker et al. 1999). This is supported by site-directed mutagenesis, which was used to identify putative active site residues in the DNase domain of ColE9 (Garinot-Schneider, Pommer et al. 1996). Three single site mutations were recognized which entirely destroyed the toxic action of the colicin, R544A, E548A and H575A. All three residues are highly conserved amongst the DNase colicins signifying that these residues are involved either in preserving the overall fold or in the mechanism of the DNase enzyme (Kleanthous, Kuhlmann et al. 1999). NMR has confirmed that the nickel ion which is bound in the crystal structure is coordinated by three histidine side chains and a phosphate molecule (Hannan, Whittaker et al. 1999).

The crystal structure of the ColE7-Im7 complex at 2.3 Å resolution (Ko, Liao et al. 1999) shows the DNase domain is a novel α/β protein with a Zn^{2+} ion bound to 3 histidine residues, a water molecule and contains a zinc finger motif (Ko, Liao et al. 1999). The three catalytically important residues identified by site-directed mutagenesis of ColE9 are conserved in ColE7 and are located near the Zn^{2+} ion. It was found that the zinc ions are not required for binding of the active domain to DNA, but for hydrolysis. Ordinarily the metal ion in the zinc finger motif coordinates with four amino-acid residues (Pommer, Kuhlmann et al. 1999; Walker, Georgiou et al. 2002); however, in the ColE9 and E7 DNase domains, the fourth ligand is either a phosphate molecule or a water molecule respectively (Hannan, Whittaker et al. 2000; Sui, Tsai et al. 2002). This could decrease the affinity of the protein for the metal ion, permitting removal of the metal ion during translocation, resulting in partial unfolding (Kleanthous, Kuhlmann et al. 1999; Hannan, Whittaker et al. 2000; van den Bremer, Jiskoot et al. 2002). Consequently, the metal ion could display another role in stabilising the DNase domain in the extracellular environment.

The RNase domains of E3, E4, E5 and E colicins display greater sequence conservation (80-90 %) than that for DNase E colicins, and kill cells by inactivating the protein biosynthetic machinery (Nomura and Witten 1967). The crystal structure of the ColE3 cytotoxic domain complexed with its cognate immunity protein to 2.4 Å resolution revealed a highly twisted central antiparallel β-sheet particularized with a short N-terminal helix (Carr, Walker

et al. 2000; Soelaiman, Jakes et al. 2001). Sequence and structural investigations combined with molecular modelling and mutagenesis studies have revealed a putative active site within the toxin containing a His-Glu catalytic pair (Soelaiman, Jakes et al. 2001; Walker, Lancaster et al. 2004). The importance of residue H513 and E517 of the RNase domain of ColE3 have been confirmed by alanine mutagenesis (Walker, Lancaster et al. 2004). Sequence comparison of the C-terminal domains of colicins E4 and E6 with those of ColE3 shows that ColE4 and E6 are homologous to ColE3 and also cleave 16S rRNA.

However, the C-terminal region of ColE5 exhibits no sequence similarity to colicin E3. *In vitro* and *in vivo* examination of ColE5 activity has demonstrated that the target of ColE5 is not ribosomes, as is the case for ColE3, but specific tRNAs (Ogawa, Tomita et al. 1999; Masaki and Ogawa 2002). Colicin D has also been shown to act as a tRNase, cleaving arginine specific tRNAs (Masaki and Ogawa 2002). In addition, the tRNase activity of colicin D has been localised to 91 residues at the C-terminus (de Zamaroczy, Mora et al. 2001).

1.4.4 Immunity protein

An important phenomenon in colicin producing cells is the need to be immune to the action of the produced colicin (Fredericq 1957); this immunity is conferred via an inhibitor protein, approximately ~10 kDa to 15 kDa in size (Ebina, Kishi et al. 1979) that is the product of the *im* gene in the colicin operon. Many immunity proteins show no sequence similarity. The E2, E8 and E9 immunity sequences showed 54-97 % similarity of their residues to each other (Riley 1993). These immunity proteins have a high affinity for, and

bind at a 1:1 ratio to the C-terminus of their cognate enzymatic colicins, thus making a tight heterodimer complex (Jakes and Zinder 1974; Oudega, Klaasen-Boor et al. 1975; Ohno, Ohno-Iwashita et al. 1977). The cognate immunity protein provides almost complete protection for the producing strain against the cytotoxic action of endogenous as well as incoming colicin (Kleanthous and Walker 2001). However, non-cognate immunity proteins can bind to the cytotoxic domain of the colicin providing variable degrees of protection. Consequently, the colicin-immunity protein interaction provides a model system for studying protein- protein interaction.

1.4.4.1 Immunity proteins for endonuclease colicins

Endonuclease colicins are secreted as heterodimers, binding tightly and specifically with an immunity protein on synthesis before being exported into the extracellular medium. Binding of the immunity protein neutralises the endonuclease activity of the colicin and thus protects the host cell from self-destruction. After binding to a sensitive *E. coli* cell, the immunity protein must be lost from the colicin-immunity protein complex at some stage to ensure that an active endonuclease is imported into the cytoplasm of target cells. Free ColE9 has the same bactericidal activity as ColE9 bound to Im9 (Wallis, Reilly et al. 1992), confirming therefore that the immunity protein is not a requirement for receptor binding or the translocation steps of colicin. Conversely, removal of Im3 from ColE3 leads to substantial loss of bactericidal activity signifying that, in addition to protecting producing cells from ColE3, Im3 may also stabilise ColE3 before entering susceptible cells (Walker, Moore et al. 2003).

The interaction of an endonuclease colicin and its cognate immunity protein (i.e. ColE9 binding to Im9) is one of the highest affinity protein-protein interactions known. This was validated by determining that the equilibrium dissociation constant (K_d) for the binding of Im9 to full-length ColE9, is 9.3 x 10^{-17} M at pH 7 and 25 °C (Wallis, Moore et al. 1995). A similar K_d value of be 7.2 x 10^{-17} M was found for the interaction between the ColE9 DNase domain and Im9 to, therefore it is assumed that the immunity protein makes energetically essential contacts with the DNase domain of the colicin only (Wallis, Leung et al. 1995). However, binding of the DNase domain to a non-cognate immunity protein (i.e. binding of ColE9 to Im8), resulted in K_d values in the range of 10^{-4} to 10^{-16} M (Wallis, Leung et al. 1995). Observation of the *in vivo* protection provided by the immunity proteins against ColE9 was in the order of Im9 > Im2 >Im8 > Im7 which reflects the difference in their *in vitro* affinities (Wallis, Leung et al. 1995).

Structure determination of the three colicin endonuclease domains bound to their cognate immunity proteins (Figure 1-7) has provided insights into how immunity proteins protect bacteria from the cytotoxic activity of endonuclease colicins. The structure of full-length ColE3 in complex with Im3 has been resolved (Soelaiman, Jakes et al. 2001) as well as the structure of only the RNase domain in complex with Im3 (at 2.4 Å resolution, (Carr, Walker et al. 2000)) were very similar structures. Furthermore, the structures of the DNase domain of ColE7 in complex with Im7 (at 2.3 Å resolution, (Ko, Liao et al. 1999; Cheng, Shi et al. 2006)) and ColE9 in complex with Im9 (at 2.05 Å and 1.7 Å resolution, (Kleanthous, Kuhlmann et al. 1999; Kuhlmann, Pommer et al. 2000)) have also been determined. Comparison of these structures reveals

few similarities; the DNase domains are αβ proteins (Kleanthous, Kuhlmann et al. 1999; Ko, Liao et al. 1999) while the RNase domain consists of predominantly β sheet (Carr, Walker et al. 2000; Soelaiman, Jakes et al. 2001). The immunity proteins for the RNase and DNase domains are also structurally unrelated. The DNase immunity proteins are distorted four-helix bundles (Chak, Safo et al. 1996; Osborne, Breeze et al. 1996) whilst the RNase immunity proteins are predominantly β sheet proteins (Zakharov, Lindeberg et al. 1999).

However, the DNase and RNase immunity protein complexes share a charge complementarity as a result of the basic nature of the endonuclease domain and the acidic nature of the immunity protein. Moreover, NMR experiments have been used as a guide for alanine scanning mutagenesis of Im9 (Osborne, Wallis et al. 1997; Li, Hamill et al. 1998), and the conserved residues that make the largest relative contribution toward E9 DNase binding were determined (Wallis, Leung et al. 1998).

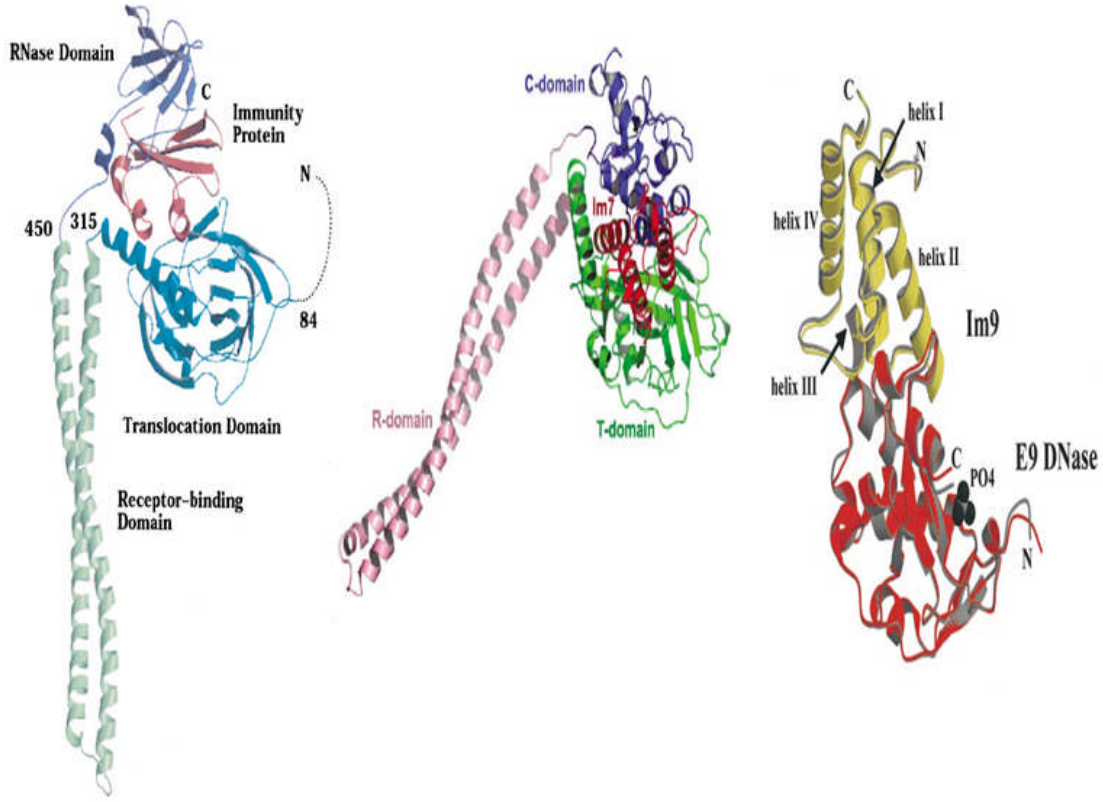


Figure 1-7: Structures of different endonuclease colicin-immunity complexes. The structures of the colicin E3-Im3 complex (left) (Soelaiman, Jakes et al. 2001), the ColE7-Im7 complex (middle) (Cheng, Shi et al. 2006) and the ColE9 DNase-Im9 complex (right) (Kuhlmann, Pommer et al. 2000) are shown.

The ColE9 DNase-Im9 complex (Figure 1-7) consists of a hydrophobic core of mainly aromatic residues from both the DNase and Im9, with multiple hydrogen bonds and salt bridges surrounding this core. The structure of the ColE7 DNase -Im7 complex shows that there are 2.17 hydrogen bonds per 100 $Å^2$ shared between the E7 DNase and Im7, which is substantially more than the average calculated for enzyme-inhibitor complexes of 1.37 hydrogen bonds per 100 $Å^2$, (Jones and Thornton, 1996). This, in conjunction with the fact that

many of the hydrogen bonds involve charged donor and acceptor groups, is likely to contribute to the high affinity of the interaction (Ko, Liao et al. 1999).

The structure of the ColE3 RNase domain in complex with Im3 shows that the RNase N-terminal helix wraps around the exposed face of the four-stranded β-sheet of Im3 (Kolade, Carr et al. 2002). On binding of the ColE3 RNase domain to Im3, there is a much greater loss of surface area as compared to binding of ColE9 or E7 DNase domains to their respective immunity proteins (2554 Å 2 compared to 1575 Å 2 and 1473 Å 2, respectively) (Kuhlmann, Pommer et al. 2000; Cheng, Shi et al. 2006). Their high affinity of the interaction is a result of the high degree of surface complementarity, which is indicative of a conformational change on formation of the protein-protein complex, but so far no structure of the free RNase is available to confirm this (Kolade, Carr et al. 2002). A recent investigation introduced mutations into Im7 and demonstrated a critical role for three residues, Leu 53, Ile 54 and Tyr 55, to lock Im7 into its unique native structure (Knowling, Bartlett et al. 2011). Leu 53 and Ile 54 were found to provide critical stabilizing interactions in the hydrophobic core of Im7, while Tyr 55 is essential for both stability and function. In contrast, Tyr 56 is crucial for colicin binding and has no role in maintaining a stable native fold (Knowling, Bartlett et al. 2011)

1.4.4.2 Mechanism of inhibition

In contrast to many enzyme antagonists, the E colicin immunity proteins do not bind directly at the active site (Kleanthous, Kuhlmann et al. 1999; Kuhlmann, Pommer et al. 2000), although there are substantial differences between the rRNase– and DNase–immunity protein complexes (Kleanthous and Walker 2001). Structural analysis of the ColE9 DNase domain in complex

with Im9 (Figure 1-7) shows that Im9 binds in a cleft in the DNase domain that is formed by a short helix, an extended strand and two loops spanning residues 72-98, that are located near the active site (Kuhlmann, Pommer et al. 2000). Based on structural and functional studies, two mechanisms of inhibition by endonuclease immunity proteins have been proposed (Kleanthous and Walker 2001). Firstly, the immunity protein inhibits nucleic acid binding at the active site by the action of steric hindrance. Secondly, the allosteric inhibition by immunity protein could be caused by conformational changes in the protein backbone of the endonuclease domain.

1.5 Colicin Binding and Import

The pathway by which colicins reach their cytotoxic target is divided into two steps. They first bind to a specific membrane receptor at the cell surface. To achieve this, colicins have parasitized proteins of the outer membranes that function to transport iron siderophores (FepA, FhuA, FhuE, Cir), or vitamin B_{12} (BtuB), or nucleotides (Tsx). Others, such as ColN have parasitized OmpF, the major porin, through which small hydrophilic solutes with a molecular weight of up to 650 Daltons can pass (Pugsley 1984). The diversity of the receptors utilized by colicins led to the classification of colicins into groups on the basis of the cell surface receptor to which they parasitize. Thus the E group colicins bind to the product of the chromosomal *btuB* gene of *E. coli* (Di Masi, White et al. 1973). In the second step of internalisation, that termed the "translocation step" colicins cross the outer membrane, the periplasmic space, and in the case of nuclease-type colicins, the inner membrane in order to reach their cellular targets. During the translocation step colicins have taken advantage of specific periplasmic proteins. According to which, colicins are

also classified in to two groups, group A colicins (e.g. A, E1, E2, E3, K, L, N, S4), are inactive against strains containing mutations in the *tol*A gene. Group B colicins (e.g. B, D, G, H, 1a, 1b, M, Q, V), are inactive against bacteria carrying mutations in the *tonB* gene. The Group A colicins (A, E1 to E9, K, L, N, and Cloacin DF13) require the Tol proteins (TolA, TolB, Pal, TolQ, and TolR) during translocation into the cell (Davies and Reeves 1975a), whereas, the group B colicins (B, D, Ia, Ib, M, 5 and 10) require the TonB, ExbB and ExbD proteins (Davies and Reeves 1975b).

1.5.1 Binding of colicin protein onto a specific receptor

The first step for a colicin passage into a susceptible cell is to bind to an outer membrane receptor. Colicin receptors are usually involved in the uptake of ligands into the cell and have been parasitized by colicins as part of their killing pathway. A number of colicin receptors have been identified, including the vitamin B_{12} receptor, BtuB (Di Masi, White et al. 1973; James, Kleanthous et al. 1996), the siderophore receptors, FepA and FhuA (Locher, Rees et al. 1998; Ferguson, Chakraborty et al. 2002), and the porins, Tsx (Bradley and Howard 1992; Pilsl and Braun 1995), OmpA (Pilsl and Braun 1995) and OmpF (Bourdineaud, Fierobe et al. 1990; Cowan, Garavito et al. 1995). All these receptors are generally used for significant biological functions, that include the passive or active transport of nutrients across the OM (James, Penfold et al. 2002). It was recently demonstrated that the energy that is required for the active transport of substrates through *tonB*-dependent outer membrane receptors is provided by the TonB/ExbB/ExbD complex, which is located in the cytoplasmic membrane (Krewulak and Vogel 2011). It has been proposed that these receptors act as gated channels, whereby channel opening

occur upon the ligand binding, leading to passage of the ligand (Lazdunski, Bouveret et al. 1998). As previously addressed the E group colicins use the BtuB receptor to gain access to its target in *E. coli* cells (Di Masi, White et al. 1973).

1.5.1.1 BtuB

BtuB is a minor but an important component of the *E. coli* outer membrane that is involved in the high-affinity transport system for vitamin B_{12}, with just about 200 copies present per cell (Figure 1-8) (Di Masi, White et al. 1973). BtuB production has been revealed to be regulated at the post-transcriptional level. The transcriptional regulation of *btuB* expression is still unclear; therefore, recently, a genomic library was screened for clones that allow *E. coli* to grow in the existence of ColE7. A plasmid carrying the *gadX* and *gadY* genes was isolated in this experiment. It was concluded through biological and biochemical analysis, that expression of GadX can directly interact with the *btuB* promoter and reduce the expression of BtuB by 57 %. It was also evident for the first time that the the btuB gene is transcriptionally repressed by the acid responsive genes *gadX* and *gadY*. BtuB protein expression was reduced by 90 % in the presence of these two genes (Lei, Syu et al. 2011).

The crystal structures (Figure 1-8) of wild-type BtuB, BtuB with bound calcium and BtuB with bound calcium and bound vitamin B_{12} have been resolved (Chimento, Kadner et al. 2003). The basic structure of BtuB consists of an N-terminal plug domain inserted in a 22-stranded β-barrel, which is very similar to FepA and FhuA. The involvement of a calcium ion in the loop ordering was determined and it has been suggested that calcium functions to order these loops, revealing the high-affinity substrate-binding site. This is

consistent with the finding that 50-100 fold reduction in binding affinity for vitamin B_{12} is observed when calcium is removed from BtuB (Bradbeer, Reynolds et al. 1986). Conformational changes occur in the barrel domain of the BtuB receptor on binding to vitamin B_{12} (Chimento, Mohanty et al. 2003). All the BtuB molecules can transport vitamin B_{12}, however, only perhaps 10-20% of the receptor molecules have been shown to assist colicin translocation. These are possibly newly synthesized BtuB molecules that are present at adhesion sites as that are associated with both the IM and OM (James, Kleanthous et al. 1996). It has been suggested through studies of the localization of BtuB-PhoA fusion proteins (James, Kleanthous et al. 1996) that residues between 327 and 399 of the 594 amino acids of BtuB receptor are required for the association of BtuB with the OM.

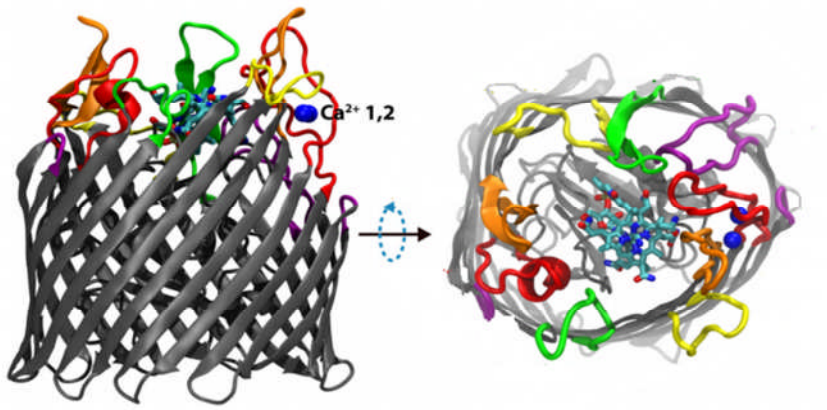


Figure 1-8: Membrane-plane and extracellular views of the Ca^{2+}/cyanocobalamin-bound structure of BtuB. The barrel and luminal domains are coloured gray, the two Ca^{2+} ions are in blue, and cyanocobalamin is shown in a stick representation coloured by atom type. The 11 extracellular loops of BtuB are drawn using a thicker representation and labelled using different colours to distinguish them from their neighbors (Gumbart, Wiener et al. 2009).

1.5.1.2 OmpF

The putative secondary receptor proposed to be used during E colicin translocation is OmpF, except for ColE1 and ColA (Dover, Evans et al. 2000). The x-ray crystallographic structures for OmpF, a trimeric porin, revealed a barrel structure with 16 stranded sheets connected with loops at the vestibule end and by short turns at the periplasmic end (Figure 1-9) (Kumar, Hajjar et al. 2010). The traverse loop (L3) of OmpF constricts the monomer pore diameter to 7 x 11 Å about half way down the β-barrel (Cowan, Garavito et al. 1995). The contacts among the three OmpF monomers are stabilized by non-covalent interactions as loop L2 bends over the wall of the barrel of the subunit. Loop L3 controls the dimension of the internal pore that folds into the barrel contracting the channel and forming a constriction region. The channel of OmpF is sufficiently large to permit insertion of an unfolded polypeptide (Fourel, Mizushima et al. 1992).

The crystal structures of the N-terminal residues of the translocation domains of ColB, E3, and Ia are unresolved, implying a low degree of secondary structure and a high degree of flexibility (Wiener, Freymann et al. 1997; Soelaiman, Jakes et al. 2001; Hilsenbeck, Park et al. 2004). It is suggested that this disordered region of the translocation domain could thread through the OmpF pore (Kurisu, Zakharov et al. 2003; Housden, Loftus et al. 2005). This theory was supported by demonstrating that OmpF channels in planar bilayers could be blocked by addition of ColE3 (Kurisu, Zakharov et al. 2003). This occlusion only occurred when ColE3 was added to the trans-side of the bilayer, expected to be analogous to the extracellular side of the outer membrane. No

occlusion was observed when ColE1 was added to the same bilayer (Kurisu, Zakharov et al. 2003). It was shown that OmpF enhances the ability of purified BtuB to protect bacteria against the endonuclease ColE9, demonstrating that either the functional receptor formed by the two OM proteins or that subsequent translocation of the bacteriocin required the recruitment of OmpF (Law, Penfold et al. 2003). Although stable binary ColE9-BtuB complexes could be readily shown *in vitro* using gel filtration and chemical cross-linking, OmpF-containing complexes could not be detected, implying that OmpF association with the BtuB-ColE9 complex, while necessary, is predicted to be weak and/or transient in nature (Law, Penfold et al. 2003). It has been shown that the BtuB-ColE9/Im9 complex recruits OmpF through a natively disordered 83 amino acid N-terminal sequence (Collins, Whittaker et al. 2002; Housden, Loftus et al. 2005).

ColN also uses OmpF during translocation. *E. coli* cells with a G119D mutation in OmpF which results in loop L3 protruding into the lumen of the OmpF channel, dividing it into two sections of reduced diameter, show resistance to ColN (Jeanteur, Schirmer et al. 1994). It was concluded that this mutation prevents ColN from entering the porin channel (Jeanteur, Schirmer et al. 1994). Mutation of G119 or G120 of OmpF to bulkier residues has also been shown to radically affect ColA activity, indicating that loop L3 also has a role in ColA recognition and/or transportation (Bredin, Simonet et al. 2003). The activity of ColA and N were also found to be affected by mutations in residues D113 and D121. However, residue D113 was found to play a more prominent role in the activity of colicin A, whereas residue D121 was more

significant for ColN activity. This specifies uptake differences of the two colicins with ColN interacting with residues positioned at the top of L3 and ColA with residues further down and on the other side of L3 (Bredin, Simonet et al. 2003).

If two different OM proteins, such as BtuB and OmpF, are required as a co-receptor by a colicin such as ColE3 to traverse the outer membrane, they must be within a definite distance of one another (Cao and Klebba 2002). It has been proposed that translocation across the OM possibly will be dependent on membrane fluidity, as it is reliant on temperature (Bourdineaud, Fierobe et al. 1990). This could hypothetically be a consequence of the requirement for OmpF and BtuB to be in close proximity in the OM.

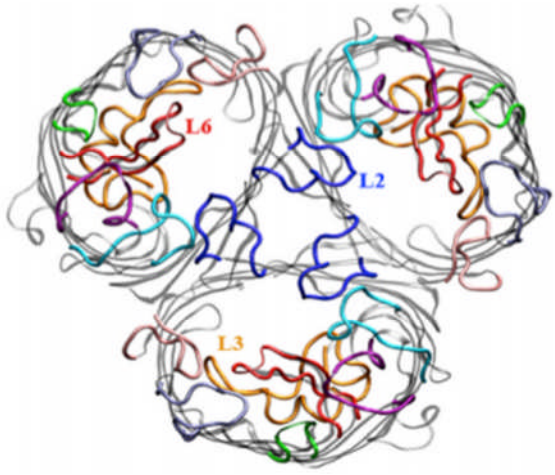


Figure 1-9: X-ray structures of an OmpF trimer is shown: loop L2 in each OmpF monomer is coloured in dark blue, L3 in orange, L4 in light blue and L6 in red (Kumar, Hajjar et al. 2010).

1.5.2 Receptor-binding domains of colicins

Despite utilising diverse OM receptors (Table 1-1), there are structural similarities between the receptor-binding domains of colicins E3 and Ia, with both comprising a coiled coil of 100 Å and 160 Å in length, respectively (Wiener, Freymann et al. 1997; Soelaiman, Jakes et al. 2001). The coiled coil of colicin Ia was proposed to allow the colicin to span the periplasmic space, which has an estimated width of 150 Å (Wiener, Freymann et al. 1997), but the coiled coil of ColE3 is too short to permit this.

The 76-residue minimum receptor-binding domain of ColE9 has been shown to involve residues 343-418 (Penfold, Garinot-Schneider et al. 2000) that forms a helical hairpin structure (Collins, Whittaker et al. 2002) and is identical in sequence to that of ColE3. In an *in vivo* biological protection assay the receptor binding domain was shown to protect cells at least as efficiently as the full-length colicin (Penfold, Garinot-Schneider et al. 2000). NMR spectroscopy has revealed that the minimum receptor-binding domain has a flexible inter-helix loop and slowly interchanging conformers (Collins, Whittaker et al. 2002). It has been proposed that to allow access of the translocation domain to the periplasm the flexible loop may act as a hinge to maintain unwinding of the helical hairpin upon receptor-binding (Boetzel *et al.*, 2002). This is supported by the loss of activity of mutants with engineered disulphide bridges near the middle or the top of the receptor-binding domain of ColE9 (Penfold, Healy et al. 2004).

The function of linker region, comprising residues 419-448, of ColE9 is unknown. This region is equivalent to the upper part of the coiled coil of

ColE3 (James, Penfold et al. 2002). It has been suggested that this region possibly will bridge the periplasmic space to contribute to the delivery of the endonuclease domain to the cytoplasm, however it has been suggested that the linker region is too short (~50 Å) to achieve this (James *et al.*, 2002). This may not be the case if colicins preferentially use the newly synthesized BtuB that has been demonstrated to associate with adhesion sites (Guihard, Boulanger et al. 1994; Penfold, Garinot-Schneider et al. 2000).

The receptor binding domain and the translocation domain of colicin B are intertwined at one end of the dumbbell structure which make it difficult to distinguish them (Hilsenbeck, Park et al. 2004). There is significant sequence homology in the receptor-binding domain between amino acids 262-282 of colicin B and the two β-strands of colicin Ia, indicating that these residues could be involved in receptor-binding. Also amino acids 1-291 of both ColB and D show significant sequence similarity. These two colicins use the same receptor, FepA, and consequently these residues are possibly involved in receptor-binding (Hilsenbeck, Park et al. 2004). The receptor-binding domain of colicins B, D, E2, E3, E6, E7 and E9 are located between residues 130-291 that is similar in all these colicins (Hilsenbeck, Park et al. 2004). Based on this sequence similarity, it was proposed that residues 343-418 could be used in binding to OmpF, rather than BtuB, supported by the fact that ColB and D, which do not bind to OmpF, do not have sequences homologous to residues 343-418 in ColE9 (Hilsenbeck, Park et al. 2004). However this suggestion disregards substantial evidence supporting the participation of residues 343-418 of ColE9 in binding to BtuB, *ie* the structure of BtuB bound to the residues

313-447 of the ColE3 receptor-binding domain (Kurisu, Zakharov et al. 2003), and the validation that the isolated residues 343-418 of ColE9 compete with vitamin B12 for binding to BtuB (Penfold, Garinot-Schneider et al. 2000).

1.5.3 Release of the immunity protein

It is known that endonuclease colicins are exported from producing cells as a high affinity complex with its cognate immunity protein to neutralise the cytotoxic activity of the colicin. In order for cell killing to occur, the immunity protein must be released, presumably before entering a target cell, but how and where this occurs is as yet undefined. It is possible that unfolding which occurs during colicin translocation is the trigger to facilitate the release of the immunity protein. However, the resolved structure of ColE3 receptor binding domain, residues 313-447, bound to BtuB that resolved at resolution 2.75 Å (Kurisu, Zakharov et al. 2003) only revealed a slight unfolding at the two ends of the coiled-coil. Also isothermal titration calorimetry (ITC) experiments studying the interaction between BtuB with ColE9 and with ColE9 with a disulfide bond across the R-domain produced almost identical Ka values, signifying that ColE9 does not unfold upon receptor binding (Housden, Loftus et al. 2005). Recently, free Im3 was removed from ColE3 simply by using anion-exchange chromatography (Zakharov, Zhalnina et al. 2006). Taken together these results suggest that unfolding is not required for immunity protein release, but this has not been proven conclusively.

The detection of free immunity protein in the media was found to occur ~20 min after receptor binding (Duche, Frenkian et al. 2006), which is the time required for enzymatic colicins to reach their cellular target (Vankemmelbeke,

Healy et al. 2005). The release of immunity protein required the presence of the translocation machinery (Duche, Frenkian et al. 2006), including the periplasmic and inner membrane Tol proteins. This is in agreement with the recent findings that release of immunity protein from the colicin complex is an energy-dependent process governed by the cytoplasmic membrane PMF that have provided a rationale for how an energized Tol system might lead to immunity protein loss and concomitant colicin uptake in sensitive cells (Vankemmelbeke, Zhang et al. 2009).

1.5.4 Translocation of group A colicins

1.5.4.1 The Tol system

The Tol/Pal system has been extensively studied; however, the normal cellular function of the *tol* system in *E. coli* is less clear. It is thought that the Tol proteins play a role in maintaining cell envelope integrity through a network of interactions spanning the cytoplasmic membrane, periplasm, and outer membrane (Lloubes, Cascales et al. 2001). Moreover, the Tol system is coupled to the proton motive force across the CM (Lloubes, Cascales et al. 2001) and thus transfers energy between CM and OM. The *tol* dependent translocation system consists of the proteins TolA, TolB, TolQ, TolR and Pal (James, Penfold et al. 2002). Strains containing a mutation in the *tol* genes are insensitive to group A colicins. Pal is a lipoprotein associated with the peptidoglycan (Mizuno 1979) and TolB is a periplasmic protein (Isnard, Rigal et al. 1994). TolB is associated with the OM through the interaction of its C-terminal β-propeller domain with Pal, while its N-terminal domain interacts with the C terminal domain of the TolA protein, TolAIII (Bouveret, Derouiche et al. 1995; Dubuisson, Vianney et al. 2002). The TolA protein is anchored in

the cytoplasmic membrane, via its N-terminal TolAI region which facilitates its interaction with TolQ and TolR (Germon, Ray et al. 2001) forming an inner membrane complex.

The production levels of the Tol-Pal have not yet been simultaneously measured in a given strain, although, it is estimated by Western blotting that TolA is present at approximately 800 copies per cell and there are 2000-3000 copies of TolR (Levengood, Beyer et al. 1991; Muller, Vianney et al. 1993). This is consistent with the results of radioactive labelling studies which suggest that the TolA:TolR:TolQ ratio is 1:2:6 (Guihard, Boulanger et al. 1994). Pal has been found to be present in great excess over the TolA protein and it is predicted that there are 10,000 to 30,000 Pal molecules per cell (Cascales, Gavioli et al. 2000). It is not yet clear if the Tol system is dependent on the formation of stable complexes of defined stoichiometry or on more subtle transient interactions between different protein partners.

The *tol* mutations induce sensitivity to dyes and detergents and their tendency to release periplasmic proteins into the medium indicates that the membrane permeability of *tol* mutants is affected. Consequently, it has been suggested that the Tol system maintains outer membrane integrity (Lazzaroni, Fognini-Lefebvre et al. 1989). Identification of the TolA-Pal and TolB-Pal interactions and the interaction of TolB and Pal with OmpA and Lpp (Clavel, Germon et al. 1998; Cascales, Bernadac et al. 2002), suggested that the Tol system could link the OM and CM to peptidoglycan. The observed interaction of TolA and TolB with porin trimers (Derouiche, Gavioli et al. 1996; Rigal, Bouveret et al. 1997; Clavel, Germon et al. 1998; Dover, Evans et al. 2000) suggested that these

proteins could be involved in porin assembly. This is supported by the decrease in the quantity of OmpF and LamB in *tol* mutants (Lazdunski, Bouveret et al. 1998). LPS translocation or assembly could also be regulated by the Tol proteins as *tol* mutants can have reduced LPS content and TolA is required for the surface expression of O-antigen and LPS (Lazzaroni, Germon et al. 1999). The Tol-Pal proteins might assist porin trimer-LPS complexes with a molecular weight greater than 50 kDa, the cut-off point for translocation across the peptidoglycan layer, to cross the peptidoglycan layer (Clavel, Germon et al. 1998). The Tol-Pal system contributes in the transport of newly synthesized OM components by bringing the CM and OM into close proximity via the TolA-Pal or TolA-TolB interactions (Cascales and Lloubes 2004).

Studies that demonstrated that the CM Tol complex, TolA, TolQ and TolR, can transduce energy dependent on the proton motive force, suggest that TolQRA could act as an ion potential-driven molecular motor (Cascales, Gavioli et al. 2000). This is supported by the recent findings that TolQ and TolR transduce energy to TolA that eventually lead to release of immunity protein as a requirement of ColE9 to transport across the periplasm (Vankemmelbeke, Zhang et al. 2009).

Several studies have now revealed that interaction of group A colicins with Tol proteins is required for translocation. Three major classes of colicin-tolerant (*tol*) mutants of *E. coli* K-12, which adsorbed colicins but were not killed by them, were isolated and studied genetically and physiologically (Nagel de Zwaig and Luria 1967). The Tol system was suggested to contribute to the cell

envelop integrity, this was based on the pleotropic phenotypes exhibited by *tol* and *pal* mutants including the hypersensitivity to drugs and detergents and the release of periplasmic contents into the extracellular medium (Nagel de Zwaig and Luria 1967; Fognini-Lefebvre, Lazzaroni et al. 1987). Tol mutants have shown a defect in the O-antigen insertion into the OM, so Tol-pal proteins interfere with the assembly of OM components (Meury and Devilliers 1999; Cascales, Buchanan et al. 2007). The *tolABQR* and *pal* mutants form OM vesicles that contain periplasmic proteins. The OM blebbing in *tol* mutants possibly reflects an incorrect assembly of the OM structure during cell growth.

E. coli strains with *tonB* mutations are tolerant to all group B colicins, although, *exbB* or *exbD* mutants retain some slight sensitivity to colicins due to cross interaction with the Tol system (Davies and Reeves 1975a; Braun and Herrmann 1993). Strains with both *exbB/D* and *tolQ/R* mutations are tolerant to group B colicins (Braun 1989). Mutations in the TonB transmembrane domain that inactivates TonB were found to prevent PMF-dependant conformational changes (Larsen and Postle 2001). Colicin D shares 96 % homology with ColB yet mutants in TonB Arg158Ser and Pro161Leu are resistant to ColD and sensitive to B, Ia, M and bacteriophage (Mora, Diaz et al. 2005). It was shown that point mutation within TolQR abolish the PMF-dependent TolA interaction.

1.5.4.2 TolB

TolB is a periplasmic protein of 408 residues after cleavage of its 21- residue signal sequence (Isnard, Rigal et al. 1994). It is primarily found in the periplasm however a small amount is also found linked with the outer membrane (Isnard *et al.*, 1994). The crystal structure of TolB has been resolved by two different research groups to 1.95 Å resolution (Abergel *et al.*, 1999) and 2 Å resolution (Carr, Penfold et al. 2000). Both structures display that the overall protein fold comprises a 131-residue (residues 34–165) N-terminal domain consisting of a 5 stranded mixed β-sheet that sandwiches two major α-helices against a C-terminal 6-bladed β -propeller domain consisting of residues 166–431 (Figure 1-10). Each blade of the propeller involves β -sheets that are radially arranged around a central tunnel. TolB protein dimerisation was indicated by yeast two-hybrid studies and *in vitro* cross-linking experiments (Dubuisson, Vianney et al. 2002; Walburger, Lazdunski et al. 2002). The isolated N-terminal domain of TolB has been shown to expose different cross-linking sites due to its capacity to dimerise and multimerise, probably owing to the adoption of different conformations (Walburger, Lazdunski et al. 2002). These findings indicate that the dimerisation appears to occur through the N-terminal domain. The physiological relevance of the dimerisation need further clarification as it was not detected in resolving the crystal structure or in previous *in vitro* and *in vivo* studies (Bouveret, Derouiche et al. 1995; Abergel, Bouveret et al. 1999; Carr, Penfold et al. 2000).

Figure 1-10: The crystal structure of TolB looking down the central barrel of the C-terminal β- propeller domain. Six repeated sequence motifs produce the six propellers of the domain (Carr, Penfold et al. 2000).

1.5.4.3 Colicin-TolB Interaction

Normally β-propeller proteins use their central tunnel to coordinate a ligand or to carry out a catalytic function that is conserved by the structural rigidity of the propellers. Consequently it has been suggested that colicin translocation domains interact with the β-propeller domain of TolB (Carr, Penfold et al. 2000). The structure of the 16-residue TolB binding epitope located in the natively disordered T-domain of the ColE9, bound to TolB was solved to a resolution of 2.0 Å (Loftus, Walker et al. 2006). The ColE9 TolB binding epitope binds to the β-propeller domain from the top side, with 15 of the 16 residues contacting TolB. No major structural changes in TolB were observed upon binding to peptide. The colicin binds within a canyon in the β-propeller domain blocking the central channel. The peptide is attached to the TolB canyon surface by the indole side chains of the two tryptophans (Trp-39 and Trp-46) with further hydrophobic interactions being made by Pro-45 and Ala-33 (Loftus, Walker et al. 2006). The distance separating the hydrophobic

docking sites in TolB for the tryptophans can only accommodate three residues in an extended conformation. As six amino acids separate the two tryptophans, the chain becomes compressed, the resulting turn pushing colicin residues toward the channel at the base of the canyon.

The interaction between ColE9 and TolB was analysed by the identification of alanine mutations in the TolB box that abolished the biological activity of the mutant ColE9 (James, Penfold et al. 2002). Three essential residues, D35, S37, and W39, of the TolB box pentapeptide sequence were in the N-terminal translocation domain of the enzymatic E colicins. Additional site-directed mutagenesis demonstrated that the TolB box sequence in ColE9 is actually larger than the pentapeptide and extends from residues 34 to 46. The affinity of the TolB box mutants for the periplasmic TolB was determined by SPR to confirm that the loss of biological activity in all mutants except one (N44A) of the extended TolB box correlates with a reduced affinity of binding to TolB. A PCR mutagenesis protocol was used to isolate mutations that restored activity to the inactive ColE9 D35A, S37A, and W39A mutants. A serine residue at position 35, a threonine residue at position 37, and phenylalanine or tyrosine residues at position 39 restored biological activity of the mutant ColE9.

An extension of the TolB box of ColA to include the equivalent residues to S40, S41, and E42 in ColE9 has been proposed based upon deletion analysis (Hands, Holland et al. 2005). Interestingly, the Tol-dependent, pore-forming ColK lacks a TolB box suggesting that its translocation might be TolB independent, or that it interacts with TolB differently compared to other TolB-

dependent colicins (Barneoud-Arnoulet, Gavioli et al. 2010). Recent findings showed that ColK requires the TolA, TolB, TolQ, and TolR proteins. Using periplasmic production of the N-terminal domain of ColK in wild-type cells and *in vivo* co-immuno-precipitation and *in vitro* pulldown experiments, an interactions between the ColK N-terminal domain and the TolA, TolB, and TolR proteins have been demonstrated. For the first time, an interaction between a colicin translocation domain and the fourth component of the Tol complex, the TolQ protein has been identified (Barneoud-Arnoulet, Gavioli et al. 2010).

1.5.4.4 Pal

Pal is a 152 amino acid lipoprotein that has a serine residue at position +2, resulting in its localisation in the OM through an N-terminal lipid moiety (Mizuno 1979). The peptidoglycan recognition sequence contained within residues 94 to 114 forms an amphipathic helix which interacts with peptidoglycan (Bouveret, Benedetti et al. 1999; Cascales and Lloubes 2004). *In vitro* and *in vivo* studies showed that Pal protein can form dimers, most likely via its C-terminal residues, which is independent of Tol proteins, Lpp and OmpA (Cascales, Bernadac et al. 2002; Cascales and Lloubes 2004). The Pal lipoprotein interacts with many cellular components, such as TolA, TolB, OmpA, the major lipoprotein and the murein layer (Figure 1-12). The analyses of the functions and interactions of the mutated or deleted residues in the Pal protein defined an N-terminal region of 40 residues that can be deleted without showing a cell-damaging effect and three independent regions required for its interaction with TolA, OmpA and TolB or peptidoglycan. Investigating the

integrity of the cells producing the various Pal lipoproteins revealed strong outer membrane destabilization when these binding regions were deleted. Furthermore, a conserved polypeptide sequence located downstream of the peptidoglycan binding motif of Pal was required for the TolA-Pal interaction and for the maintenance of the outer membrane (Cascales and Lloubes 2004).

In vivo cross-linking and immune-precipitation experiments confirmed that TolB interacts with Pal (Bouveret, Derouiche et al. 1995). Insertion of two residues in the β-propeller domain of TolB, abolished the interaction of TolB with Pal, and reduced the interaction with OmpA, suggesting that the TolB β-propeller domain may be involved in binding to Pal and OmpA (Abergel, Bouveret et al. 1999). It was also identified that the C-terminal β-propeller domain of TolB is important for the interaction with Pal (Ray, Germon et al. 2000). The regions of interaction of Pal with TolB have been limited by mutagenesis analysis to residues 89-104 and 126-130 (Clavel, Germon et al. 1998). A peptide including residues 89-130 of Pal is able to interact with TolB and with peptidoglycan (Bouveret, Benedetti et al. 1999). Conversely, the peptide is unable to bind to both the peptidoglycan and TolB at the same time *in vivo*, which is consistent with the region involved in binding to TolB overlapping with the binding site for peptidoglycan. Moreover, dimerisation of Pal is not essential for the interaction with TolB (Cascales and Lloubes 2004).

The interaction of TolB with Pal plays a role in colicin translocation and such interaction was shown to be prevented by binding of ColE3 to TolB. Point mutations have been identified in Pal that renders *E. coli* cells insensitive to

colicins (Bouveret, Rigal et al. 1997; Clavel, Germon et al. 1998). TolB could act as a shuttle between Pal in the outer membrane and TolQRA in the inner membrane (Bouveret, Benedetti et al. 1999). It has been shown that the TolB-Pal complex is disrupted by colicin binding causing local instability of the OM as a prelude to colicin import (Bouveret, Benedetti et al. 1999). Ca^{2+} ions which bind within the β-propeller channel of TolB switch the surface electrostatics from negative to positive, allowing the negatively charged T-domain of ColE9 to bind TolB with an affinity equivalent to that of Pal and competitively displace it (Loftus, Walker et al. 2006).

Regarding the interaction of Pal with the OM proteins, cross-linking experiments have revealed that TolB and Pal can be cross-linked with the outer-membrane proteins, OmpA and Lpp (Clavel, Germon et al. 1998; Cascales, Bernadac et al. 2002). The OmpA binding sequence of Pal has been localised to residues 44-61 (Cascales and Lloubes 2004). Association of TolB with Pal is essential for OmpA to interact with these two proteins. Consequently it has been suggested that TolB and Pal are part of a multiprotein complex that connect the peptidoglycan to the OM (Clavel, Germon et al. 1998).

1.5.4.5 TolA

TolA is a 44 kDa inner membrane protein, which can be divided into three domains (Figure 1-12), although no crystal structure of the full-length protein has yet been resolved (Webster 1991). Domain I, consisting of the N-terminal 47 amino acids, encloses a hydrophobic segment (21 amino acid) anchoring the protein in the inner membrane (Webster 1991). Formaldehyde crosslinking

showed that this domain interacts with TolR and TolQ (Derouiche, Benedetti et al. 1995). Secondary structure estimates and circular dichroism measurements specify that the central domain II is mainly α-helical (Levengood, Beyer et al. 1991; Derouiche, Lloubes et al. 1999). The three domains are separated by glycine residues, from domain I by five, and from domain III by three glycine residues. A single helix formed by this region would be approximately 35 nm in length, enabling the protein to span the periplasm. Molecular modelling of domain II, proposes that the domain could involve a three-stranded coiled coil structure and this has been suggested to act by tethering the anchoring domain I to the functional domain III (Webster 1991).

A crystal structure of domain III of TolA is now available, in complex with domain I of the phage minor coat gene 3 protein (Lubkowski, Hennecke et al. 1999). Domain III of TolA is a novel fold involving three antiparallel β strands with four helical motifs. NMR studies have revealed that interaction with ColA induces a significant structural change in domain III of TolA, including an increase in the domain flexibility (Deprez, Blanchard et al. 2002). Similar results have been reported in the interaction of C-terminal domain of TolA and N-terminal domain of phage g3p (Deprez, Lloubes et al. 2005). It has been suggested that this increase in flexibility could disrupt the interaction of TolA with Pal, contributing to the disruption of the Tol/Pal system, permitting translocation of ColA (Deprez, Blanchard et al. 2002).

TolA mutational analysis have shown different uptake properties with ColA and E1, moreover, some *tolA* point mutations have been isolated that showed tolerance toward ColA but not to colicins E1, 2, 3 or K (Schendel, Click et al. 1997; Dubuisson, Vianney et al. 2002). The TolA binding site was originally proposed to be located between residues 30-39 of ColE3, yet mutational analysis within the anticipated sequence revealed a critical role for their interaction with TolB but not with TolA (Escuyer and Mock 1987; Benedetti, Lazdunski et al. 1991). Further mutagenic investigation narrowed the TolA binding sequence of ColA to residues 52-97 which was found also to have homology with a Pal sequence and a similar motif found in ColN and K (Journet, Bouveret et al. 2001; Cascales and Lloubes 2004). Alanine-scanning mutagenesis and SPR have shown an extended sequence in the N-terminal TolA box of ColN (Gokce, Raggett et al. 2000) Although, *tolA* mutants are tolerant to enzymatic colicins, a TolA box motif was not found in the enzymatic E colicins and no direct interaction was detected with the TolA protein. The TolA binding motif, which consisted of four conserved residues (SYGK/E) located in the C-terminal region of Pal, was also present in g3p and colicin A, but was not found in the enzymatic E colicins. The tyrosine residue of the SYGK sequence present in Pal and the YGT motif of bacteriophage g3p were found to be significant for formation of hydrogen bonds (Cascales and Lloubes 2004). Alanine substitution of the tyrosine residues in the SYNT and PYGR motifs in ColA, located between residues 52-97 of the TolA binding sequence, showed colicin tolerance and OM defects. The tyrosine residue at position 58 resulted in a reduction in the interaction with TolA and abolished the killing activity of the mutated ColA (Pommier, Gavioli et al. 2005).

Moreover, alanine mutagenesis and quantitative binding experiments presented the precise identification of residues involved in TolA binding of ColN. The tyrosine residue at position 62 (Y62), is found to be absolutely required for the full binding, additionally the binding site was shown to be extended with a region of at least 20 amino acids involved in TolA III binding with ColN (Raggett, Bainbridge et al. 1998). Mutations in the TolA C-terminal domain eliminated the formation of a TolA-TolB complex and have been shown to be important for the passage of ColA and E3 but not E1, as E1 does not require TolB interaction (Walburger, Lazdunski et al. 2002).

1.5.4.6 TolR

TolR is an inner-membrane protein (142-residues), which can be separated into three domains (Figure 1-12). Domain I, consisting of the N-terminal 43 residues, spans the inner membrane (Muller, Vianney et al. 1993). Domain II is positioned in the periplasm and appears to be involved in homo-dimerisation (Journet, Rigal et al. 1999). The C-terminal domain III, consisting of residues 117-142, has been proposed to form an amphipathic α-helix, which could interact with proteins such as TolQ and TolA in the CM (Lazzaroni, Vianney et al. 1995; Journet, Rigal et al. 1999). TolRII-III and TolRI-II proteins are unable to complement *tolR* cells indicating that domains I and III are involved in TolR function and therefore the interactions between TolR and TolQ and TolA are likely to be significant for TolR functionality (Journet, Rigal et al. 1999).

Overproduction of the TolRII domain in the bacterial periplasm induces tolerance to ColA, indicating that this domain interacts with ColA (Journet, Rigal et al. 1999). *In vivo* and *in vitro* cross-linking studies have confirmed that domain II of TolR is the one that interacts with colicins A and E3 (Journet, Bouveret et al. 2001) and was recently found to interact with the colicin K T-domain (Barneoud-Arnoulet, Gavioli et al. 2010). The interaction between TolR and ColA has only been detected using formaldehyde cross-linking, yet no interaction could be detected using a yeast two-hybrid screen or spectro-fluorometric analysis, suggesting that the interaction between TolR and ColA is weak and transient or may be dependent on other *in vivo* factors (Journet, Bouveret et al. 2001).

TolRII cannot interact with TolA alone but the N-terminal domain of ColA cross-linked to TolRII can interact with TolA at the same time to form a TolRII-ColA-TolA complex (Journet, Bouveret et al. 2001). Deletion analysis and cross-linking experiments verified that residues 7-14 of ColA were involved in the interaction with TolR (Journet, Bouveret et al. 2001). The sequence of the putative TolR box region is not well conserved in other colicins, except for the presence of some glycine residues. Overlapping was observed between the TolB and TolR boxes of ColA (residues 11-15), consistent with the fact that all colicins which require TolB for translocation, also require TolR. This may rule out a simultaneous interaction between colicin, TolB and TolR, although the colicin-TolB and colicin-TolR interactions are probably closely linked (Journet, Bouveret et al. 2001).

1.5.4.7 TolQ

TolQ is an integral CM protein (230 residues), estimated to span the membrane three times (Kampfenkel and Braun 1993). The small loop and N-terminus are located in the periplasm, the large loop and C-terminus, following the first transmembrane region, are located in the cytoplasm (Vianney, Lewin et al. 1994). Mutational analysis has showed that the transmembrane regions and fragments of the cytoplasmic loop are significant for TolQ activity (Kampfenkel and Braun 1993; Vianney, Lewin et al. 1994). The predicted sequences of transmembrane regions are also highly conserved, again demonstrating that these regions may be important in the TolQ functionality.

1.5.5 Tol proteins net interactions

The multi-protein Tol-PAL system of *E. coli* is organized into two complexes, one near the OM between TolB and PAL and one in the CM between TolA, TolQ, and TolR. In the cytoplasmic membrane, all of the Tol proteins have been shown to interact with each other (Journet, Rigal et al. 1999). A mutation in the third transmembrane region of TolQ induced tolerance to ColA but not ColE1 (Vianney, Lewin et al. 1994). TolQ is required for uptake of both colicins A and E1; however TolR is only essential for uptake for colicin A, showing that this mutation was disturbing the interaction between TolQ and TolR. This was confirmed by the detection of suppressor mutations in TolR that restored colicin sensitivity to the *tolQ* mutant (Lazzaroni, Vianney et al. 1995). Four out of five of these suppressor mutations were located in the transmembrane helix of TolR, indicating that the third transmembrane domain of TolQ interacts with the transmembrane region of TolR, which was

confirmed by cross-linking experiments (Journet, Rigal et al. 1999). The fifth mutant was positioned at the C-terminus of TolR in the region predicted to involve an amphiphilic helix. It has been proposed that TolR C-terminal helix interacts with TolQ and TolA in the CM (Lazzaroni, Vianney et al. 1995; Journet, Rigal et al. 1999). Cross-linking experiments have shown that the TolR N-terminal transmembrane domain interacts with TolA N-terminal transmembrane domain (Derouiche, Benedetti et al. 1995), while cross-linking studies could not detect any complex between a TolR dimer and TolA (Journet, Rigal et al. 1999). As proposed previously, the C-terminal domain of TolR plays a role in the TolR-TolA interaction, this is in agreement with the observation that TolR domain III partially co-fractionates with the membranes (Journet, Rigal et al. 1999).

Mutation of the N-terminal region of TolA resulted in disruption in the interaction with TolQ or TolR (Germon, Clavel et al. 1998). Suppressor mutant analyses and cross-linking experiments have localized the TolA interactions with TolQ and TolR to the first transmembrane domain of TolQ and have shown that the third transmembrane domain of TolQ interacts with the transmembrane domain of TolR (Germon, Clavel et al. 1998). The transmembrane domain of TolR was shown to be involved in the TolA-TolR and TolQ-TolR interactions, while TolR central and C-terminal domains appear to be involved in TolR dimerization (Journet, Rigal et al. 1999). Phenotypic studies clearly showed that the three domains of TolR (N terminal, central, and C terminal) and the level of TolR production are important for

ColA transport and for the maintenance of cell envelope integrity (Journet, Rigal et al. 1999).

1.5.5.1 Variation of Tol protein binding between pore-forming or nuclease colicin

A functional translocon is formed when the translocation domain of an enzymatic Group A colicin interacts with the Tol proteins mediating the release of immunity protein which is necessary for the activity of endonuclease colicins (Duche, Frenkian et al. 2006; Vankemmelbeke, Zhang et al. 2009). Interaction with Tol proteins requires a specific sequence located at the N-terminus of the colicin, in the translocation domain. This is designated as TA for the translocation domain of ColA (Benedetti, Frenette et al. 1991) and TE9 for the translocation domain of ColE9. The TA domain encloses three binding sequences involved in interactions with the TolA, TolB, and TolR proteins (Journet, Bouveret et al. 2001). Mapping of the ColA and E1 domains required for the import process identified specific conserved sequence in the N-terminal region for binding to Omp, TolC, TolB and TolR (Benedetti, Frenette et al. 1991). Further studies verified a direct interaction of the N-terminal domain of colicins with the Tol system. Colicins A and E1 interact with TolA (Benedetti, Frenette et al. 1991; Bouveret, Rigal et al. 1997); while colicins A and E3 interact with TolB (Bouveret, Rigal et al. 1997; Bouveret, Rigal et al. 1998); and ColA interacts with TolR via a region that clearly overlaps with that involved with TolB binding, thus suggesting a non-simultaneous interaction with TolB and TolR during ColA translocation (Bouveret, Rigal et al. 1998; Journet, Bouveret et al. 2001).

The pentapeptide sequence of the T-domain that interacts with TolB is referred to as the TolB box. Initial mutagenesis studies showed that the TolB box of the ColE9 is located between residues 35-39 and consists of the sequence **DGSGW** which is essential for the interaction of the colicin with TolB protein and is fundamental for cell killing (Garinot-Schneider, Penfold et al. 1997). Sequence alignment of the translocation domains of the enzymatic colicins E2, E3, E6, E7 and E9 revealed a longer conserved sequence of the TolB box (Garinot-Schneider, Penfold et al. 1997; Carr, Penfold et al. 2000). Subsequent experiments confirmed that the TolB box sequence in ColE9 extends from residues 34 to 46 The crystal structure of the periplasmic TolB protein has been determined (Carr, Penfold et al. 2000), and the protein-protein interaction between its β-propeller domain and the T-domain TolB box of ColE9 was found to be essential for E colicin translocation (Figure 1-11).

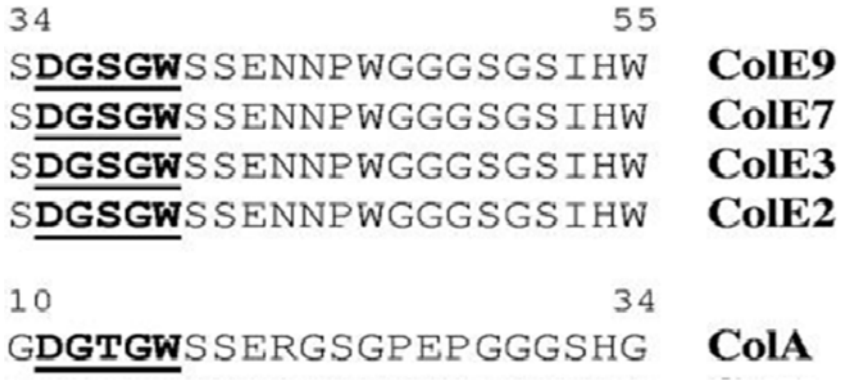


Figure 1-11: Alignment of the TolB box region of the Tol-dependent colicins (Hands, Holland et al. 2005).The pentapeptide TolB box sequences, consisting of residues 35 to 39 in the E colicins and residues 11 to 15 in ColA, are underlined.

Cross-linking studies have previously been used to show that ColA interacts with TolA (Bouveret, Rigal et al. 1997; Derouiche, Benedetti et al. 1997), but no interaction has been demonstrated between an enzymatic colicin and TolA, by either SPR or gel filtration (Hands, Holland et al. 2005). Using a series of deletion and point mutants in the N-terminal domain of colicin A, Bouveret et al., 1998 localized the regions responsible for the interactions with TolA between residues 34–107. Journet et al., 2001, mapped these limits further by testing the interaction of new deletion mutants with TolA to deduce that the region of interaction with TolA in ColA is between residues 52 and 97, but did not implicate any specific residues in the interaction. Sequence homology has been shown between the N-terminal region of ColA and ColK that are involved in the interaction with the C-terminal domain of TolA (Pilsl and Braun 1995). Although ColE1 interacts with TolA differently than group A colicins (Schendel, Click et al. 1997), ColN was shown to interact with the C-terminal domain of TolA like ColA (Raggett, Bainbridge et al. 1998). Consistent with the findings from the crystal structure of ColN (Vetter, Parker et al. 1998), the crystal structure of ColM also revealed that the N-terminal region exhibits a high degree of flexibility and the lack of secondary structure (Zeth, Romer et al. 2008). This might be a reason why the electron density could not be resolved for the first 83 residues of ColE3 (Soelaiman, Jakes et al. 2001) and the first 22 residues and 39-83 residues of ColIa (Wiener, Freymann et al. 1997; Soelaiman, Jakes et al. 2001). This region has been anticipated to include the sequences important for interaction of the colicin with TolA (James, Penfold et al. 2002).

Summarizing all these results have resulted in a model for the translocation of group A colicins, taking into account the localization of the Tol proteins in the cell envelop along with the structural organization of colicins (Cascales, Buchanan et al. 2007).

1.5.6 Translocation through the outer membrane

The mechanism by which colicins, once bound to their receptor, traverse the outer membrane is not yet completely defined. Two hypotheses have been proposed in an attempt to elucidate such mechanism (Cao and Klebba 2002). The first, the Trans-porin "Nail" hypothesis, suggests that binding of the tip of the helical hairpin of a colicin R-domain to the receptor permits colicin penetration into the membrane by transition through the β-barrel of the receptor. Although it is likely that the hairpin of the receptor binding domain possibly will fit into the receptor barrel, the cytotoxic domains are too large to cross through any known OM channels unless they are unfolded (Cao and Klebba 2002). The alternative hypothesis suggests that the cytotoxic domain possibly will translocate through the membrane at another site, further away from the initial binding site (Cao and Klebba 2002). This seems reasonable for ColE3 and Ia having extended structures which would allow translocation of the cytotoxic domain away from the binding site (Cao and Klebba 2002).

Some available evidence suggests that on binding of colicins to the OM receptor at least partial unfolding may take place. Denaturation of ColA by urea has been shown to decrease the time taken to depolarise the CM, as shown by the decreased lag time for onset of the efflux of cytoplasmic

potassium (Bénédetti *et al.*, 1992; Bourdineaud *et al.*, 1990a). Isothermal titration calorimetry (ITC) indicates that a structural rearrangement takes place on formation of the OmpF-ColN complex (Evans, Labeit et al. 1996). It has been proposed that proteins can become less ordered on reaching the membrane due to a local increase in pH and in the dielectric constant (Bychkova, Dujsekina et al. 1996). Colicin unfolding would also increase its length, allowing the cytotoxic domain to reach the CM, with the receptor-binding domain still bound to its outer-membrane receptor. Cells treated with ColA can be rescued by the addition of trypsin that causes membrane depolarization to be halted (Benedetti, Lloubes et al. 1992), indicating that part of the colicin is still accessible to the external medium even after the pore-forming domain has introduced into the CM.

A model for ColE9 translocation across the OM has been put forward (Cascales, Buchanan et al. 2007). Following receptor binding to BtuB, ColE9 recruits OmpF by its natively unstructured segment in the T domain forming a BtuB-OmpF-colicin translocon (Housden, Loftus et al. 2005). Then this part of the T domain passes into the cell through an OmpF pore to interact with TolB (Loftus, Walker et al. 2006), bringing the cytotoxic domain of ColE9 close enough to cross the OM through another OmpF pore (Zakharov, Zhalnina et al. 2006). ColN is a much shorter molecule when compared with other colicins and therefore this restricts the characteristics of the access for its cytotoxic domain. The receptor binding domain of ColN originally binds to OmpF, also the pore-forming domain has been shown to associate with OmpF (Dover, Evans et al. 2000). A model of ColN binding to OmpF receptor, has been

suggested in which the receptor-binding domain sits like a plug above the channel of OmpF with positively charged residues in a solvent-exposed cleft of the receptor-binding domain interacting with negatively charged loops of OmpF (Vetter, Parker et al. 1998). Both the pore-forming and translocation domains of ColN are anticipated to be involved in interacting with OmpF as the binding affinity of the receptor-binding domain alone for OmpF is much weaker than the full-length colicin (Evans, Labeit et al. 1996). Knowledge of the ColN structure, coupled with the structure of its receptor, OmpF, and previously available biochemical data, limits the numerous possibilities of translocation and leads to propose a model in which the translocation domain inserts itself through the OmpF porin pore, the receptor-binding domain remains in contact with the membrane and the pore-forming domain inserted along the outer wall of the trimeric porin channel (Vetter, Parker et al. 1998).

1.5.7 Colicin Translocon

A colicin translocon model has been proposed (Cascales, Buchanan et al. 2007), as most colicins require at least two OM proteins for cell entry: such as a BtuB surface receptor, which does not participate in translocation and OmpF for cell penetration. A BtuB-OmpF-colicin translocon was shown to form when ColE9 binds to BtuB through the natively unstructured region of the T-domain (Housden, Loftus et al. 2005). The structure of BtuB bound to residues 313-447 of the receptor binding domain of ColE3 (R135) shows that only 27 residues between residues I369-T402 of R135 and 29 residues mostly at the top of the plug domain of BtuB are involved in the BtuB-R135 complex, with 24 % and 6.3 % of R135 and BtuB, respectively, buried on formation of the complex (Kurisu, Zakharov et al. 2003). Slight conformational changes are

observed in the plug domain of BtuB, but these cannot account for translocation of the colicin, indicating the role of BtuB is only as a cell surface receptor, with other membrane proteins being involved in translocation. The R135-BtuB structure provoked the suggestion that as an alternative of colicin being directly translocating through BtuB, this receptor is only used to concentrate the colicin on the surface, where it can then "fish" for a secondary receptor (Zakharov, Eroukova et al. 2004). The translocation domain would bind to this secondary receptor as OmpF, forming a colicin-translocon (Figure 1-12).

The events that take place during ColE3 import through the OM BtuB/OmpF translocon have been proposed (Sharma, Yamashita et al. 2007). Once ColE3 binds to BtuB, OmpF is recruited into the translocon through interaction of ColE3 unstructured T-domain. Unfolding of the receptor binding domain triggers the penetration of the T-domain though the OM to interact with the Tol proteins. Simultaneously with this, Im3 is released and the catalytic RNase domain is transported through OmpF towards the CM (Zakharov, Zhalnina et al. 2006). Proteolytic cleavage of colicin occurs between the C and R domains allowing access of the C domain into the cytoplasm, where it acts cytotoxically as an endoribonuclease.

A potential role of the linker region of ColE9 which was suggested to act as a spacer between the receptor-binding domain and the translocation domain, this was speculated as the cytotoxic domain or translocation domain passes through the porin, whilst the receptor-binding domain is still attached to its receptor.

Also the long helix of colicin Ia T-domain that has no obvious function has been suggested to act as a spacer (Wiener, Freymann et al. 1997). ColA mutants with residues 98-108 or 154-172 deleted are still active, and it has been proposed that these regions could act as spacers (Bouveret, Rigal et al. 1998; Journet, Bouveret et al. 2001).

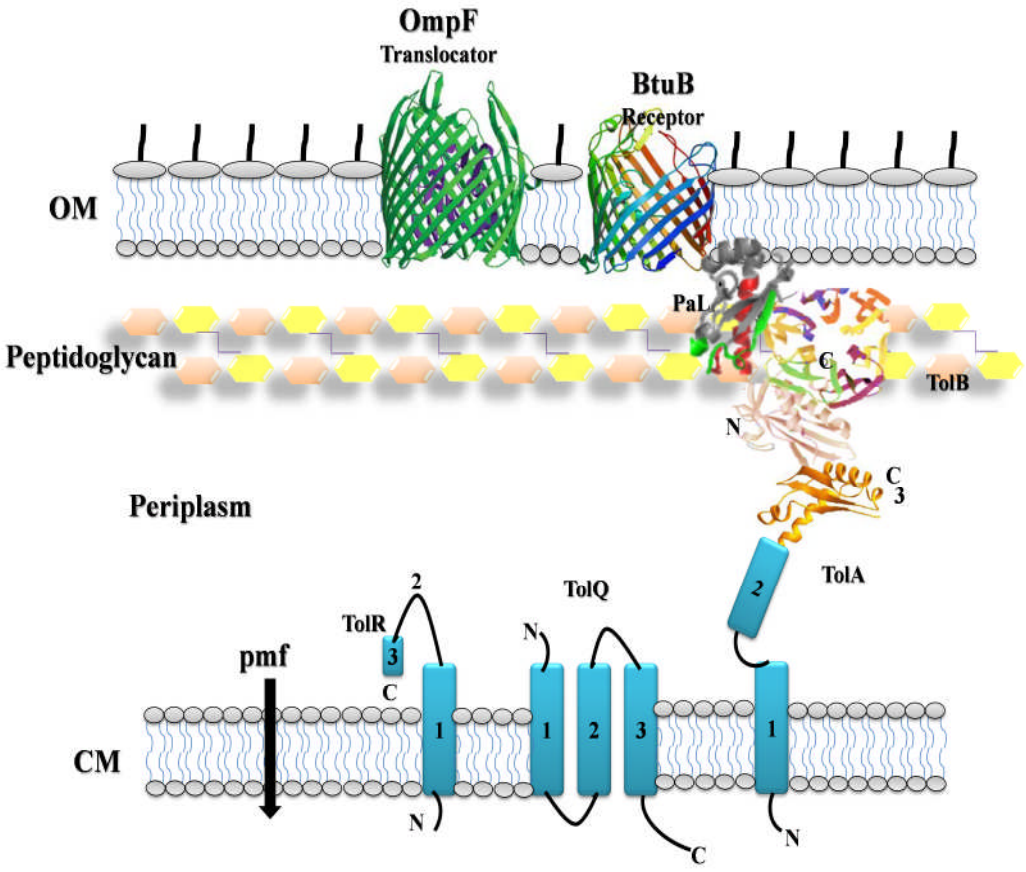


Figure 1-12: Localization of the BtuB, OmpF and the Tol–Pal proteins in the cell envelope of Gram-negative bacteria (adopted from (Lazzaroni, Germon et al. 1999; Kleanthous 2010).

1.5.8 Tol-dependent translocation model

Movement through the OM requires the formation of functional colicin translocon which is mediated by binding to a specific surface receptor and then recruitment of a second outer membrane porin, either OmpF or OmpC (Sharma, Yamashita et al. 2007). The second step of colicin killing, takes place during crossing of the OM and the periplasmic space, during translocation. In the case of nuclease colicins, there is an additional requirement to cross the CM, in order to reach their cellular targets (Figure 1-12). All E-group colicins require the porin OmpF for their translocation except for ColE1 (Cavard 1994). It is hypothesized that the T-domain of E colicins may cross the outer membrane via the central channel of the trimeric porin OmpF and therefore is able to interact with Tol periplasmic proteins (James, Kleanthous et al. 1996; Lazdunski, Bouveret et al. 1998). It has been shown that the BtuB-ColE9/Im9 complex recruits the OmpF through a natively disordered 83 amino acids N-terminal sequence (Collins, Whittaker et al. 2002; Housden, Loftus et al. 2005). The interaction of a nuclease colicin T domain with TolB leads to immunity protein release (Duche, Frenkian et al. 2006; Vankemmelbeke, Zhang et al. 2009) which then permits the C-terminal domain to unfold, and penetrate through OmpF (Zakharov, Zhalnina et al. 2006). It has been suggested that the inserted C-terminal domain is then separated from the R domain by an action of a protease and consequently cross the OM (de Zamaroczy, Mora et al. 2001; Shi, Chak et al. 2005), however, this is still controversial. Reaching the cellular target, the cytotoxic domain interacts directly with the CM in the case of pore-forming colicin or has to cross the CM to reach their target in the cytoplasm of the attacked cell for nuclease

colicins (James, Penfold et al. 2002). The cytotoxic domain of nuclease colicins use the other components of the Tol pathway to pass through the CM and gain entry into the cytoplasm through formation of voltage-independent ion channels in planar lipid bilayers (Mosbahi, Lemaitre et al. 2002).

However, this hypothesis raises several questions such as where the energy to drive the colicin through the barrel would come from and whether the diameter of the β-barrel of the receptors is large enough to accommodate the colicin in the folded or unfolded state.

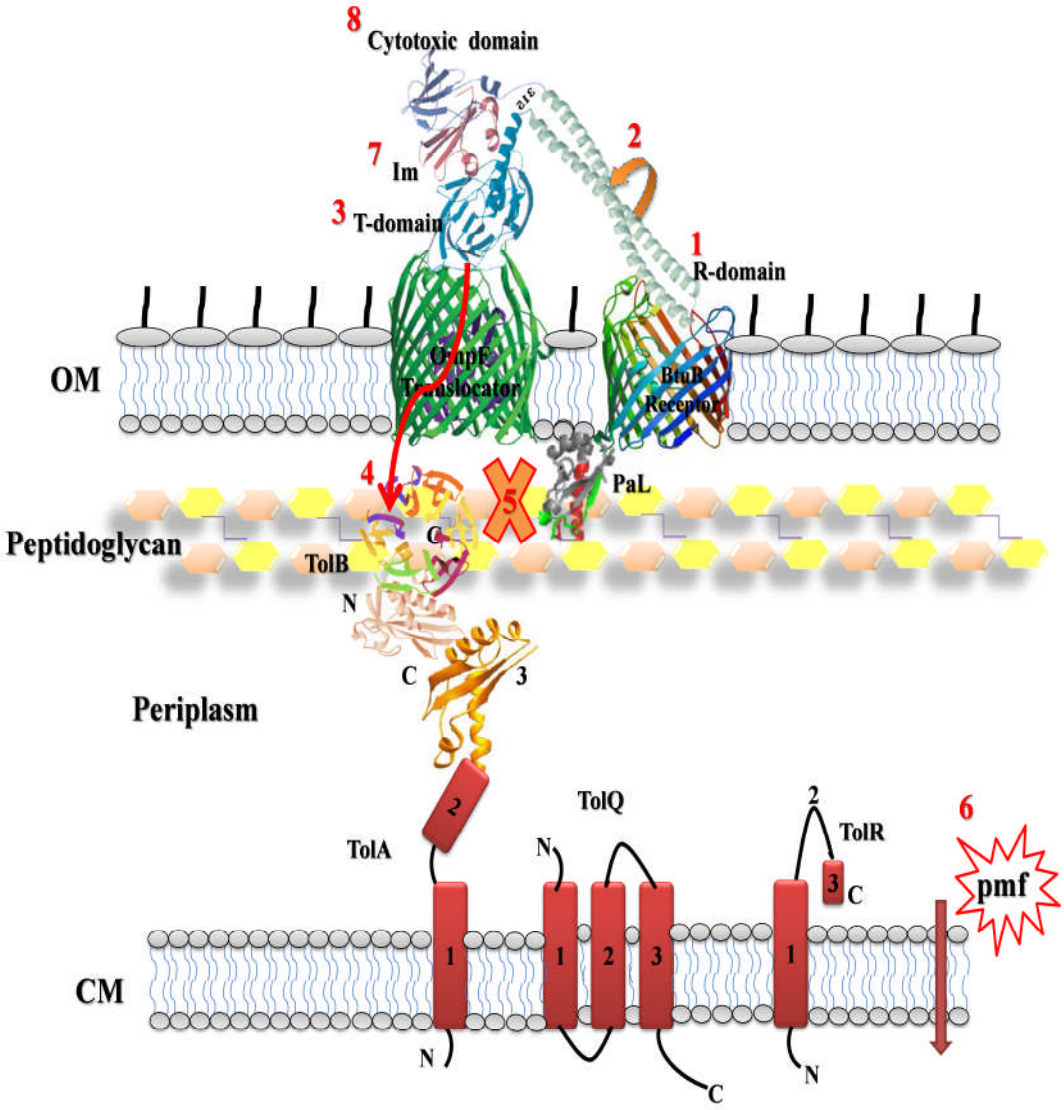


Figure 1-13: Schematic representation of translocation of endonuclease colicin. Colicin R-domain binding to BtuB receptor allows unfolding and binding of the T-domain to OmpF and then insertion into the periplasm where the T-domain can interact with TolB displacing it from Pal allowing the energized Tol system to pull-down the cytotoxic domain after releasing the immunity protein.

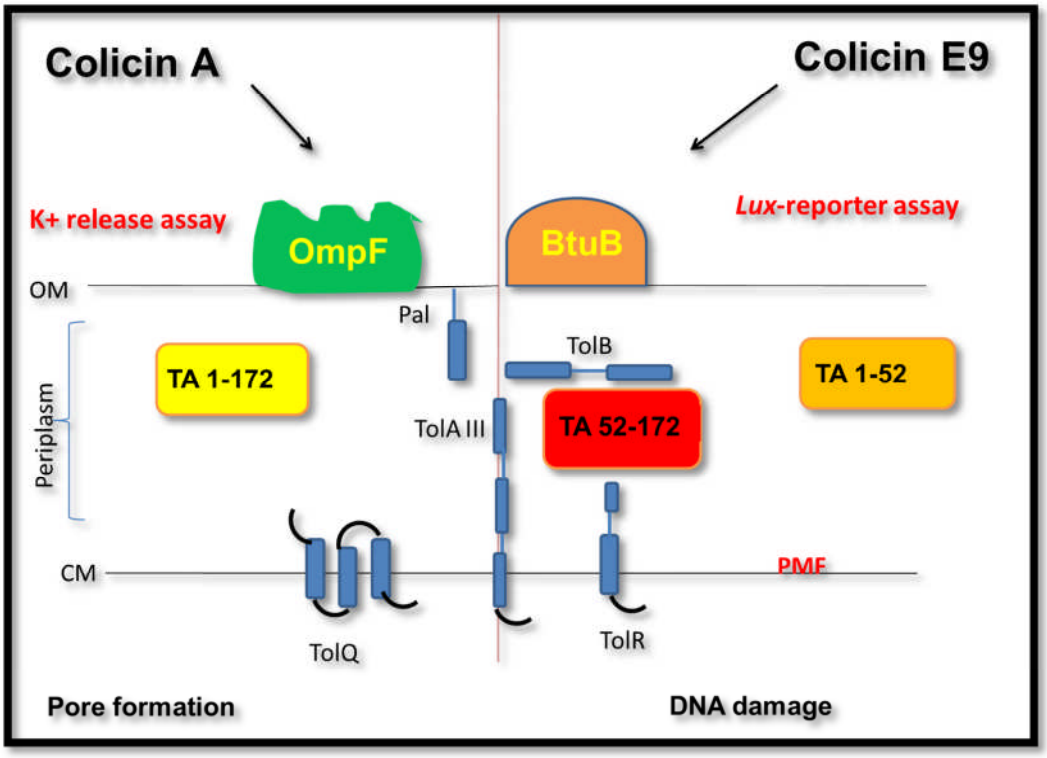
1.6 Aims of the study

The overall aims were to investigate the sequence of events that take place during colicin translocation. By engineering unique periplasmically- expressed colicin polypeptides containing mutations, we hoped to be able to investigate the protein-protein interactions that occur in the periplasm during colicin translocation and also to identify key residues that are essential for the translocation process. Furthermore, immunity protein release during the process of translocation is a requirement for nuclease colicins, in this work a challenge of developing a sensitive real-time assay takes place to investigate the release of immunity protein.

Previously described methods for expressing and secreting colicin domains into the cell periplasm were used to monitor the interaction between the Tol system and externally added colicins. The production of the translocation domain of colicin in the periplasm of the *E. coli* cells has determined specific interaction with the Tol proteins (Bouveret, Rigal et al. 1998; Pommier, Gavioli et al. 2005). Using this 'periplasmic expression technique' to perturb the function of the Tol system in the cell envelope, interactions of secreted colicin domains with the Tol proteins can be monitored by the patterns of sensitivity/resistance of the cells to externally added colicins using the previously developed *lux*-reporter assay (Vankemmelbeke, Healy et al. 2005) and the potassium release assay (Boulanger and Letellier 1988).

This study mainly concentrates on investigations into the role of the Tol proteins in the translocation process of colicins and the dynamics of the interaction between different colicins and the Tol system, the data collected and the consequences observed from these experiments, showed that the

expression and secretion of the translocation domains of colicins E9 and A protect cells from externally added ColE9 and A through interactions of those domains with Tol proteins which prevent subsequent interactions of Tol system with the translocation domains of the externally added colicins. In addition competitive inhibition of TolA showed a larger effect on cell resistance than inhibition of TolB providing further insights into the role of different Tol proteins in the translocation of different colicins. This will provide further insights in determining some details that take place as the colicin reaches its target. A further, long term aim of the study was to develop a real time fluorescent assay which allows studying the immunity protein release and the role of the Tol proteins during this process.



CHAPTER TWO

2 Materials and methods

This chapter describes the basic methods and procedures used together with the required conditions, materials, reagents and equipment. All chemical reagents were purchased from Sigma Aldrich unless otherwise stated.

2.1 BACTERIAL GROWTH MEDIA

All bacterial strains grown for the purpose of DNA manipulation were cultured using Luria Broth (LB) medium supplemented with the required antibiotic unless otherwise stated. All bacterial strains grown for the purposes of protein expression and purification were cultured using 2 x yeast tryptone (2YT) medium supplemented with 100 $\mu g ml^{-1}$ ampicillin unless otherwise stated.

2.1.1 Luria-Bertani medium

Luria-Bertani (LB) broth was prepared as described by (Sambrook, Fritsh et al. 2001) and consists of 10 g bacto-tryptone (Oxoid), 5 g bacto-yeast extract (Oxoid) and 5 g NaCl made up to 1 L with deionised water. The pH was adjusted to 7.0 with 5 M NaOH and sterilisation was achieved by autoclaving at 121 °C and 15 psi for 20 min.

Luria-Bertani agar was made with the addition of bacteriological agar No.1 (Oxoid) to a final concentration of 1.5 % ($^{w}/_{v}$).

2.1.2 2YT medium

2 x yeast tryptone (2YT) broth was prepared as described by (Sambrook, Fritsh et al. 2001) and consisted of 16 g bacto-tryptone (Oxoid), 10 g bacto-yeast extract (Oxoid) and 5 g NaCl made up to 1 L with deionised water. The pH was adjusted to 7.0 with 5 M NaOH and sterilisation was achieved by autoclaving at 121 °C and 15 psi for 20 min.

2YT agar was made with the addition of bacteriological agar No.1 (Oxoid) to a final concentration of 1.5 % ($^{w}/_{v}$).

2.1.3 Minimal medium + Glucose

Minimal medium broth was prepared as described (Sambrook, Fritsh et al. 2001) and contains 2 % Casamino Acids, 0.2 % glucose and 1 X M9 Salts (See below for recipe for 10 X M9 Salts), 1 mM $MgCl_2$ and 1 mM thiamine.10 x M9 salt was prepared as described by (Sambrook, Fritsh et al. 2001) and consisted of 60 g Na_2HPO_4, 30 g KH_2PO_4, 5 g NaCl, and 10 g NH_4Cl made up to 1 L with deionised water. The pH was adjusted to 7.4 with 5 M NaOH and sterilisation was achieved by autoclaving at 121 °C and 15 psi for 20 min.

2.2 BACTERIAL STRAINS

The bacterial strains used in this thesis are described in Table 2-1.

Table 2-1: Bacterial strains used in this study are described with their origin and genotypes.

Bacterial Strain	Description / Genotype	Source/Reference
E. coli BL21 (DE3)	Protein expression strain of *E. coli*. Genotype: F-, *omp*T, *hsd*S_β(r_β-m_β-), *dcm*, *gal*, (DE3) *ton*A	Invitrogen
E. coli DH5α	General laboratory strain of *E. coli*. Genotype: F-, ø80d*lac*ZΔM15, Δ(*lac*ZYA-*argF*)U169, *deo*R, *rec*A1, *end*A1, *hsd*R17(rK^-, mK^+), *pho*A, *sup*E44, λ–, *thi*-1, *gyr*A96, *rel*A1	Invitrogen
E. coli DPD1718	General laboratory strain of *E. coli*. Genotype: recA promoter region to the *Photorhabdus luminescens* luxCDABE reporterintegrated into the lacZ locus of *E. coli* DPD1692	(Vankemmelbeke et al., 2005)
E. coli JM83	General laboratory strain of *E. coli*. Genotype: F^-, *ara*, Δ(*lac-pro*AB*), rps*L, phi80*lac*Z ΔM15	(Yanisch-Perron, Vieira et al. 1985)

2.3 Plasmids and cloning vectors

The plasmids used in this study together with a description and their source of origin are listed in Table 2-2.

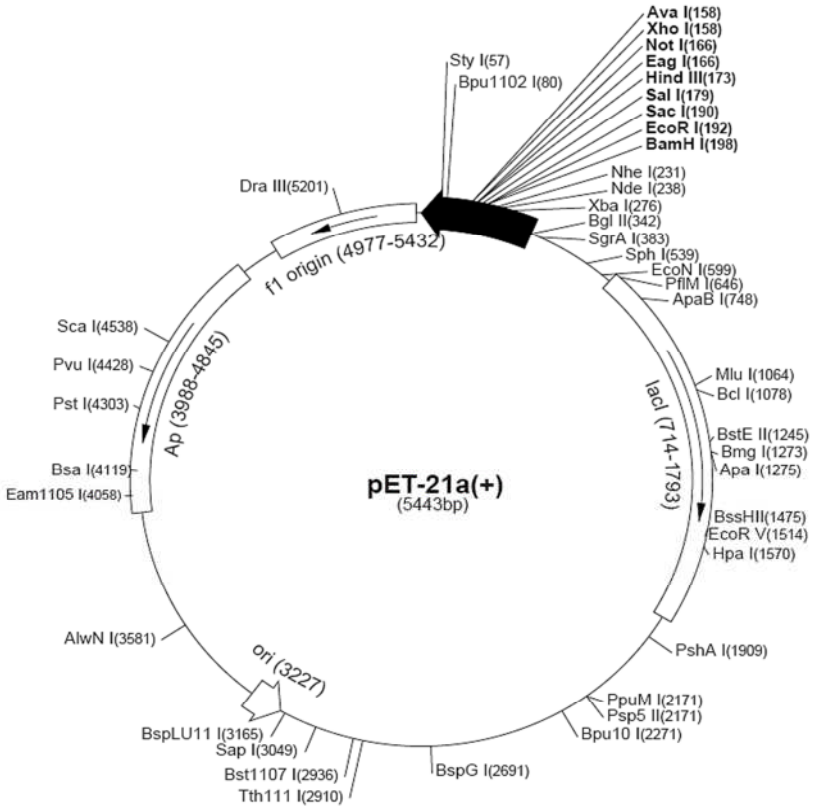
Table 2-2: Plasmids and cloning vectors.

Plasmid	Description	Source / Reference
pBAD/gIIIC	T-tailed vector for cloning PCR products	Invitrogen
pET-21a	*E. coli* protein expression vector	Novagen

The two plasmids used to create recombinant clones were pBAD/gIIIc and pET-21a. Recombinant clones were identified by screening on LB agar supplemented with 100μgml^{-1} ampicillin.

Genes were cloned into the *E. coli* expression vector pET-21a. Expression from this plasmid is under the control of T7 bacteriophage transcription and translation signals. Recombinant proteins expressed from pET-21a carry a C-terminal hexahistidine tag to assist in affinity chromatography. pBAD/gIIIc expression vectors designed for regulated, dose-dependent recombinant periplasmic protein expression and purification in *E. coli*. The regulatory protein, AraC, is provided on the pBAD vectors allowing regulation of P_{BAD} in presence of L-arabinose. Vector maps are shown in (Figure 2-1and Figure 2-2). The sequences of the important plasmids constructed during this study can be found in the appendices.

(A)



(B)

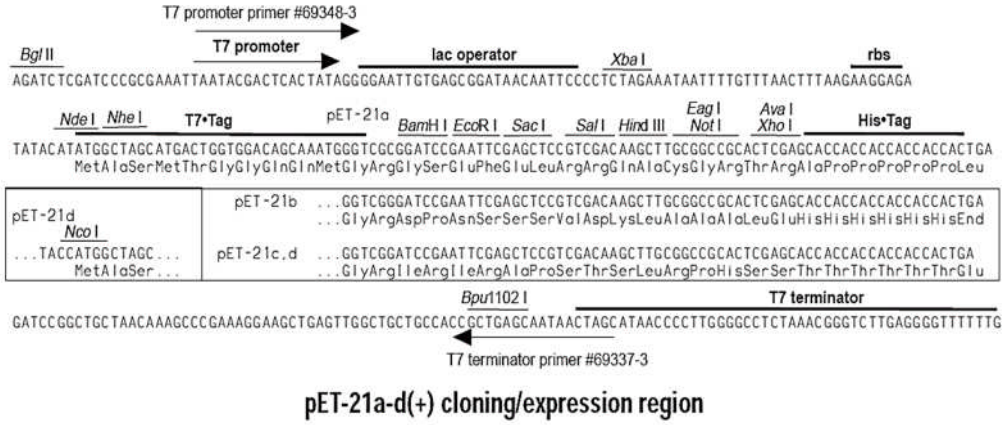


Figure 2-1: (A) Shows a vector map of pET-21a (Novagen) with the multiple cloning sites in black and the location of the coding sequences. The sequence of the multiple cloning sites, the location of the promoter and the hexahistidine tag are shown in detail in (B).

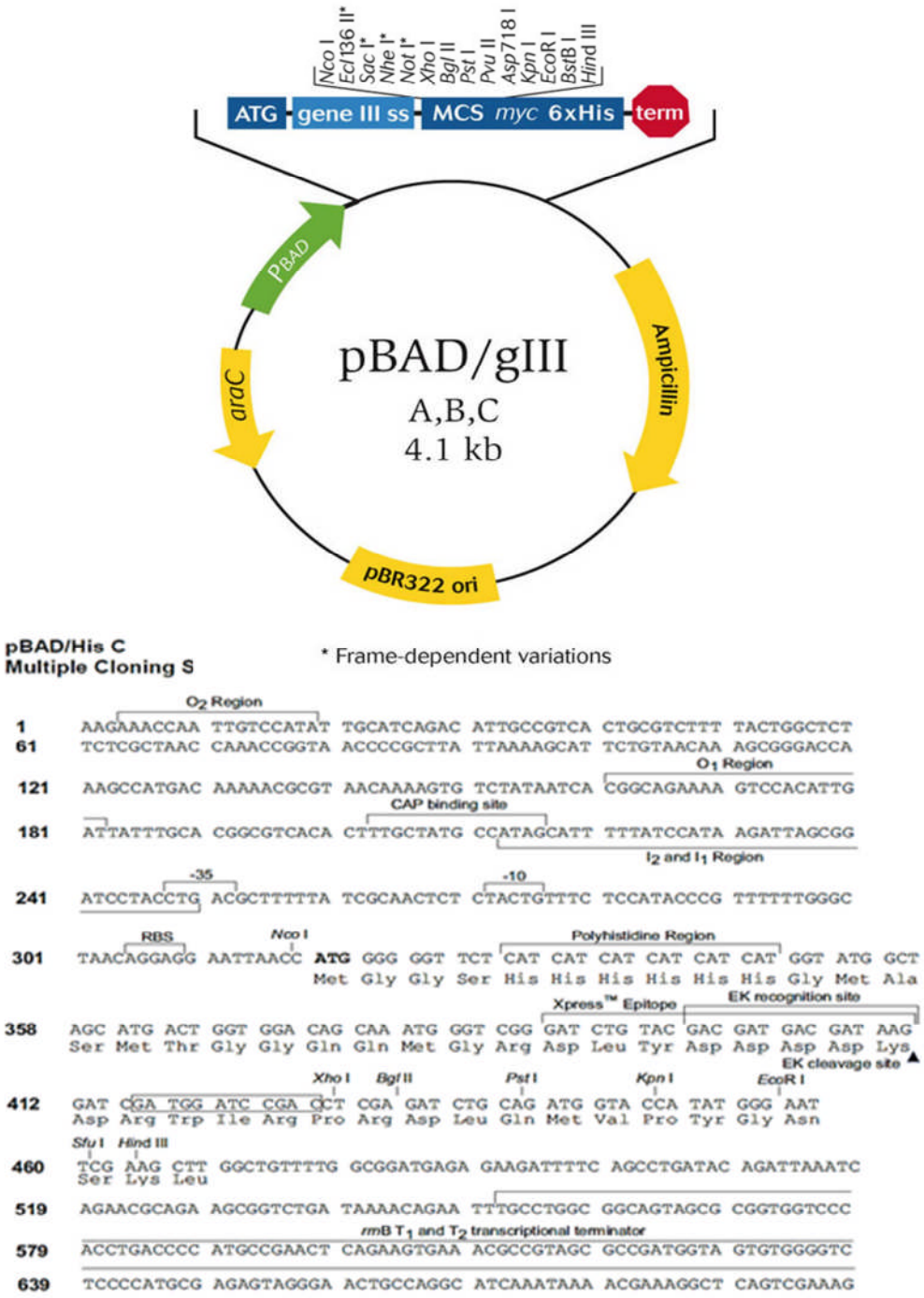


Figure 2-2: Vector map of pBAD/gIII A, B, C.

2.4 DNA MANIPULATIONS

2.4.1 Polymerase chain reaction

Polymerase chain reaction (PCR) was used for the amplification of target sequences and screening for target sequences in recombinant clones. Site-directed mutagenesis was also accomplished using modified PCR protocols or Quick Change site directed mutagenesis protocol (Stratagene, US).

Polymerase chain reactions were carried out according to (Saiki, Scharf et al. 1985) using an Eppendorf Mastercycler Gradient thermocycler. Pwo DNA polymerase (Roche) with proof-reading activity was used for amplification of DNA to be used in cloning procedures, while High-fidelity DNA polymerase (Roche) was used for colony PCR and screening recombinant plasmids for the presence of the target sequence. Reaction volumes were varied from 10 µl to 50 µl according to requirements. A negative control lacking template DNA was carried out in all cases to rule-out cross-contamination of reaction tubes and the various reagents. The reagents used in a standard 50 µl reaction are listed in Table 2-3.

Table 2-3: A Standard PCR reaction

Volume	Components
25 µl	Ultrapure water (Sigma)
5 µl	x10 PCR reaction buffer (containing 1.5 mM $MgCl_2$)
5 µl	dNTPs (2 mM)
5 µl	Forward primer (10 mM)
5 µl	Reverse primer (10 mM)
5 µl	Template DNA (50 $ngµl^{-1}$)
0.4 µl	DNA polymerase
50 µl	**Total reaction volume**

Typical reactions consisted of a denaturing step of 95 °C for 1 min followed by 35 cycles of 95 °C for 45 s, 55 °C for 1 min and 72 °C for 2min. An incubation step at 72 °C for 30 min was used to ensure full length fragments were synthesized after all the PCR cycles were completed. A standard annealing temperature (T_a) of 55 °C was used in most cases, however the T_a was adjusted to improve yield or reduce non-specific annealing if required. For larger PCR products, the extension step at 72 °C was increased from 30 s with a 1 min extension time allowed for every kilobase of amplified DNA product.

2.4.2 Oligonucleotide primers

Table 2-4 lists the oligonucleotide primers used in the PCR reactions during this study. Restriction sites used for cloning are shown underlined in red. Oligonucleotides were designed using Vector NTI 10.3 software (Invitrogen) and synthesized by Sigma Genosys.

Table 2-4: Oligonucleotide primers for PCR. Codons that do not match the target sequence and result in an amino acid change are underlined.

Primer	Oligonucleotide Sequence 5′-3′	Description
RJ18	GCTCGAGCCGGGGAACTTTCACAGTCCA	Reverse primer to introduce *Xho*I site in ColA TA_{1-172}
CNP25	GCCATGGCTGGATTTAATATGGT	Forward primer to introduce *Nco*I site in ColA TA_{1-172}
YZ42	GGCTGGAGCTCAGCACGTGGGAGTGGT	Forward primer to mutate Y58 to A in ColA
YZ43	ACCACTCCCACGTGCTGAGCTCCAGCC	Reverse primer to mutate Y58 to A in ColA
YZ44	GAAACCAGGGGATTCGGCTAACACCCCGTGGGGA'	Forward primer to mutate E18 to A in ColA
YZ45	TCCCCACGGGGTGTTAGCCGAATCCCCTGGTTTC	Reverse primer to mutate E18 to A in ColA
FA1	AGCTCAGCCAATAACCCTTGGC	Forward primer to mutate E18 to A in ColA that contain the ColE9 TolB box residues

FA2	GCCAAGGGTTATTGGCTGAGCT	Reverse primer to mutate E18 to A in ColA that contain the ColE9 TolB box residues
Fa3	CGGCCATGGAACCAGGGGATTCG	Forward primer to clone ColA TA_{52-172} and introduce an *Nco*I restriction site
FA4	CCCTCGAGCATTACCGTCACAGA	Reverse primer to clone ColA TA_{1-52} and introduce an *Xho*I restriction site
FA5	AATTCATCGATGGTTCCTTACGGCGCAGCGGCTGCAGCGGTTTTAAATTCCCTG	Forward primer to introduce five alanine mutations in TA_{1-1-72} R92-R96A
FA6	CAGGGAATTTAAAACCGCTGCAGCCGCTGCGCCGTAAGGAACCATCGATGAATT	Reverse primer to introduce five alanine mutations in TA_{1-1-72} R92-R96A
pBAD-F	ATGCCATAGCATTTTTATCC	Forward primer for cloning and sequencing inserts in the pBAD/gIIIc vector. Used also for sequencing
pBAD-R	GATTTAATCTGTATCAGG	Reverse primer for cloning and sequencing inserts in the pBAD/gIIIc vector. Used also for sequencing
T7 promoter	TAATACGACTCACTATAGGG	Sequencing of DNA fragments cloned into the pET-21a vectors
T7 terminator	CTAGTTATTGCTCAGCGGTGG	Sequencing of DNA fragments cloned into the pET-21a vector

2.4.3 Isolation of plasmid DNA

A 10 ml overnight culture of *E. coli* DH5α was grown in LB broth supplemented with the antibiotic required by the selective marker on the plasmid. Plasmid DNA was extracted using the Wizard Plus SV Miniprep kit (Promega) following the manufacturer's instructions. Large scale circular plasmid DNA isolation was conducted using spin columns QIAGEN midi prep kit (Appendix C). Plasmid mini prep kits (Promega) were used to purify small scale circular plasmid DNA according to the manufacturer's guidelines. Purified DNA samples were stored at -20 °C.

2.4.4 Restriction digests

Endonuclease digestion of plasmid DNA was carried out according to the methods of (Sambrook, Fritsh et al. 2001). Restriction enzymes were purchased from New England BioLabs and Roche and were used with appropriate buffers.

2.4.5 Ligation of DNA fragments

DNA fragments were ligated using the Rapid DNA Ligation kit (Roche) following the manufacturer's instructions. Recombinant plasmids produced by ligations were transformed into *E. coli* DH5α unless stated otherwise.

2.4.6 Agarose gel electrophoresis

PCR amplicons and DNA fragments were separated by agarose gel electrophoresis as described by (Sambrook, Fritsh et al. 2001). Fragments were sized by comparing them to 1 kb and 100 bp ladders purchased from New

England BioLabs. DNA was extracted from agarose gels using the QIAEX II gel purification kit (Qiagen) following the manufacturer's protocol.

2.4.7 Quantification of DNA

Genomic and plasmid DNA samples were quantified on a NanoDrop ND-1000 spectrophotometer by measuring absorbance at 260 nm. The purity of samples was estimated by the ratio of absorbance of nucleic acids (260 nm) to amino acids (280 nm).

2.4.8 DNA sequencing

Plasmid DNA was diluted to a final concentration of 100 ngμl^{-1}. A 15 μl sample was sent to Geneservice Ltd (Nottingham) for sequencing with in-house stock primers by the chain termination method (Sanger, Nicklen et al. 1977). Recombinant clones derived from pET-21a were sequenced with the T7 promoter and T7 terminator primers. Clones derived from pBAD/gIII were sequenced using the pBAD forward and reverse primers. All the sequencing primers used are listed in Table 2.4. Chromatograms were analysed using the Lasergene 7 (DNAStar) and Vector NTI 10.3 (Invitrogen) software packages.

Primers used for sequencing of recombinant plasmids by Geneservice Ltd are listed in Table 2-4.

2.5 Transformation of bacteria

2.5.1 Preparation of chemically competent *E. coli* JM83 cells

A 50 ml culture of *E. coli* BL21 (DE3) or LMG194 or DPD1718 in LB broth was grown at 37 °C. Cell density was monitored by measuring absorbance at 600 nm. When the culture reached an OD_{600} of 0.4-0.6 it was cooled on ice for 10 min prior to harvesting by centrifugation at 6,000 xg for 10 min at 4 °C. Cells were re-suspended in 10 ml of sterile ice cold 100 mM $CaCl_2$, 20 % ($^{v}/_{v}$) glycerol. Cells were then incubated on ice for a further 15 min before harvesting again as described above. Cells were finally resuspended in 2 ml of sterile ice cold 100 mM $CaCl_2$, 20 % ($^{v}/_{v}$) glycerol and incubated on ice for 1 - 2 h after which, they were divided into 100 µl aliquots and stored at -80 °C to be used when required.

2.5.2 Heat shock transformation of *E. coli* with plasmid DNA

Approximately 50 ng of plasmid DNA was added to 100 µl of chemically competent *E. coli* DH5α (Invitrogen), *E. coli* BL21 (DE3), *E. coli* LMG194 or *E. coli* DPD1718. After incubation on ice for 30 min, the cells were then heat-shocked at 42 °C for 45 s and placed on ice for 2 min. The cells were then incubated at 37 °C with shaking for 1 h after the addition of 400 µl of pre-warmed LB broth before 200 µl of the transformants were plated on LB agar plates with antibiotic selection and grown for 18 h at 37 °C.

2.5.3 Preparation of electro-competent E. coli cells

In order to make electro-competent *E. coli* JM83 cells, an overnight culture was diluted 1:100 into fresh LB supplemented with appropriate antibiotics and

incubated at 37 °C with shaking (200 revolutions per min; rpm) until OD_{600} reached between 0.5-1.0. Following rapid chilling of the culture on ice for 30 min, the cells were subsequently harvested by centrifugation at 4,000 ×g for 15 min at 4 °C. The supernatant was discarded and cell pellet was re-suspended in an equal volume of pre-chilled sterile distilled H_2O. The cell suspension was again centrifuged and the supernatant was discarded. This process was repeated using 4 ml of ice cold, sterile 10 % ($^{v}/_{v}$) glycerol solution. The cells were pelleted by centrifugation and finally resuspended into 0.4-0.6 ml of pre-chilled sterile 10 % ($^{v}/_{v}$) glycerol. Aliquots of 55 µl cells were stored at -80 °C until required.

2.5.4 Transformation of *E. coli* cells by electroporation

Electroporation of plasmid DNA into *E. coli* cells was carried out as previously described (Dower, Miller et al. 1988). An aliquot of 55 µl of electrocompetent cells was thawed on ice and 1-2 µl of plasmid DNA was added. The mixture was incubated on ice for 1 min before transferring into a pre-chilled 2 mm electroporation cuvette (BIO-RAD). A Bio-Rad Gene Pulsar was used, according to manufacturer's instructions, to deliver an electric pulse of 2.5 kV. The cuvette was immediately removed and 1 ml of LB broth was quickly added to the electroporation cuvette. After incubation for approximately 1-2 h at 37 °C, bacterial suspension was plated out on to selective LB agar plate.

2.6 PROTEIN EXPRESSION

Protein expression was carried out on small scale cultures (5 ml) when confirming the expression of a polypeptide of the expected size, and on a larger scale (1 L) for purifying an expressed protein.

2.6.1 Screening transformants for expression of a recombinant protein

A single colony was used to inoculate 5 ml of either minimal media or 2YT media supplemented with 100 µg / ml ampicillin. The culture was grown at 37 ºC with shaking until the OD_{600} reached 0.4 – 0.6. At this point, arabinose (0.02 % $^{w}/_{v}$) was added to pBAD/gIIIc based plasmids, and IPTG (1 mM) was added to pET21a based plasmids, to induce expression. The culture was incubated for a further 2-3 h at 37 ºC with shaking. A 20 µl sample of the culture was then analyzed by SDS-PAGE as described in below section 2.6.3, and compared to a control culture that was not subjected to arabinose or IPTG induction.

2.6.2 Expression of recombinant proteins for purification

Protein expression on a large scale was carried out for the purpose of protein purification for further experimental studies. A single colony of freshly transformed *E. coli* BL21 (DE3) carrying the relevant plasmid was used to inoculate two 5 ml cultures of 2YT media. The cultures were incubated at 37 ºC with shaking for 2 h. Each 5 ml culture was used to inoculate separate flasks of 500 ml of either RM media or 2YT media supplemented with 100 $\mu g ml^{-1}$ ampicillin. These cultures were incubated at 37 ºC with shaking until the OD_{600} reached 0.4 – 0.6, at which point, arabinose or IPTG was to induce

expression. The cultures were then incubated with shaking at a lower temperature of 30 ºC for 2 to 3 h. To ensure maximal productivity of recombinant proteins during production culture it is typical to encourage an initial phase of rapid cell proliferation to achieve high biomass followed by a stationary phase. Low temperature induced growth arrest is associated with many positive phenotypes including increased productivity, sustained viability and an extended production phase (Kumar, Gammell et al. 2008). The time allowed for protein induction depended on the protein's expected yield and solubility. Cells were harvested by centrifugation in a Beckman Avanti J20I at 10,000 xg for 12 min at 4 ºC. Cell pellets were stored at -20 ºC.

2.6.3 SDS-PAGE analysis of protein samples

Samples were boiled for 2 min in protein loading buffer [100 mM Tris-HCl, 4 % ($^{w}/_{v}$) SDS, 20 % ($^{v}/_{v}$) glycerol, 0.2 % ($^{w}/_{v}$) bromophenol blue, 4 % ($^{v}/_{v}$) 2-mercaptoethanol]. Proteins were then separated by SDS-PAGE using a discontinuous Tris/glycine buffer with 12 or 16 % ($^{w}/_{v}$) acrylamide resolving gels and 4 % ($^{w}/_{v}$) acrylamide stacking gels containing 0.1 % ($^{w}/_{v}$) SDS (Laemmli 1970). Gels were stained with Coomassie blue and the approximate molecular weight of polypeptide bands was estimated by comparison of their mobility compared to that of a broad range (7 – 175 kDa), pre-stained marker protein mixture (New England BioLabs).

2.7 Protein purification

Large scale cultures were used to purify expressed proteins for further characterisation. The purification protocol consisted of nickel chelate chromatography followed by gel filtration chromatography.

2.7.1 Nickel-chelate chromatography

Nickel chelate chromatography was used as the first step in purifying proteins that contain a C-terminal hexahistidine tag that is introduced during the cloning of the target gene. Nickel bound to a nickel chelate column (5 ml HiTRAP chelating column (GE Healthcare, UK)) binds hexahistidine tagged proteins which can then be washed to remove many contaminating proteins before elution from the column with gradient buffer containing imidazole.

A cell pellet harvested from one or two 500 ml cultures was obtained as described in section 2.6.2. The pellet was re-suspended in 30 ml NiC buffer (10 % $^{v}/_{v}$ glycerol, 25 mM NaH_2PO_4 pH 7.4, 50 mM NaCl) containing a Complete EDTA-free Protease Inhibitor Cocktail Tablet (Roche), and an additional 300 µl of 100 mM PMSF was added to inhibit any contaminating cysteine proteases. Cell lysis was achieved either by sonication on ice at an amplitude of 8 µm for 20 s, followed by 40 s cooling, for a total of 40 min using a Soniprep 150 (MSE), or by using the bugbuster lysis buffer. A 5 ml HiTRAP Chelating HP affinity column (GE Healthcare) was charged with 20 ml of 50 mM $NiSO_4$, using a BioLogic LP (Bio-Rad Laboratories). The 5 ml/1ml columns were equilibrated with 25 ml/5 ml of 1 x PBS + 50 mM NaCl, pH 7.4, charged with 25 ml / 5 ml of 50 mM $NiSO_4$ and washed with 25 ml / 5 ml of 1 x PBS + 50 mM NaCl, pH 7.4. Protein samples were filtered using a sterile 0.2 µm filters before being applied to the column which were then washed with 25 ml / 5 ml of 1 x PBS + 50 mM NaCl, pH 7.4 and the his-tagged proteins were eluted using an increasing gradient of imidazole (1- 100 % 1 M imidazole in 1 x PBS + 50 mM NaCl, pH 7.4) in a volume of 50 ml / 10

ml. The elution was monitored at OD280 and by running samples of the fractions on SDS-PAGE. Protein-containing fractions were pooled and then dialysed in 5 L potassium phosphate buffer (50 mM K_2HPO_4, 50 mM KH_2PO_4, pH 7.0) at 4 ºC for a minimum of 16 h.

2.7.2 Gel filtration chromatography

In order to produce high-purity samples of recombinant protein for the purpose of crystallization, the initial metal-chelate chromatography was followed by gel filtration to remove the remaining contaminating species. A Sephacryl S-100 High Resolution Gel filtration column (GE Heathcare) attached to an Äkta Explorer (Pharmacia Biotech) was washed with 640 ml of degassed and filtered Millipore water and equilibrated with 480 ml of degassed and filtered GF Buffer (20 mM Tris-HCl pH 7.0, 0.5 M NaCl) at a flow rate of 1 $mlmin^{-1}$. Pooled fractions from metal-chelate chromatography could be loaded onto the column without the need for dialysis but as a large volume would reduce the resolution between separated proteins a maximum sample volume of 16 ml was used. Proteins were eluted using 320 ml of GF Buffer at a flow rate of 0.4 $mlmin^{-1}$. Fractions were collected in volumes of 5 or 10 ml and UV absorbance at 280 nm and conductivity were monitored to determine which fractions contained the desired protein. SDS-PAGE was used to confirm the presence, and assess the purity, of eluted proteins. These fractions were then pooled and dialysed.

2.7.2.1 Standard protein dialysis

Proteins purified by metal-chelate chromatography (as described above in section 2.7.1) to be used in biological activity assays were dialyzed twice at

4°C against 2 L phosphate-buffered saline (pH 7.4; PBS). Complete EDTA-free tablets (Roche) were added to the dialysis buffer as protease inhibitors to avoid any breakdown of the protein during dialysis. Protein labeled with a fluorophore was shielded from light during dialysis to avoid photo-bleaching.

2.7.2.2 Estimation of protein concentrations

The absorbance of proteins at 280 nm was measured using a NanoDrop ND-1000 spectrophotometer. Samples were blanked against the appropriate dialysis buffer. The molar extinction coefficient (ε) and molecular weight of each protein were determined using Vector NTi 10.3 (Invitrogen). This data was used in conjunction with the ND-1000 software package to determine protein concentrations. The Beer – Lambert law was used to confirm the concentration: A_{280} = ε x path length x concentration.

For all proteins molar extension coefficients (ε) in cm^{-1} M^{-1}, at 280 nm were calculated from the formula: ε cm^{-1} M^{-1} = (5,500 * number of tryptophan residues) + (1490 * number of tyrosine residues) + (125 * number of disulfide bonds).

2.8 Assays for antimicrobial activity

2.8.1 Stab test

A single colony of *E. coli* DH5α, freshly transformed with the relevant plasmid, was "stabbed" onto an LB agar plate using a plastic pipette tip and grown overnight at 37 ºC. The bacteria were lysed by exposure to chloroform vapor for 2 min. The plate was dried for 10 min in a laminar flow cabinet after which, 8 ml of 0.7 % ($^{w}/_{v}$) agarose (Invitrogen) at 50 ºC was inoculated with

100 µl of an overnight culture of the indicator organism and poured as an overlay onto the plate and allowed to set. The overlay was allowed to grow for a minimum of 16 h at 37 ºC though zones of growth inhibition could often be observed after 4 -16 h.

2.8.2 Agar diffusion assay

Purified proteins were tested for antimicrobial activity by spotting 10 µl aliquots onto LB agar plates at a range of concentrations. Buffers were also tested for antimicrobial activity, together with a positive control. After the protein sample had dried the plate was overlaid with a sensitive indicator strain as previously described (section 2.7.1).

2.8.3 Liquid cell assay

The cell lysis assay is a variation on turbidity assays used to measure the effect of antimicrobial protein on an indicator organism. This assay measured the lysis and inhibitory growth effect on an *E. coli* culture by monitoring optical the density.

An overnight culture of the indicator organism was grown in LB broth with shaking at 37 ºC. The culture was diluted to an OD_{600} of ~ 0.02 and 4 ml was grown with shaking at 37 ºC until it reached an OD_{600} of ~ 0.08. Aliquots of 180 µl of cells were added to a 96 well plate and grown in a microtiter plate (Nunc), until they reached an OD_{600} of ~ 0.1. Proteins were diluted in PBS to 10 X the concentration to be tested. A multichannel pipette was used to add 20 µl of protein sample to each well. All proteins were tested in triplicate and PBS

was used as a negative control. The 96 well plate was incubated at 37 ºC for 24 h with a 5 s shaking step between optical density readings.

2.9 Sub-cellular fractionation of *E. coli* cells

To investigate the sub-cellular localisation of pFA4 in *E. coli* cells, a traditional method of cell fractionation was used to prepare cytoplasmic, periplasmic, inner membrane and outer membrane fractions of the cell. *E. coli* cells were grown overnight at 37 °C in LB broth supplemented with 100µg/ml ampicillin. Periplasmic proteins were prepared by a modification of the method of Nossal and Heppel (Nossal and Heppel 1966) or a chloroform extraction method (Ames, Prody et al. 1984). Briefly, cells from 100 ml overnight cultures were harvested at 13000 xg for 2 min and the pellet was re-suspended in 1 ml of EB buffer (10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 25 % sucrose), and washed twice in the same buffer. Finally, the pellet was re-suspended in EB buffer and incubated for 10 min on ice. The preparation was centrifuged at 13,000 xg for 4 min, following rapid resuspension in 0.4 ml of ice cold water and incubation on ice for a further 10 min, followed by centrifugation at 13000 xg for 2 min. The upper fraction of the supernatant consisting of periplasmic proteins was transferred to a fresh eppendorf and stored at -20 °C. After collection of the periplasmic fraction, the cell pellets were re-suspended into 0.4 ml Tris-HCl (pH 7.5) and sonicated to release the cytoplasmic contents. Non-disrupted cells were removed by centrifugation at 5,000 xg for 1 min. The upper clear supernatant was transferred to a fresh eppendorf and centrifuged at 17,000 xg for 30 min. The supernatant was collected as the cytoplasmic protein fraction and stored at -20 °C. The

remaining pellet was re-suspended in 0.2 ml of 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$ and 0.2 ml of 10 mM Tris-HCl pH7.5, 10 mM $MgCl_2$ and 4 % Triton X-100. The sample was incubated at 37 °C for 30 min and then centrifuged at 17,000 xg for 30 min. The supernatant was collected as the cytoplasmic membrane fraction and stored at -20 °C. The final pellet (yielded after collection of the cytoplasmic membrane fraction) was deemed the outer membrane protein-enriched fraction. This pellet was re-suspended by brief sonication in 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$ and then 0.5 ml of 10 mM Tris-HCl pH7.5, 10 mM $MgCl_2$ and 2 % Triton X-100 was added. The suspension was incubated at 37 °C for 30 min and then centrifuged at 17,000 xg for 30 min. This step was repeated to remove any remaining soluble proteins to ensure the purity of fraction. Final pellet was re-suspended in 0.2 ml 10 mM Tris-HCl pH 7.5 and stored at -20 °C.

CHAPTER THREE

3 Investigating the *in vivo* interaction of colicin translocation domains with Tol proteins

3.1 Introduction

Colicins begin their journey into an *E. coli* cell by initially binding with high affinity to an OM receptor (Di Masi, White et al. 1973). Most receptors used by colicins are monomeric, 22-strand β-barrel proteins that habitually transport small metabolites. In addition, outer membrane porins such as OmpF often act as co-receptors for colicins and are referred to as translocator proteins. The events that take place after colicin localization to the cell surface have not been fully elucidated but presumably require the assembly of what has been described as a translocon (Kurisu, Zakharov et al. 2003). The translocon assembly is thought to be facilitated by an unstructured region of the T-domain, which recruits the OM translocator protein to deliver at least part of the colicin T-domain into the periplasm of the target *E. coli* cell, where it can recruit the Tol proteins, allowing the entry of the cytotoxic domain through the OM (Cao and Klebba 2002; Housden, Loftus et al. 2005). It is still yet to be demonstrated if the entire colicin molecule is translocated into the cell but recent evidence has suggested that the cytotoxic domain is cleaved through FtsH mediated catalysis before it crosses the cytoplasmic membrane (Chauleau, Mora et al. 2011). The *tol*-dependent translocation system consists of the proteins TolA, TolB, TolQ, TolR and Pal (James, Penfold et al. 2002), which are all important for group A colicin entry. *E. coli* strains containing a mutation in the *tol* genes are tolerant of group A colicins. The outer membrane-associated lipoprotein Pal interacts with the periplasmic TolB protein (Bouveret, Derouiche et al. 1995). A number of recent studies of the Tol-Pal proteins have provided useful information on the structure/function relationships between Tol proteins and colicin biology (Cascales, Buchanan et

al. 2007). One approach to study *in vivo* interactions between colicins and components of the Tol–Pal or TonB systems has been the expression and secretion of colicins and/or colicin domains to the cell periplasm followed by colicin challenge of those cells from the external mileau. The aim of this technique is to perturb the function of individual proteins of the Tol or Ton systems to determine their role in the uptake of the colicin molecule. This can be extended to include mutated colicins to investigate important residues in these processes. Also it is a useful tool to examine the diverse interaction between colicins or bacteriophage g3p protein of filamentous bacteriophages with the translocation machineries. The first observation that the periplasmic production of interacting proteins perturbed the Tol–Pal system was from strains that were infected with filamentous bacteriophages which displayed a *tol* phenotype. These strains behaved like *tol* mutants showing a tolerance to group A colicins; having leaky phenotypes and hypersensitivity to drugs and detergents (Zinder 1973; Smilowitz 1974) that they normally would be resistant too. As these bacteriophages require the TolA-Q-R proteins to infect sensitive cells, it was shown that expression of g3p and secretion into the periplasm could create leakiness of cell contents through inhibition of the normal functioning of the Tol proteins. This approach was extended to examine the role of Tol proteins on colicin biology (Cascales, Buchanan et al. 2007). Thus by analogy, expressed and secreted colicin translocation domains should interact with the cell's translocation machinery in the periplasm, thereby disturbing the normal functioning of this cellular system and rendering the producing cells less sensitive or 'tolerant' to colicins of the same group attacking the cells from the external environment. Moreover, proteins of the

Tol or TonB systems could be secreted to the periplasm and subsequently preventing colicin action either by directly trapping the imported colicin to non-functional Tol or by disturbing the function of the envelope systems due to the presence of non-functional protein-interactions in the periplasm, and thus preventing the translocation process. Overexpressed TonB fragments suggested that TonB interfered with the receptors interaction in *E. coli* and yet abolished sensitivity of cells to bacteriophage φ80 and colicin M, both of which use FhuA as the receptor, these results were obtained using the periplasmic production technique (Howard, Herrmann et al. 2001). Secretion of TolAIII, colicins or g3p domains in the periplasm of wild-type cells produce a tol-associated phenotype (Henry, Pommier et al. 2004), and it has been postulated that these domains may alter outer membrane integrity by altering the stability of the Tol-Pal interactions (Bouveret, Journet et al. 2002). Moreover, sequestration of active TolA-TolQ-TolR complexes found to be due to the periplasmic overexpressed TolR molecules (Journet, Rigal et al. 1999). Using this technique allowed identification of an interaction between TolABR and ColK T-domain and for the first time an interaction with TolQ protein (Barneoud-Arnoulet, Gavioli et al. 2010). Generally cells secreting the T-domains of group A colicins become tolerant specifically to group A colicins, while cells secreting the T-domains of group B colicins become tolerant specifically to group B colicins. Cells producing Tol or TonB domains become tolerant specifically to colicins or phages that require the same translocation machinery for cell entry (Bouveret, Rigal et al. 1997; Bouveret, Journet et al. 2002).

One of the problems of studying enzymatic colicins in the past has been the lack of a rapid and quantifiable assay for the determination of cell killing that would be necessary to study the effects of periplasmic expression of Tol proteins or colicin domains. An approach for monitoring cell killing through DNA damage was developed that incorporated a SOS promoter-*lux* fusion reporter system. Furthermore, because the reporter assay enables a quantitative assessment of colicin activity, the effect of individual mutations on the biological activity of colicin (Vankemmelbeke, Healy et al. 2005) can be compared. The reporter assay has recently been used to show the importance of the Tol proteins in the release of immunity protein from the catalytic domains of enzymatic colicins (Vankemmelbeke, Zhang et al. 2009).

This chapter describes a method for expressing and secreting colicin domains into the cell periplasm to monitor the interaction between the Tol system and externally added colicins. Using this 'periplasmic expression technique' to perturb the function of the Tol system in the cell envelope, interactions of secreted colicin domains with the Tol proteins can be monitored by the patterns of sensitivity/resistance of the cells to externally added colicins. It is shown that expression and secretion of the translocation domains of colicins E9 and A protect cells from externally added ColE9 and A through interactions of those domains with Tol proteins which prevent subsequent interactions of these proteins with the translocation domains of the externally added colicins. In addition competitive inhibition of TolA has a larger effect on cell resistance than inhibition of TolB providing further insights into the role of different Tol proteins in the translocation of different colicins.

3.2 Materials and methods

3.2.1 Bacterial strains, growth conditions and media

E. coli DPD1718 was used as the host strain for plasmid expression. Bacterial cultures were grown at 37 °C in Luria-Bertani (LB) broth in a shaking incubator or on LB agar plates supplemented with ampicillin (100 μgml^{-1}) and chloramphenicol (30 μgml^{-1}) to select for recombinant clones.

3.2.2 Cloning of ColA TA_{1-172}

Polymerase chain reaction (PCR) amplification was carried out as described in section 2.3.1. Briefly, a 500 bp DNA fragment encoding residues $_{1-172}$ of the translocation domain of ColA (TA_{1-172}) was amplified from plasmid pNP339 using primers CNP25 (forward) and RJ18 (reverse). The restriction enzyme sites, *Nco*I and *Xho*I, were introduced by the forward and reverse primer, respectively.

The purified PCR product was digested with *Nco*I and *Xho*I restriction enzymes and a fragment of approximately 0.5 kb encoding TA_{1-172} was obtained through gel extraction (as detailed in section 2.3.6). Following extraction, the 500 bp DNA fragment was ligated into *Nco*I and *Xho*I digested pBAD/gIIIC vector, according to the manufacturer's recommendations, to produce pFA4 and transformed into *E. coli* DH5α by transformation (section 2.4.2). The pFA4 was sequenced using the pBADF and pBADR primers (as described in section 2.4.8).

All generated constructs and their protein analysis are described in appendix B.

3.2.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

3.2.3.1 Sample preparation

Cultures of *E. coli* DPD1718 containing pFA4, or the empty vector pBAD/gIIIC, were grown at 37 °C overnight, with shaking. The cultures were diluted 100-fold in LB medium with 0.2 % glucose, supplemented with (100 μgml^{-1}) ampicillin and (30 μgml^{-1}) chloramphenicol and then incubated at 37 ºC with shaking until an OD_{600} of 0.4-0.6 was reached. Each culture was then divided into two, one of which was induced with L-arabinose (0.0002-2 % final concentrations) and the other was left unmodified. The cultures were further grown at 37 °C with shaking and 1 ml of the sample was taken from each tube after 1, 2 and 3 h. Samples were centrifuged at 13,000 rpm for 2 min and the cell pellet was re-suspended in 100 µl of 1x SDS loading buffer (100 mM Tris-HCl, 4 % ($^{v}/_{v}$) SDS, 20 % ($^{w}/_{v}$) glycerol, 0.2 % ($^{v}/_{v}$) bromophenol blue, 4% ($^{w}/_{v}$) 2-mercaptoethanol) and boiled for ~ 3 min at 100 °C followed by centrifugation for 1-2 min at 13,000 x*g* speed.

3.2.3.2 SDS-PAGE analysis

Proteins were separated by SDS-PAGE in a 16 % resolving gel using Tris/glycine buffer (appendix A) (Laemmli 1970). Twelve microliters of each sample was loaded into each well and the SDS-PAGE gels were run at 150 V. Gels were stained with Coomassie blue and the size of the protein bands was determined by comparison with a pre-stained protein standard marker (NEB, 7-175 kDa range).

3.2.3.3 Tricine-SDS PAGE

Tricine–SDS-PAGE is the preferred electrophoretic system for the resolution of small proteins. The PAGE was prepared using a 16 % resolving gel overlaid with a 4 % stacking gel with a 10 % 'spacer gel' between the stacking and resolving gels this will considerably sharpens the bands for proteins and peptides of 1–5 kDa (Schagger 2006). The samples were prepared as described in section 2.6.1.

3.2.4 Localization of TA fragments and quantitation of relative expression levels

Cells expressing the various TA or TN fragments were grown in LB medium containing 0.02 % arabinose and harvested at an optical density of 0.8 at 600 nm. Then, 1.5 ml portions of the cultures were the cells osmotically shocked (as described in section 2.9), except that a general protease inhibitor (EDTA free; Sigma-Aldrich, Germany) was added to the solutions used. The cell pellets were resuspended directly in SDS-PAGE sample buffer, while the supernatants were precipitated by the addition of trichloroacetic acid (TCA) to a final concentration of 5 %. The precipitates were collected by centrifugation, washed with 90 % acetone in water, and resuspended in SDS-PAGE sample buffer.

3.2.5 Formaldehyde cross-linking and immunoprecipitation

Cells were formaldehyde cross-linked and co-immuno-precipitated essentially as described previously (Lloubes, Cascales et al. 2001). In brief, exponentially growing cells containing approximately $2x10^9$ cells/ml were harvested, washed

with 20 ml of 10 mM sodium phosphate buffer (NaPi , pH 6.8), and re-suspended in NaPi buffer supplemented with 1 % formaldehyde (v/v). After incubation at room temperature for 20 min, the cross-linking reaction was stopped by the addition of 0.3 M Tris-HCl, pH 6.8, and the cells were washed once in 20 mM Tris-HCl (pH 6.8). The cell pellet was then subjected to solubilisation for 30 min at 37 °C in TES (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1 % SDS) in the presence of protease inhibitors (Complete; Roche), and diluted 15-fold in TNE (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl) supplemented with 1 % Triton X-100. After incubation for 2 h at room temperature with vigorous shaking, the extract was centrifuged for 15 min at 18,000 x*g* to remove unsolubilized material. Supernatants were then incubated overnight at 4 °C with the primary penta-His-tag antibody (Qiagen, UK), coupled to protein A/G-plus agarose beads (Santa Cruz biotechnology, UK). Beads were then washed twice with TNE supplemented with 1% Triton X-100, once in TNE supplemented with 0.1 % Triton X-100 and 0.1 % Tween, and once in TNE supplemented with 0.1 % Triton X-100. The immune-precipitated material was then heated at 70 ºC for 10 min in loading buffer prior to analysis by SDS-PAGE and immuno-blotting.

3.2.5.1 Immunoblot analysis

For immunoblot analysis, proteins separated by 16 % SDS-PAGE were transferred onto a nitrocellulose membrane (Amersham Biosciences) in semi-dry blotting buffer as described in appendix A, using a Trans-Blot SD semidry transfer cell (BIO-RAD) at a constant current of 14 mA for approximately 30 min. The membranes were incubated in blocking buffer that contains 1 %

Bovine serum albumin (BSA) (Sigma) in PBS for 30 min at room temperature. The membranes were then probed with mouse anti-pentahistidine antibody (Qiagen, UK) diluted 1:10,000 in blocking buffer and incubated overnight with shaking at 4 °C. The following day the membrane was washed three times, 15 min each, with PBS containing 0.05 % Tween-20 (PBS-Tween), and incubated into secondary antibody (anti-mouse IgG conjugated to alkaline-phosphatase) (Sigma, UK) at the concentration of 1:30,000 for 2 h at RT. After 2 h the membrane was washed twice with PBS-Tween for 15 min each time. The membrane was then developed using the BCIP (5-bromo-4- chloro-3-indolylphosphate)–Nitro Blue Tetrazolium liquid substrate (PerkinElmerTM) or exposed to EZ-ECL (Biological industries, UK) for visualization of immunoreactive proteins for the HRP conjugated antibody. The membrane was finally washed with dH_2O and a digital image of the membrane was taken using a GS-800 calibrated densitometer (BIO-RAD).

3.2.6 Luminescence reporter assay

E. coli DPD1718 containing a fusion of the promoter region of *recA* of *E. coli* to the *Photorhabdus luminescens luxCDABE* integrated into *lacZ* locus of *E. coli* DPD1692, was used as a luminescent reporter strain (Davidov, Rozen et al. 2000). The assay was performed as described previously (Vankemmelbeke, Healy et al. 2005). Briefly, an overnight culture of the luminescent reporter strain was diluted 1:100 with minimal media (as detailed in section 2.1.3) supplemented with (100 $\mu g ml^{-1}$) ampicillin and (30 $\mu g ml^{-1}$) chloramphenicol, and incubated at 37 °C with shaking until OD_{600} ~ 0.4. The cells were then diluted 1:2 with minimal media in a total volume of 100 µl in the wells of a 96-well microtiter plate (Nunc), and 5 nM of purified ColE9 was added to each

well. The luminescence was then determined over 3 h at 37 °C with readings taken every 300 or 600 s, cell density was recorded via OD_{492} values, using a microtiter plate luminometer (Lucy1, Anthos Labtech, Salzburg, Austria). Prior to undertaking the analysis, the Luminometer, microtiter plate and medium were pre-warmed to 37 °C in an effort to prevent unwanted stress to the cells.

Luminescence values are mostly presented as relative luminescence units (RLU). The gamma value is defined as the luminescence induced for any given sample concentration minus the luminescence of the control cells at the same time point divided by the luminescence of the control cells at that time point: (Lsample - Lcontrol)/Lcontrol (Vankemmelbeke, Healy et al. 2005). The activity of the ColE9 protein was calculated by dividing the gamma value of the cells treated with ColE9 by the gamma value of control cells and expressed as a percentage. Protection against ColE9 by over-expression of a recombinant protein in the periplasm was assessed by dividing the gamma value of the induced culture with that of the uninduced control culture. All assays were performed in triplicate, with three to six replicates for each condition.

3.2.7 Potassium Release assay

The potassium release assay detects variation in the K^+ content of bacterial cells (K^+_{in}) (Bourdineaud, Howard et al. 1989). This assays is used to measure the effect of pore-forming colicins such as ColA and ColN on an indicator organism by measuring the changes of the K^+ concentration in the external medium (K^+_{out}) that are caused by cell killing with a K^+ selective electrode.

An overnight culture of the indicator organism was grown in LB broth with shaking at 37 ºC. The culture was diluted to an OD_{600} of ~ 0.01 and 100 ml was grown in LB supplemented with 10 mM KCl, shaking at 37 ºC until it reached an OD_{600} of ~ 0.5-0.6. Cells were collected by centrifugation at 3000 x*g*, for 15 min at room temperature and washed with 100 mM sodium phosphate buffer, pH 7.0, then re-suspended in 1 ml of the same buffer containing 5 % ($^{v}/_{v}$) glycerol and stored on ice with vortexing every 15 min. Aliquots of the cells corresponding to approximately 5 x 10^9 cells were injected into a glass vessel containing 50 ml of 100 mM sodium phosphate buffer (pH 7.0) and maintained at 37 ºC with constant magnetic stirring. Samples of colicin proteins were injected when the temperature and potassium fluxes were equilibrated. Potassium concentration measurements were performed with a K^+-specific electrode using a 781 pH/Ion meter (Metrohm UK Ltd), and were recorded and printed using a Custom DP40-S4N printer (Metrohm UK Ltd) connected to the ion meter. Potassium release data were analysed using an Excel spreadsheet (Microsoft 2010).

3.3 Results:

3.3.1 Construction of a clone expressing ColA TA_{1-172}

The T-domain of ColA was selected for periplasmic protection experiments to investigate colicin translocation as it is known to have TolA, TolB and TolR boxes (Bouveret, Rigal et al. 1998). The PCR product encoding residues 1 to 172 of the translocation domain of ColA (TA_{1-172}) was digested with *Nco*I and *Xho*I and then ligated into the pBAD/gIIIc vector after digestion with the *Nco*I and *Xho*I, resulting in pFA4 (Figure 3-1). In order to study the effect of periplasmic expressed colicin domains against the externally added colicin it was necessary to engineer a recombinant clone containing the translocation domain of ColA in a vector that adds a signal sequence to the N-terminal of the recombinant protein; the pBAD g/IIIc vector was chosen because the expression from the vector promoter is tightly regulated by arabinose.

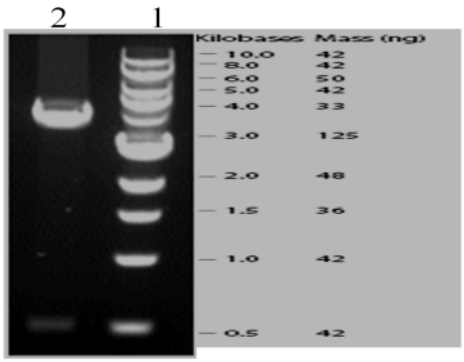


Figure 3-1: Gel electrophoresis of double digests of the translocation domain of ColA and pBAD/gIII vector using *NcoI*/*XhoI*. (1) is the DNA marker, (2) is the double digest showing a band at 4.1 kb representing the pBAD vector and another band of 500 bp representing the pFA4 (TA_{1-172}).

3.3.2 Characterization of the production of recombinant TA_{1-172} in *E. coli* pFA4

Before any *in vivo* studies can be undertaken it was important to determine the expression of the DNA encoding the recombinant TA_{1-172} in pFA4. The DNA fragment encoding TA_{1-172} is under the control of the arabinose inducible promoter in pFA4, resulting in arabinose-inducible over-production of TA_{1-172} in the periplasm of the producing cells. To select the best conditions, different arabinose concentrations ranging from 0.00002 to 2 % arabinose, and various lengths of time for induction (0-4 h post induction) during the growth of the cells were used. Figure 3-2-A shows that at 0.00002 % arabinose very little recombinant protein was produced whereas at 0.002 % and above a large amount of protein was produced. There appeared little difference, by SDS-PAGE, in protein concentrations expressed from 0.02 and 0.2 % arabinose and therefore to reduce any possible toxic effects caused by high concentrations of arabinose 0.02 % arabinose was selected as the optimum concentration to be used in the *in vivo* experiments. Regarding the selected time, analysed samples that were induced for 1, 2, and 3 h were compared to the uninduced samples, three hours was selected for the optimal induction time (Figure 3-2-B).

Once the optimal conditions were selected, the expression was repeated with 0.02 % arabinose over 3 h, SDS-PAGE analysis revealed an intense protein band of ~17 kDa, corresponding to the predicted molecular weight of TA_{1-172} (Figure 3-3). pFA4 plasmid DNA was sequenced and further confirmed that this 17 kDa polypeptide corresponded to TA_{1-172} by mass-spectroscopy.

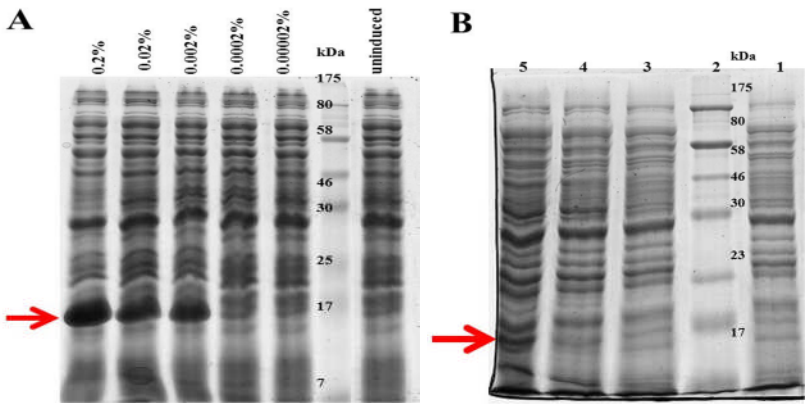


Figure 3-2: Analysis of the periplasmically constructed TA_{1-172}. (A) SDS-PAGE comparing the induction of TA_{1-172} using various L-arabinose concentrations, different arabinose concentrations from 0.00002-0.2 % used for protein induction, compared to uninduced cells. (B) SDS-PAGE comparing the induction using 0.02 % L-arabinose for several time points from 1-3 h, lane 3-5 respectively. Lane 1 is uninduced culture; lane 2 is protein marker (7-175 kDa). Samples for SDS-PAGE analysis prepared as described in section 3.2.3.1.

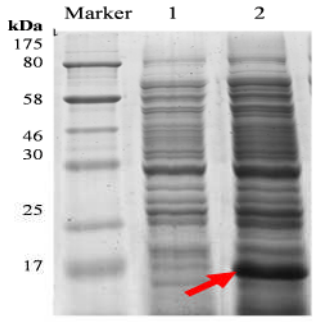


Figure 3-3: Analyses of protein by Coomassie blue stained SDS-PAGE showing the expression of pFA4 from *E. coli* cells upon induction using the optimized conditions. Uninduced cells are shown in lane 1, induced cells with 0.02 % L-arabinose for 3 h are in lane 2. Arrow indicates the induced TA_{1-172}. Molecular weight markers (7-175 kDa) are shown on the left.

3.3.3 Growth curve analysis of *E. coli* cells expressing TA_{1-172}

To show that the presence of the pFA4 in cells did not affect the growth characteristics of the *E. coli* DPD1718 cells, the OD_{600} of the culture of *E. coli* DPD1718 was compared with that of *E. coli* DPD1718 expressing pFA4 and the cells expressing the empty vector (Figure 3-4). Cells were grown from an overnight culture by diluting in LB media supplemented with 100 μgml^{-1} ampicillin and 30 μgml^{-1} chloramphenicol. No effects of expressing the T-domain of ColA on the growth of the *E. coli* DPD1718 was observed as all cultures have shown similar patterns of growth.

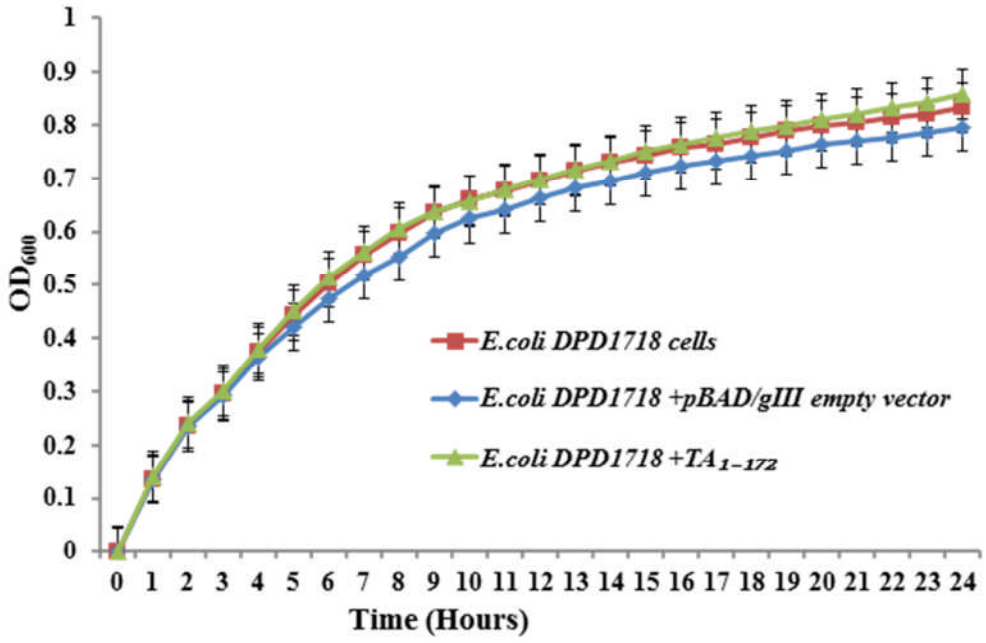


Figure 3-4: Growth characteristics of *E. coli* DPD1718 compared to *E. coli* DPD1718 (pFA4) and *E. coli* DPD1718 expressing the empty pBAD/gIII vector. The cells were grown and OD_{600} were measured every hour, were these cells induced with 0.02 % arabinose at mid-log. The experiment detailed in section 3.2.6, each experiment was repeated three times and each sample was run in triplicate and a representative example is shown.

The range of arabinose concentrations used in determining the optimal conditions of the protein expression were also tested to select the optimal condition for the *in vivo* experiments with the *lux*-reporter strain (Figure 3-5). Low concentrations of arabinose ($2x10^{-4}$ and $2x10^{-5}$) had very little effect on DNA damage of cells caused by ColE9 compared with cells that were uninduced, whereas high concentrations of arabinose (2×10^{-2} and 2×10^{-1}) were toxic to the cells and little or no growth was observed (Table 3-1). The optimum concentration of arabinose that did not affect growth but had a profound effect on cell killing by ColE9 was 0.02 % arabinose (Figure 3-5). Assuming that this concentration is giving the maximum protein production in the *lux* reporter cells, whereas the fractionation experiments have localized the produced protein in the periplasmic fractions. This concentration of arabinose was chosen for future experiments.

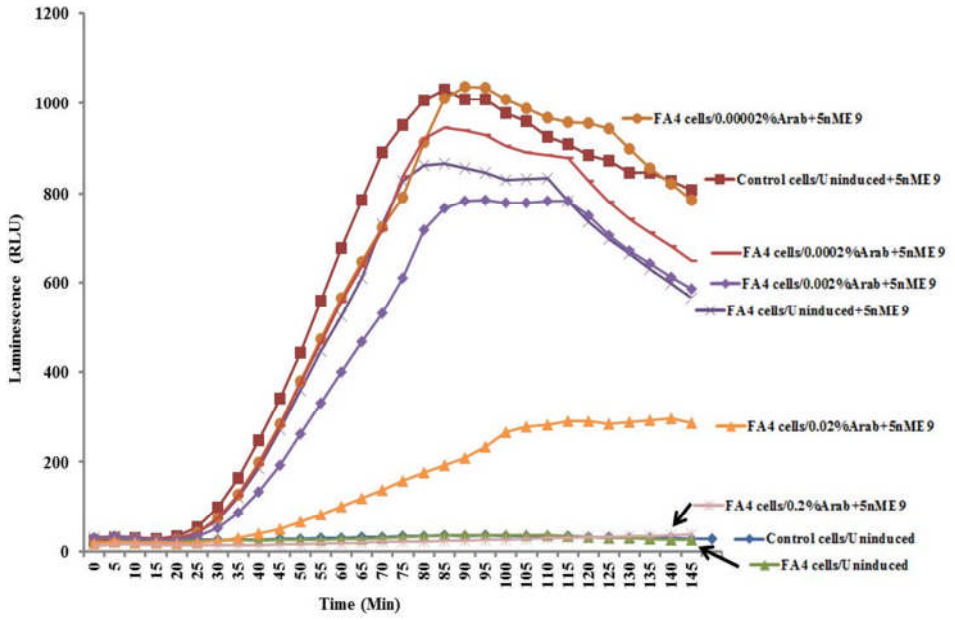


Figure 3-5: *In vivo* detection of the periplasmically constructed TA_{1-172} protective effect upon induction using various arabinose concentrations. Analysis of DNA damage caused by addition of ColE9, represented as relative luminescence units. Experiments detailed in section 3.2.6.

Table 3-1: L-arabinose effect on lux induction in *E. coli* cells expressing TA_{1-172}		
Constructs + 5nM ColE9	**Lux induction (%)**	**Percentage of protection against ColE9**
Control cells uninduced	100	0
FA4 uninduced	100	0
FA4 + 0.00002% arabinose	99.96	0.04
FA4 + 0.0002% arabinose	98.95	1.05
FA4 + 0.002% arabinose	66.48	33.52
FA4 + 0.02% arabinose	11.24	88.76
FA4 + 0.2% arabinose	100 (Slow growth)	0 (Slow growth)
FA4 + 2% arabinose	No growth	No growth

3.3.4 Complementary analysis of the periplasmic expressed TA_{1-172} using mass spectrometry

Mass spectrometry is a powerful analytical technique for protein analysis and for the study of biomolecules in general it can be used to identify, characterize, and quantify proteins with high sensitivity, even in complex samples. This technique was used for further analysis of the overproduced TA_{1-172} protein. The protein was first purified using size exclusion chromatography (as described in section 2.7.1). The purified protein samples were then analysed using SDS-PAGE and the selected protein band was cut out and sent for mass spectrometric analysis (Protein & Nucleic Acid Chemistry Laboratory, University of Leicester). The data confirmed that the overproduced protein is the TA_{1-172} (Figure 3-6).

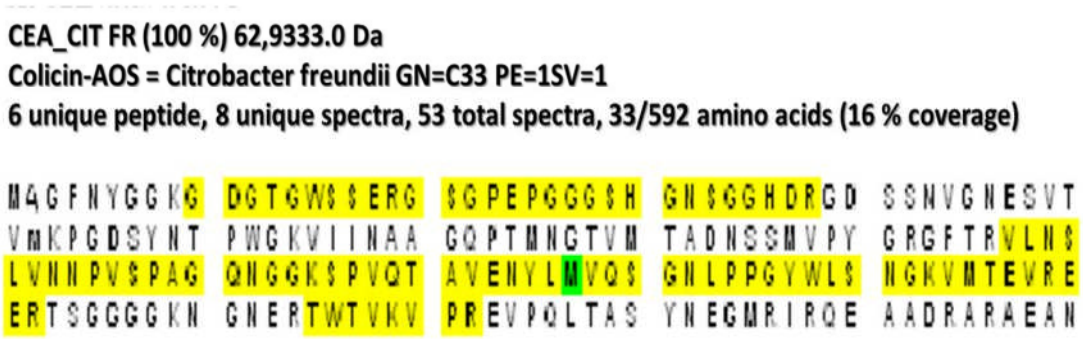
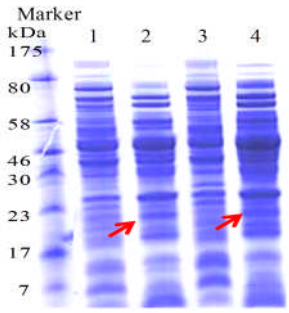


Figure 3-6: Mass spectroscopic analysis of purified overproduced TA_{1-172} confirming the presence of the expected residues of the translocation domain of ColA. The protein sequence confirmed on a purified protein by ion-exchange chromatography, protein fractions were analysed on SDS-PAGE, the expected protein band (~17 kDa) was cut and sent for analysis.

3.3.5 Expression and secretion of the lysostaphin gene to the cell periplasm does not protect cells from exogenous colicin E9 or A

The *Lux*-reporter assay revealed the protection conferred by periplasmic expression of TA_{1-172} against exogenous colicin E9, which is indicated by the level of DNA damage detected by the level of luminescence. Two control constructs were tested to validate the results obtained in this experiment. These were (1) an empty vector control using the pBAD/gIII expression vector containing no insert control, and (2) pBAD/gIII expressing an unrelated 172 amino acids constituting the lysostaphin targeting domain that has no propensity to bind to Tol (Bardelang, Vankemmelbeke et al. 2009). *E. coli* DPD1718 cells expressing the empty vector or the lysostaphin targeting domain (named pFA14) were grown and induced with 0.02 % arabinose for 3 h, after which the cells were challenged with exogenous ColE9 (Figure 3-7-B), assuming that these cells expressing similar amount of protein in the cells periplasm as they are induced with the same amount of arabinose and for the same time length. Cells expressing the empty vector or the lysostaphin domain were completely sensitive to ColE9 induced DNA damage when compared to the *E. coli* DPD1718 strains that were expressing the TA_{1-172} protein in the periplasm (Figure 3-7-B). Aliquots of the induced and uninduced lysostaphin domain expressing cells were also analysed by SDS-PAGE and compared to cells expressing pFA4 (Figure 3-7-A). Protein analysis was carried out to ensure that the lysostaphin domain is present in the periplasm before the *in vivo* assay was carried out.

(A)



(B)

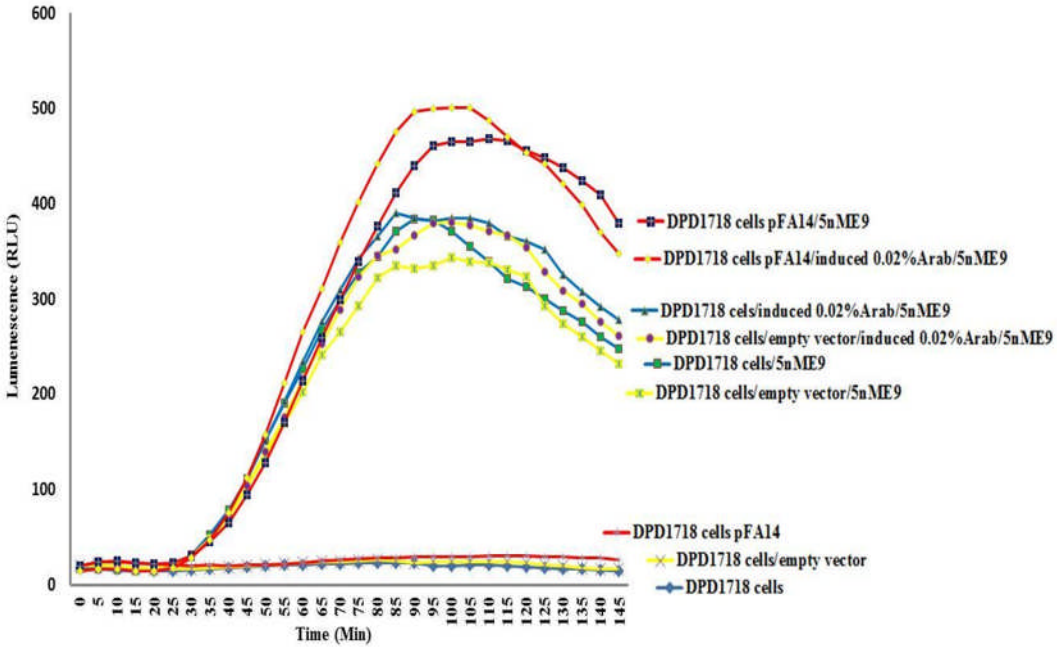


Figure 3-7: Characterization of control cells used for Lux-reporter assay. (A) Coomassie blue stained SDS-PAGE to compare the production of the expressed TA_{1-172} (lane 3 uninduced and lane 4 induced (arrow at ~17 kDa), to the produced lysostaphin T-domain pFA14 (lane 1 uninduced and lane 2 induced (arrow at ~19 kDa)). (B) Lux-reporter assay to compare the response of the different control *E. coli* reporter strains to external ColE9 killing activity by measuring the relative luminescence units as DNA damage indicator. The experiment carried out as detailed in section 3.2.6.

3.3.6 *In vivo* monitoring of the effect of the over-expressed WT ColA TA_{1-172}

3.3.6.1 Protection against ColE9

Monitoring the DNA damage in TA_{1-172} producing cells after the addition of ColE9 by observing the induced luminescence indicated the level of protection conferred by over-expressing TA_{1-172} in the periplasm. It is reasonable to assume that the observed 86 % protection against ColE9 induced DNA damage is the result of the over-expressed TA_{1-172} domain binding to cellular Tol proteins and thus making them unavailable for translocation of any exogenous colicin. Because TA_{1-172} can bind to both the TolA and TolB proteins, we attempted to assess their relative importance by constructing plasmids that expressed truncated variants of TA_{1-172} that consisted of only the TolA box (named pFA15) or the TolB box (named pFA16) (Figure 3-8, A and B, respectively) assuming that similar amount of proteins are expressed within the cell periplasm. Separating the TolA and TolB boxes of ColA showed the relative contribution of each box to cell protection. The results showed 78.9 % protection against ColE9 DNA damage with cells expressing the TA_{52-172} and only 22.23 % with cells expressing $TA_{1-52,}$ indicating that the major contribution to protection results from occupying TolA by the expressed TolA box when comparing these data to that shown by cells expressing both TolA and TolB boxes in TA_{1-172} which offers 86.35 % protection (Figure 3-9, A and B).

The result of this experiment is surprising because there is no evidence that ColE9 interacts directly with TolA during translocation. Gamma values were calculated at a time point of 50 min. The percentage of activity and protection were calculated by dividing the gamma value of each time point to WT ColE9 and taking off the activity of 100 % (as described in section 3.2.6).

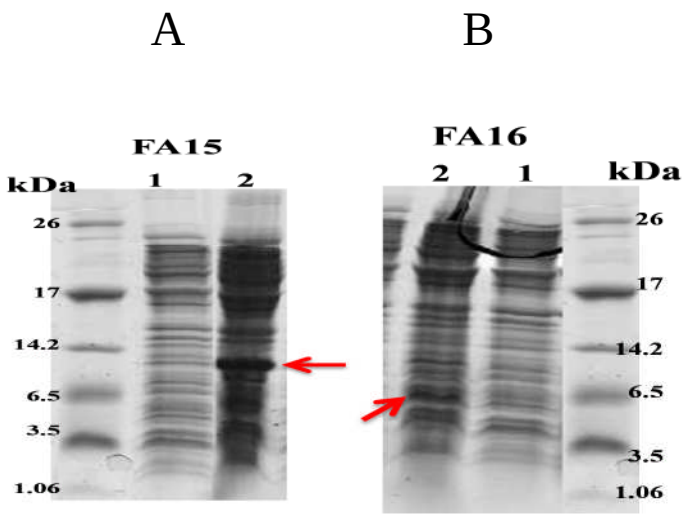
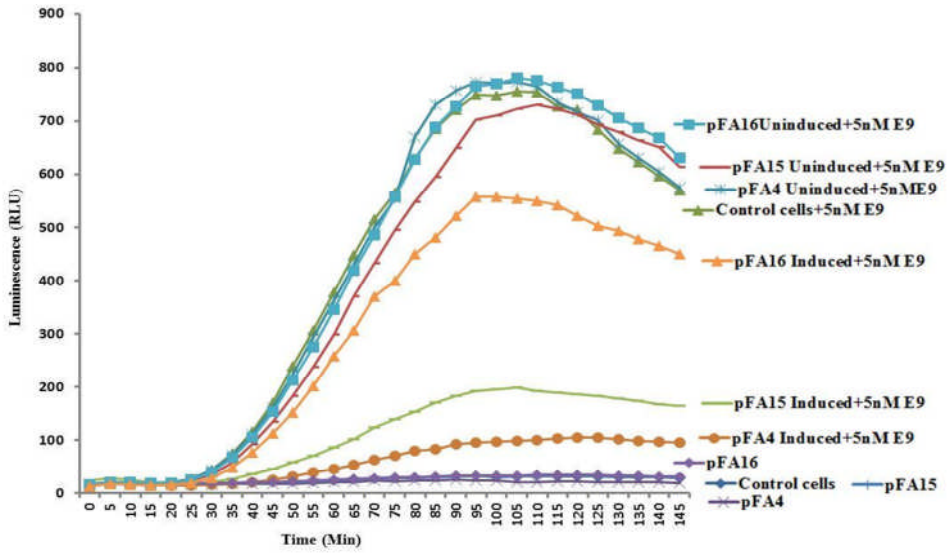


Figure 3-8: Analysis of small truncated polypeptides of FA15 and FA16 by Tricine-SDS-PAGE. On the left, FA15 (ColA TolA box) lane 1 is uninduced, lane 2 is induced with 0.02 % L-arabinose for 3 h showing a protein of ~12.88 kDa. On the right, FA16 (ColA TolB box), lane 1 is uninduced and lane 2 is induced with 0.02 % L-arabinose for 3 h showing a protein of ~5 kDa. Low range protein markers were used (26-1.06 Da).

(A)



(B)

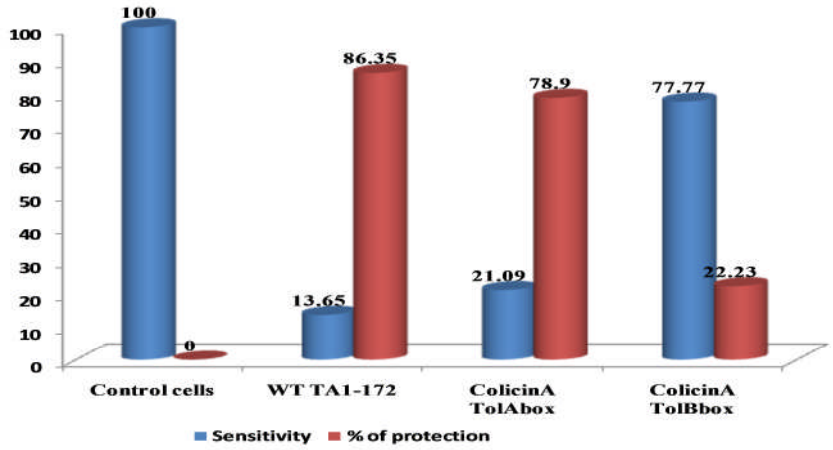


Figure 3-9: *In vivo* periplasmic protection assay of WT TA, TolA box and TolB box. (A) Lux reporter assay showing protection against exogenous added ColE9. (B) Bar chart represents the calculated percentage of protection against different expressed truncated polypeptide. Cells grown to mid-log and induced with 0.02 % L-arabinose for 3 h and then cells diluted 1:1 with minimal media were ColE9 added to a final concentration of 5 nM, the luminescence measured as DNA damage indicator.

3.3.7 Detection of protein-protein interaction; *in vivo* cross linking and immunoprecepitation

3.3.7.1 *In vitro* assay

It has been shown previously by surface plasmon resonance (SPR) that the TolA and TolB boxes of ColA interact with TolA and TolB respectively, (Hands et al 2005). It was assumed that the tolerance of *E. coli* cells to ColE9 after expression and secretion of Tol boxes to the cell periplasm was due to steric hindrance of Tol preventing exogenously added colicin from targeting and interacting with Tol. *In vitro* cross linking of purified proteins was carried out initially to demonstrate if there was a physical interaction of $TA_{1\text{-}172}$ with TolA and/or TolB.

Three µM of each of the $TA_{1\text{-}172}$, TolB, and TolAIII proteins were mixed in equal ratios in different combinations and incubated with or without 1% formaldehyde for an hour at room temperature. SDS-PAGE analysis demonstrated the presence of interacting proteins in formaldehyde treated samples compared with samples not treated with formaldehyde indicating complex formation. These were revealed by the presence of higher molecular weight bands on SDS-PAGE. Products of ~72 kDa ($TA_{1\text{-}172}$, with TolB and TolAIII) , ~52 kDa (TolB with TolAIII), and 28 kDa ($TA_{1\text{-}172}$ with TolAIII) were observed following formaldehyde treatment. The molecular weight of the individual $TA_{1\text{-}172,}$ TolB and TolAIII proteins are 17 kDa, 44 kDa and 10 kDa, respectively (Figure 3-10).

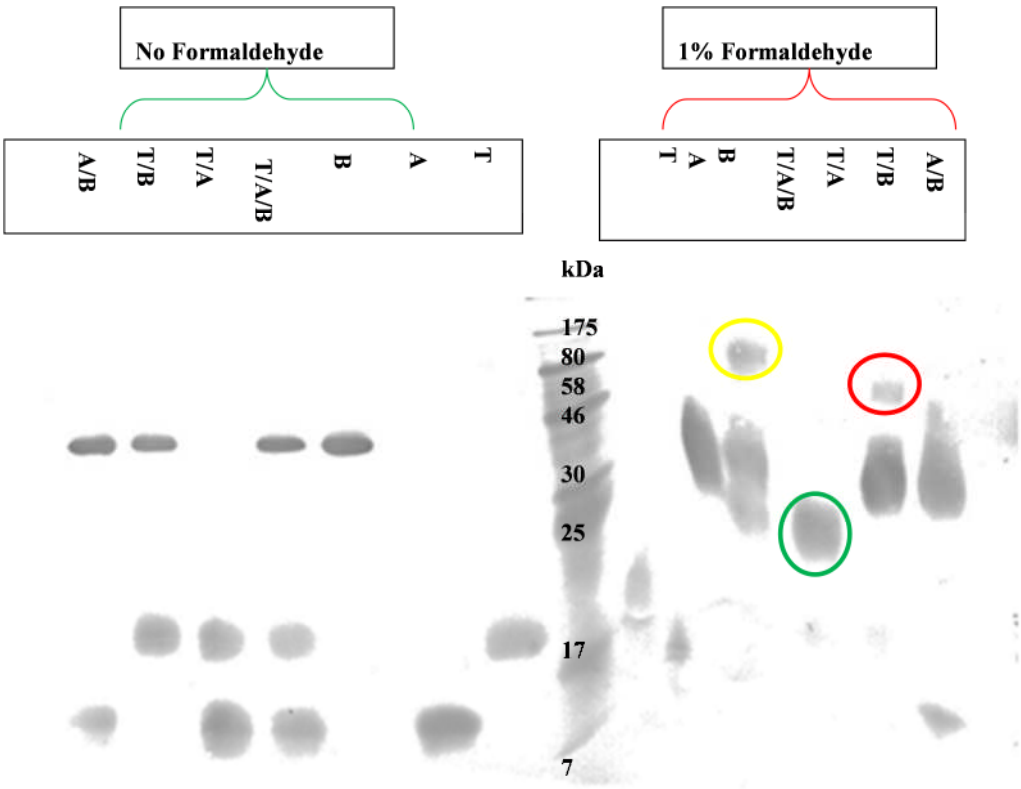


Figure 3-10: Immuno-blotting analysis of *in vitro* formaldehyde cross-linked purified proteins TolAIII, TolB and ColA TA_{1-172}. (T) represents TA_{1-172}, (A) is TolAIII, (B) is TolB, (T/A) is cross-linked TA_{1-172} and TolAIII, (T/B) cross-linked TA_{1-172} and TolB and (T/A/B) is cross-linked TA_{1-172} and TolAIII and TolB. Proteins were mixed in 1:1 ratio and cross-linked with 1 % formaldehyde for 20 min at room temperature, analysis done using 16 % SDS-PAGE that was subjected to western blotting using anti-his tag antibodies, a cross-linked protein band around 52 kDa circled in red, and around 28 kDa circled in green, and at 72 kDa circled in yellow. (7-175 kDa) protein marker was used.

3.3.7.2 *In vivo* cross linking assay

Although the *in vitro* cross-linking showed a trimeric complex between TolA, TolB and TA_{1-172} it was necessary to show the presence of, at least, a dimeric complex of TA_{1-172} with TolA and/or TolB. Therefore, cross-linking and immunoprecipitation was carried out on cells overexpressing TA_{1-172} (pFA4) to study the *in vivo* interaction of TA with the Tol proteins in the periplasm. Individual, non-complexed bands of TolB and TA_{1-172} were present in extracted samples that were not treated with formaldehyde whereas, a larger band of approximately 80 kDa was present in an induced sample that was formaldehyde treated indicative of complex formation between binding partners (Figure 3-11). Mass spectroscopy on the 80 kDa band confirmed the presence of TA_{1-172} and TolB; however TolA was not detected in this sample.

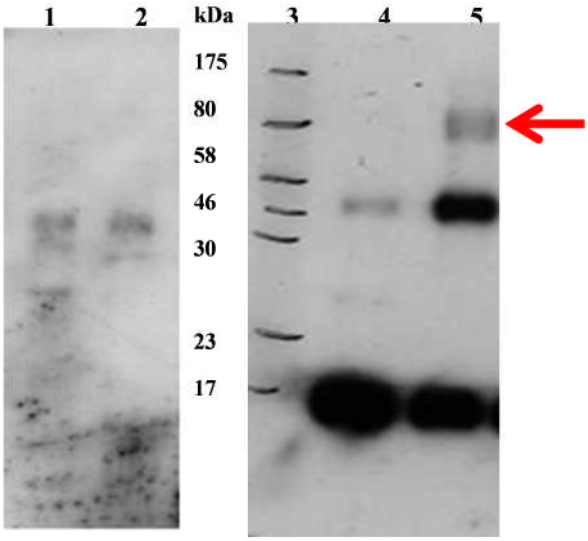


Figure 3-11: Immuno-blotting analysis of *in vivo* formaldehyde cross-linking of *E. coli* cells expressing TA_{1-172}. Cultures of induced and uninduced cells were subjected to formaldehyde cross-linking. Control experiments were carried out without formaldehyde cross-linking. Lane 1 and 5 are uninduced and induced cells, respectively, cross-linked with 1 % formaldehyde. Lane 2 and 4 are uninduced and induced cells, respectively, without formaldehyde. Lane 3 is a protein marker (7-175 kDa). The red arrow shows the protein complexed bands (~50 and 80 kDa) that were sent for mass spectroscopic analysis.

3.3.7.3 *In vivo* detection of the protection against ColA

The *lux* reporter assay allows the very sensitive detection of the DNA damage induced in *E. coli* DPD1718 cells by ColE9. We have demonstrated that over-expression of TA_{1-172} in the periplasm provides significant protection against ColE9-induced DNA damage and that it appears that the TolA box contributes significantly to this protection, even though there is no evidence for a direct interaction between ColE9 and TolA during translocation. We thus sought to investigate the level of protection conferred by TA_{1-172} against colicins such as ColA or ColN that interact with both the TolA and TolB proteins but kill sensitive cells by depolarisation of the inner membrane. Since the action of pore-forming colicins does not result in DNA damage, we could not use the *lux* assay in these experiments. Leakage of potassium ions from *E. coli* cells has been used previously to detect the killing activity of ColA and N (Boulanger and Letellier 1988; Bourdineaud, Boulanger et al. 1990).

The potassium release assay is an *in vivo* approach based on intracellular K^+ efflux measurements from *E. coli* cells as a reporter of membrane alteration induced by pore-forming colicins. Consequently, ColA lethality as measured by the amount of potassium in the external media was determined in cells expressing TA_{1-172} in the periplasm. Base line measurements of K^+ efflux in *E. coli* control cells with and without the addition of ColA were recorded as direct comparisons. The results demonstrated that periplasmically expressed TA_{1-172} showed almost total protection against ColA activity as no potassium efflux was detected in the external media compared to the control reading (Figure 3.10) Similarly when TA_{52-172} was expressed and secreted to the cell periplasm no potassium (K^+) was released upon treatment with ColA (Figure 3-12),

presumably indicating that binding of cellular TolA by the TolA box of TA prevents the pore-forming activity of external ColA resulting in the release of K^+. These results are consistent with the data on the protection of cells against ColE9.

Periplasmic expression of TA_{1-52} (ColA TolB box,) suggesting that similar amount of proteins produced in the periplasm, showed reduced K^+ release compared with control cells not expressing any colicin translocation domain in the periplasm but much larger K^+ release compared with the cells expressing and secreting $TA_{52-172,}$ indicating the importance of TolA in the translocation of ColA. It is interesting that the level of protection offered by the different Tol boxes of ColA is dependent on the exogenously added colicin indicating that enzymatic and pore forming colicins have different structure/function specificities and methods of translocation despite relying on similar proteins for cell entry.

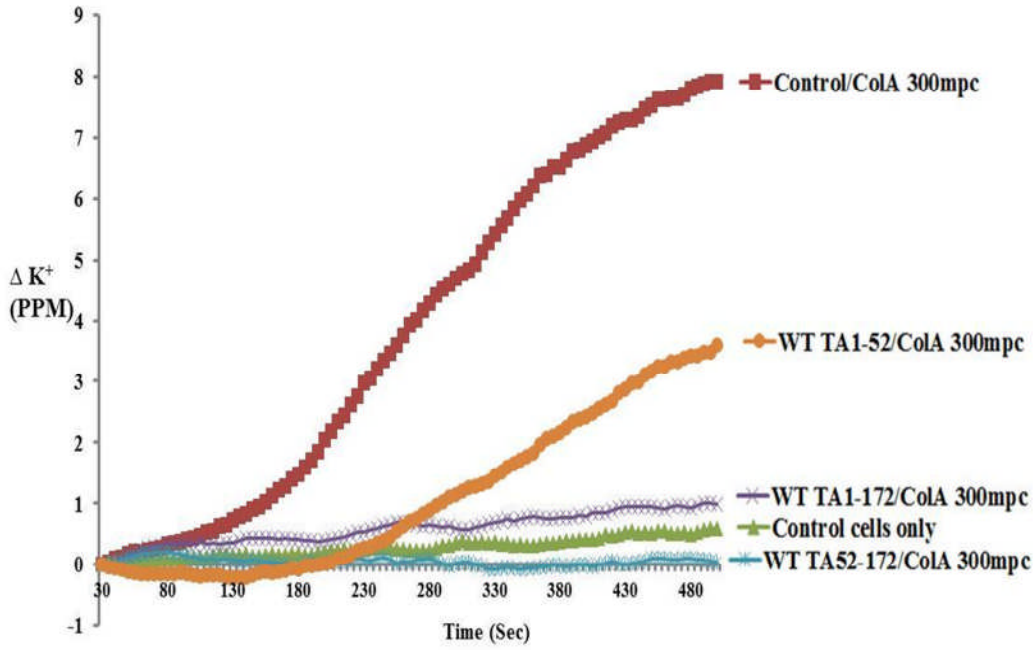


Figure 3-12: Comparison of potassium release to check the *in vivo* periplasmic protection conferred by the overexpressed TA_{1-172} domain and that conferred by expressing either the individual TolA box (TA_{52-172}) or TolB box (TA_{1-52}) by measuring potassium release in the external media by addition of ColA. The potassium release was measured using a specific potassium electrode.

3.3.8 Introducing residues of TolB box of ColE9 into TA_{1-172}

The TolB box of ColE9 binds to TolB, with a K_d of ~1 µM (Loftus, Walker et al. 2006), whilst the TolB box of ColA binds to TolB with a significantly lower affinity of 10 µM (Gokce, Raggett et al. 2000). Different binding affinities suggest different *in vivo* interactions. To investigate this, the TolB box of ColA was replaced with that of ColE9 and the truncated polypeptide was expressed in the periplasm of *E. coli* cells that were then challenged with either ColE9 or ColA.

The residues of the ColA TolB Box were substituted with those of ColE9 (Figure 3-13) either in TA_{1-172} (named pFA5) or in TA_{1-52} (named pFA17). The truncated polypeptides were expressed in the periplasm of *E. coli* reporter strains (as visualized in tricine-SDS-PAGE (Figure 3-14)) and challenged with ColE9. DNA damage was measured using the *lux* reporter assay. Measuring the percentage of protection conferred against ColE9 showed a higher level of protection, up to as much as 97 % with cells expressing pFA5, which represents a ten-fold difference in the interaction of TolB box of ColA and ColE9 with the periplasmic TolB protein. Swapping only the seven residues of ColE9 (pFA17) raised the protection to almost 69 % when compared to expressing TolB box of ColA (pFA16) that showed slight protection (Figure 3-9). Also comparing the result of protection by expressing pFA17 to that of expressing the TolA box only showed a tenfold increase (Figure 3-15, A and B). This indicates that both the TolA and TolB proteins are important for the ColE9 translocation process. Furthermore, these results are consistent with the explanation of the lower percentage of protection when the ColA TolB box was expressed in the periplasm (Figure 3-9). Consequently the protection

conferred by expressing these domains against ColA activity was measured using the potassium release assay. The result displayed a total protection presented by no potassium detected in the external media of cells expressing pFA5 when ColA added (Figure 3-16).

ColA-G**DGTGW**SSERGSGPEP

ColE9-S**DGSGW**SSENNPWGEP

Figure 3-13: Sequence alignment of the TolB box of ColE9 and ColA. Those residues of the TolB box of ColA that were exchanged with analogous residues of the TolB box of ColE9 are shown in yellow.

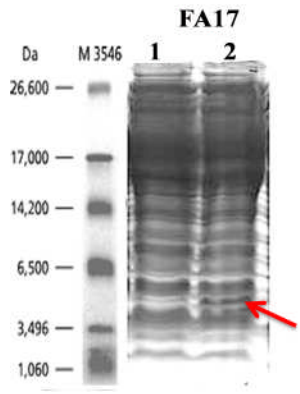
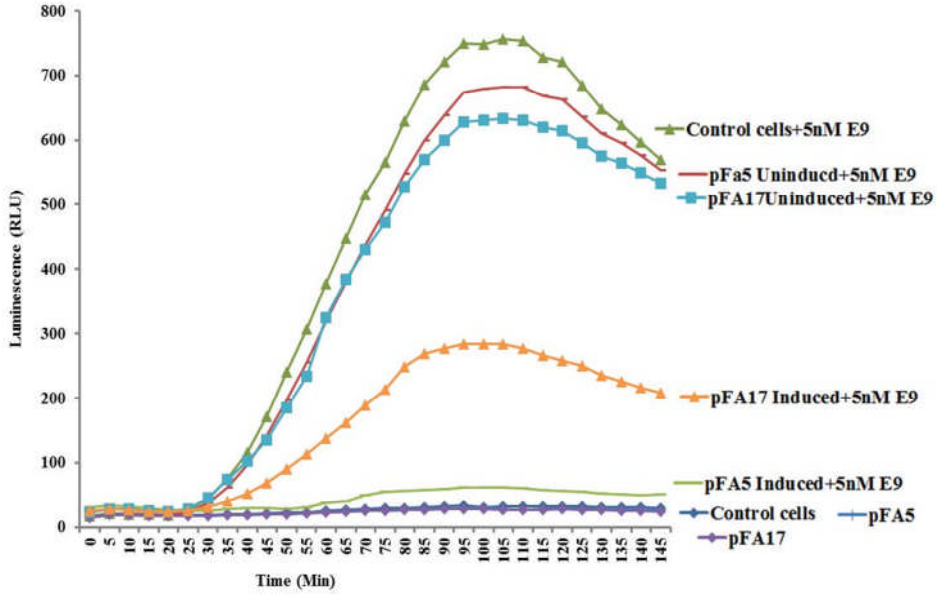


Figure 3-14: Analysis of the induced small polypeptides of FA17 by Tricine-SDS-PAGE. Culture of FA17 induced with 0.02 % L-arabinose for 3 h, showing an expressed protein (~5145 Da) in lane 2, compared to uninduced culture in lane 1. Low range protein marker was used (26-1.06 Da).

(A)



(B)

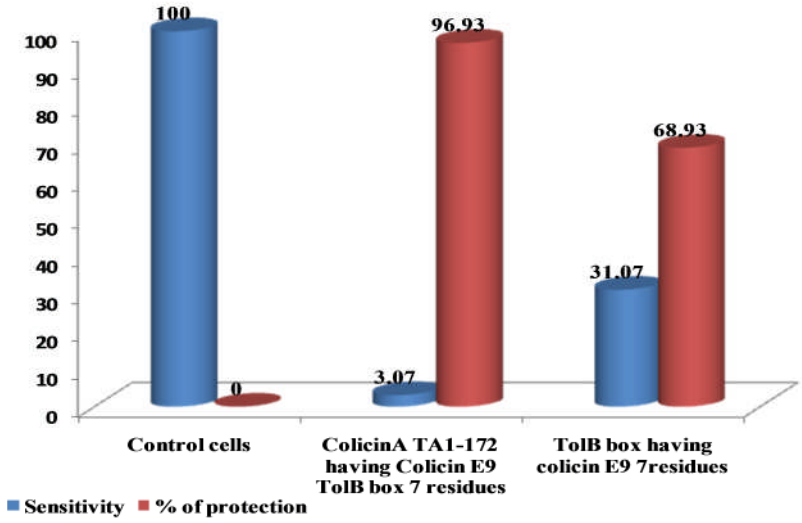


Figure 3-15: Determining the *in vivo* protection conferred by the periplasmic expression of TA_{1-172}, or TA_{1-172} that contains the seven residues of the TolB box of ColE9. (A) Showing the relative luminescence units (RLU) in the *lux* reporter assay to measure protection against exogenous added ColE9. (B) Bar chart represents the calculated percentage of protection comparing different

expressed truncated polypeptides. Cells grown to mid-log and induced with 0.02 % L-arabinose for 3 h and then cells diluted 1:1 with minimal media were ColE9 added to a final concentration of 5 nM, the luminescence measured as DNA damage indicator.

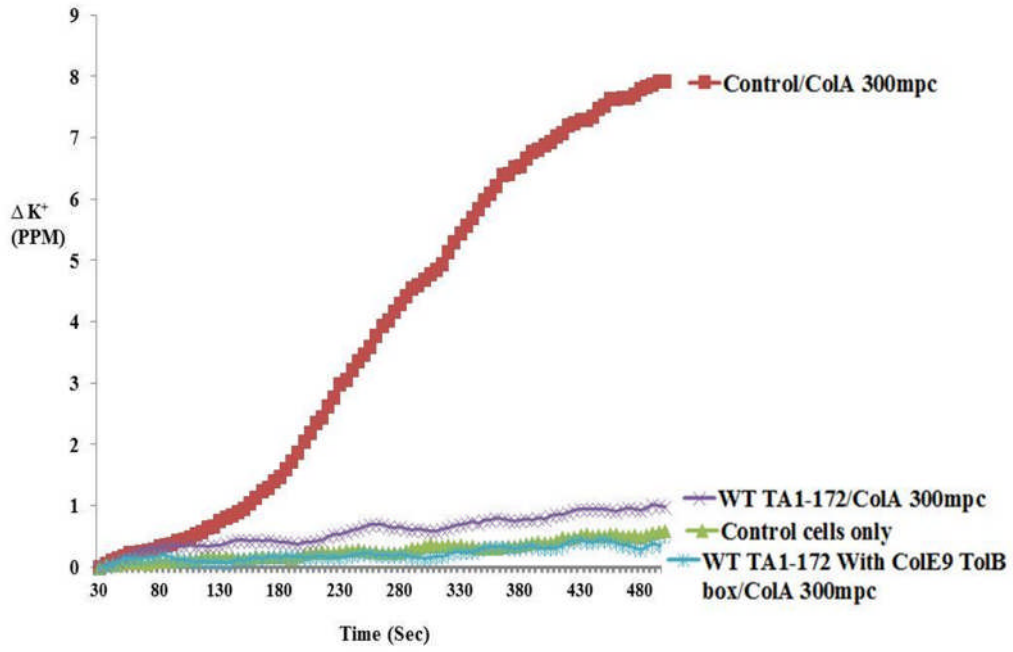


Figure 3-16: Potassium release assay checking the *in vivo* periplasmic protection conferred by the overexpressed TA_{1-172} compared to the protection conferred by TA_{1-172} containing the TolB box residues from ColE9. Periplasmic protection was determined by measuring the potassium ion release into the media as a reporter of cell killing by ColA, using a specific potassium electrode

3.3.9 Comparing the interaction of TolA box of ColN to that of ColA

Swapping the TolB box of ColA with that of ColE9 showed *in vivo* variability in the interaction with TolB protein, therefore, to detect if Tol proteins interact differently with other colicins, the TolA box of ColA was swapped with the equivalent domain of ColN. Using the periplasmic expression technique combined with a sensitive reporter assay the similarities and/or differences in the interaction of colicin translocation domains with Tol proteins were determined.

The ColA TolB box was fused with residues 44 to 66 of the TolA box of ColN (named pFA19) (Figure 3-17). Cells producing the truncated T-domain were challenged with ColE9 (Figure 3-18, A and B), and the results showed that the level of protection was less by 11 % when compared to that from expressing $TA_{1\text{-}172}$. Challenging the same cells with ColA and ColN, using the potassium assay (Figure 3-19), showed almost around 30 % difference in the potassium released into the external media with both ColA and ColN. These findings provide further support for the suggestion of differences in the interaction of colicins and Tol proteins. Although, the assay does not show the nature of the interaction, the results indicate that the TolA box of ColA interacts with Tol proteins in a different way to that of the ColN TolA box ColA.

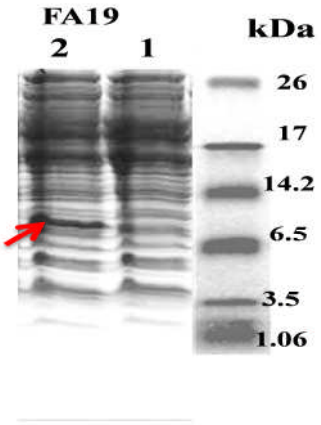
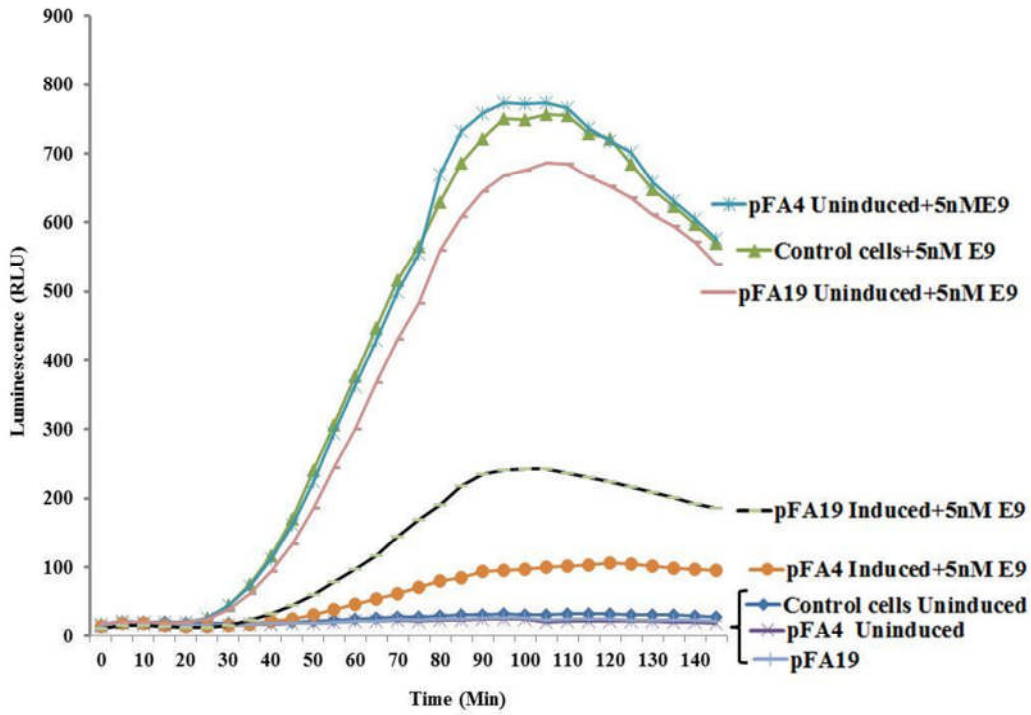


Figure 3-17: Analysis of induced FA19 by Coomassie stained Tricine-SDS-PAGE. The arrow indicates the induced protein band (~10 kDa) in lane 2, compared to the uninduced culture in lane 1. Low range protein marker was used (26-1.06 Da).

(A)



(B)

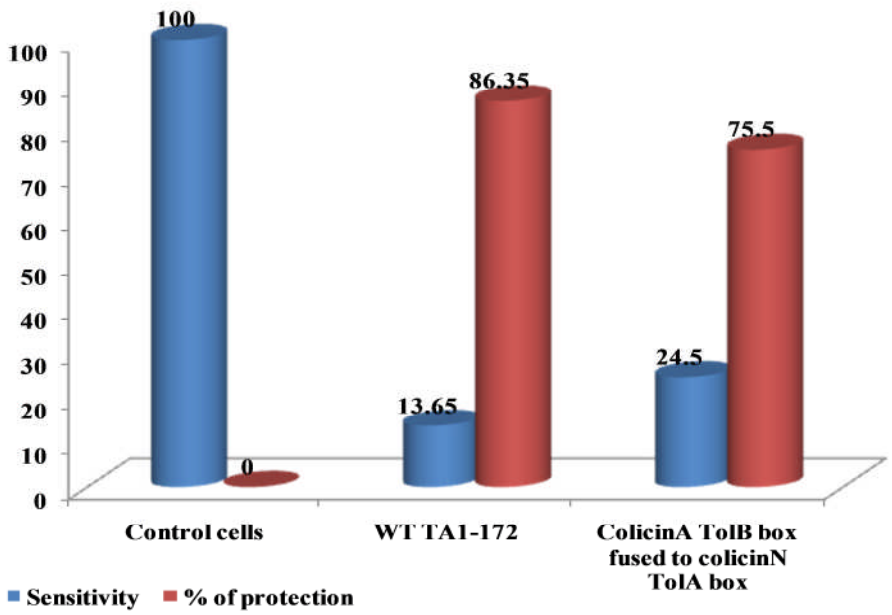


Figure 3-18: Analysis of *in vivo* periplasmic protection comparing TA_{1-172} to the truncated polypeptide consist of TA TolB box (TA1-52) fused to ColN TolA box (TN44-66). (A) Showing relative luminescence units (RLU) by *lux* reporter assay measuring the protection against exogenous ColE9. (B) Bar chart compares the calculated percentage of protection of the different expressed truncated polypeptides. Cells grown to mid-log and induced with 0.02 % L-arabinose for 3 h and then cells diluted 1:1 with minimal media were ColE9 added to a final concentration of 5 nM, the luminescence measured as DNA damage indicator.

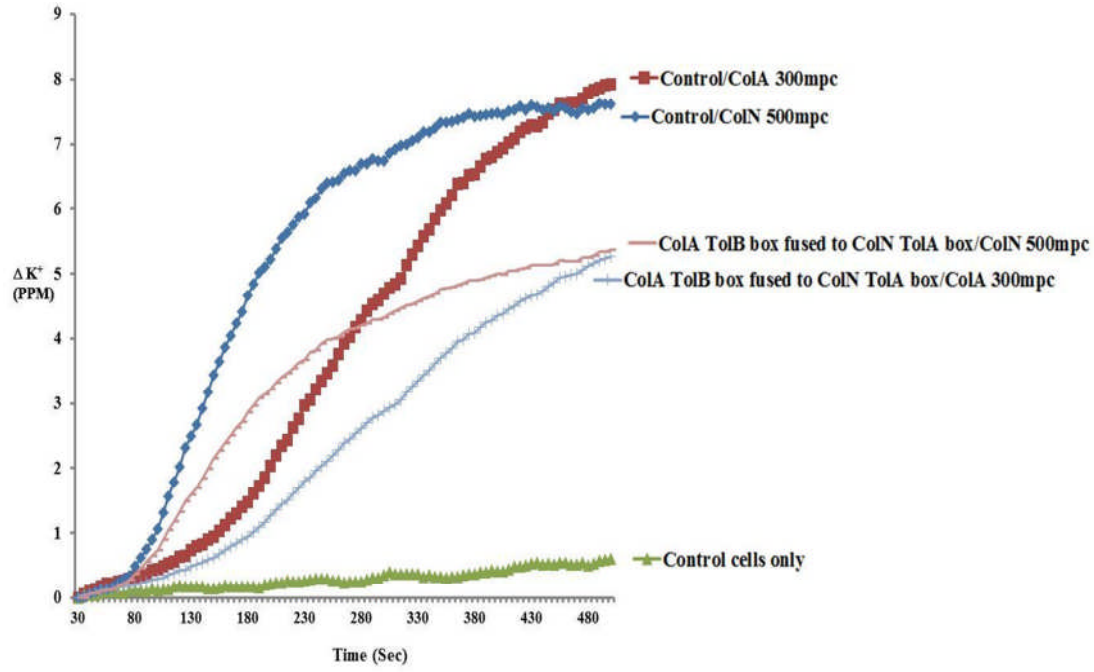


Figure 3-19: Potassium release assay measuring *in vivo* periplasmic protection against ColA and ColN killing. The protection provided by the overexpressed TA_{1-172} was compared to the protection conferred by expressing TA_{1-52} fused to the TN_{44-66}. The amount of potassium measured using specific electrode in the external media after addition of exogenous colicins.

To further investigate the difference in the *in vivo* interaction shown between the translocation domain of ColA fused to the TolA boxes of ColA and ColN, we engineered a construct which expresses the TolA box of ColN alone. The small polypeptide with an estimated molecular weight of ~5 kDa was detected using Tricine-SDS-PAGE (Figure 3-20). Reporter cells expressing the TN_{44-66} polypeptide were challenged with ColE9. No protection was shown compared to the TA_{1-172} and TA_{52-172} (Figure 3-21, A and B).

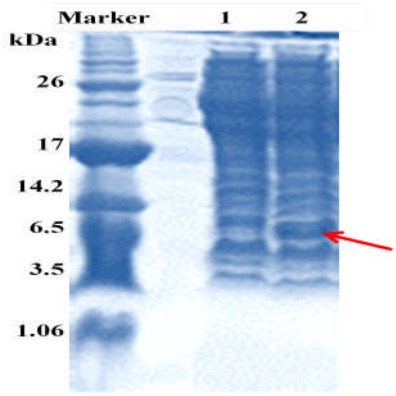
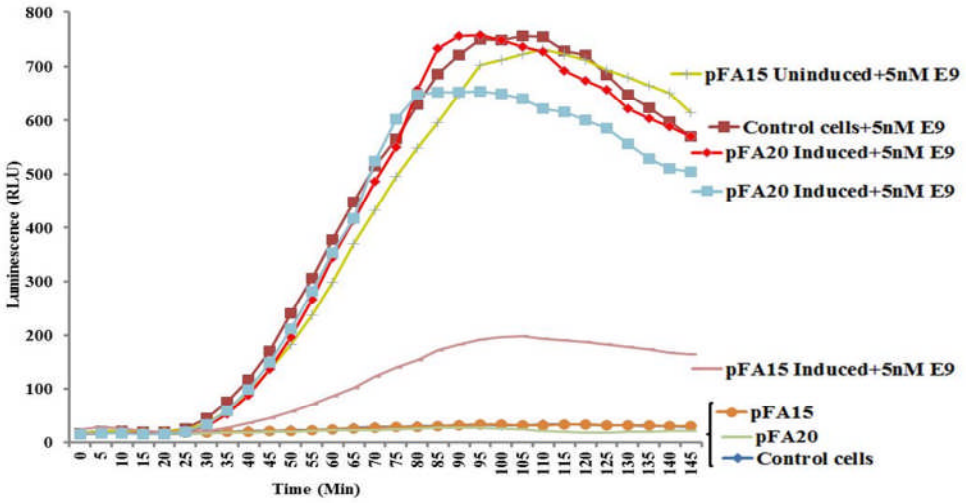


Figure 3-20: Tricine-SDS-PAGE Commassie blue stained gel visualizing the small fragment of expressed TN_{44-66}.A 16 % resolving gel was separated from the 4 % stacking gel by a 10 % spacer gel. A low range protein marker was used (26-1.06 Da). Lane 1 is the uninduced FA20 and lane 2 is the culture induced with 0.02 % arabinose for 3 hrs at 37 ºC The red arrow indicates an induced protein band of around 5.2 kDa.

(A)



(B)

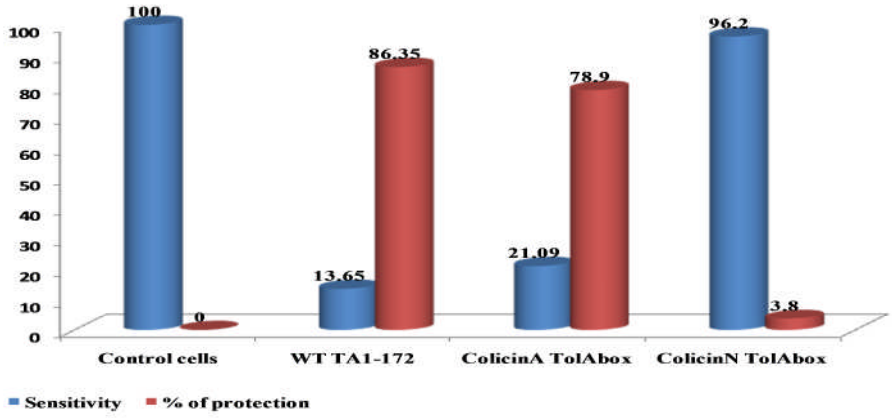


Figure 3-21: Analysis of *in vivo* periplasmic protection conferred by comparing $TA_{1\text{-}172}$, or $TA_{52\text{-}172}$ to the ColN TolA box ($TN_{44\text{-}66}$). (A) Showing relative luminescence units (RLU) by the *lux* reporter assay indicating the protection against exogenous ColE9. (B) Bar chart compares the calculated percentage of protection of the different expressed truncated polypeptides. Cells grown to mid-log and induced with 0.02 % L-arabinose for 3 h and then cells diluted 1:1 with minimal media were ColE9 added to a final concentration of 5 nM, the luminescence measured as DNA damage indicator.

Expressing the TolA box of ColN in the periplasm of the reporter strain showed no protection against ColE9 in the *lux* assay. Additionally, the activity of ColA and ColN was also measured against the reporter cells expressing TN_{44-66}. Comparing the amount of potassium released in the external media of the cells treated with exogenous ColA showed similar amounts as the cells treated with ColN (Figure 3-22), the assay results showed similar protection against ColA and ColN. These results indicated a different pattern of *in vivo* protein-protein interactions reflected by the different level of protection shown against ColA, E9 and N, specifying a different role for TolA protein.

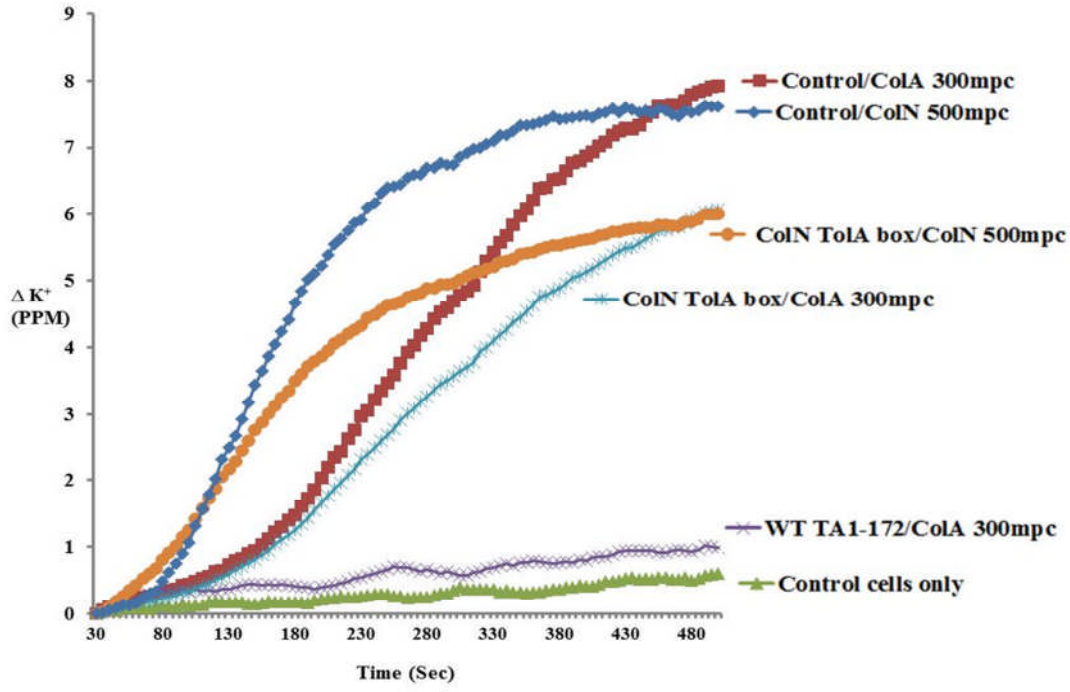
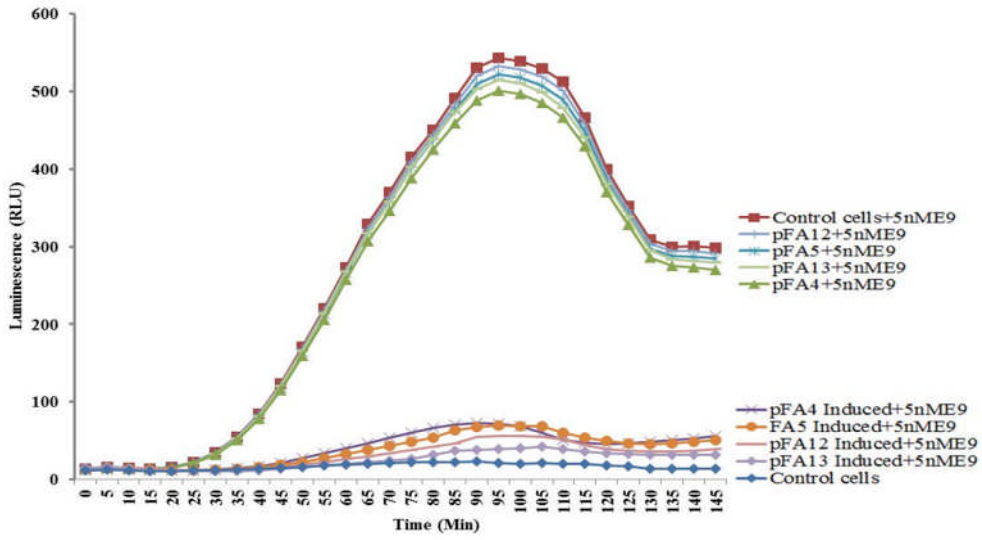


Figure 3-22: Potassium release assay checking *in vivo* periplasmic protection provided by the overexpressed TA_{1-172} compared to the protection conferred by expressing the ColN TolA box TN_{44-66}. The amount of potassium measured using specific electrode in the external media after addition of exogenous colicins

3.3.10 Checking the effect of the extra amino acids in the expressed TA

It is known that TA_{1-172} contains a TolB box between residues 7-20 and a TolA box between residues 52-107 (Bouveret, Rigal et al. 1998) so, all the previous experiments described used TA_{1-172}. In order to exclude any involvement of the C-terminal 65 amino acids of this construct in the interaction with the Tol proteins, we engineered a construct which only expressed the N-terminal 107 residues of ColA (TA_{1-107}). This construct allowed us to compare the level of protection conferred by TA_{1-172} and TA_{1-107}. The similar level of protection conferred by expression of these two polypeptides (Figure 3-23) indicates no involvement of the C-terminal 65 residues of TA_{1-172} in the *in vivo* interaction with Tol protein.

(A)



(B)

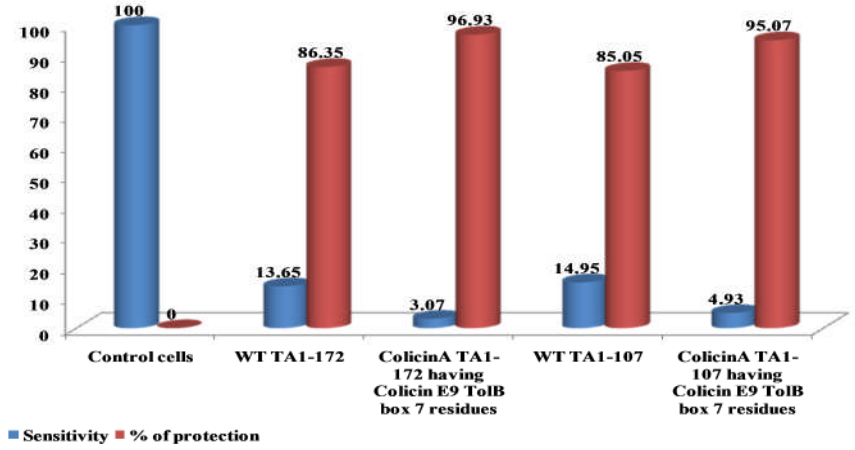


Figure 3-23: Analysis of *in vivo* periplasmic protection conferred by comparing $TA_{1\text{-}172}$ to $TA_{1\text{-}107.}$ (A) Showing relative luminescence units (RLU) by *lux* reporter assay representing protection against exogenous ColE9. (B) Bar chart compared the calculated percentage of protection of the different expressed truncated polypeptides. Cells grown to mid-log and induced with 0.02 % L-arabinose for 3 h and then cells diluted 1:1 with minimal media were ColE9 added to a final concentration of 5 nM, the luminescence measured as DNA damage indicator.

3.4 Discussion

This study has used the concept of combining the periplasmic protection technique (Pommier, Gavioli et al. 2005) and the *lux* reporter assay (Vankemmelbeke, Healy et al. 2005) to dissect the translocation process of ColE9 and illustrates important aspects of the interaction of different colicins with Tol proteins. The results showed that the periplasmic protection assay provides some useful *in vivo* information. The translocation domain of ColA that contains the TolA and TolB boxes was fused to a signal sequence to regulate its expression and secretion into the periplasm of the *E. coli* reporter cells (Vankemmelbeke, Healy et al. 2005). Measuring the DNA damage caused by external ColE9 was used to determine the effect of periplasmically expressed proteins on blocking the entry of the added ColE9.

Expressing colicin domains in the cell periplasm did not affect the growth characteristics of the cells and therefore any differences of biological activity, as measured by *lux* induction, were not due to differences in growth rate. In addition, the expression and secretion of a similar sized polypeptide derived from lysostaphin (Bardelang, Vankemmelbeke et al. 2009), a polypeptide of no known role in Gram negative cell envelope biogenesis or colicin translocation, had no effect on biological activity of exogenously added ColE9 or ColA and allowed us to dismiss the notion that any protection by internally expressed colicin domains is not due to the expression of a specific polypeptide effects. The protection conferred to *E. coli* cells expressing $TA_{1\text{-}172}$ after treatment with ColE9 was 86.35 % compared to a total activity of ColE9 against the control cells. Treatment of these cells with ColA showed almost total activity as measured by the amount of potassium released in the external media of the

treated cells. The different level of protection observed against ColA and ColE9 could be explained by the different translocation requirement and the different sequence of both colicins. These experiments suggested that an *in vivo* interaction between the TA_{1-172} and Tol proteins was providing protection against external ColE9, therefore, constructs were engineered in which the TolB and TolA boxes of TA_{1-172} were separated and the truncated polypeptides expressed in the periplasm of the reporter cells and challenged with ColE9. The results showed that expressing the TolA box (TA_{52-172}) accounted for the majority of protection against ColE9 (around 78.9 %), and the total protection against ColA. Expressing the TolB box (TA_{1-52}) showed only 22.23 % protection against ColE9 and almost 50 % against ColA. The lower protection shown by expressing the TolB box of ColA presumably reflects differences in the affinity of binding of TolB by the TolB boxes of ColE9 and ColA, which are reported to be ~ 1 µM (Loftus, Walker et al. 2006) ColA and 10 µM (Gokce, Raggett et al. 2000) respectively. Different binding affinities suggests different *in vivo* interactions; in order to investigate this, the TolB box of ColA was replaced with that of ColE9 and the truncated polypeptide was expressed in the periplasm of *E. coli* cells that were challenged with either ColE9 or ColA. The protection increased in both assays showing 96.93% against ColE9 and total protection against ColA when the TA_{1-172} with substituted TolB box residues. Expressing only the smaller TolB box polypeptide containing the substituted residues from ColE9 showed higher protection (68.93 %) against ColE9 indicating the significance of the different affinity of interaction. The interaction of the ColE9 TolB box with TolB may thus serve predominantly to hold the ColE9 molecule sufficiently long in the required position at the cell

surface for immunity protein to be released (Vankemmelbeke, Zhang et al. 2009) and thus allow translocation to take place.

Moreover it is known that ColE9 does not have a TolA box and there is no evidence of any interaction between the ColE9 translocation domain and the TolA protein (Carr, Penfold et al. 2000). The significant level of protection against ColE9 conferred by expressing the TolA box of ColA in the periplasm indicates an important role for TolA in the ColE9 translocation process. It is reasonable to speculate that energy transferred to a TolB-bound ColE9, probably through a conformational change caused by TolA-TolB interaction, results in immunity protein release and the consequent translocation of the cytotoxic domain to its target. This would explain the requirement for an energized TolA in the absence of any evidence for a direct interaction of a ColE9 with TolA, as previously proved for immunity protein release requirement (Vankemmelbeke, Zhang et al. 2009).

Periplasmic protection is presumed to result from the expressed TolA or TolB boxes interacting with their cognate tol proteins and thus making them unavailable for the translocation of externally added ColE9. In order to investigate this further, *in vivo* cross linking using formaldehyde was carried out on the cells expressing $TA_{1\text{-}172}$. Analysis of the induced and the uninduced samples showed protein bands that were comparable to the bands shown upon analysis of *in vitro* cross linked purified proteins (Figure 3-10).

The interaction between Tol proteins and different colicin domains was studied in a different way in which the TolA box of ColA was swapped with the ColN TolA box and *E. coli* cells expressing the resulting protein where challenged

with ColE9. The level of protection was reduced to 75.5 % when compared to the TA_{1-172} , while challenging the same cells with either ColA or ColN showed only 30 % protection. In contrast, expressing only the TN_{44-66} (ColN TolA box) showed no protection against ColE9. Challenging these cells with ColA or ColN also showed similar results. It might be expected that we would observe protection when cells were treated with ColN, however it is already known that the interaction between TolA and the full length ColN (K_d of 18μM) is much weaker than that of the isolated TN (K_d of 1μM) (Raggett, Bainbridge et al. 1998). Previous observation showed that the pore-forming domain of ColN is very similar to that of ColA; their amino acid sequences are highly similar, they show the same channel properties in lipid bilayers (Wilmsen, Pugsley et al. 1990) and similar threshold membrane potential for opening the channel *in vivo*. The lower molecular mass of ColN (42 kDa) that requires only OmpF/TolAQ as translocator components, may explain the rapid translocation of ColN which is revealed by the short lag time before the K^+ efflux, while ColA showed a delay in the onset of K^+ release which may be mainly due to the binding, translocation and insertion steps.

It is already clear that the nature of the interaction between group A colicins and the TolA protein is very variable (Schendel, Click et al. 1997; Bouveret, Rigal et al. 1998). Therefore, expressing the ColN TolA box in the periplasm results in no protection against ColE9, whereas expression of TA_{52-172} results in 78.9 % protection against ColE9 (Figure 3-9). The similarity of the TolA binding sequence between ColA and ColN that was previously analysed (el Kouhen, Fierobe et al. 1993) does not appear to be reflected on a similar manner of interaction with the TolA protein (Figure 3-21). All these results,

regardless of similarity and differences between group A colicins, highlight diverse *in vivo* interactions between the TolA protein and the truncated polypeptides that have been used in these experiments.

Moreover, the *in vivo* involvement of the residual sequence of the TA, which was not related to Tol interaction, was investigated by comparing the protection conferred on *E. coli* reporter cells that express TA_{1-172} or TA_{1-107} against ColE9. The results showed a comparable level of protection was conferred by the two polypeptides, suggesting that the C-terminal 65 amino acids of TA_{1-172} are not involved in the *in vivo* interaction with Tol proteins. Such findings are consistent with the previous suggestion that deletion of these residues does not affect the interaction with Tol proteins but affect the conformation of the translocation domain of ColA leading to less efficient translocation process (Bouveret, Rigal et al. 1998).

The work in this chapter provides evidence of the value of using the periplasmic protection technique and the *lux* reporter assay in investigating the translocation process of group A colicins.

CHAPTER FOUR

4 *In vivo* mutational analysis of periplasmically expressed ColA translocation domain

4.1 Introduction

Mutational analysis has been used to dissect the functional domains of colicins and their interaction with receptors and Tol proteins (Cascales, Buchanan et al. 2007). Mutations in any of the *tol* genes make the bacterium hypersensitive to different agents and drugs, and results in the release of periplasmic contents into the medium (Webster 1991). *E. coli* strains with *tonB* mutations are tolerant to all group B colicins, although, *exbB* or *exbD* mutants retain some slight sensitivity to colicins due to cross interaction with Tol system (Davies and Reeves 1975a; Braun and Herrmann 1993). Mutational analysis aids the identification of specific conserved residues in either Tol proteins or in colicin domains (Wallis, Leung et al. 1998; Walker, Lancaster et al. 2004; Cascales, Buchanan et al. 2007).

Site-directed mutagenesis provides a powerful tool for studying structure-function relationships of colicins with their translocation machinery. Moreover, the combined periplasmic technique with the *lux* reporter assay has shown interesting *in vivo* results in the previous chapter. In this chapter, the overall aims were to investigate the sequence of events that take place during colicin translocation. By engineering unique periplasmically-expressed colicin polypeptides containing mutations, I hoped to be able to investigate the protein-protein interactions that occur in the periplasm during colicin translocation and to identify key residues that are essential for the translocation process.

4.2 Materials and methods

4.2.1 Site-directed mutagenesis:

The Quickchange II-E technique (Stratagene, UK), was used to allow the site-specific mutation of nucleotides within a circular plasmid. This, rapid three-step procedure generates mutants in a single reaction without the need for a specialized vector or restriction sites, or a requirement for multiple transformations or any gel extraction. The QuickChange II-E site directed mutagenesis method was performed using *PfuUltra* high-fidelity DNA polymerase, dsDNA and two oligonucleotide primers, both containing the desired mutation which are complementary to opposite strands of the dsDNA target sequence. The primers are extended during temperature cycling (section 2.4.2, table 2.3), and allow the generation of a mutated plasmid containing a staggered nick. The PCR product was then treated with *Dpn* I endonuclease to digest the remaining parental DNA template and to select for the mutation-containing newly synthesized DNA strand. Subsequently, transformation was carried out into XL-I blue competent cells that were plated on an LB plate containing ampicillin (100 $\mu g ml^{-1}$).

All generated constructs and their protein analysis are described in appendix B.

4.3 Results:

The periplasmic expression technique described in chapter 3.1 used to disturb the function of the Tol system in *lux* reporter strains results in protection against ColE9 if the expressed translocation polypeptide binds to and titrates out important Tol proteins. Periplasmic expression can also be used to identify key residues by introducing mutations into the expressed polypeptides and observing any reduction in the level of protection against ColE9. I used this method to investigate the effect of mutations in specific residues of the TolA box and the TolB box of TA_{1-172} that is related to the Tol protein they bind to.

4.3.1 Single alanine mutant in E18 TolB box of TA_{1-172}

An alanine substitution of residue E18 was introduced into pFA4 (TA_{1-172}), and into pFA5 (the truncated TA_{1-172} that contains the seven residues of ColE9 TolB box). The resulting plasmid constructs were, named pFA6 and pFA8, respectively. Cultures of *E. coli* DPD1718 pFA6 and *E. coli* DPD1718 pFA8 induced with arabinose were compared to uninduced control cultures by SDS-PAGE analysis. The results clearly show proteins of expected size in the induced samples (Figure 4-1). Proving the expression of the constructed proteins, the cultures of *E. coli* DPD1718 containing the plasmids were challenged with ColE9 using lux reporter assay (Figure 4-2, A). It is clearly shown that the E18A mutation in pFA6 seems to affect the protection by almost 7 %, whereas the same mutation in pFA8 showed no effect on the level of protection conferred against ColE9 (Figure 4-2, B), once more this represents the difference of the nature and affinity interaction of ColE9 and ColA to TolB.

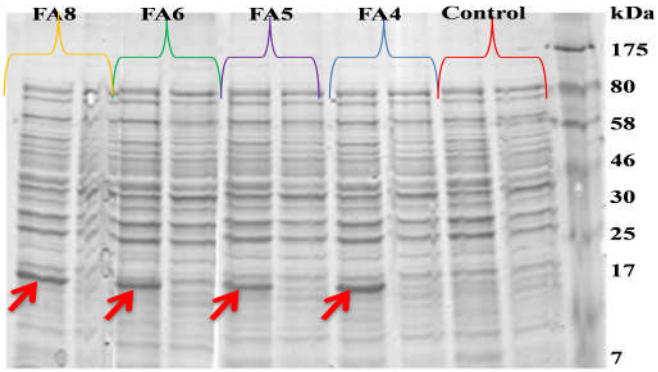
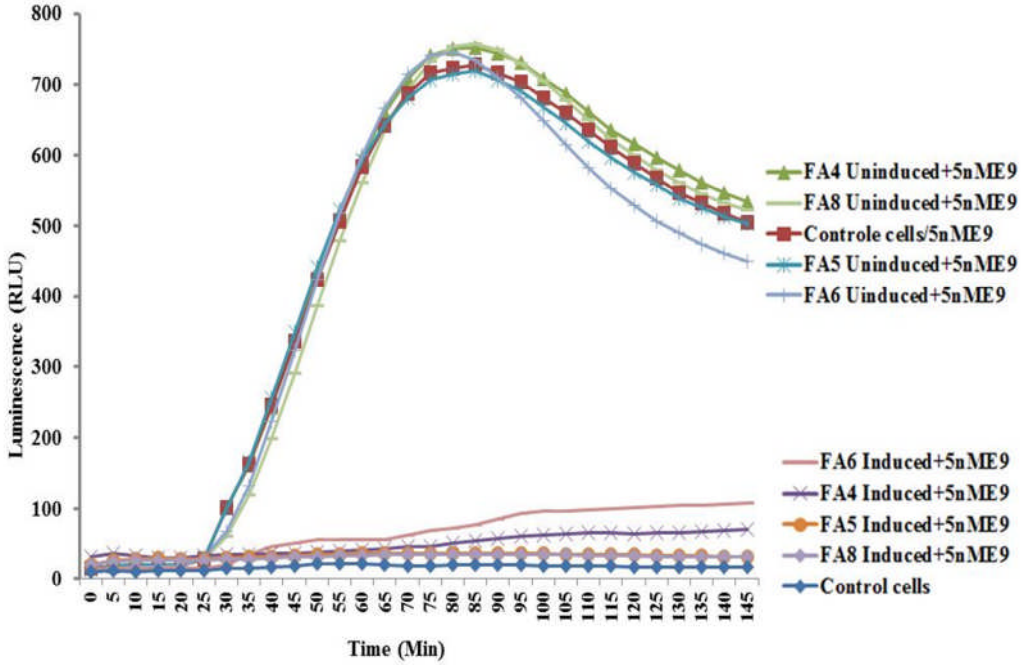


Figure 4-1: Analysis of alanine mutant in TolB box of TA. Coomassie blue stained SDS-PAGE detecting the periplasmic expression of pFA4 and pFA5 compared to their related mutated constructs pFA6 and pFA8, respectively. The polypeptide of around 17 kDa in each of the constructs represents the induced protein compared to a control *E. coli* DPD1718 cells. (7-175 kDa) protein marker was used.

(A)



(B)

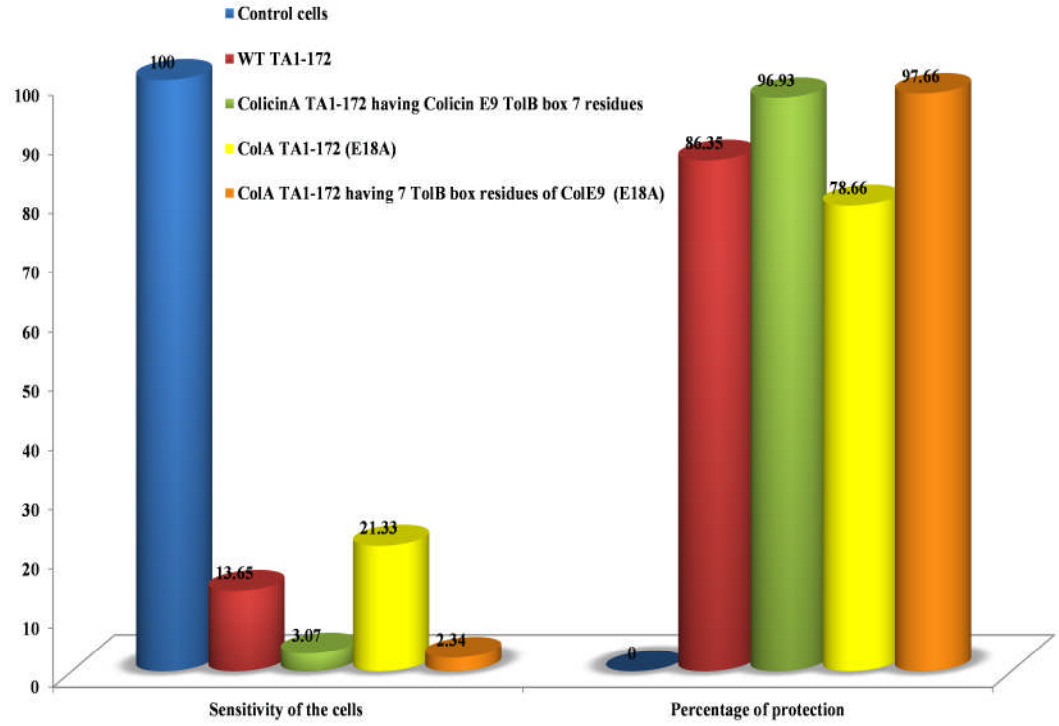


Figure 4-2: Analysis of the effect of alanine mutations in the TA-TolB box on the *in vivo* interaction with Tol proteins. (A) The *lux* reporter assay was used to detect the protection conferred by expressing these constructs (B) Bar chart represented the percentage of protection (right side) calculated from the induction of DNA damage induced by ColE9 in *E. coli* cells expressing the different constructs (left side). Cells grown to mid-log and induced with 0.02 % L-arabinose for 3 h and then cells diluted 1:1 with minimal media were ColE9 added to a final concentration of 5 nM, the luminescence measured as DNA damage indicator.

Using a spot test, the activity of ColA was tested against the *E. coli* cells producing the mutated polypeptides expressed in the periplasm, compared to *E. coli* cells expressing $TA_{1\text{-}172}$. No differences were detected using the overlay method between the protection conferred by expressing the $TA_{1\text{-}172}$ and the mutated TA, as a similar zone of inhibition was observed (Figure 4-3, B and C). Control *E. coli* cells show total activity against all the different concentrations of colicin used (Figure 4-3, A). Additionally ColE9 was tested using the spot test assay and colicin B was also used as a positive control. The activity of ColE9 was similar to ColA activity in the overlay method.

The results clearly show that the presence of the ColE9 TolB box enhanced the binding of the translocation domain of ColA to the Tol machinery in the periplasm as only higher concentrations of colicin showed zones of inhibition (Figure 4-3, D and E).

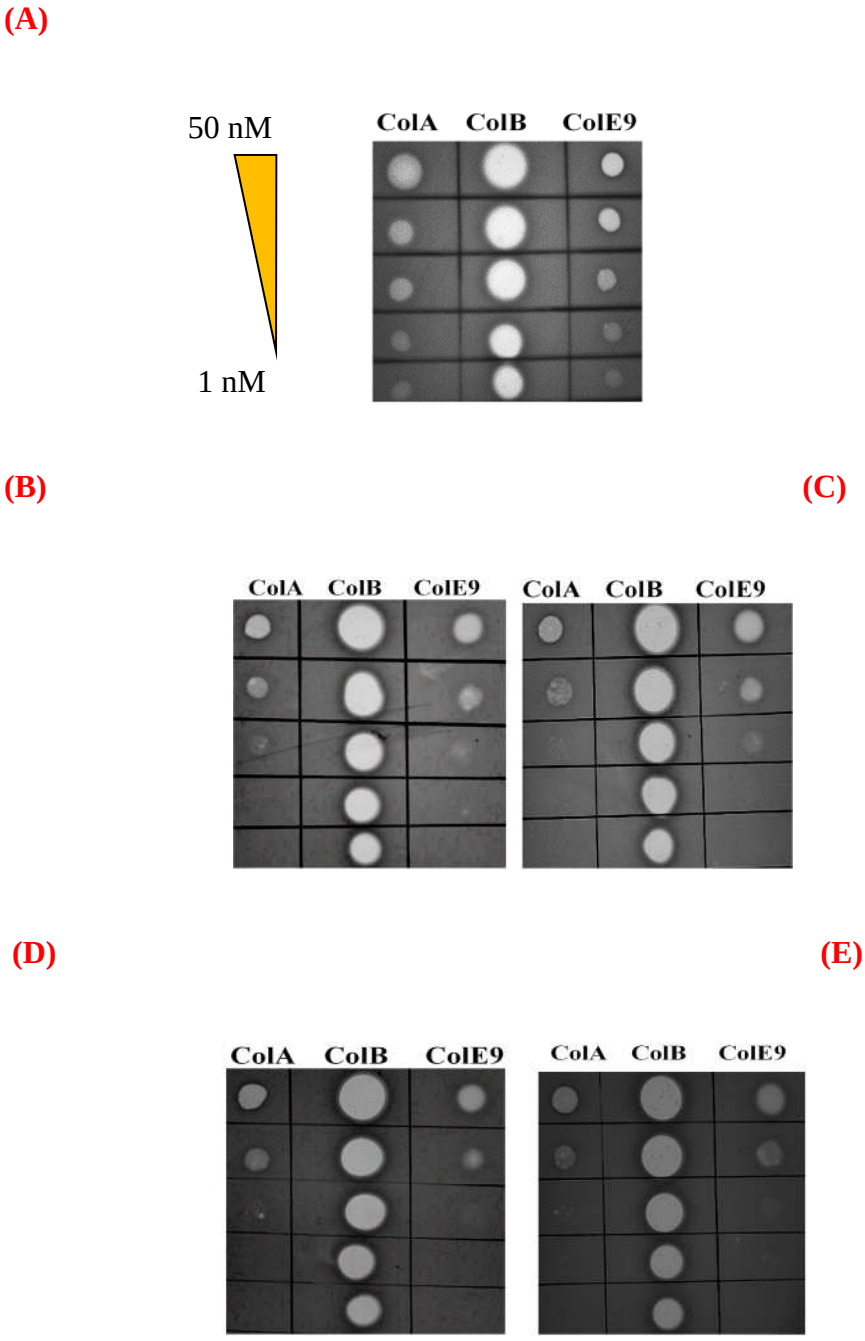


Figure 4-3: Determination of the effect of various alanine mutants on TA variants on the activity of different colicins *in vitro*. Spot test performed using purified ColA, ColB and ColE9. 2 µl of each protein at a range of concentrations between 50 and 1 nM were spotted onto agar seeded with *E. coli* DPD1718 containing plasmid indicated; (A) control cells; (B) pFA4; (C) pFA6; (D) pFA5; (E) pFA8.

4.3.2 Single alanine mutant in Y58 TolA box of ColA TA_{1-172}

An alanine mutation was introduced into TA_{1-172} TolA box at position 58 by site directed mutagenesis (as described in section 4.2.1), replacing the tyrosine residue that was previously suggested to have an important role in binding to the TolA protein (Pommier, Gavioli et al. 2005). Alanine mutations were introduced into both WT TA_{1-172} and into TA_{1-172} that has residues of ColE9 TolB box, named pFA7 and pFA9, respectively. Arabinose-induced expression of the polypeptides encoded by plasmids pFA7 and pFA9 was confirmed by SDS-PAGE (Figure 4-4). The sensitivity of *E. coli* DPD1718 cultures containing pFA7 or pFA9 to ColE9 was determined with the *lux* reporter assay (Figure 4-5, A). Expression of the Y58 mutant polypeptide encoded by pFA7 in *E. coli* DPD1718 restored 58 % of ColE9 activity compared with pFA4 (Figure 4-5, B). Even with the presence of the ColE9 TolB box residues, the level of protection was reduced to 50 %. This is presumed to be the result of reduced binding of the Y58A mutant polypeptide to TolA in the periplasm which allows more ColE9 to enter the cells and induce DNA damage, even though ColE9 does not bind directly to TolA.

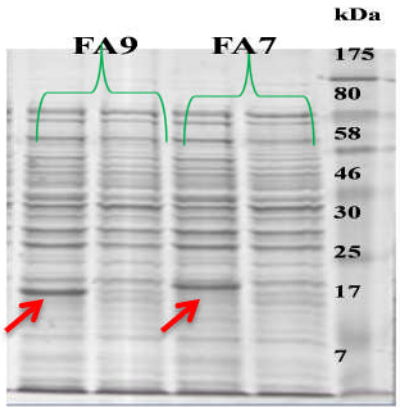
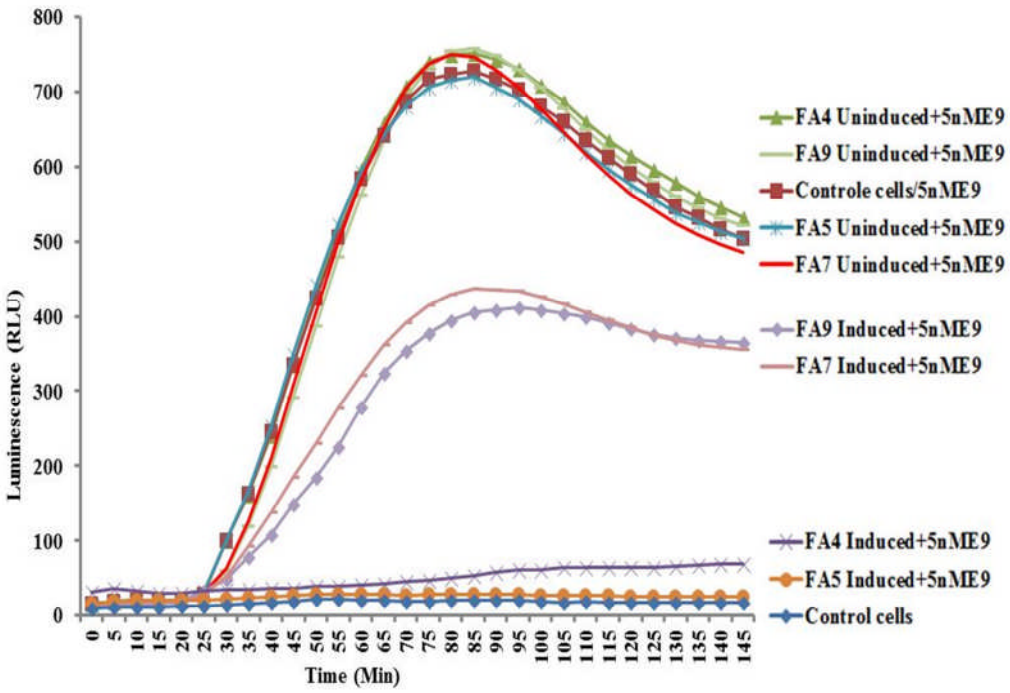


Figure 4-4: Analyses of periplasmically expressed TA variants by SDS-PAGE. Coomassie blue stained gel detected the periplasmic expression by pFA7 and pFA9 of a polypeptide of around 17 kDa. The red arrows represent the induced protein compared to uninduced culture that showed no proteins. (7-175 kDa) protein marker was used.

(A)



(B)

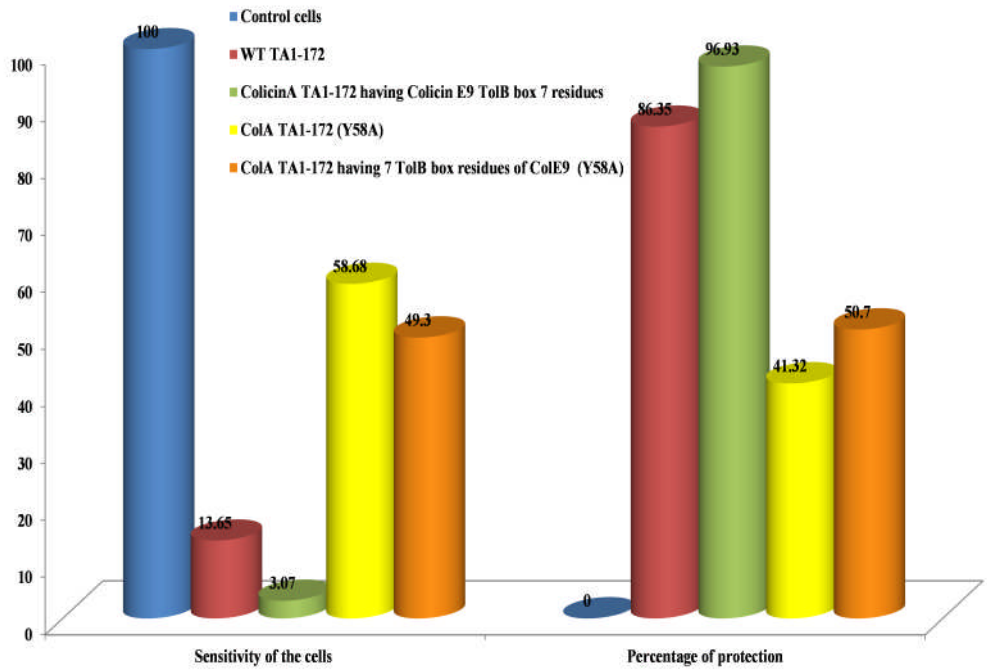


Figure 4-5: Detection of the effect of alanine mutants in the TA-TolA box. The *lux* reporter assay detected the *in vivo* protection conferred by expressing pFA7 and pFA9. (A) Relative luminescence units measuring the DNA damage caused by ColE9; the luminescence of both induced and uninduced cultures were compared for each construct. (B) Bar chart represented the percentage of protection (right side) calculated from the RLU as an indicator of the colicin activity for each different construct. The protection conferred by the mutated TA variants was compared to that of pFA4 and pFA5. Cells grown to mid-log and induced with 0.02 % L-arabinose for 3 h and then cells diluted 1:1 with minimal media were ColE9 added to a final concentration of 5 nM, the luminescence measured as DNA damage indicator.

Activity of ColA against *E. coli* DPD1718 cells expressing the Y58 mutants in both constructs pFA7 and pFA9 was determined using a spot test (Figure 4-6). The results showed some restored activity at lower concentration compared to WT $TA_{1\text{-}172}$ (pFA4) results, while ColE9 showed zones of inhibition with all the concentrations tested. The TAY58A that contained ColE9 TolB box residues also showed activity; however, at concentration of 2-1 nM the zones were hazy compared to the TAY58A control cells.

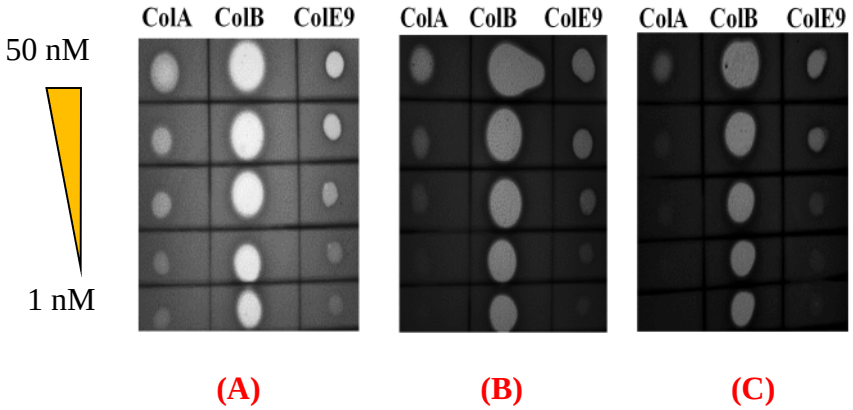


Figure 4-6: Determination of the effect of the TolA box alanine mutants on *in vitro* colicins activity. Spot test performed using purified ColA, ColB and ColE9. 2 µl of each protein at a range of concentrations between 50 and1nM were spotted onto agar seeded with *E. coli* DPD1718 containing the plasmid indicated (A) control cells; (B) pFA7; (C) pFA9.

4.3.3 Combined mutants in TolA and TolB boxes

From the results obtained by mutating either the TolA box or TolB box individually, it was interesting to check the effect of combined mutants of both boxes. The TA_{1-172} construct containing both the E18A and Y58A mutations was named pFA10, while a mutant in TA that has the ColE9 TolB box residues was named pFA11. The expression of these proteins in these constructs was analysed by SDS-PAGE (Figure 4-7)

The protection conferred by expression of either pFA10 or pFA11 against ColE9 was determined using the *lux* assay (Figure 4-8, A), and against purified ColA, using a range of concentrations (1 to 50 nM), in the overlay assay (Figure 4-8, B). The data showed that the induction of mutated pFA10 affected the interaction with Tol proteins as the measured luminescence showed 86 % restored activity of ColE9 when the mutant polypeptide was expressed in *E. coli* DPD1718 cells compared with TA_{1-172}. 75 % restored activity was observed with pFA11. Similar zones of inhibition showed in the spot test compared to the control cells, except for the hazy zones shown for the construct that contains the ColE9 TolB box (Figure 4-9). The additive effect of the individual alanine mutant in either TolA or TolB boxes was less than the effect of collective mutants. The results suggested that the dual mutants affected the interaction with the Tol protein *in vivo* which resulted in restored activity of the tested colicins in both methods. The mutations may also have an effect on the protein conformation, which could explain the less reduced protection against the challenge colicins.

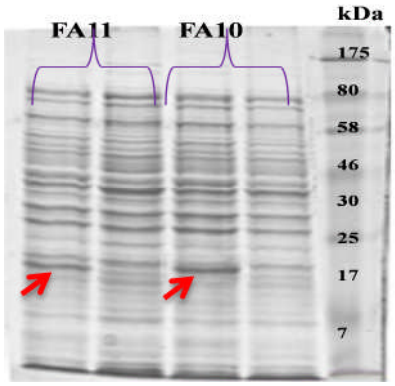
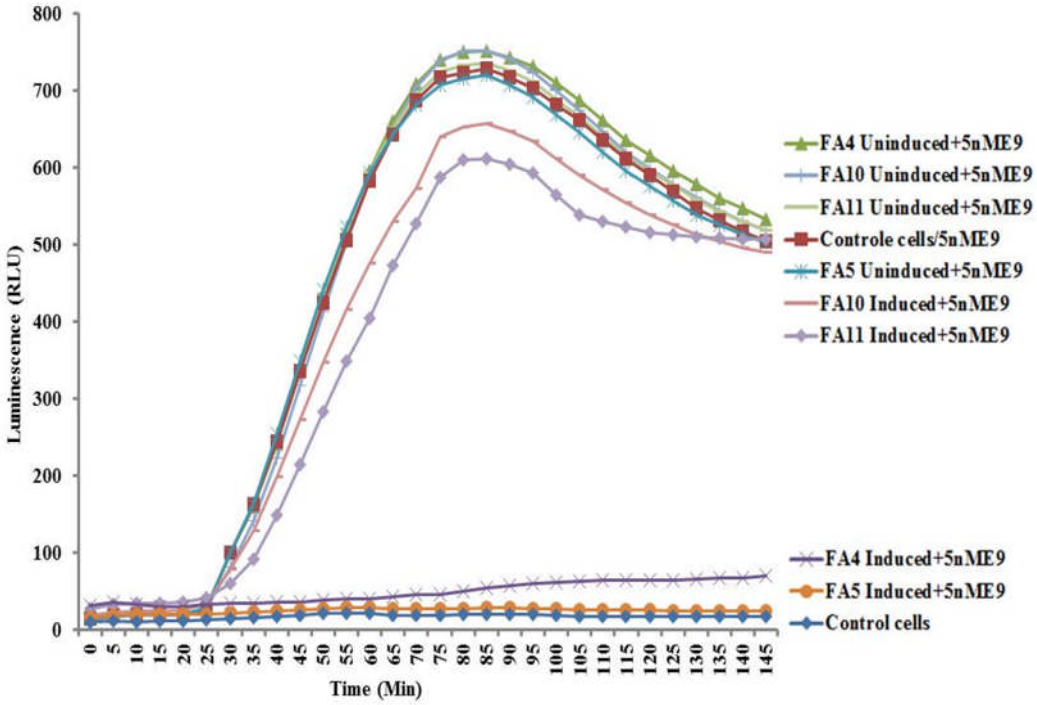


Figure 4-7: Analyses of overproduced pFA10 and pFA11 by SDS-PAGE. Coomassie blue stained gel detected the periplasmic expression of pFA10 and pFA11. The red arrows indicate a polypeptide band of around 17kDa that represents the induced protein. (7-175 kDa) protein marker was used.

(A)



(B)

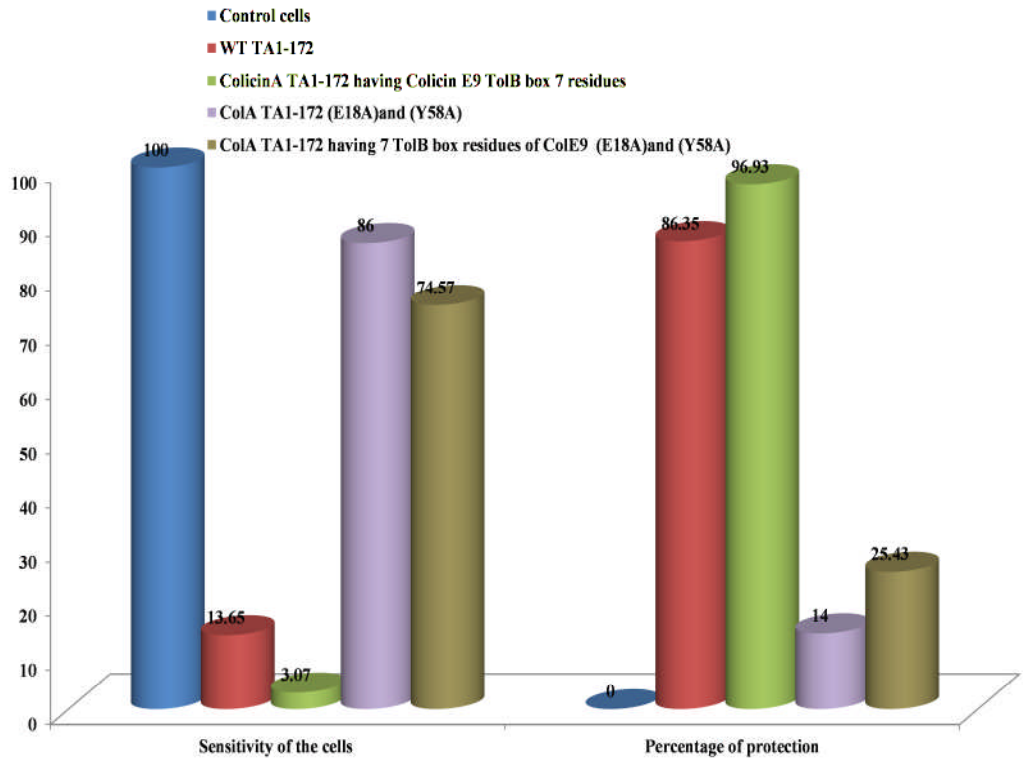


Figure 4-8: Determination of the effect of periplasmically expressed TA containing combined TolA and TolB box alanine mutants. The *lux* reporter assay was used to detect the *in vivo* protection conferred by expressing pFA10 and pFA11. (A) Relative luminescence units measuring the DNA damage caused by ColE9; the luminescence of both induced and uninduced cultures were compared for each construct. (B) Bar chart representing the percentage of protection (right side) calculated from the RLU as an indicator of the colicin activity of the different constructs. The protection was compared to that of pFA4 and pFA5 constructs. Cells grown to mid-log and induced with 0.02 % L-arabinose for 3 h and then cells diluted 1:1 with minimal media were ColE9 added to a final concentration of 5 nM, the luminescence measured as DNA damage indicator.

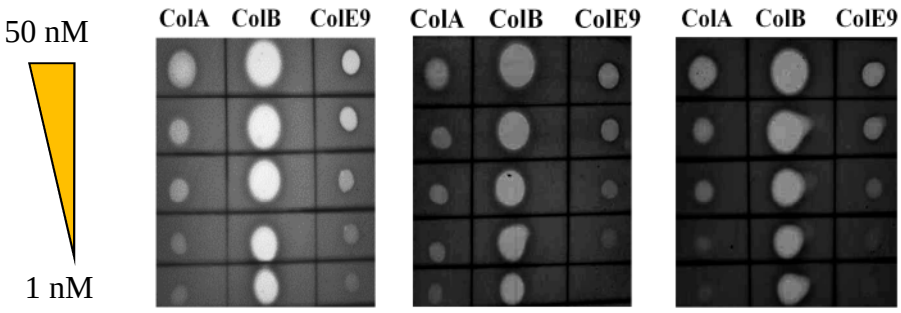


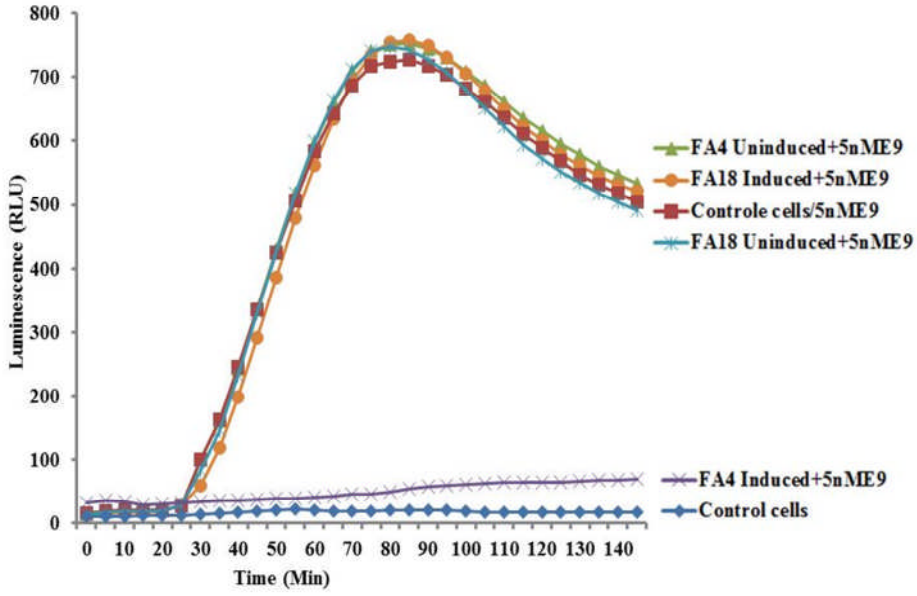
Figure 4-9: Analysis of the effect of the combined TolA and TolB box alanine mutants on the activity of different colicins *in vitro*. Spot test was performed using purified ColA, ColB and ColE9. 2 µl of each protein at a range of concentrations between 50 and 1 nM were spotted onto agar seeded with *E. coli* DPD1718 containing the plasmid indicated (A) control cells; (B) pFA10; (C) pFA11.

4.3.4 Mutations in TolA residues that interact with TA_{1-172}

The crystal structure of TolAIII in complex with the TolA box of ColA has recently been solved (unpublished work by our group). This has revealed that Leu375, Leu376, Asp377, Ile378 and Pro380 residues of TolAIII are important in the binding interface between TolAIII and the TolA box of ColA. Therefore, it was decided to mutate the residues in the TA TolA box that interact with these TolAIII residues.

Using site directed mutagenesis, ColA TolA box (R62-69A) residues related to the interaction with the five residues in TolAIII were constructed to test if this alters the extent of periplasmic protection conferred against ColE9 possibly by disrupting interaction of the mutated TA with TolA (TolAIII). The expression of the mutated TA polypeptides was analysed by SDS-PAGE and compared to that of WT TA. Using the *lux* assay, it was shown that expressing the TA TolA mutant showed no protection against ColE9, indicating the loss of interaction of the periplasmically expressed protein with cellular TolA protein (Figure 4-10 A and B). The results obtained using the potassium release assay (described in section 3.2.1) was in agreement with the *lux* assay (Figure 4-11). These results indicate the importance of these residues in the TolA box in the interaction with the TolA protein during colicin translocation.

(A)



(B)

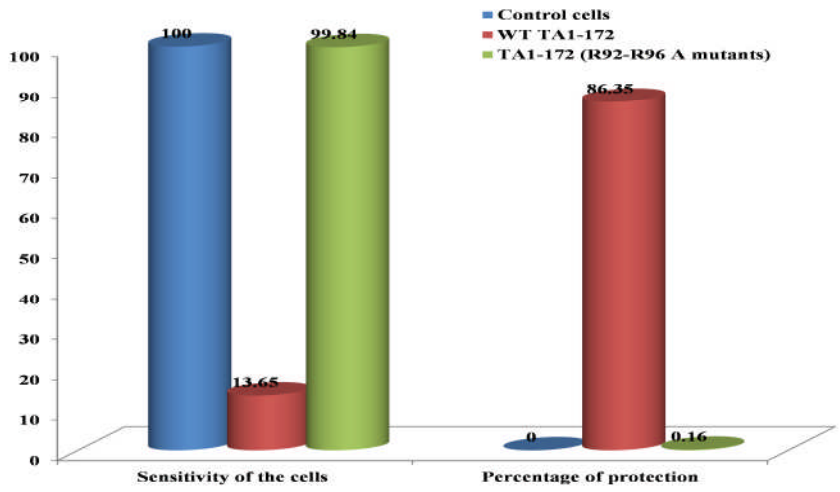


Figure 4-10: Detection of the effect of alanine mutants in TA_{1-172} on the interaction with Tol proteins. The *lux* reporter assay detected the *in vivo* protection conferred by expressing pFA18 compared to pFA4. (A) Relative luminescence units measuring the DNA damage caused by ColE9; the luminescence of both induced and uninduced cultures were compared for each construct. (B) Bar chart represented the percentage of protection (right side)

calculated from the RLU as an indicator of the colicin activity for different construct. Cells grown to mid-log and induced with 0.02 % L-arabinose for 3 h and then cells diluted 1:1 with minimal media were ColE9 added to a final concentration of 5 nM, the luminescence measured as DNA damage indicator.

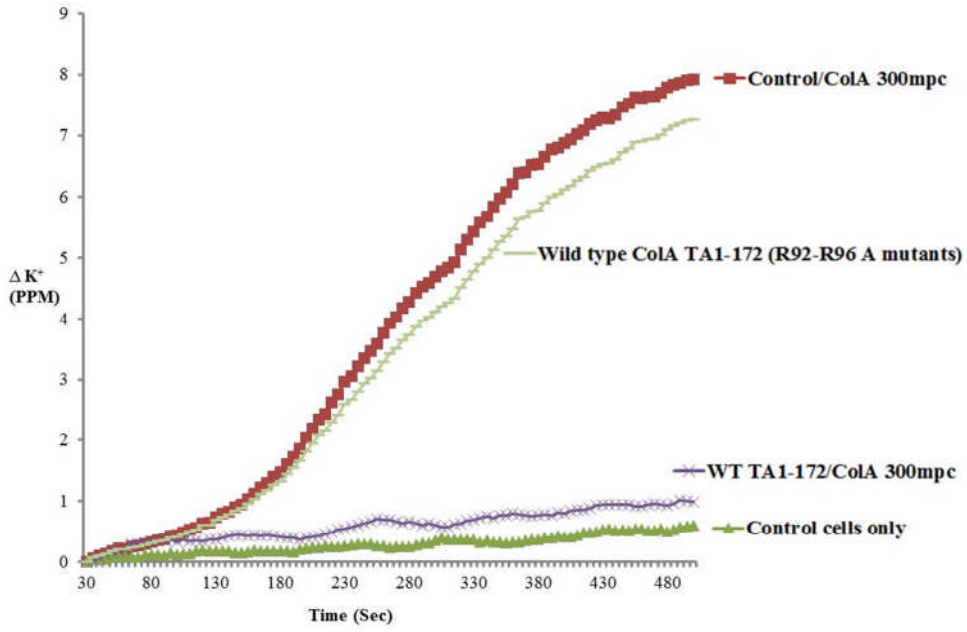


Figure 4-11: Detection of the effect of the mutated TA on the *in vivo* periplasmic protection using the potassium release assay. The activity of added ColA was detected by the amount of potassium released into the external media. The protection against ColA provided by the overexpressed WT TA (pFA4) was compared to that of the TA TolA box R62-969A mutant (pFA18).

4.4 Discussion

This chapter describes an investigation of the level of protection against external colicins conferred by the introduction of specific mutations in the expressed TA.

Pommier et al., (2005) suggested that residues Y58 and Y90 of two homologous regions of TA may be involved in TolA binding. The E18A mutant of ColA was shown to be inactive due to its inability to bind TolB, which is consistent with the E42A mutant in ColE9. The result of an *in vitro* spot test of the protection provided by either single mutations resulting in TAE18A, or TAY58A or by combining the two mutations against killing by the ColA and E9 is presented (Figure 4-3, Figure 4-6, Figure 4-9). In order to test the activity of the mutant proteins and the protection in a very sensitive and quantitative way, the *lux* reporter assays was used. The result of the *lux* reporter assay following treatment with ColE9 in the presence of periplasmic expressed proteins at a time point of 50 min, demonstrated that the induced protein prevented the translocation of ColE9 to around 78 % which was 10 % less than $TA_{1\text{-}172}$. The E18A mutant in the TolB box of ColE9 showed similar results to the non-mutated protein (Figure 4-2), signifying the tighter binding of the TolB box of ColE9 to the periplasmic TolB protein.

The TA protein appeared to interact with the Tol proteins and inhibit the translocation of ColE9, whilst mutated TA failed to show substantial protection against ColE9, in particular the TolA box mutant which was previously determined to abolish the activity of ColA in an *in vitro* assay (Journet, Bouveret et al. 2001; Pommier, Gavioli et al. 2005). This result supports the previous finding of the importance of the TolA Tyrosine residue

at position 58, by showing that the *in vivo* interaction between TolA and the Tol protein is affected when this residue is mutated to alanine. These results indicate the significance of the Tyr58 residue for TolA binding, although lower activity was detected *in vivo* as compared with the reporter assay, which is a very sensitive assay. A 10-15 fold lower colicin activity was seen with the TAY58A mutant in the *in vivo* assay while the reporter assay showed around 45 fold reduced activity (Figure 4-5).

Combined mutants in both the TolA and TolB boxes showed a reduced effect of the level of protection (Figure 4-8), although, the single TolB box mutant does not show a noticeable effect individually (Figure 4-2). This could be explained by the presence of the two mutations leading to conformational changes that might result in a reduced ability to interact with the Tol proteins in the periplasm, allowing the colicin to be translocated to its target and induce killing.

The investigation was extended to study the effects of TolA box mutants of the five residues responsible for the interaction with the TolAIII domain of TolA. The level of protection against colicin killing was determined using both the potassium release assay (Figure 4-11) and the *lux* reporter assay (Figure 4-10). The luminescence reporter assay using TA containing the R62-69A mutations demonstrated a substantial loss of protection against both externally added ColA and ColE9. The diminished protection was presumably the result of the loss of the interaction between the mutant TA and the TolAIII protein, allowing the translocation of externally added colicins that show complete activity

CHAPTER FIVE

5 Development of a real time fluorescence assay to study immunity protein release from the nuclease colicin-immunity protein complex

5.1 Introduction

It is known that ColE9 (60 kDa), upon synthesis, forms a high affinity interaction with its cognate immunity protein, Im9 (~10 kDa), which is encoded by the same colicin operon. The formed heterodimeric complex protects the producing cells against DNA damage and potential suicide prior to releasing the complex in the surrounding environment. An additional function of the immunity protein in protecting the colicin against proteolytic cleavage at the cell surface during its import has been suggested (Duche, Issouf et al. 2009). The nature of the complex formation between ColE9 and its cognate Im9 protein and other colicin-immunity complexes has been well characterized, and, in the case of ColE9/Im9, a very high affinity interaction has been revealed under physiological conditions, with a dissociation constant of the order of 10^{-14} m (Wallis, Leung et al. 1995). In spite of the high avidity of such interaction, ColE9 DNase domain seems to have only a marginally stabilizing effect on Im9 (Hann, Kirkpatrick et al. 2007).

Considerable progress is being made to unravel the early events that take place after receptor binding, where it has been shown that the ColE9 NDR (natively disordered region) enters the periplasm through the OmpF lumen where it interacts with TolB, possibly displacing it from its interaction with Pal (Housden, Loftus et al. 2005; Bonsor, Grishkovskaya et al. 2007; Yamashita, Zhalnina et al. 2008). It was also demonstrated that the receptor binding and translocation domains remain in contact with their binding partners in the outer membrane and the periplasm, respectively, when the DNase domain gains access to its target (Duche 2007). In contrast, the molecular mechanisms that govern the loss of the immunity protein from the colicin complex during cell

entry of the DNase domain are incompletely documented. Investigations of immunity protein loss from the colicin E2-Im2 complex concluded that receptor binding alone does not lead to immunity protein release and that a functional *tol* translocation complex is required to launch immunity protein release (Duche, Frenkian et al. 2006).

Because of the strength of the interaction between the colicin and its cognate immunity protein, removal of the immunity protein from the complex requires a considerable amount of energy. Recent work in our lab has shown a direct energy requirement for immunity protein release from the nuclease ColE9-Im9 complex upon cell entry which assigned an essential role of TolA and TolB in this process (Vankemmelbeke, Zhang et al. 2009). The presented data demonstrated a role for the individual Tol proteins and addressed the issue of energy requirements for immunity protein release. A disulfide-"locked" colicin construct and domain deletion mutants were used to show that entry of the N-terminal 80 residues disulphide of the colicin translocation domain and its interaction with TolB are critical factors for immunity protein release. The engineered disulfide-locked ColE9, BH29, was developed previously in our lab wherein a pair of cysteine mutations introduced into the receptor binding domain of ColE9 (Y324C and L447C) lead to the formation of a bond located near the top of the R domain. After DTT reduction, this disulfide-locked ColE9 was as active as the WT ColE9 (Penfold, Healy et al. 2004). On oxidation with diamide, no biological activity was seen with BH29 due to formation of the disulphide-lock; however diamide had no effect on the activity of ColE9 itself. The loss of biological activity of the disulfide-containing mutant colicin was not due to an indirect effect on the properties of the translocation or the DNase

domains of the mutant colicins. The data was consistent with a requirement for flexibility and unfolding of the coiled-coil R domain after binding to the OM BtuB receptor (Penfold, Healy et al. 2004).

BH29 is a perfect tool to synchronize cell killing caused by ColE9, because it is easy to initiate the conversion of the inactive oxidized form of ColE9 to the active reduced form by the addition of DTT which can be controlled and clearly defined. In order to study the molecular requirements for immunity protein release a rapid, sensitive and constant real-time fluorimetric assay to study the release of immunity protein from colicin complex is required. Such an assay will help in increasing our knowledge about the uptake process of this antibiotic toxin. The assay is based on the principles of fluorescence resonance energy transfer (FRET) (Patterson, Piston et al. 2000; Patterson, Day et al. 2001). The energy transfer which happens when the fluorescence donor comes in close contact with the fluorescence acceptor results in a quenching of the fluorescence (Figure 5-1). In order to apply this, both the ColE9 DNase and Im9 proteins were labelled with fluorescent dyes such that the immunity protein acts as the fluorescent acceptor and the ColE9 DNase the fluorecent donor. Both free proteins will be fluorescent when they are some distance apart, but when complexed together there will be quenching of the fluorescence (Zakharov, Sharma et al. 2008; Vankemmelbeke, Zhang et al. 2009). *In vivo*, upon release of the immunity protein after cell binding of the fluorescent colicin complex, an increase in donor (colicin) fluorescence should be observed as a result of the release of the quencher Im9. FRET is a useful tool to quantify molecular dynamics such as protein-protein interactions, and

hopefully can aid the study of both colicin uptake and immunity protein release.

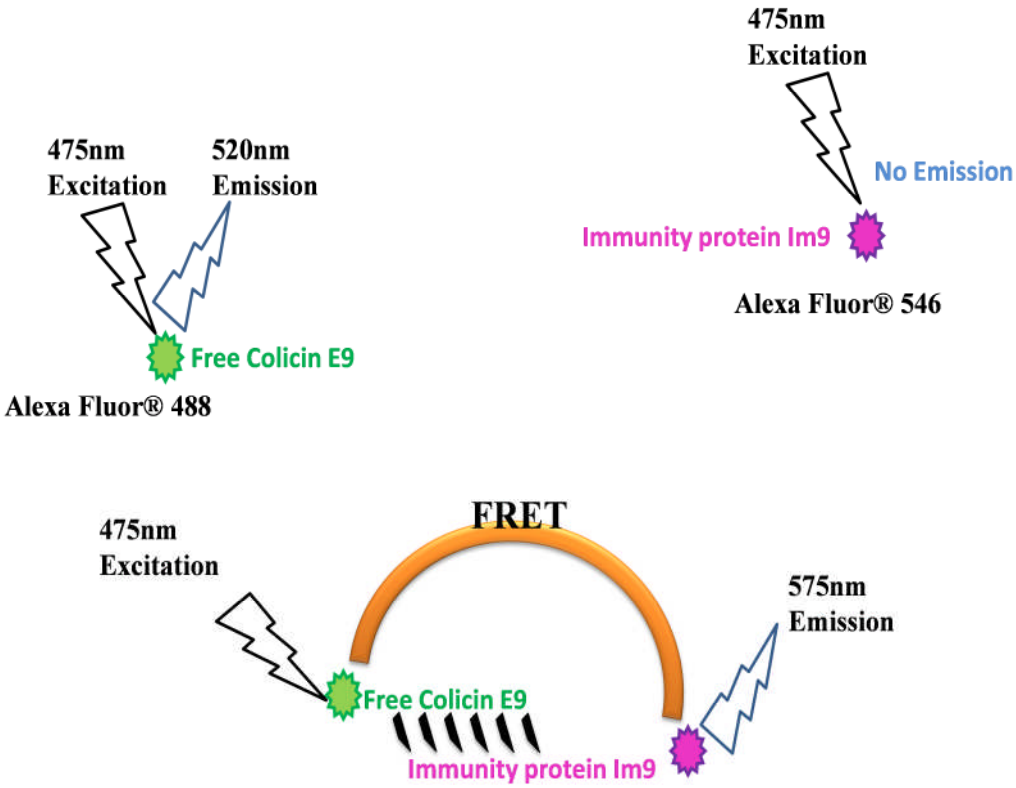


Figure 5-1: Schematic representation of FRET. FRET occurs between acceptor and donor fluorescently labelled proteins when the two proteins are in close proximity. The cartoon is adopted from Wikipedia.

Upon complex formation, quenching of the donor fluorescence is generated, however, release of the acceptor protein from the complex would allow the donor fluorescence signal to appear once again. Applying the assay on cells treated with labelled complex would provide more information about the dynamics of immunity protein release.

5.2 Materials and methods

5.2.1 Protein labelling

5.2.1.1 Protein Thiol content assessment: Ellman's test (5, 5'-dithiobis-2-nitrobenzoic acid or DTNB)

Spectrophotometric assay of the thiol content was carried out using Ellman's reagent (Hermanson 1996), that was incubated to a final concentration of 10 μM with serially diluted cysteine solution: 0, 10, 20, 40, 80 and 100 μM in 20 mM Tris HCl [pH 8.0]. The samples were quantified using a Nanodrop ND-100 spectrophotometer by measuring the absorbance of visible light at 412 nm. The concentration was plotted against the OD_{412} readings, and the thiol concentration deduced, according. Alternatively the thiol concentration was calculated using an extinction coefficient of 14,150 M^{-1} cm^{-1} for TNB in dilute buffer solutions.

5.2.1.2 Labelling of FA3 protein

FA3 protein ($ColE9^{s-s}$ with Cys at 469) was dialyzed against PBS to remove DTT and its concentration was then assayed using an ND-100 spectrophotometer using the molar extinction coefficient of 47440 moL^{-1} cm^{-1}. Incubation with a 25-fold molar excess of Alexa-Fluor 488-C5-maleimide was carried out in the dark for 2 h at room temperature, followed by extensive dialysis against PBS to remove the excess of the labelling dye. The extent of labelling was then checked using (εAlexa Fluor 488 = 77100 cm^{-1} M^{-1}) and OD280 (εFA3 = 47440 cm^{-1} M^{-1}, pre-labelling).

5.2.1.3 Labelling of Immunity protein

MV16 protein (Im9 with C23S/S6C for labelling) was unfolded in 3 M Gn-HCl/PBS and then incubated with a 25-fold molar excess of Alexa-Fluor 546-labeled C5-maleimide. Incubation was carried out in dark for 2 h at room temperature, followed by extensive dialysis against PBS to remove the excess labelling dye. The extent of labelling was then checked via comparison to the unlabeled protein at OD557 (εAlexa Fluor 546 = 111000 cm^{-1} M^{-1}) and OD280 (εIm9 (C23S/S6C) = 9,970 cm^{-1} M^{-1}, prelabeling).

5.2.1.4 Diamide oxidation

Labelled FA3 protein samples were dialyzed overnight against phosphate-buffered saline (pH 7.4; PBS) to remove the dithiothreitol (DTT). Samples were incubated with 1 mM diamide (N,N,N',N'-tetramethylazodicarbamide) for 30 min at ambient temperature before extensive dialysis against PBS. The amount of dialyzed protein was quantified on a NanoDrop ND-1000 spectrophotometer by measuring absorbance at 260 nm.

All labelled samples were handled with additional precautions and covered with aluminium foil continually.

5.2.2 Characterization of the protein post labelling

5.2.2.1 *In vitro* monitoring of double strand cleavage

The assay was performed in 50 mM Tris HCl pH 8.0 buffer with 40 mM $MgCl_2$. The reaction was started by the addition of ~200 ng per assay of linearized DNA (*NdeI*-digested pUC18) to labelled or unlabelled, free or complex protein. After incubation for 1 h at 37 ºC the reaction was stopped by adding loading buffer, samples were then loaded and run in a 1 % ($^{w}/_{v}$) agarose

gel with a 1 kb ladder. A negative control was also included containing DNA alone. The amount of protein in the assay was about 750 ng (Wallis, Reilly et al. 1992).

5.2.2.2 DNase activity assessed by FRET assay

A double stranded FRET DNA substrate was generated by annealing two fluorescently labelled primers: FAM (5'-CCACAGGTAGCGACAG-3') and TAMRA (3'-GGTGTCCATCGCTGTC-5') (Shi, Chak et al. 2005). After mixing the complementary oligonucleotides together at a 1:1 molar ratio, and diluting to a final concentration of 1 pmol/µl in 10 mM Tris [pH 8.0], 1 mM EDTA and 50 mM NaCl, the two primers were annealed using the thermocycler program for the simple protocol which includes a denaturation step at 95 °C for 5 min followed by 70 cycles of annealing at 95 °C for 1 min. Cleavage of the generated substrate was followed either via conventional digestion and gel electrophoresis or in real-time by the FRET properties of the substrate. The assay was set up using varied concentration of the wild type free ColE9 and free FA3, with or without cognate Im9 (2 nM).

Protein samples were subsequently mixed with the DNA FRET substrate using 10 mM Tris [pH 8.0] and 8 mM $MgCl_2$ buffer, incubated at 25 °C for 30 min-1 h, and analysed in a 15 % non-denaturing polyacrylamide gel electrophoresis. For the real-time analysis, enhanced fluorescence, as a result of cleaving the fluorogenic DNA substrate, was monitored for 1200 sec (with 10 sec intervals) at 516 nm after excitation at 486 nm using an LS 55 luminescence spectrometer (Perkin Elmer) at 25 °C.

5.2.2.3 15 % non-denaturing polyacrylamide gel

A non-denaturing polyacrylamide gel for electrophoresis of small DNA fragments was prepared using 40 % PAGE (19:1 acrylamide: Bisacrylamide) mixed with 10 x TBE buffer (108 g Tris, 55 g boric acid and 40 ml of 0.5 M EDTA), 10 % ($^{w}/_{v}$) APS TEMED and dH_2O.

5.2.2.4 *In vitro* endonuclease activity assay by spot test

The cytotoxic activity of the mutant protein was determined by spotting 2 µl samples of purified ColE9 protein on an LB plate supplemented with 30 $\mu g ml^{-1}$ of chloramphenicol overlaid with a soft agar lawn of the sensitive indicator strain *E. coli* DPD1718 cells that were grown from an overnight culture to mid-log (0.4-0.6 OD_{600}). Lysis of bacterial cells was detected as a clear zone in a lawn of sensitive bacteria.

5.2.3 FRET measurements

5.2.3.1 Fluorescence spectra

Fluorescence emission spectra were determined using a LS 55 luminescence spectrometer (Perkin Elmer), at 20 °C and 1cm quartz fluorimeter cuvette. Excitation-emission spectra of Alexa-Fluor 488 labelled colicinE9 protein in complex with Alexa-Fluor 546 labelled MV16 was measured at 475 nm and the emission spectrum recorded from 480-600 nm (with band width for both excitation and emission = 5 nm).

5.2.3.2 *In vitro* FRET assay: Immunity protein release assay

This was applied using LS 55 luminescence spectrometer (Perkin Elmer), The release of Im9 from the ColE9-Im9 complex (≈175 ng) was monitored in 100 µl (using 1cm quartz fluorimeter cuvette) of 3 M Guanidin-HCl /phosphate-

buffered saline at 20 °C. The fluorescence yield was measured at 517 nm with excitation at 475 nm and the bandwidth for both excitation and emission was 5 nm.

5.2.3.3 *In vivo* FRET assay: Fluorescence cell based immunity release assay

E. coli LMGpAG1 cells from an overnight culture were grown to mid-log phase (OD_{600} of 0.4-0.6) in LB medium supplemented with 100 μgml^{-1} ampicillin. Oxidized, free Alexa-Fluor 488-labeled FA3 or FA3 pre-incubated with Alexa-Fluor 546-labelled MV16 was added to 1 ml of cells to a final concentration of 10 nM. The $FA3^{AF488}$ / $Im9^{AF546}$ complex was formed not long prior to the assay taking place by mixing the two labelled proteins at a 1:1 molar ratio and incubating for 1 h on ice. Receptor binding to *E. coli* cells was allowed for 7 min at 37 °C, after which the cells were spun for 2 min at 6,000 rpm, to remove unbound colicin, and then re-suspended in 100 µl of pre-warmed LB medium. At this stage the cell-bound relative fluorescence units (RFU) were measured, and then DTT to a final concentration of 2 mM was added; and the RFU of the cell pellet (resuspended in pre-warmed LB medium) wasmeasured in 96-well plates (optical bottom) in triplicate by using a Victor2 1420 multilabel plate reader (Wallac) controlled by the Wallac 1420 software, using a filter with bandwidth of 25 nm.

5.2.3.4 Visualising binding of fluorophore labelled protein to bacteria

The ability of protein labelled with Alexa-Flour 488-C5-maleimide to bind to bacteria was assessed using fluorescence microscopy. A 10 ml overnight culture of *E. coli* LMGpAG1 was grown in LB broth at 37 ºC with shaking. The cells were diluted by taking 100 µl of the culture and adding to 900 µl of

sterile PBS. A 100 µl sample was removed from this and a 5 nM final concentration of labelled free ColE9 DNase protein was added and mixed by pipetting. The cells were incubated with the protein at 37 ºC for 5 min. To remove excess protein and protein aggregates, the cells were centrifuged at 4000 xg for 2 min and resuspended in 100 µl of fresh, sterile PBS. Finally, 20 µl of the washed cells were mounted on a standard microscope slide and covered with a glass cover slip.

5.2.3.5 Fluorescence Microscopy

Fluorescence microscopy were carried out on a Nikon Labophot-2 binocular microscope fitted with Olympus 40x PL, Olympus SPLAN 100x / 1.25 oil, Nikon Plan 40x / 0.7 DL and Nikon 100 x / 1.25 oil Ph4 DL objective lenses. A Nikon Super High Pressure Mercury Lamp (Model HB-10101AF) provided a high intensity UV light source while a Nikon EFD-3 epifluorescent microscopy filter was used to select the desired wavelengths of light for excitation of fluorophores. Images were captured using a Nikon Digital Eclipse DXM-1200 camera and viewed using the Nikon ACT-1 Digital Sight software package.

5.3 Results:

5.3.1 Construction of ColE9 mutant

A cysteine residue was introduced in the DNase domain of pBH29 in order to allow efficient labelling with the Alexa-Fluor maleimide dye. pBH29 was used as the template since this codes for ColE9 with a disulfide at the distal end of its receptor binding domain (Penfold, Healy et al. 2004). This disulfide is crucial for the synchronization of cell killing by ColE9.

5.3.1.1 Cloning and mutagenesis

In order to substitute one residue with another at a specific site in the ColE9 gene the technique site-directed mutagenesis was used. A cysteine residue at position 469 was generated to allow enhanced labelling of the protein with the selected fluorescent dye. In this approach, a primer (MV80) containing the desired mutation was used with the T7 terminator primer to create a PCR product of 800 bp (Figure 5-2) using pBH29 as the template. The 1st PCR product was used as mega-primer in a second stage PCR with pBH29 and T7 promoter as the reverse primer. The expected size of the second stage PCR product was approximately 2 kb (Figure 5-3).

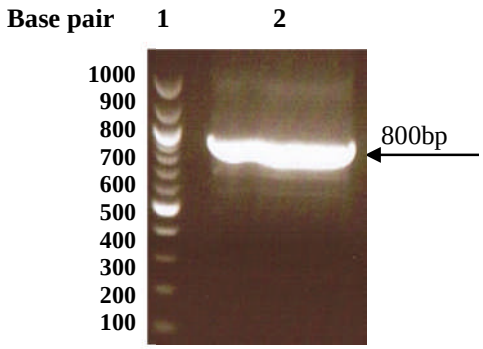


Figure 5-2: Gel electrophoresis of a polymerase chain reaction for generation of a 800 bp amplicon using *p*BH29 as a template. The 800 bp product (lane-2) (mega-primer) was subsequently used as primer for the 2nd PCR product. A 100 bp ladder (lane-1) was used as a marker (1000-100 bp).

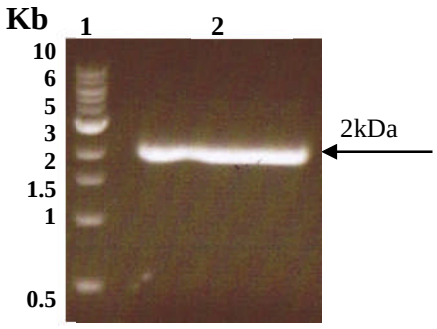


Figure 5-3: Gel electrophoresis of a polymerase chain reaction for generation of a 2 kb amplicon (lane-2) corresponding to the DNase ColE9-Im9 complex mutant using pBH29 as a template along with mega-primer. A 1 Kb ladder (lane-1) was used as a marker (10-0.5 kb).

Purification of the 2nd stage PCR product was carried out using a Qiagen purification kit, followed by double digestion using the restriction enzymes *NdeI* and *XhoI* of the PCR product and of pET21a vector (Figure 5-4). The digested PCR product was ligated into pET21a and transformed into *E. coli* DH5α. Four colonies were chosen randomly from the transformation plates. Liquid cultures of these colonies were prepared. The plasmid DNA was isolated, some of which was sent for sequencing after it was digested with *NdeI* and *XhoI* and run on 1 % agarose gel to confirm the presence of the cloned gene. Figure 5-5 shows clearly that the insert is present in all the samples along with the control. This result was further supported by sequencing which confirmed the presence of the required mutation at position 469.

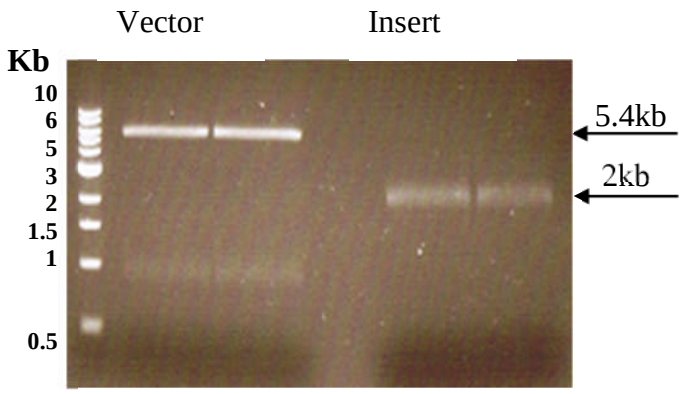


Figure 5-4: Gel electrophoresis of the double digested pET21a vector and PCR product insert with *Nde*I and *Xho*I. A 1Kb ladder was used as a marker (10-0.5 kb).

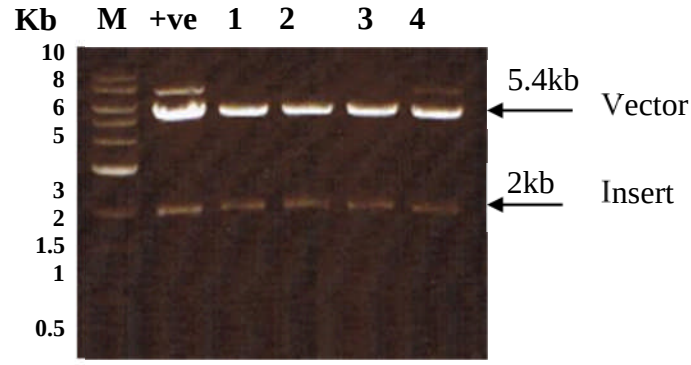


Figure 5-5: Gel electrophoresis of the double digested of purified DNA from the chosen transformed colonies with *Nde*I and *Xho*I. A 1Kb ladder was used as a marker (10-0.5 kb).

5.3.1.2 Protein expression

The constructed mutated ColE9, named pFA3, sequence was confirmed by sequencing. pFA3 was then transformed into *E. coli* BL21 (DE3) for expression and production of protein. A selected colony was grown overnight and subsequently used to grow to mid-log at 37°C before induction with 1mM IPTG at 30°C. Prior to carrying out purification, a small aliquot was checked on SDS-PAGE for protein production compared with the uninduced cells that were used as a mutant control (Figure 5-6)

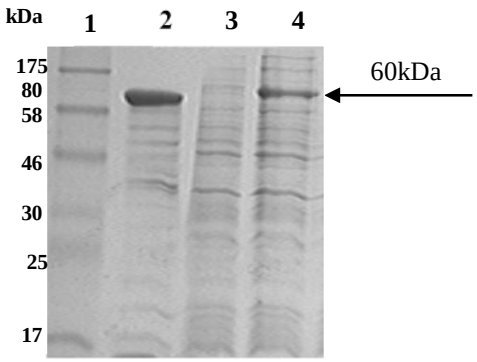


Figure 5-6: Expression of the mutant ColE9/Im9 complex from pFA3. SDS-PAGE of the recombinant expressed protein (lane-4, 60kDa protein) was used to compare to the uninduced culture (lane-3) and the wild type ColE9-Im9 as a control (lane-2), while (lane-1) contained a (7-175 kDa) protein marker.

5.3.2 Preparation of free ColE9 and Im9 proteins for labelling

The mutant ColE9 protein encoded by pFA3 (protein FA3) was expressed in a large scale in *E. coli* host strains, using 1 mM IPTG as the inducer.

5.3.2.1 FA3 purification

The protein was purified from harvested cells using nickel-affinity chromatography (as detailed in section 2.7.1). The purified fractions were dialysed in potassium phosphate buffer, pH 7.4 and analysed by SDS-PAGE (Figure 5-7 and Figure 5-8).

5.3.2.2 Purification of Im9

A previous clone that encoding the cognate immunity protein of ColE9 (named pMV16 (Vankemmelbeke, Zhang et al. 2009)) was used to overexpress the Im9 protein to be purified for labelling. Nickel-affinity chromatography was carried out for Im9. The selected fractions from the UV traces was analysed by SDS-PAGE for the expected Im9 (Figure 5-9 and Figure 5-10).

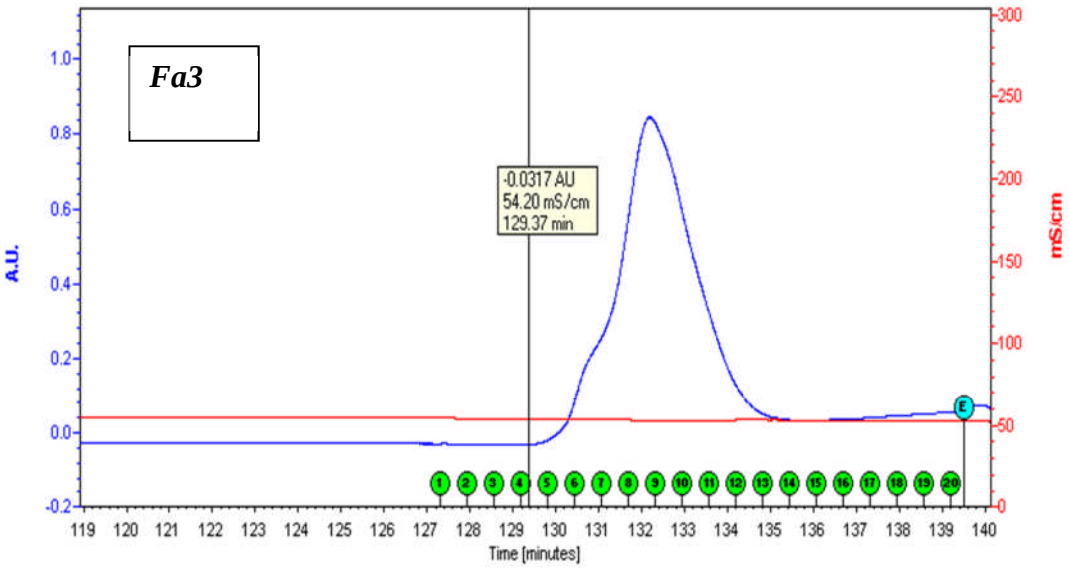


Figure 5-7: UV trace taken during his-tag purification of ColE9-Im9 complex. The sharp peak centred at approximately 132 minutes is the ColE9/Im9 complex. Fractions from 5-13 were collected to analyse by SDS-PAGE, see (Figure 5-8).

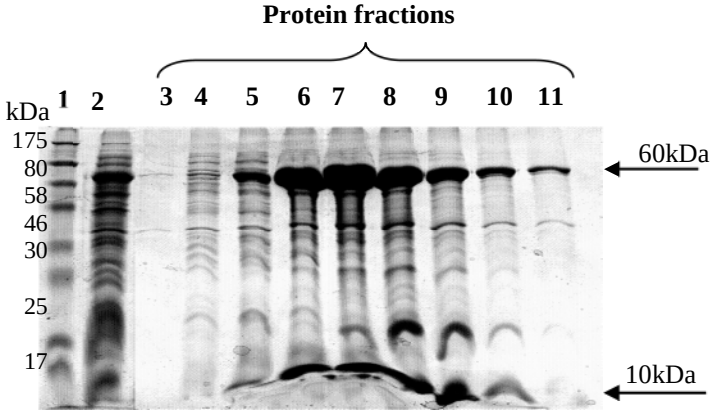


Figure 5-8: Coomassie-stained 16 % SDS-PAGE gels showing purified ColE9 protein. *E. coli* BL21 (DE3) contains plasmid pFA3 that was induced with IPTG, lysed, soluble proteins were applied to nickel-affinity columns and the selected fractions were loaded on a 16 % SDS-PAGE gel using (7-175 kDa) marker.

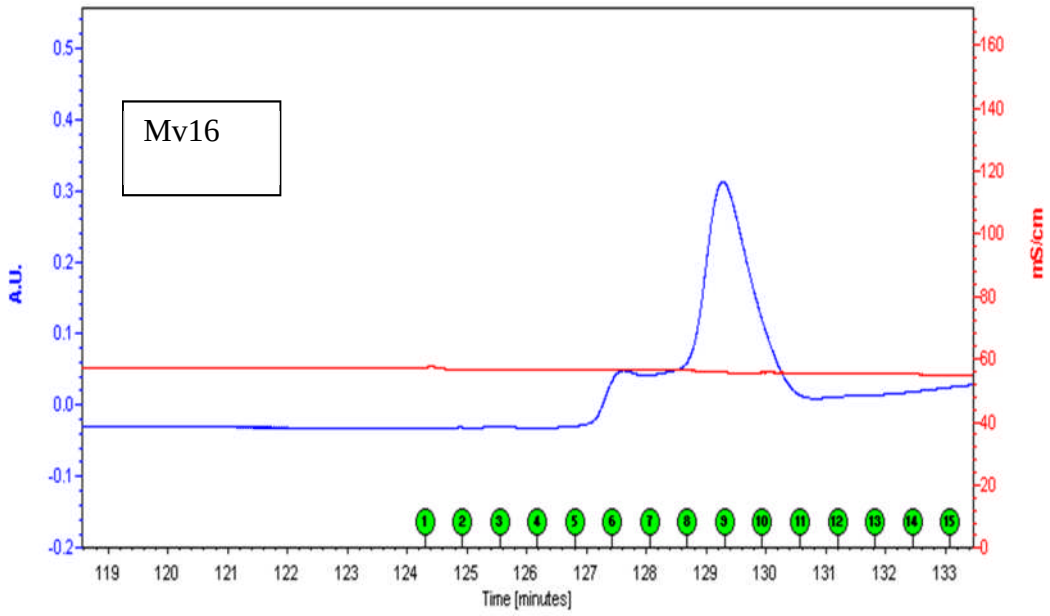


Figure 5-9: UV trace taken during his-tag purification of Im9 protein. The sharp peak centred at approximately 130 min is the Im9 protein (as fractions 5-12 verified by SDS-PAGE see below Figure).

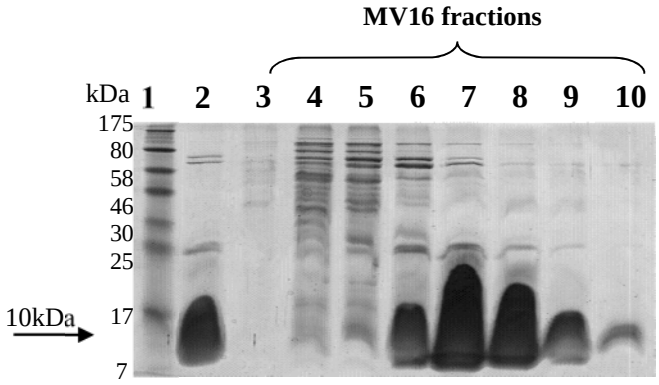


Figure 5-10: Coomassie-stained 16 % SDS-PAGE gel of fractions from the nickel-affinity column of purified Im9 protein. The predominant protein mastering at 10 kDa as expected for Im9 is indicated by the arrow. Protein marker (7-175 kDa) is shown in lane 1.

5.3.2.3 ColE9 Complex separation and Purification

The use of a his-tag (the polyhistidine tag is used for protein purification by metal affinity chromatography and for immunodetection) as the initial purification step has thus far provided initial separation of the ColE9/Im9 complex from other cellular proteins. The purified ColE9/Im9 complex was subjected to further purification using gel filtration to strip the Im9 (detailed in section 2.7.2) (Figure 5-11). This was carried out to separate the ColE9 from its immunity protein to allow labelling each protein separately to permit studying the immunity protein release. Fractions related to the ColE9 protein peak after stripping the immunity protein were checked on an SDS-PAGE (Figure 5-12), which showed a pure band at 60 kDa representing the ColE9 and the band that represent the Im9 protein was not present indicating a successful stripping of the colicin complex.

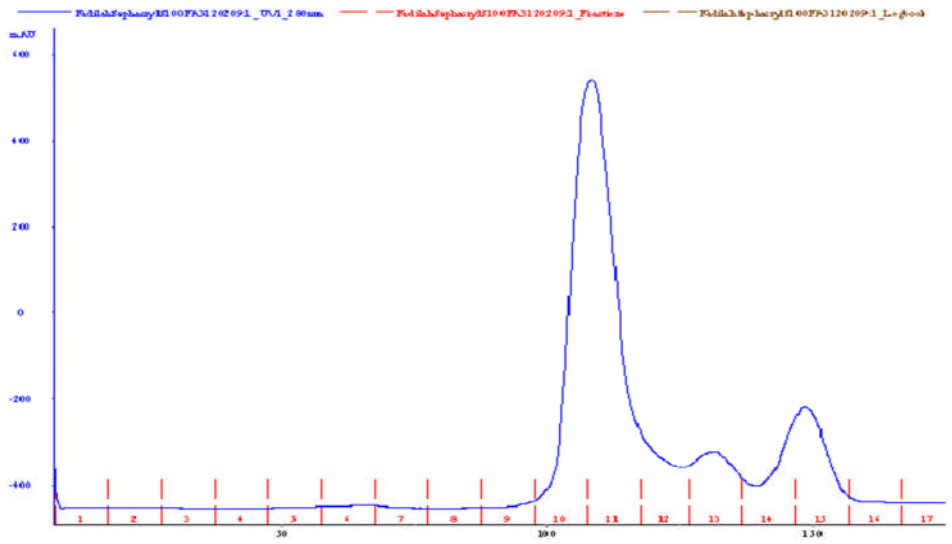


Figure 5-11: UV trace taken during size exclusion chromatography of the ColE9/Im9 complex. The sharp peak centred at approximately 110 ml is the ColE9 protein after stripping Im9 protein (as verified by SDS-PAGE, see below). The small peak centred at approximately 150 ml is the stripped Im9.

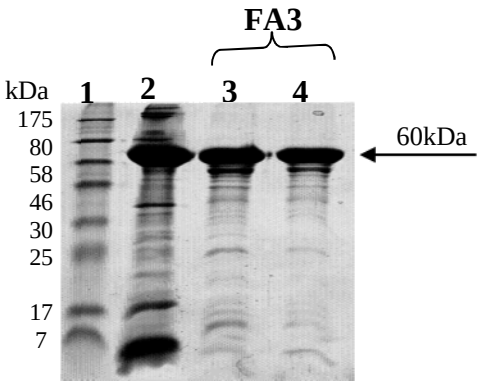


Figure 5-12: Coomassie-stained 16 %SDS-PAGE gels showing purified ColE9 mutant protein after gel filtration to allow stripping of Im9 (lane 1 = protein markers (7-175 kDa), lane 2 = ColE9/Im9 complex after his-tag purification, lanes 3 and 4 = elution fractions corresponding to ColE9 peak seen at approximately 110 mL elution from Figure 5-11.

5.3.3 Characterization of purified protein

5.3.3.1 Determination of Thiol group content by Ellman's assay

Thiol content determination was carried out prior to labelling the protein in order to check whether the single cysteine in the DNase domain of FA3 would be accessible for labelling and also to evaluate whether the disulfide in the receptor binding domain is suitably oxidized so as not to interfere with the labelling. Measuring thiol groups on proteins was carried out using a chemical compound Ellman's reagent (5, 5'-dithiobis-(2-nitrobenzoic acid DTNB). The reaction between the reagent and a thiol group leads to the release of 2-nitro-5-thiobenzoate (TNB^-) that ionizes to the dianion TNB^-2 at neutral and alkaline pH, where this anion has a yellow colour. The addition of one mole of thiol to the reagent leads to release of one mole of TNB. The released TNB was quantified using a spectrophotometer by measuring the absorbance at 412 nm. Quantification of FA3 was performed by measuring the sample at OD_{412} (that was = 0.078) at the same time, and its thiol content was deduced from a standard curve of increasing cysteine-HCl concentrations (Figure 5-13). The protein thiol content was also calculated from OD_{280} readings (1.77 mg/ml) and εFA3 = 47440 cm^{-1} M^{-1} and Ellman's reagent extinction coefficient of 14,150 M^{-1} cm^{-1}. Both methods gave comparable results: 36.6 µM and 37 µM respectively.

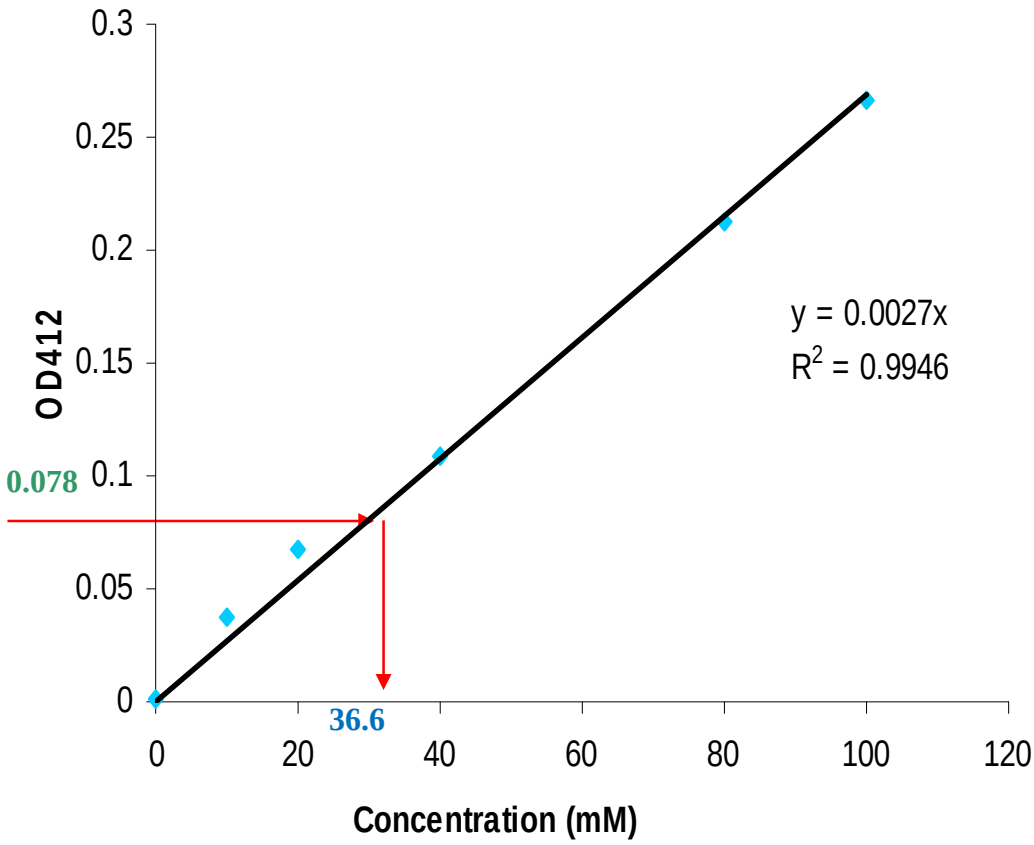


Figure 5-13: Thiol content determination. The OD_{412} readings were measured for serially diluted cysteine solutions mixed with Ellman's reagent (10 µM), and plotted against the cysteine concentrations. FA3's thiol content was deduced directly from the plot.

5.3.3.2 Checking the activity of purified FA3

In order to check whether the introduction of the cysteine in the DNase domain of FA3 had affected the cytotoxic activity, a spot test was performed. *In vitro* endonuclease activity of WT ColE9 and FA3 (5nM) was assessed using a spot test, represented as a clear zone on a lawn of *E. coli* DPD1718 which is sensitive to ColE9. Figure 5-14 (zone-A) represent the activity of FA3, compared to the wild-type ColE9 which used as control (zone-B).

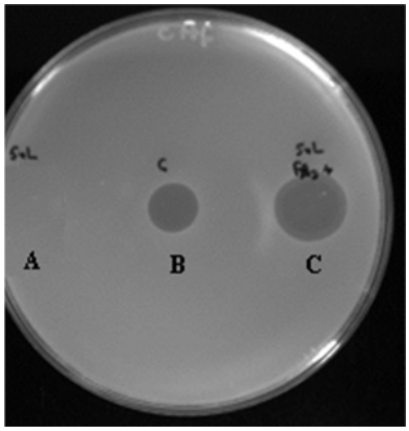


Figure 5-14: Results of a spot test performed with *E. coli* DPD1718 cells. ColE9 mutant protein activity was compared to results with wild-type ColE9. Zone C is 5 nM Protein FA3 and Zone B is 5 nM wild-type as a positive control and A is 5 µl of buffer used as negative control that has no killing activity. The clear zones indicate the cell death caused by the colicin.

5.3.4 Labelling and characterizing the fluorescent colicin

Once the mutated protein was purified and its activity checked primarily by spot test, it was subjected to labelling with fluorescent dye as detailed (in section 5.2.1). The labelled protein was first checked on SDS-PAGE and compared to un-labelled FA3, which indicated that FA3 is efficiently labelled and its activity is clearly not affected by the fluorescent label (Figure 5-15). In order to check that the labelling of FA3 had not unduly compromised its activity a number of *in vitro* and *in vivo* activity assays were carried out.

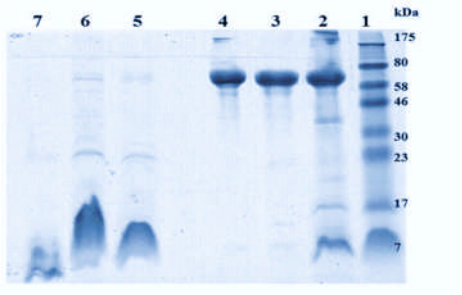


Figure 5-15: SDS-PAGE analysis checking labelled $FA3^{AF488}$ and $Im9^{AF546}$. In lanes 2 and 5 show expressed protein from an *E. coli* culture used as the source of both FA3 (60 kDa) and Im9 (10 kDa) respectively. In lanes 3 and 6 show unlabelled, purified proteins; in lanes 4 and 7 show labelled, purified $FA3^{AF488}$ and $Im9^{AF546}$. Lane 1 shows the protein marker (7-175 kDa).

5.3.4.1 Studying the association of Im9 protein to ColE9; *In vitro* assay

It is known that ColE9 non-specifically cleaves DNA yet the presence of the Im9 protein neutralizes the cytotoxic effect as it is tightly bound to it (Cao and Klebba 2002). The biological activity of the resulting $Im9^{AF546}$ was ascertained by monitoring the cleavage of double strand pUC18 DNA by FA3. Linearized pUC18 was used to test the *in vitro* ColE9 DNase activity of FA3 and the protective effect of immunity protein (Figure 5-16). Complete cleavage was observed after 1 h at 37 °C [pH 8.0] with free ColE9, Free purified FA3 (ColE9 Cys-469) and labelled $FA3^{AF488}$ as shown in Figure 5-16 lanes; 2, 3 and 6, respectively. The DNase activity of these free ColE9 proteins was abolished to a similar extent after addition of either Im9 or labelled $Im9^{AF546}$, which indicates that Im9 effectively bound (at 1:1 molar ratio) to the labelled ColE9 and protected the DNA substrate against its activity. This indicates that the fluorescenct label is not interfering with the protein-protein interaction between ColE9 and Im9.

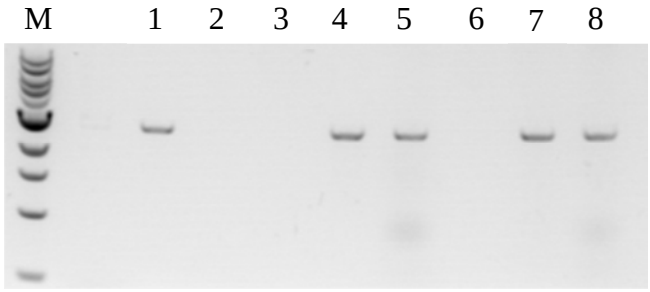


Figure 5-16: *In vitro* endonuclease assay that was inhibited by addition of Im9 to ColE9. Plasmid pUC18 (200 ng lane-1 as negative control) was incubated for 1 h at 37 °C with 750 ng of each protein. The digested DNA was resolved by agarose gel (1 %) electrophoresis. The gel lanes contained free ColE9 (lane-2); free unlabelled protein (lane-3), free ColE9 with unlabelled Im9 (lane-4), and with Alexa Fluor 546 labelled Im9 (lane-5); free Alexa Fluor 488 labelled ColE9 protein (lane-6); in complex with unlabelled Im9 (lane-7); and in complex with Alexa Fluor 546 labelled Im9 (lane-8). A 1Kb ladder was used as a marker (10-0.5 kb).

5.3.4.2 Validation of *in vitro* association of unbound ColE9 to purified Im9

To validate complex formation between $FA3^{AF488}$ and $Im9^{AF546}$, the association of unbound $FA3^{AF488}$ to the purified $Im9^{AF546}$ which was incubated for 30 min in phosphate buffer at a 1:1 molar ratio, was analysed using size-exclusion chromatography. Unbound and unlabelled FA3 (60 kDa) migrate as a single A_{280} peak that eluted at 8 ml. Unbound $FA3^{AF488}$ showed the same A_{280} peak and an additional A_{493} peak indicative of the Alexa 488 label. $FA3^{AF488}$ bound to $Im9^{AF546}$ also gave a similar trace, indicating that the protein is not affected after labelling and its association to $Im9^{AF546}$ was confirmed by the absorbance at A_{557} at the position where the colicin complex elutes, indicating the binding between both proteins (Figure 5-17).

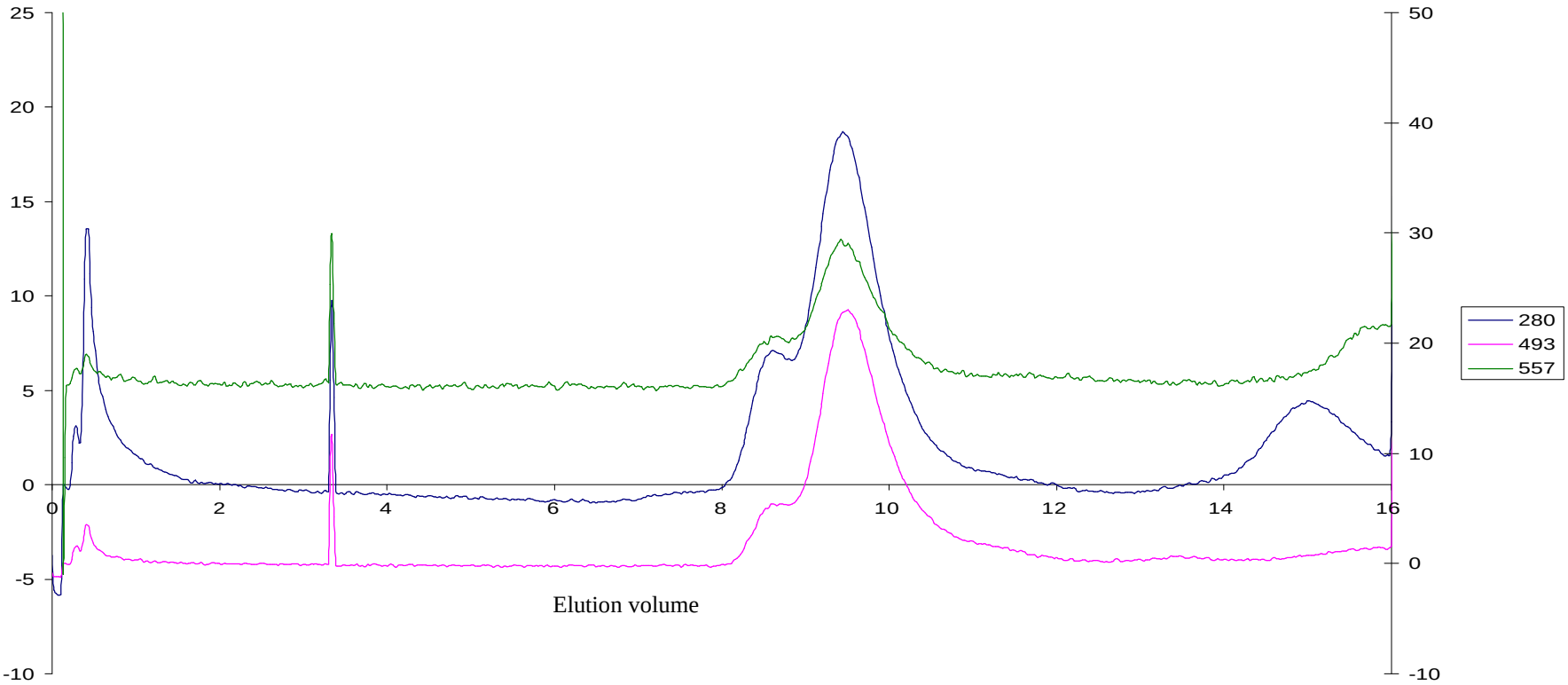


Figure 5-17: Gel-filtration chromatography to study ColE9 association with Im9. Physical association was observed using a Superdex 75 column and PBS at room temperature. ColE9 migrates as a single peak at an elution volume of 8 ml.

5.3.4.3 Complementation analysis of Im9 association and DNase activity by FRET

Of the FRET pairs available (Shi, Chak et al. 2005), FAM (6-carboxyl-fluorescein) and TAMRA (6-carboxyl-tetramethyl-rhodamine), were adopted in the FRET substrate for another sensitive real-time kinetic assay to study the DNase activity of un-labelled colicin constructs. The annealed oligonucleotide substrate was mixed with 2 nM ColE9 DNase or FA3 and compared to ColE7 as a control (Figure 5-18). Visualizing the gel under UV light showed the annealed oligonucleotide substrate while the ColE9 DNase and FA3 successfully cleaved it as compared to ColE7.

The excitation/emission spectra of the fluorogenic DNA substrate was determined (Figure 5-19). The activity of free FA3 was compared to ColE9/Im9 complex detected by this FRET assay (Figure 5-20). In the assay a fluorophore and quencher-labelled oligonucleotide used as the substrate showed no fluorescence emission as it was monitored with time. For a control to compare with Ala 575 mutants, an inactive ColE9, and WT free ColE9 or ColE9/Im9 complex were selected for the measurement of enzyme and cell killing activity. Cleavage of the fluorophore-labeled oligonucleotides by the DNase domains gave increasing fluorescence emission intensities. The measured intensities showed that free FA3 was active, compared to ColE9 wild-type enzyme activity, while the FA3/Im9 labelled complex showed no activity (compared to WT ColE9/Im9 complex) indicating a successful association of the FA3 with Im9.

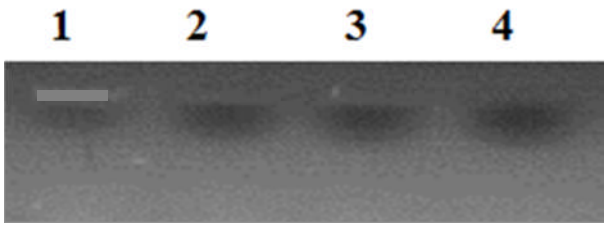


Figure 5-18: Gel electrophoresis of non-denaturing polyacrylamide visualized under UV light. Lane 1 show annealed oligonucleotide related to the DNA substrate only, lane 2 is mixed with 2 nM ColE7, lane 3 is mixed with 2 nM WT free ColE9 DNase and lane 4 is 2 nM FA3.

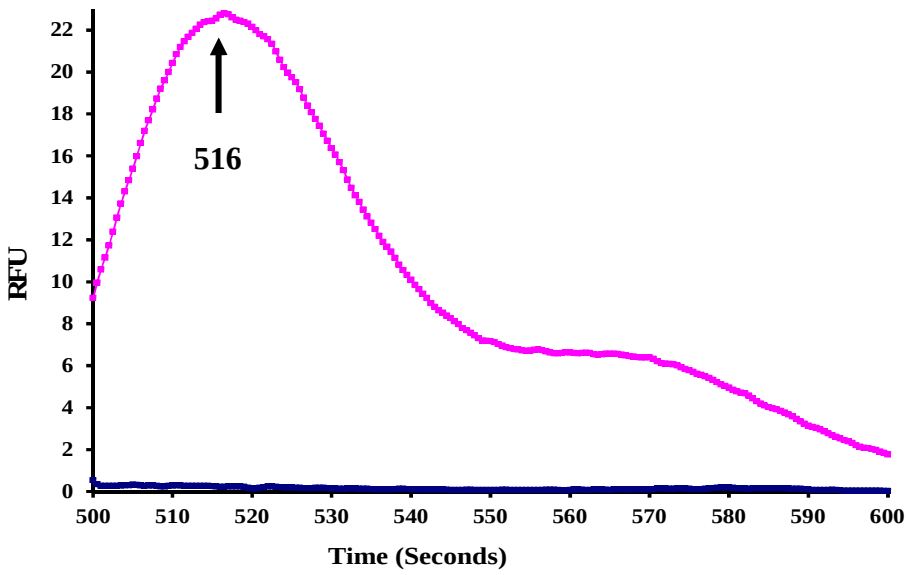


Figure 5-19: Emission spectra for the fluorogenic double-stranded DNA. The spectrum of the substrate was measured at 486 nm. Maximum fluorescence measurements were detected at 516 nm (pink line compared to the buffer only blue line). The excitation and emission band width were 5 nm.

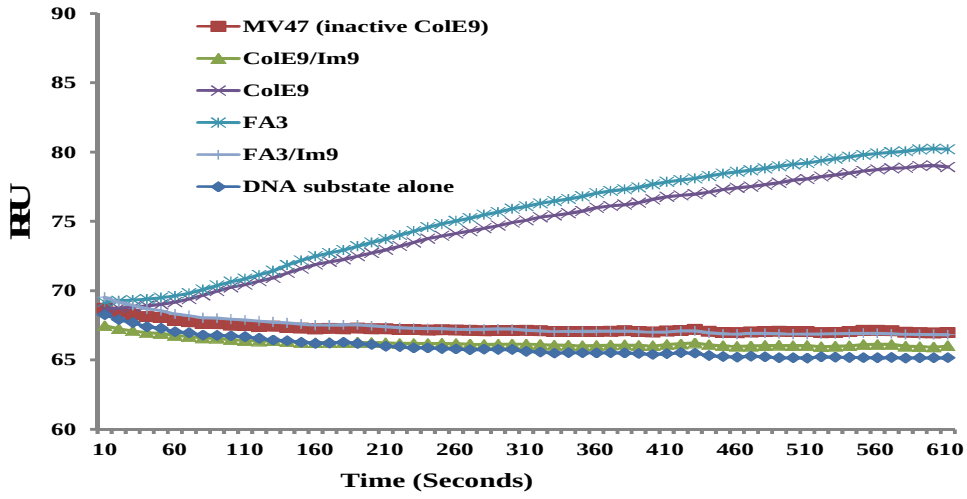


Figure 5-20: Monitoring DNA substrate cleavage by ColE9. 100nM DNA substrate was mixed with 10 nM of each protein construct. No fluorescence was detected by monitoring the DNA substrate alone (control).

5.3.4.4 *In vivo* ColE9 activity using the *lux* reporter assay

The *in vivo* cytotoxic activity of the ColE9 constructs was measured, using *E. coli* DPD1718 cells as a reporter strain, to ensure that labelling the proteins had not affected its cytotoxicity. Treatment of the reporter strain with either the free unlabelled FA3, unlabelled FA3 complex, or labelled free FA3 and labelled complex $FA3^{AF488}/Im9^{AF546}$ generated similar amounts of luminescence. Each protein sample was measured in the absence and presence of 2 mM DTT (Figure 5-21). DTT was added to reduce the disulphide bond and restore the colicin activity. Gamma values were calculated at around 45 min; where FA3 activity was similar to that of the wild type free ColE9 and the ColE9 complex; each of which showed around 60 % activity. The results indicate that labelling the proteins did not impede the *in vivo* activity and that the labelled protein is suitable for further *in vivo* assays.

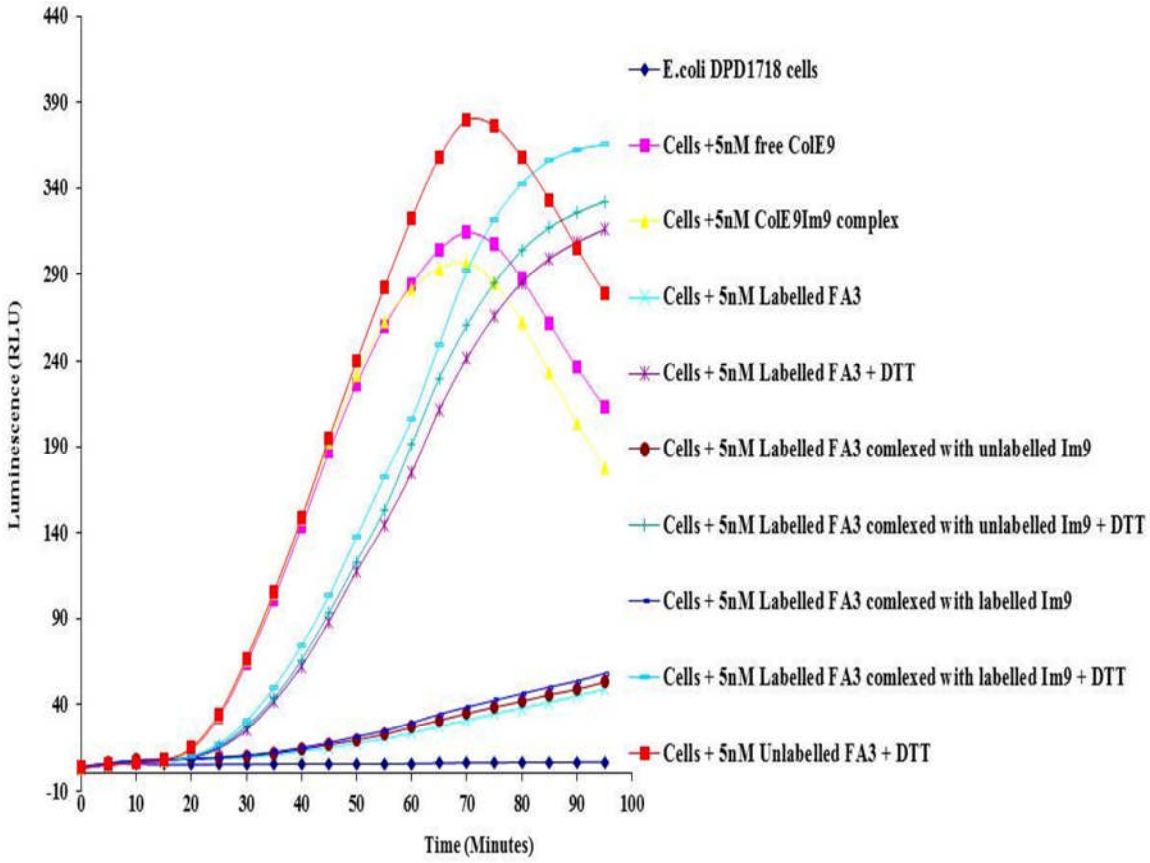


Figure 5-21: *Lux* reporter assay of the activity of the oxidized and reduced form of either labelled or unlabelled free FA3, or in complex with either unlabelled or labelled immunity proteins, compared to the activity of wild type ColE9. Gamma values were calculated at a time point of 50 min. The percentages of colicin activity were calculated from the gamma value of each time point.

5.3.5 FRET

5.3.5.1 Fluorescence measurement

Spectral characterization of the alexa-labelled constructs was carried out in order to determine the FRET efficiency upon complex formation. Alexa Fluor® 488 has an excitation maximum at 493 nm and an emission maximum at 515 nm. $FA3^{488AF}$ was incubated with $Im9^{546AF}$ at various molar ratios 1:1.15, 1:1.2 and the FRET efficiency, *E*, calculated from the equation $E=1-I_{DA}/I_D$, where I_{DA} is the intensity of the donor $ColE9^{488AF}$ in the presence of the acceptor $Im9^{546AF}$ while I_D is the intensity of the donor alone $ColE9^{488AF}$ (Figure 5-22). *E* was also calculated either in the presence or absence of DTT, but both values were comparable indicating that DTT has no effect and the different dilution ratios used showed almost similar efficiency indicating a saturation of the FRET complex (Table 5-1). Excitation of the $FA3^{AF488}$ alone at 475 nm showed a peak at 516 nm, the peak fluorescence signal at 516 nm reduced almost 50 % when the $FA3^{AF488}/Im9^{AF546}$ complex was used. A small peak shown at 575 nm represents the $Im9^{AF546}$ spectra when the complex is excited at 475 nm (as both donor and acceptor has the same excitation spectra).

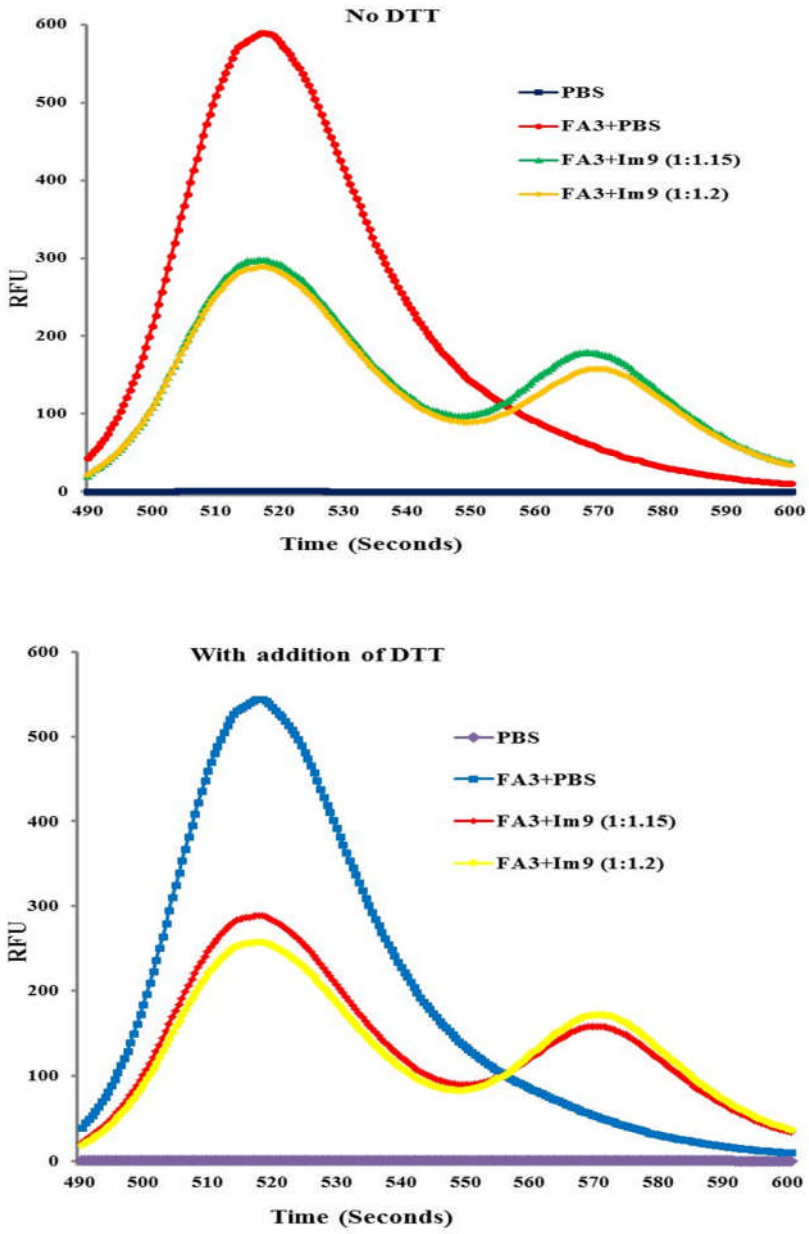


Figure 5-22: Excitation-emission spectra of $FA3^{AF488}$/ $Im9^{AF546}$. Excitation was carried at 475 nm and fluorescence emission was recorded between 490-600 nm in PBS. The excitation and emission band width were both 5 nm. The spectra of $FA3^{AF488}$ (176 nM) either alone or in complex with $Im9^{AF546}$ at different molar ratios (1:1.15) and (1:1.2), was used to calculate *E* in absence or presence of 2 mM DTT. PBS used as control. RFU= relative fluorescence units.

Table 5.1- Average percentage of quenching efficiency (%E)		
$FA3^{AF488}$: $Im9^{AF546}$ ratios	No DTT	With 2 mM DTT
1:1.15	64.94	63.53
1:1.2	64.23	63.2

5.3.5.2 *In vitro* monitoring of immunity protein release

Complex formation between donor and acceptor fluorophore molecules causes fluorescence quenching, yet the fluorescence was restored as the complex was dissociated. The release of $Im9^{546AF}$ from $FA3^{488AF}$ was monitored for 20min as the complex was denatured in 3 M Gn-HCl/ PBS at 20 °C. The measurements were done at 517 nm with excitation at 475 nm. Increased fluorescence at 517 nm was observed as a result of releasing $Im9^{546AF}$ from the complex with $FA3^{488AF}$, therefore the quenching effect of the acceptor was reduced and fluorescence intensity increased (Figure 5-23). The increase in the fluorescence was detected in the first three minutes then the fluorescence stabilized with time.

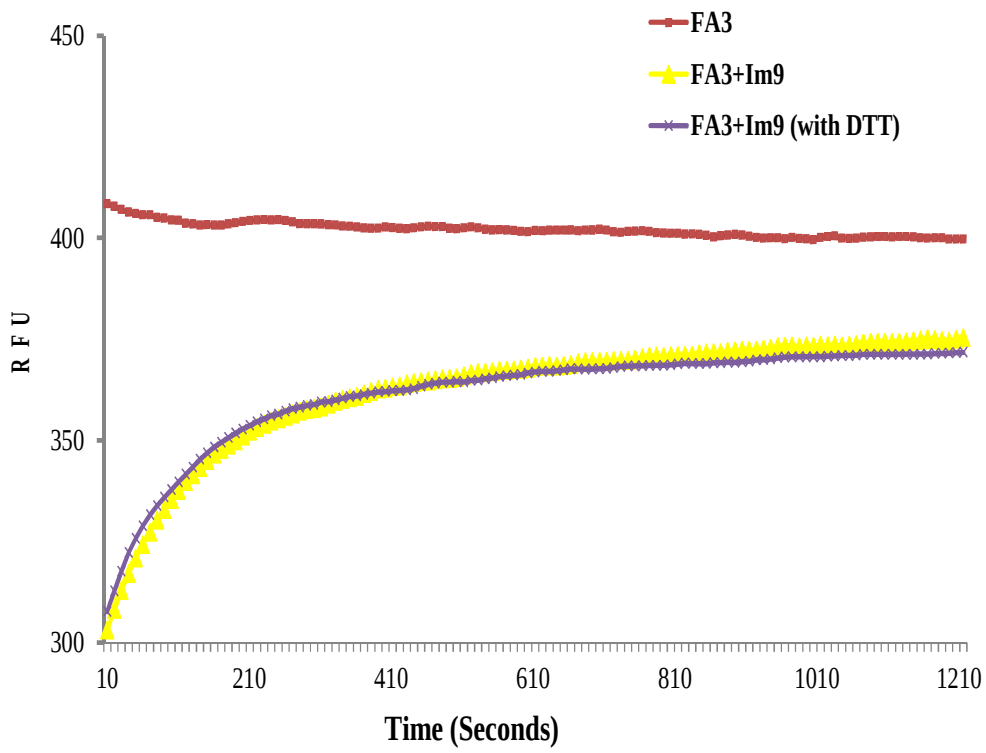


Figure 5-23: *In vitro* FRET assay for Im9 release. Fluorescence measured at 517 nm (excitation at 475 nm) resulted from releasing the acceptor ($Im9^{AF546}$) from donor ($FA3^{AF488}$). Monitoring was carried out in 100 µl Gn-HCl phosphate buffer using 750 ng of $FA3^{AF488}$/ $Im9^{AF546}$ complex. Fluorescence of donor $FA3^{AF488}$ alone (Red), was compared with the quenched $FA3^{AF488}$ after releasing the acceptor ($Im9^{AF546}$) (Yellow and Purple for without and with DTT, respectively).

5.3.5.3 Fluorescence cell based assay: Optimization of the assay

A cell based assay was used to study the dynamics of Im9 protein release during the translocation process of ColE9 cytotoxic domain to its site of action in the cells. The *E. coli* LMGpAG cells were mixed with 10nM of oxidized $FA3^{AF488}$ or $FA3^{AF488}/Im9^{AF546}$. An aliquot of the cells were fixed to a microscopic slide and observed to check for bound $FA3^{AF488}$ fluorescence (Figure 5-24), to ensure that the fluorescent protein is bound to the target cells. Optimization of the *in vivo* detection was carried out to optimise the fluorescence signal. Three different growth media were used (LB, Tris-HCl and Minimal media), Single readings were taken and compared to the readings of control cells. Although no difference regarding the fluorescence was observed between the three growth media used, the cells were growing well in LB media compared to their growth in other media. Although there was a high background with LB, which could be subtracted, it was the preferred media. Comparing the RFU detected from cell-bound $FA3^{AF488}$ from the 1:10 ratio, revealed a very weak fluorescence signal when compared to the cells alone. The ratio of cells was therefore increased to 1:1 in an attempt to enhance the fluorescence signal (Figure 5-25). Cell-bound relative fluorescence units were measured either in the presence or absence of DTT.

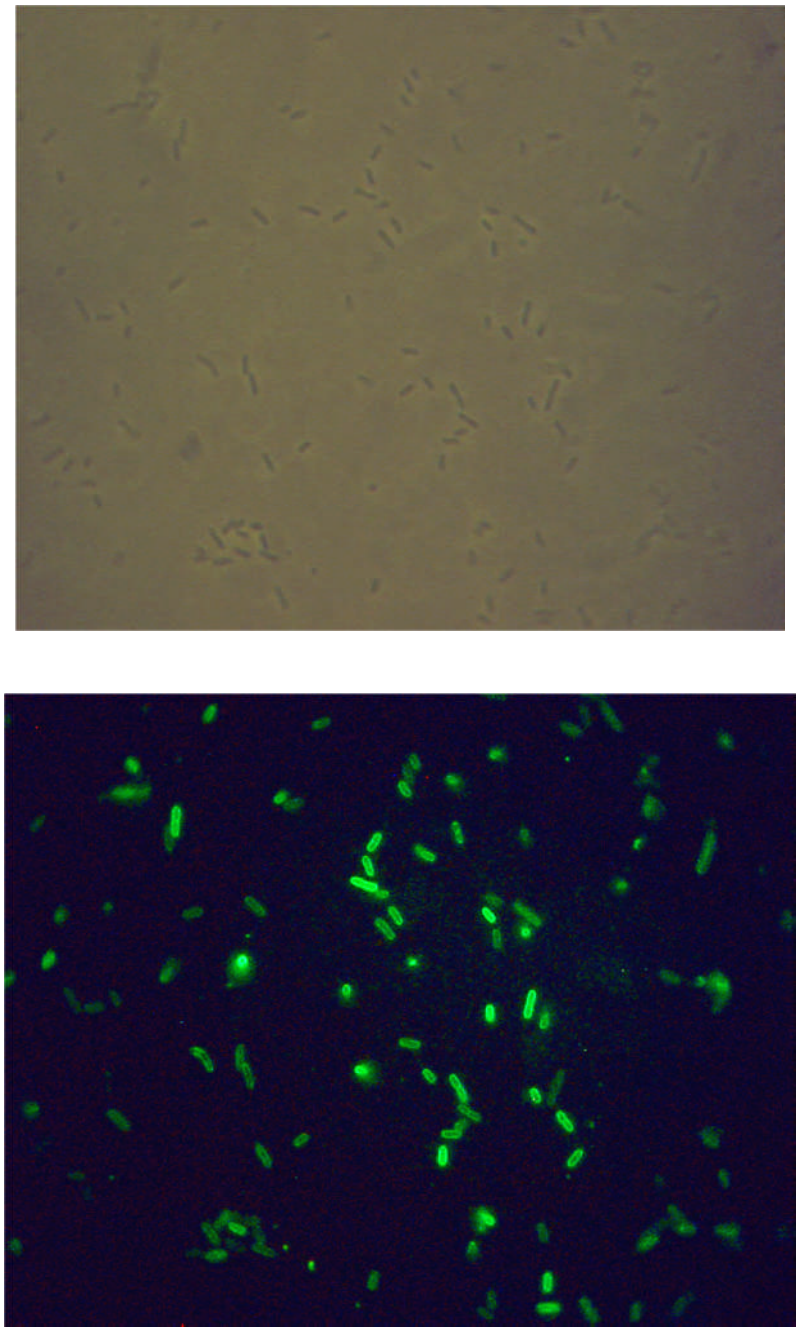


Figure 5-24: Microscopic detection of $FA3^{AF488}$ binding to cells of *E. coli* LMGpAG1. The microscopic images showed cell-bound $FA3^{AF488}$ (green fluorescent image at the bottom) and a phase contrast image of control cells at the top. *E. coli* LMGpAG1 cell were grown to mid log phase, incubated with $FA3^{AF488}$ for 7 min at 37 ˚C. Cells were then spread on a glass slide and analysed under blue light (excitation 450 nm-490 nm).

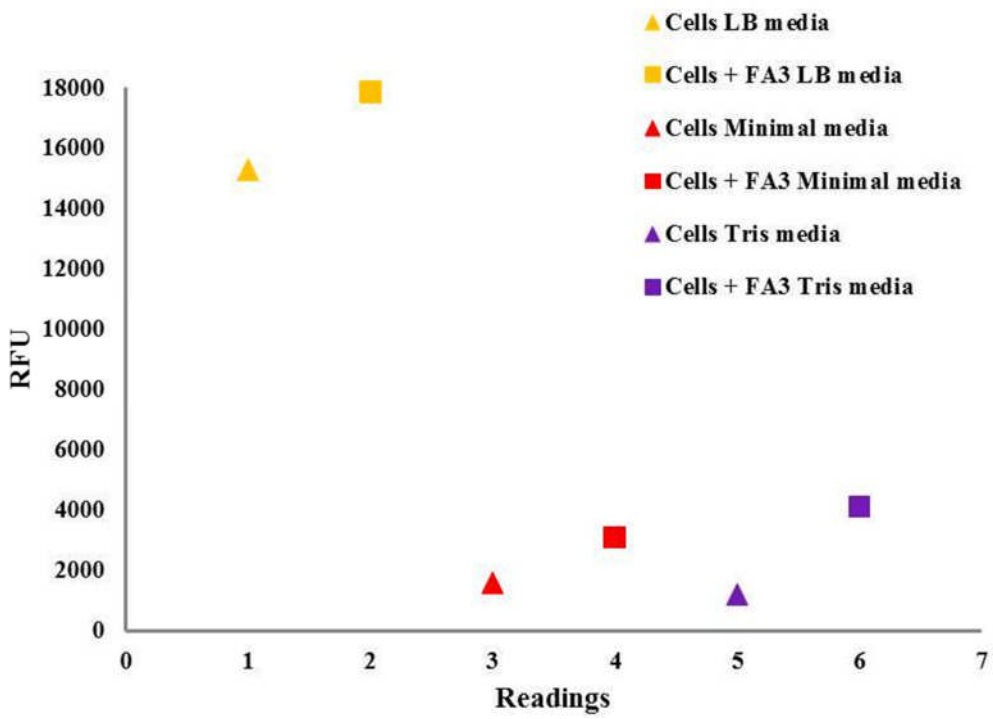


Figure 5-25: *In vivo* FRET assay. Optimization of fluorescence cell based assay using three different growth media. Cell-bound $FA3^{AF488}$ was diluted 1:10 in different growth media. Cells alone were used as a control. $FA3^{AF488}$ was added to a final concentration of 10 nM.

5.3.5.4 Fluorescence cell based assay: *In vivo* monitoring of Im9 release

Using the optimized conditions, a comparison was made of the fluorescence detected from the $FA3^{AF488}$ $Im9^{AF546}$ complex, $FA3^{AF488}$ and un-treated cells as a control. The background-subtracted release expressed as relative fluorescence units, were measured for $FA3^{AF488}$ alone and in complex with $Im9^{AF546}$. Continuous measurements of the fluorescence $FA3^{AF488}$ showed lower fluorescence signal compared to the $FA3^{AF488}$/$Im9^{AF546}$ complex although quenching was expected (data not shown). The results obtained from the assay were not satisfactory; therefore, a step back was essential to determine the reason for the problem with the assay.

Diamide oxidation was carried out to avoid the presence of any reduced protein and to ensure that inactive protein is used in the assay. The results were compared to those obtained with the same protein samples prior to diamide oxidation (Figure 5-26, A and B). A higher fluorescent signal was obtained after diamine oxidation indicating that some protein was in a reduced state and biologically active. To ensure that the inability to observe any quenching is not caused by the loss of the fluorescently labelled colicin complex from the cell membrane, and, in addition to confirm that in the experimental setup the immunity protein is indeed released from the *E. coli* cells, the cell supernatant was also analysed. Addition of DTT resulted in enhanced fluorescence when the cell supernatant was measured.

5.3.5.5 Fluorescence cell based assay: Manipulation of incubation time

The time of incubating the cells with fluorescent $FA3^{AF488}$ was increased to 30 min to investigate if this increased binding to the cell surface. Fluorescence in

the supernatant was also measured (Figure 5-27, A and B). DTT was added prior taking the fluorescence measurements.

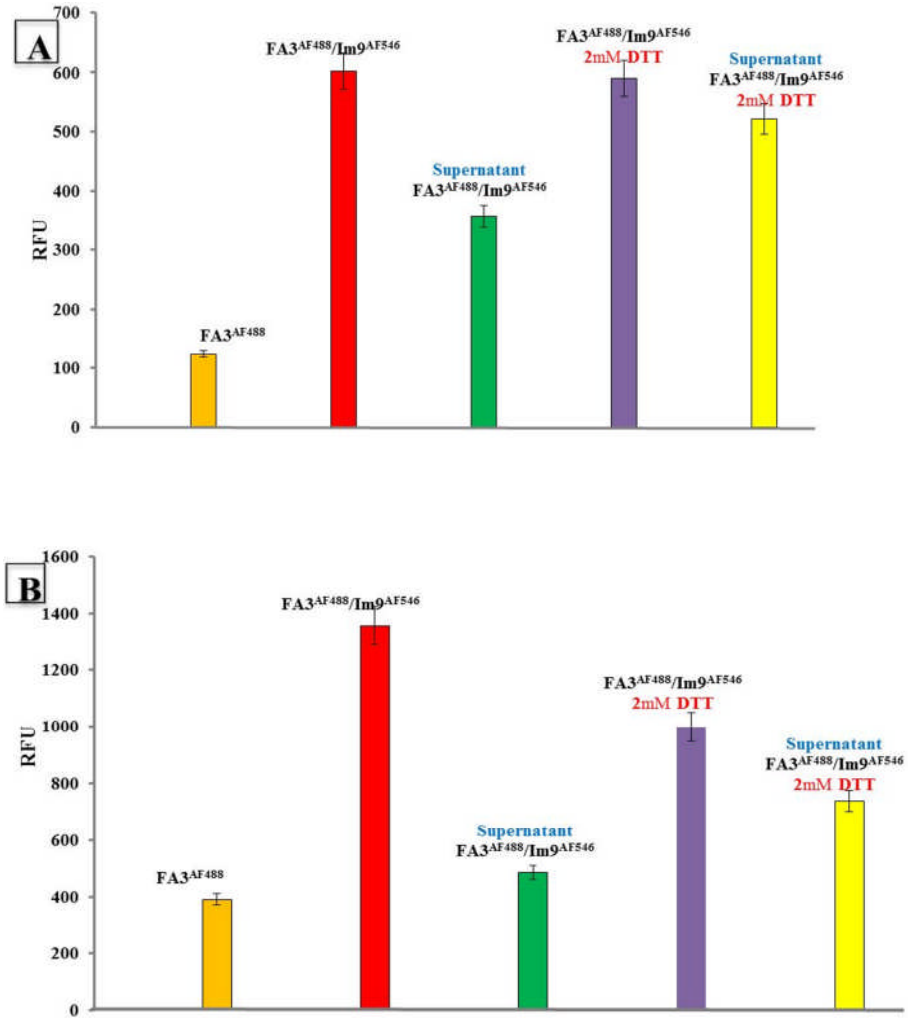


Figure 5-26: *In vivo* FRET assay monitoring immunity protein release from cell bound $FA3^{AF488}$ / $Im9^{AF546}$ complex and $FA3^{AF488}$ alone. Untreated cells were used as a control, diluted 1:1 in LB media. The background-subtracted release (relative fluorescence units) is shown for *E. coli* LMG194 (pAG1) cells pre-incubated with $FA3^{AF488}$ / $Im9^{AF546}$ complex (10 nm) in the presence and absence of 2 mm DTT after a 30 min incubation period at 37 °C. [A] In this experiment the dialyzed protein in PBS was used; [B] In this experiment the diamide oxidized protein was used.

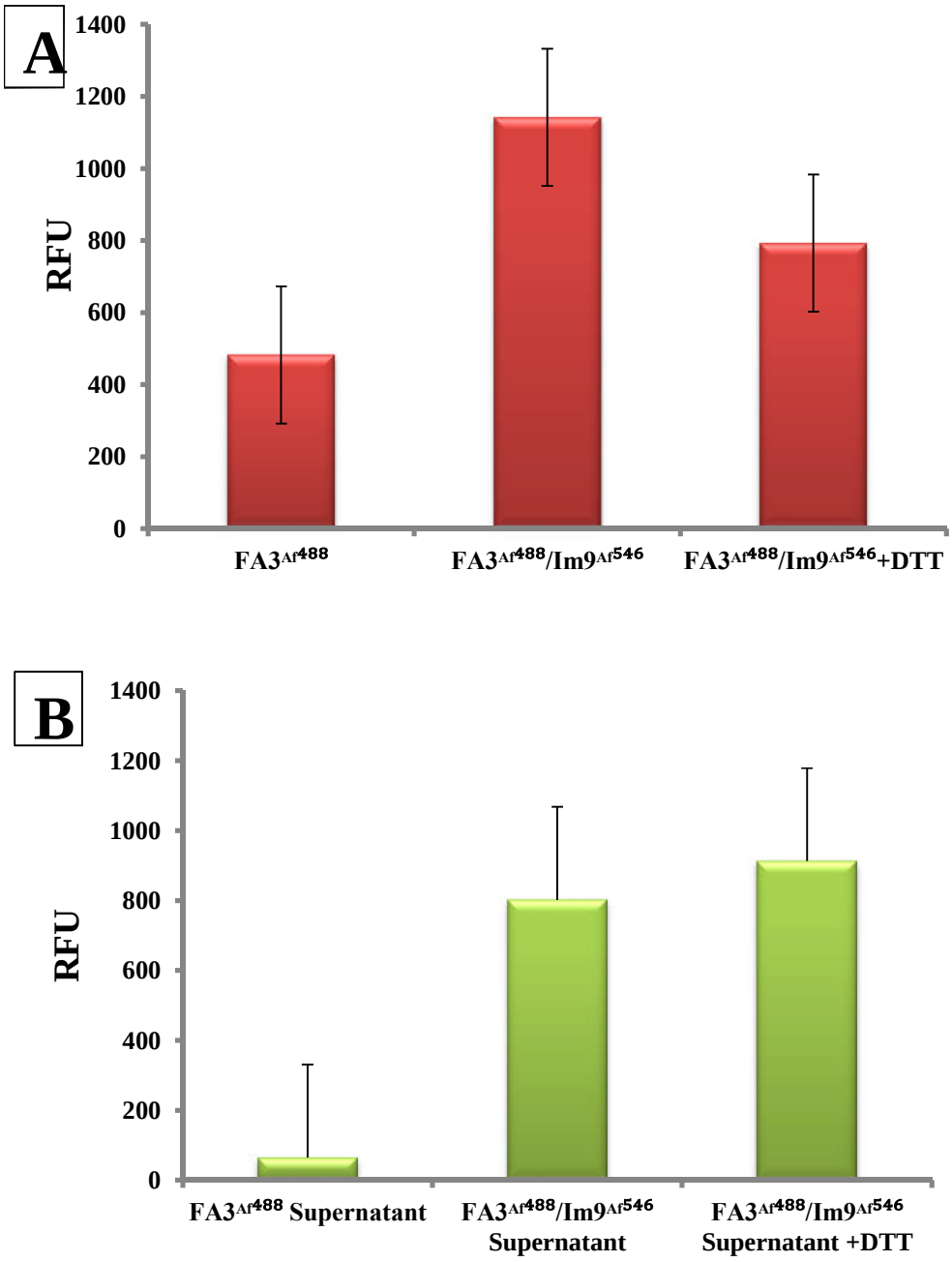


Figure 5-27: Monitoring immunity protein release from $FA3^{AF488}/Im9^{AF546}$ cell bound complex after 30 min pre-incubation with *E. coli* LMG194 (pAG1). Comparison of fluorescence signals detected using either the $FA3^{AF488}/Im9^{AF546}$ complex or $FA3^{AF488}$ alone, either in the absence or presence of DTT (A) represent RFU from cells; (B) is the RFU from the supernatant. Untreated cells were used as a control diluted 1:1 in LB media.

5.3.5.6 Fluorescence cell based assay: Characterization of different bacterial cells

E. coli LMG194 (pAG1) was compared with *E. coli* DPD1718 (pAG1) cells in the assay to rule out an effect of the bacterial strain on the assay. Similar results were observed comparing the two different bacterial strains (Figure 5-28).

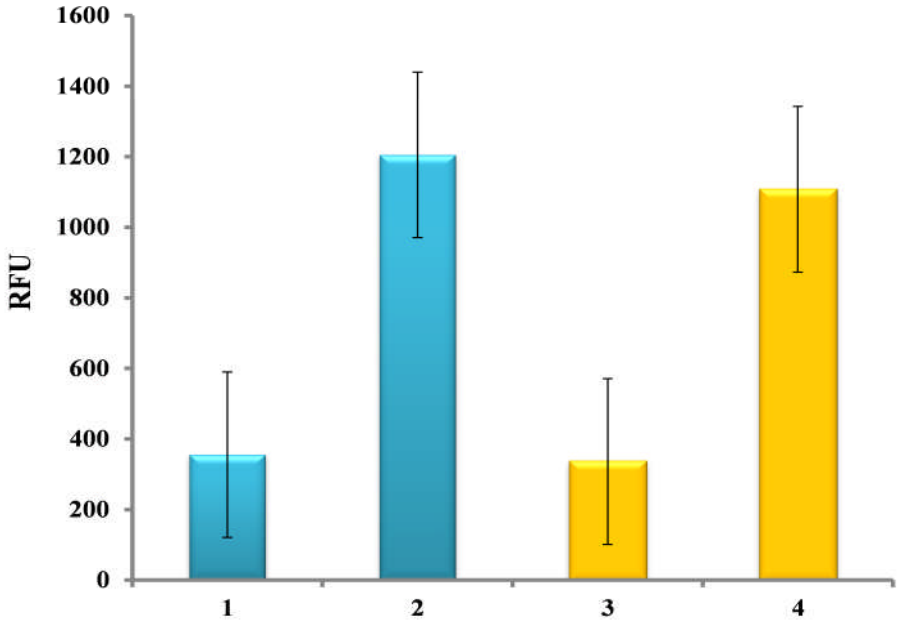


Figure 5-28: Monitoring immunity protein release from the $FA3^{AF488}$/$Im9^{AF546}$ complex and from $FA3^{AF488}$ *in vivo*. Untreated cells were used as a control diluted 1:1 in LB media in presence of 2 mM DTT. The background-subtracted released RFU is shown for *E. coli* LMG194 (pAG1) cells pre-incubated with (1) $FA3^{AF488}$, (2) $FA3^{AF488}$/$Im9^{AF546}$ complex or for *E. coli* DPD1718 (pAG1) cells pre-incubated with (3) $FA3^{AF546}$, (4) $FA3^{AF488}$ /$Im9^{AF546}$ complex.

5.3.5.7 Modification of the labelled flourophores

In order to get reliable data for continuous FRET assay, further optimization is required to develop the fluorescence assay; FA3 and Im9 were labelled with different fluorescent dyes that would have a stronger fluorescent signal. Alexa-Fluor 546 was used to label FA3 and Alexa-Fluor 594 was used for labelling Im9. The newly labelled proteins were subjected to validation assays to ensure its activity as shown for the previous labelled proteins. Diamide oxidation was carried out to ensure the oxidation of the disulphide bond in the $FA3^{AF546}$. An enhanced fluorescence signal was detected, however, no quenching effect was shown with $FA3^{AF546}$ /$Im9^{AF594}$ (Figure 5-29). Measuring the supernatant of the $FA3^{AF488}$ samples showed fluorescence; washing of the cells after incubation with the labelled protein was carried out to ensure the removed of the excess protein.

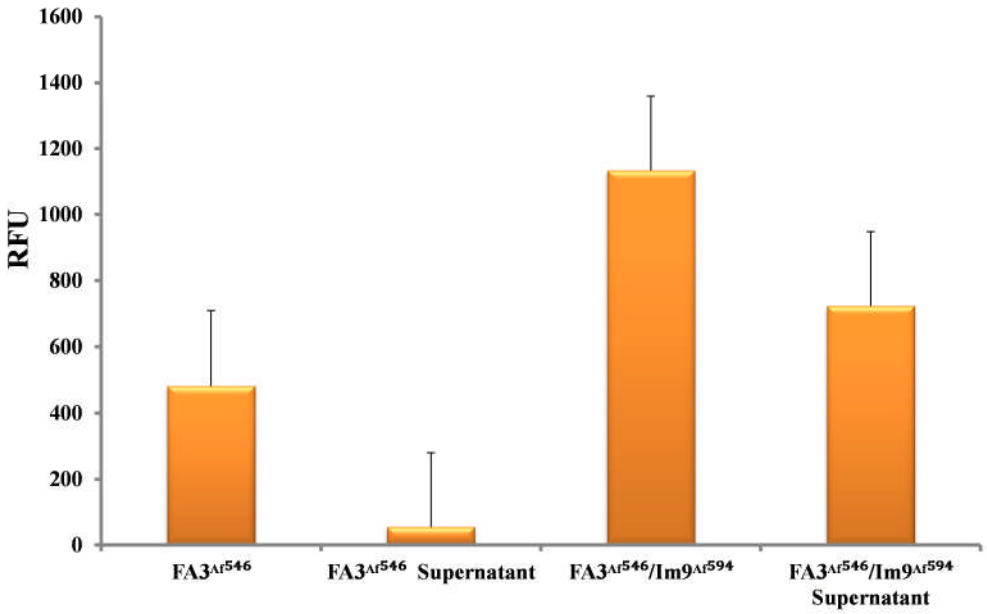


Figure 5-29: Monitoring immunity protein release from cell-bound $FA3^{AF546}/Im9^{AF594}$ complex and from $FA3^{AF546}$ *in vivo*. Untreated cells were used as a control diluted 1:1 in LB media. The background-subtracted released RFU is shown for *E. coli* LMG194 (pAG1) cells pre-incubated with either $FA3^{AF546}$or $FA3^{AF546}/Im9^{AF594}$ complex (10 nm) for 7 min at 37 °C.

5.4 Discussion

This study has used the concept of fluorescence energy transfer (FRET) in developing a quantitative, real-time assay for assessing the rate of release of Im9 from a ColE9-Im9 complex that is bound to *E. coli* cells. ColE9 was specifically labelled with Alexa Fluor 488 (FRET donor) while Im9 was labelled with Alexa Fluor 546 (FRET acceptor). Following ColE9-Im9 complex formation, the strong binding affinity between the two proteins brought the donor and acceptor fluorophore molecules into close proximity, thereby facilitating the appearance of a FRET emission signal. Determination of FRET efficiency (*E*) was accomplished using the donor AF488 FA3 emission intensities in the presence ($AF546_{both}$) and absence ($AF546_{only}$) of acceptor AF546 Im9, calculated from the equation $E = 1-I_{DA}/I_D$. Using this equation, the FRET efficiency was found to be around 64 % when the fluorophores interacted.

To establish the best conditions for the probe fluorescence intensities, the emission intensities ($AF488_{em}$ and $AF546_{em}$) of the fully conjugated probes were measured separately. The concentration of probes was 10 nM $FA3^{AF488}$/$Im9^{AF546}$ mixed in 1:1.15 ratios, respectively. The plasmid pAG1 was introduced into the *E. coli* LMG194 and *E. coli* DPD1718 cells to increase the level of the BtuB receptor to about 20-40 % of the OM proteins (Gudmundsdottir, Bradbeer et al. 1988); this will help to increase the detection sensitivity of the assay. Theoretically, the fluorescence of the labelled $FA3^{488AF}$ should increase as the colicin complex binds to the cell surface and subsequently $Im9^{546AF}$ is released. Denaturation of ColE9/Im9 complex was monitored for the increase intensity of the florescence over time *in vitro*.

As mentioned earlier, BH29 has an engineered disulfide bond in its receptor-binding domain (via two strategically positioned cysteine mutations) (Penfold, Healy et al. 2004). It is biologically inactive in the oxidized form and its activity is restored upon treatment with DTT, allowing a control of colicin activity that is independent of cell binding. This construct binds to sensitive *E. coli* cells but remains inactive until the disulfide bond is reduced, at which point the colicin resumes its biological activity. The activity of the FA3 prior to labelling was detected *in vitro* using a lawn of an *E. coli* sensitive strain. A clear zone of inhibition was observed when compared to the WT ColE9 that was used as a control. Furthermore, the biological activity of the resulting $FA3^{AF}$-$Im9^{AF}$ complex was ascertained using the pUC18 cleavage assay. The DNase activity of free FA3 and $FA3^{AF}$ were inhibited to a similar extent by the addition of either Im9 or $Im9^{AF}$ at a 1:1 molar ratio. Complex formation between $Im9^{AF}$ and $FA3^{AF}$ was confirmed using size exclusion chromatography (Superdex 75 10/300 GL, GE Healthcare). Also, the *in vivo* biological activity of the $FA3^{AF}$-$Im9^{AF}$complex was examined using our *lux* reporter assay for DNA damage. Treatment of the reporter strain *E. coli* DPD1718 with reduced unlabelled FA3, $FA3^{AF}$-Im9 or $FA3^{AF}$-$Im9^{AF}$ generated similar amounts of luminescence (Figure 5-21), indicating that the fluorescence label on the immunity protein was not impeding the biological activity of the colicin complex. A small amount of background luminescence was routinely observed in these experiments when using the disulfide-locked colicin complexes. This is likely to have been caused by trace amounts of reduced protein remaining after the dialysis process so diamide oxidation was performed to ensure total oxidation of any reduced traces.

The catalytic activity of free FA3 was further measured by a fluorescent method using a fluorophore and quencher-labeled oligonucleotide as the substrate. In the assay a fluorophore and quencher-labelled oligonucleotide used as the substrate that showed no fluorescence emission as it was monitored with time. An inactive ColE9 His575-Ala mutant, was compared with WT free ColE9 or ColE9/Im9 complex, along with ColE7, that was used in the original study that used this assay (Shi, Chak et al. 2005). His 575 is a conserved, catalytic residue in the HNH motif that is located in the C-terminal DNase domain of ColE9 (Garinot-Schneider, Pommer et al. 1996; Pommer, Kuhlmann et al. 1999). Cleavage of the fluorophore-labeled oligonucleotides by the proteins gave increasing fluorescence emission intensities. The measured intensities showed that free FA3 was active, compared to the wild-type ColE9 enzyme activity, while the FA3/Im9 labelled complex showed no activity, compared to wild-type ColE9/Im9 complex, indicating a successful association of the labelled FA3 with labelled Im9.

The data obtained from the in vitro release assay indicated a successful FRET assay that upon denaturation show a fluorescence signal due to release of $Im9^{AF}$ from the $Fa3^{AF}/Im9^{AF}$ complex (Figure 5-23). It proved difficult to develop this into an in vivo assay of Im9 release from the ColE9-Im9 complex. A high background was observed with LB media, although growth of the *E. coli* cells was better in this media.

A low level fluorescence signal was detected from cell-bound $FA3^{AF}$, although it was confirmed by microscopical examination. $FA3^{AF}/Im9^{AF}$ hypothetically should show less fluorescence signal due to quenching effect of Im9, yet an additive signal was detected. This may have been due to the failure of the

filters used to detect just the donor fluorescence without a contribution of the acceptor. However, failure of quenching can be excluded as the *in vitro* result showed successful quenching by the Im9 acceptor. Moreover in a semi-continuous assay measuring the fluorescence in the supernatant alone, an increase in the amount of Im9 protein released was observed (Vankemmelbeke, Zhang et al. 2009). In the previous study $Im9^{AF}$ released from *E. coli* LMG194 (pAG1) cells was measured after a 30 min incubation period in the presence and absence (control) of DTT. In the presence of DTT, the released $Im9^{AF}$ increased ~3-fold.

Increasing the incubation time to 30 min to allow additional time for oxidized $FA3^{AF488}$/$Im9^{AF546}$ to bind to *E. coli* LMG194 (pAG1) cells did not show any improvements in the detected fluorescence signal. Changing the bacterial cells used in the assay from *E. coli* LMG194 (pAG1) cells to *E. coli* DPD1718 (pAG1) cells did not affect the assay results (Figure 5-28).

In order to overcome the observed problems with the fluorescence assay, a further modification was carried out to try the effect of a different fluorescent label for both $Fa3^{AF}$ and $Im9^{AF}$. Alexa-Fluor 594 was used as the acceptor and Alexa-Fluor 546 as the donor probe; the fluorescence efficiency for this pair of FRET probes increased to 73 %. Validation and characterization assays on the newly labelled proteins were carried out as previously shown for the $FA3^{AF488}$ and $Im9^{AF546}$. FRET analysis using the $FA3^{AF546}$/Im9AF594 complex showed very similar results to the previous labelled FRET probes (Figure 5-29). It appears likely that the problem was technical as the filter used detected an additive fluorescent signal with both combined probes. It is possible that the detection filter also picked up $Im9^{AF}$ emission or the sensitized emission of the

donor and that would explain the increase in fluorescence with the complex compared to the free protein. To verify this more the RFU of $Im9^{AF}$ protein was measured on Victor with the filters used for the cell-based assay. A similar result was obtained which supports the suggestion of a technical error. Because of time constrains and the success in other areas of research, this problem was not investigated further. Developing a successful continuous fluorescence assay would provide useful information to study the dynamics of immunity protein release during the translocation of a colicin molecule to its cellular target.

CHAPTER SIX

6 General discussion:

A summary of the proposed mechanisms for the translocation of both pore-forming and endonuclease colicins is shown in (Figure 1.12). Generally, there are two major requirements for colicin translocation (James, Kleanthous et al. 1996; Housden, Loftus et al. 2005; Zakharov, Zhalnina et al. 2006; Cascales, Buchanan et al. 2007); (1) translocation of the cytotoxic domain across the OM and (2) crossing the periplasm to reach the outer surface of the cytoplasmic membrane. The main difference between pore-forming and endonuclease colicins is that the cellular target of pore-forming colicins is the CM (Dankert, Uratani et al. 1982), where they cause membrane depolarization and eventually cell death, whilst the endonuclease colicins have to cross both the outer and cytoplasmic membranes in order to reach their target chromosomal DNA or RNA (Mosbahi, Lemaitre et al. 2002).

The proposed systems that pore-forming and endonuclease colicins use in order to fulfil these requirements include an interaction of the colicin with TolB and/or TolA, which disrupts the TolB-Pal, TolA-Pal and TolB-TolA interactions, ultimately disrupting the integrity of the outer membrane (Lloubes, Cascales et al. 2001). Crossing the periplasm requires recruitment of Tol proteins. In the case of ColA it binds to TolB and then forms a complex with TolB and TolA (Zhang, Li et al. 2010). The association of TolA with TolR, via domain III of TolR, then allows an interaction between ColA and domain II of TolR, this system known to be linked to the pmf allowing transduction of energy between the inner and outer membranes (Cascales, Gavioli et al. 2000; Vankemmelbeke, Zhang et al. 2009), which then

effectively pull the colicin cytotoxic domain towards the cytoplasmic membrane.

It has been shown that ColE9 enters the periplasm through the OmpF lumen and then interacts with TolB (Housden, Wojdyla et al. 2010), possibly displacing TolB from its interaction with Pal. It was also confirmed that both the R-domain and T-domain of ColE2 remain in contact with their binding partners in the OM and the periplasm, respectively, when the DNase domain gains access to the cytoplasm (Duche 2007).

In the case of an enzymatic E colicin it is clear that the immunity protein has to be released from the colicin-immunity protein complex to allow cell killing. Given the very high affinity of the interaction between a colicin and its cognate Immunity protein, the energy requirement to break this interaction presents a formidable challenge. When, where and how the process occurs remains unclear. Release of the immunity protein from the ColE9-Im9 complex was revealed to be an energy-dependant process that is linked to the cytoplasmic membrane PMF, and involves an energized Tol system (Vankemmelbeke, Zhang et al. 2009). Energy dependent conformational change of TolA is found to be essential for its role in nuclease colicin uptake (Germon, Ray et al. 2001). It was also demonstrated that the DNase domain of E colicins can form channels in an artificial membrane (Mosbahi *et al.*, 2002) which suggests that this might be the mechanism ColE9 uses to cross the CM to reach the target DNA.

The structural organization of colicins and the interacting proteins which they use have been well documented (James et al., 2002), and much progress is

being made to unravel the events that take place during the journey of a colicin molecule to its cellular target, however, very little is known about the biophysical mechanisms that are employed by colicins to reach their cellular targets. Insufficient information is available mainly about the nature of the interaction the Tol proteins have with colicins and the release of immunity proteins, in the case of endonuclease colicins, this still needs further clarification. The work of this project is aimed to answer questions about the colicin translocation mechanism.

It is known that the translocation pathway used by a colicin to enter a target cell is specified by its N-terminal translocation domain (Benedetti, Frenette et al. 1991). It was only known that TolAIII interacts with TA and TE1, before the development of the periplasmic production technique as an approach to study the periplasmic interactions of colicins *in vivo* (Benedetti, Lazdunski et al. 1991). Additionally, this method could help to explain the nature of the interactions and to identify new protein partners that interact with colicins in the target cell. Previous studies have used this approach with the translocation domains of a number of colicins, TA, TB, TE3, and TK, and demonstrated that expressing these T-domains specifically in the periplasm disrupted the corresponding translocation machinery, showing an *in vivo* interaction with some of its proteins (Bouveret, Rigal et al. 1997; Bouveret, Rigal et al. 1998).

Concerning ColE3, interaction with TolB was proved to cause the disruption of the Tol system (Bouveret, Rigal et al. 1997). Moreover, cross-linking experiments used to detect the reduced amount of TolB–Pal complex when TE3 is produced in the periplasm indicated that TE3 and Pal interact with the same region of TolB (Bouveret, Rigal et al. 1997). On the contrary, the

disruption by TA was apparently only due to the interaction with TolA (Walburger, Lazdunski et al. 2002). It is known that residues in the 52–107 region of TA are involved in TolA interaction (Bouveret, Rigal et al. 1998). However, in this case, the interaction was only detected *in vitro* by formaldehyde cross-linking using purified proteins.

Upon periplasmic production of TB, a specific *tonB* phenotype was observed, indicating that TB interacted with proteins of the TonB–ExbB–ExbD system (Bouveret, Rigal et al. 1998). Furthermore, the genetic requirements for colicin K uptake were clarified and showed that colicin K requires the products of the *tolA, tolB, tolQ, and tolR* genes, and for the first time evidence of complex formation between a colicin T-domain and the TolQ protein has been identified using a combination of fractionation, *in vivo* co-immuno-precipitation, and *in vitro* pull-down experiments coupled to phenotypic analyses to characterize ColK import (Barneoud-Arnoulet, Gavioli et al. 2010).

In this work I aimed to provide further insights into the role of the ColN-terminal domain of *tol*-dependent colicins and the nature of their interaction with the Tol machinery. The level of protection provided by the periplasmic overproduced TA was determined *in vivo* against DNase colicins using the very sensitive *lux* reporter assay of DNA damage (Vankemmelbeke, Healy et al. 2005), or against the pore-forming colicins using the potassium release assay (Bourdineaud, Boulanger et al. 1990; Bredin, Simonet et al. 2003). Full length TA was expressed in the reporter strains which were challenged with ColE9 after which the gamma values were calculated and the percentage of protection measured as a proportion of these values (Vankemmelbeke, Healy et al. 2005).

Lux induction of control cells expressing the empty vector, and cells expressing the unrelated lysostaphin T-domain revealed no difference compared to *E. coli* DPD1718 cells containing no vector (Figure 3.7). This experiment was designed to check if the presence of an unrelated protein domain in the periplasm could disturb the normal function of the envelope system and affect colicin entry. All previous studies showed that expressing either part of Tol proteins or colicin N-terminal domains render the *E. coli* cells tolerant to the exogenous colicins (Pommier, Gavioli et al. 2005). The presence of the unrelated lysostaphin domain does not have an effect on the Tol system indicated by the total sensitivity of the challenged *E. coli* cells to group A colicins. Similar results were obtained by testing the activity of ColA using the potassium release assay (Figure 3-12).

A summary of all the previously known interactions between colicins and Tol (or TonB) proteins is difficult because all the different techniques used have detected only some of all of the known interactions. For example, the TA–TolA interaction has never been detected *in vivo* in *E. coli*, in contrary with the interactions of TE3–TolB or TA–TolR. Yet, a number of colicin interactions with TolA has been shown using overlay, surface plasmon resonance, and yeast two hybrid methods for colicins A and E1 (Benedetti, Lazdunski et al. 1991; Bouveret, Rigal et al. 1998; Walburger, Lazdunski et al. 2002). A reporter assay has detected a strong interaction *in vivo* with both TolA and TolB (Figure 3-8). Showing that the ColE9 TolB box, that has high affinity for binding to TolB, can displace the interaction between ColA TolB and Pal has provided additional information on the uptake of ColE9. Though TolA is required for ColE9 translocation, no TolA box has been detected in

ColE9. *In vivo* cross-linking and co-immuno-precipitation have detected a complex of TA-TolA-TolB (Figure 3-11). *In vitro* cross-linking of the purified proteins detected the same complex and a complex between TA and TolB as well, but no interaction was detected between TA and TolA in the absence of TolB (Figure 3-10).

All the protein-protein interaction results illustrate that ColE9 interacts directly with TolB and requires TolA interaction through TolB to be translocated. So occupying TolA only protected the challenged cells from ColE9 activity as translocation of this colicin is coupled to the pmf that is required for immunity protein release (Levengood, Beyer et al. 1991; Vankemmelbeke, Zhang et al. 2009). One important aspect concerns the ability of periplasmic TA to protect cells against colicins, and this peptide was found to be at least 25 times more active to protect cells against ColE9 than ColA, this was shown by measuring the sensitivity of cells expressing TA variants against ColA, E1 and E9 (Pommier, Gavioli et al. 2005). This may reflect the relative affinity of the interaction of the colicin translocation domains to TolA, indicating a higher binding. This suggestion is supported by the work in this thesis which shows that introducing the TolB box residues of ColE9 into the truncated TA polypeptide increases the level of protection conferred against ColE9 (Figure 3-15).

The interaction between TolB and colicin has been studied for ColE3 using purified proteins (Bouveret, Rigal et al. 1997), for ColE9 using the yeast two-hybrid technique (Carr, Penfold et al. 2000), and for ColA using both purified proteins and the yeast two-hybrid methods (Bouveret, Rigal et al. 1998; Walburger, Lazdunski et al. 2002). It seems that the two domains of TolB are

necessary for the interaction with ColA translocation domain, although, for colicins E3 and E9 the interaction takes place with only the C-terminal domain of TolB (Carr, Penfold et al. 2000; Walburger, Lazdunski et al. 2002).

Alignment of the TolB box region of all *tol*-dependant colicins has shown a conserved pentapeptide TolB box binding region in ColE9 (Figure 1-11) (Hands, Holland et al. 2005), that was later shown to be extended outside this region and indicated to have a higher affinity toward the periplasmic TolB (Collins, Whittaker et al. 2002; Hands, Holland et al. 2005). Expressing the TolB box of ColA in the *E. coli* periplasm and comparing it to the TolB box with swapped residues of ColE9 showed a higher affinity represented by the higher protection conferred against ColE9 lethality (Figure 3-15). The requirement to release the immunity protein from the DNase domain of ColE9 presumably explains the tight binding of ColE9 with the TolB protein as a means to link with cellular pmf via a TolB-TolA complex. The translocation of ColA does not involve such a requirement. Related results obtained with the potassium release assay, using *E. coli* cells expressing either TA,TA with the swapped ColE9 residues, and the expressed TolA box only, showed total protection against ColA activity as no potassium was released in the external medium after treating the cells with ColA (Figure 3-16).

Expressing the TolB box of TA showed limited protection against ColA as the level of potassium increased on the addition of ColA to these cells; ColN was used as a control in these experiments as it is known that ColN does not interact with TolB during its import. Addition of ColN to these cells showed total release of potassium measured in the external media compared to the control cells. Results from both *in vivo* assays showed that the expressed TA

polypeptide variants interacted with the Tol proteins (detected as reduced activity of added colicins) and highlight different interactions for each of the boxes in the T-domain of ColA (Figure 6-19).

The E18A mutant of ColA was shown to be inactive *in vivo* due to its inability to bind TolB, which is consistent with the properties of the equivalent E42A mutant in ColE9. The E18A mutant in the TolB box of ColA showed similar levels of protection to the non-mutated protein. This is presumably the result of the higher affinity of binding of the TolB box of the externally added ColE9 to the periplasmic TolB protein, compared with that of the TolB box of the periplasmically expressed TA domain.

The TA protein appeared to interact with the Tol proteins and inhibit the translocation process of ColE9, whilst the TolA box mutant, which was previously determined to abolish the activity of ColA in the *in vitro* assay (Journet, Bouveret et al. 2001; Pommier, Gavioli et al. 2005) failed to show substantial protection against ColE9 in particular (Figure 4-5). Pommier et al., (2005) suggested that residues Y58 and Y90 of two homologous regions may be involved in TolA binding (Pommier, Gavioli et al. 2005). In a previous study, analysis of the function of the two potential TolA binding motifs of ColA, SYNT and PYGR, located between residues 52 and 97 of the TolA binding sequence have shown that the activity was abolished by a Y58 mutation, yet with the Y90 mutants a reduced activity was detected (Journet, Bouveret et al. 2001).

The result of an *in vitro* spot test of the protection provided by introducing either the TAE18A or TAY58A, or the combined mutations, against killing by

the ColA and E9 are not very quantitative (Figure 4-6). In order to test the activity of the mutant proteins and the level of protection in a more sensitive and quantitative way, the *lux* reporter assay was used (Figure 4-5). The results of the *lux* reporter assay following treatment with ColE9 in presence of periplasmic expressed proteins at time point of 50 min, demonstrated that the induced protein prevented the translocation of ColE9 by around 78 %, which was only 10 % less than WT TA. The importance of the TolA tyrosine residue at position 58 for the activity of ColA is supported by the demonstration that the protection conferred is reduced from 86 % with the WT TA to 41 % with the TAY58A mutant. These results indicated the importance of the Tyr 58 residue for TolA binding which support the *in vitro* findings of the previous study by Pommier, et al (2005) using the very sensitive *lux* reporter assay. Whilst a 10-15 fold lower colicin activity was reported with the TAY58A mutation in the study of Pommier, the *lux* reporter assay revealed around 45 fold reduced activity. This result is in agreement with the observation that TAY58A affects the interaction with TolA (Pommier, Gavioli et al. 2005)

The dissociation constants for the binding of the N-terminal domains of ColA and ColE1 to the C-terminal domain of TolAIII were determined to be in the micro-molar range (Derouiche, Benedetti et al. 1997), while similar K_d were found for the binding of TolA to the translocation domain of ColN (Raggett, Bainbridge et al. 1998). Interestingly, heteronuclear NMR experiments have shown that interaction of the structured TolAIII domain with the natively disordered TA domain results in an increase in disorder of TolAIII. This feature is rather unusual and was not observed with the N-terminal domain of g3p binding to TolAIII (Deprez, Blanchard et al. 2002). There is no clear

homology in the regions of the translocation domains of $T_{52–97}$ and $TN_{44–66}$ that are involved in the interaction with TolA, suggesting different characteristics of their interactions, which can explain the different techniques required to detect them. It appears that ColE3 and ColE9 do not interact directly with TolA, although TolA is required for the translocation of these colicins. Consequently, using the periplasmic expression technique with the reporter assay and potassium release assay may highlight similarities and/or differences in the interaction of translocation domains with the Tol.

In this work, a construct was engineered that expressed a fusion protein consisting of the ColA TolB box fused with the TolA box of ColN. *E. coli* cells expressing the fusion protein in the periplasm were challenged with ColE9, and the different percentage of protection that is observed when compared to the wild type TA (Figure 3-18). Although, the assay does not show the nature of the interaction, the results confirm that the TolA box of ColA interacts with Tol proteins in a different way than the ColN TolA box of ColN. This conclusion was supported by the protection conferred against ColA and ColN using the potassium release assay (Figure 3-19).

ColN has a lower relative molecular mass (42 kDa) than the other pore-forming colicins (Pugsley 1987), and uses a major outer membrane protein, OmpF, as a unique receptor (Bourdineaud, Fierobe et al. 1990). The carboxy-terminal half of ColN exhibits significant homology to the C terminus of ColA (Pugsley 1987). Although they show the same channel properties in lipid bilayers (Wilmsen, Pugsley et al. 1990), the requirements for insertion into the inner membrane differ (van der Goot, Didat et al. 1993). ColN exhibits a very short lag time before cell killing compared to other pore-forming colicins,

which explains the faster potassium release detected in comparison to ColA (Figure 3-18 and Figure 3-21). Even though comparable protection was observed by potassium release assay against ColA and ColN in the potassium release assay, a reduced level of protection (11 fold) against ColE9 was observed using the reporter assay. All *in vivo* results indicate considerable differences in the way that colicins parasitize the Tol protein system.

Although the precise amino acid residues involved in TolA binding by ColN have been identified (Raggett, Bainbridge et al. 1998; Gokce, Raggett et al. 2000), sequence homology and mutagenesis studies have not been able to identify significant similarity with other group A colicins. Full length ColN has a much weaker binding affinity to TolA (18 μM) than the isolated TN domain (1 μM). The variation in the binding affinity indicated structural changes during the receptor binding step (Raggett, Bainbridge et al. 1998; Gokce, Raggett et al. 2000).

$TA_{1\text{-}172}$ includes the TolB box between residues 7-20 (Bouveret, Rigal et al. 1998; Journet, Bouveret et al. 2001). Bouveret et al (1998) also localized the regions responsible for the interactions with TolA between residues 52–107. The residues from 108-172 have not been shown to be involved in any interaction with the TolA protein, although, ColA Δ154-172 mutants were suggested to affect the conformation of the ColA T-domain rather than affecting the interaction with Tol proteins, thereby making the translocation less efficient (Bouveret, Rigal et al. 1998). So for further clarification $TA_{1\text{-}172}$ and TA were compared for the level of protection conferred when expressed in the periplasm (Figure 3-22), the purpose of this experiment was to investigate the involvement of these extra residues in the Tol interactions. Similar results

were obtained with both polypeptides, indicating no role for residues $TA_{108-172}$ in the *in vivo* interaction with Tol proteins.

The presence of mutations in both the TolA and TolB boxes showed an additive effect on the reduced level of protection against ColE9, although, the TolB box mutant on its own did not show significant effect. This was supported by the results of the *in vitro* spot test assays. This could be explained if the presence of the two mutations may (1) lead to conformational changes that might affect the ability to interact with the Tol proteins, or (2) if the TolB box is not essential for interaction with Tol proteins in the periplasm providing there is a functional TolA box.

Previous mutational analysis of the TolA or TolB box sequences of ColA was shown to result in reduced or abolished killing activity (Bouveret et al., 1997), although the mutant colicins can still bind tightly to sensitive cells, and their killing activity (pore formation or nuclease activity) is not impaired when tested *in vitro* (Mende and Braun, 1990; Buchanan et al., 2007). Mutations in the corresponding target cell protein can restore killing activity by these T-domain colicin mutants, as shown for TonB (Helleret al., 1988; Schoffler and Braun, 1989; Bell et al., 1990; Braun et al., 2002), confirming that an interaction between the colicin and the TonB protein is necessary for killing to occur. In the case of the Group A colicins, the affinity of binding between the translocation domains and components of the Tol apparatus has been measured. Isothermal titration microcalorimetry (ITC), surface plasmon resonance (SPR) and tryptophan fluorescence have been used to measure binding of the TolAIII to the T domain of ColN, which does not require TolB for cell killing (Raggett et al., 1998; Gokce et al., 2000).

In the case of the enzymatic E colicins, as ColE9, direct binding of residues in the unstructured part of the N-terminal translocation domain to TolB was demonstrated by SPR with the ColE9-Im9 complex, with a K_d ~ 14 μM (Hands et al., 2005). The affinity of binding of free ColE9 to TolB was determined to be 1 μM (Loftus, Walker et al. 2006). Binding of the ColA T domain to both TolA and TolB has been detected, using *in vitro* cross-linking or detection of binding partners with monoclonal antibodies (Bouveretet al., 1998; Journet et al., 2001). The Tol-dependent pore-forming colicin K does not possess a TolB box, yet a recent study demonstrated that the colicin K N-terminal domain interacts with components of its import machinery, including the TolB and TolQ proteins (Barneoud-Arnoulet, Gavioli et al. 2010).

Thus, the T domains of Group A colicins bind to one or more members of the Tol family of proteins in the periplasm in an essential step of colicin import. In fact, a portion of the natively disordered region of the ColE9 T domain was crystallized bound in the pocket of the TolB β-propeller domain where the Pal normally binds (Loftus et al., 2006), confirming the physical interaction between a segment of the toxin and its periplasmic binding partner in the target cell. These conclusions were confirmed in this work by *in vivo* crosslinking and mass spectroscopy. Thus, at least two distinct binding steps, high-affinity binding by the R domain to an outer membrane receptor and an interaction of the T domain with one or more periplasmic proteins, occur before the final lethal step in target cell killing by a group A colicin molecule.

The investigation was extended to study the effects of TolA box mutations of the five residues (62-69) that were identified to be located at the interface with

the TolAIII domain in the crystal structure of the TA-TolAIII complex (Chan, Li unpublished work). The level of protection conferred by the mutant TA domain against ColE9 and ColA was determined using both the potassium release assay (Figure 4-12) and the *lux* reporter assay (Figure 4-11). The *lux* reporter assay using TolA R62-69A mutated protein demonstrated a substantial reduction in the level of protection against both ColA and ColE9. The diminished protection conferred by the mutant TolA indicates a loss of the interaction between the mutant TolA box and the TolA cellular protein, thus allowing the translocation of externally added colicins. This is in agreement with recent results obtained using the *lux* assay on the reporter strain that express TolAIII L375-P380 in the periplasm which showed little protection was conferred against ColE9 (unpublished data by our group). Defining these residues at the interface between TolA box and TolAIII is an important breakthrough in our understanding of colicin translocation and may help to identify similar residues in other group A colicins.

Release of immunity protein during cell killing

After binding of the ColE9-Im9 complex to the BtuB receptor of a sensitive *E. coli* cell the immunity protein is lost from the colicin complex by means that are poorly understood. In an attempt to study this process further we developed a fluorescence energy transfer (FRET)-based method for assessing the rate of release of Im9 from the ColE9-Im9 complex. The aim was to develop a quantitative, real time based assay, based on a recent study in which a semi-continuous, sensitive fluorescence assay was used to study the molecular requirements for immunity protein release (Vankemmelbeke, Zhang et al. 2009).

The ColE9 used in these studies, ColE9$^{s\text{-}s}$ has an engineered disulfide bond in its receptor-binding domain (Penfold, Healy et al. 2004) to control its biological activity. ColE9$^{s\text{-}s}$ binds to sensitive *E. coli* cells but remains inactive until the disulfide bond is reduced by the addition of DTT, at which point the colicin resumes its biological activity. ColE9$^{s\text{-}s}$ was specifically labelled with Alexa Fluor 488 (FRET donor) whilst Im9 was labelled with Alexa Fluor 546 (FRET acceptor). Following ColE9$^{s\text{-}s}$-Im9 complex formation, the strong binding affinity between the two proteins brought the donor and acceptor fluorophore molecules into close proximity, thereby facilitating the appearance of a FRET emission signal. The efficiency of the FRET was calculated from the equation E=1−IDA/ID, which was found to be around 64 % when the fluorophores interacted (Table 5-1).

To establish the optimum conditions for the FRET assay, the emission intensities ($AF488_{em}$ and $AF546_{em}$) of the fully conjugated probes were measured separately. Theoretically, the quenched fluorescence of the labelled FA3^{488AF} by Im9^{546AF} should increase as the colicin complex binds to the cells surface and subsequently Im9^{546AF} is released. Separation of the donor and acceptor fluorophores was first detected *in vitro* in a denaturation buffer as the fluorescence of the ColE9^{AF}/Im9^{AF} complex was monitored with time and showed a rapid increase in the intensity of the florescence within thin the first few minutes (Figure 5-23).

The aim of the assay was to allow the increase in fluorescence due to release of Im9^{AF} from FA3^{AF}/Im9^{AF} complex bound to *E. coli* LMG194 (pAG1) cells in different growth media *in vivo* to be measured. A very low fluorescence signal was detected from cell-bound FA3^{AF} (Figure 5-25), which was confirmed by

microscopical examination (Figure 5-24). The fluorescent signal from $FA3^{AF}/Im9^{AF}$ hypothetically should be reduced due to the quenching effect, yet an additive signal detected (Figure 5-26). Increasing the incubation time to 30 min allowed an additional period for oxidized $FA3^{AF488}$ /$Im9^{AF546}$ to bind to *E. coli* LMG194 (pAG1) cells, but did not show any improvement in the detected fluorescence signal (Figure 5-27). Changing the bacterial strain used in the assay from *E. coli* LMG194 (pAG1) cells to *E. coli* DPD1718 (pAG1) cells also did not improve the assay (Figure 5-28). The additive fluorescence signal that was observed could be due to detecting a combination of fluorescence signal of both the acceptor and the donor; this could be overcome by using a different filter for the cell based assay. Developing a successful continuous fluorescence assay would provide useful information to study immunity protein release dynamics during the translocation of colicin molecule to its cellular target and this could also highlight details of the translocation process as it could be also combined with the periplasmic protection technique for future studies. The available time did not allow further optimization and development of this assay.

Future work

The results obtained from combining either the *lux*-reporter assay, or the potassium release assay, with the periplasmic expression technique indicated an interaction between expressed TA polypeptides and components of the Tol system in the *E. coli* periplasm, In-cell NMR spectroscopy has gained recent popularity since it provides a means to analyse the conformational and functional properties of proteins inside living cells and at atomic resolution. High-resolution in-cell NMR spectroscopy (Selenko and Wagner 2007) was originally established in bacterial cells and based on a rationale that relies on protein over-expression and sample analysis within the same cellular environment, so it could be useful to test the nature of the interaction occurring in the cell periplasm between colicin T-domains and Tol proteins. Also over-producing soluble PAL in the periplasm may give protection against ColE9 by preventing TolA-TolB complex formation. Similarly, expressing the soluble domains of TolQ and/or TolR, or the dual OmpF binding sites of the ColE9 T domain, in the periplasm and investigating if they provide protection against ColE9 may also identify important protein-protein interactions that could be further investigated by the protein cross-linking methods described in (Barneoud-Arnoulet, Gavioli et al. 2010). Moreover, combining the periplasmic expression technique and *lux*-reporter assay could further combined with the fluorescently labelled Im9 which could highlight the important Tol protein required for the release process of Im9 from its complex upon ColE9 entry to its target. Answer to all these questions along with our findings may provide valuable information to help dissect the translocation of colicins.

REFERENCES

Abergel, C., E. Bouveret, et al. (1999). "Structure of the Escherichia coli TolB protein determined by MAD methods at 1.95 A resolution." Structure **7**(10): 1291-1300.

Ames, G. F., C. Prody, et al. (1984). "Simple, rapid, and quantitative release of periplasmic proteins by chloroform." Journal of Bacteriology **160**(3): 1181-1183.

Bardelang, P., M. Vankemmelbeke, et al. (2009). "Design of a polypeptide FRET substrate that facilitates study of the antimicrobial protease lysostaphin." The Biochemical journal **418**(3): 615-624.

Barneoud-Arnoulet, A., M. Gavioli, et al. (2010). "Interaction of the colicin K bactericidal toxin with components of its import machinery in the periplasm of Escherichia coli." J Bacteriol **192**(22): 5934-5942.

Benedetti, H., M. Frenette, et al. (1991). "Individual domains of colicins confer specificity in colicin uptake, in pore-properties and in immunity requirement." J Mol Biol **217**(3): 429-439.

Benedetti, H., C. Lazdunski, et al. (1991). "Protein import into Escherichia coli: colicins A and E1 interact with a component of their translocation system." EMBO J **10**(8): 1989-1995.

Benedetti, H., R. Lloubes, et al. (1992). "Colicin A unfolds during its translocation in Escherichia coli cells and spans the whole cell envelope when its pore has formed." The EMBO journal **11**(2): 441-447.

Bonsor, D. A., I. Grishkovskaya, et al. (2007). "Molecular mimicry enables competitive recruitment by a natively disordered protein." Journal of the American Chemical Society **129**(15): 4800-4807.

Boulanger, P. and L. Letellier (1988). "Characterization of ion channels involved in the penetration of phage T4 DNA into Escherichia coli cells." The Journal of biological chemistry **263**(20): 9767-9775.

Bourdineaud, J. P., P. Boulanger, et al. (1990). "In vivo properties of colicin A: channel activity is voltage dependent but translocation may be voltage independent." Proceedings of the National Academy of Sciences of the United States of America **87**(3): 1037-1041.

Bourdineaud, J. P., H. P. Fierobe, et al. (1990). "Involvement of OmpF during reception and translocation steps of colicin N entry." Molecular Microbiology **4**(10): 1737-1743.

Bourdineaud, J. P., S. P. Howard, et al. (1989). "Localization and assembly into the Escherichia coli envelope of a protein required for entry of colicin A." Journal of Bacteriology **171**(5): 2458-2465.

Bouveret, E., H. Benedetti, et al. (1999). "In vitro characterization of peptidoglycan-associated lipoprotein (PAL)-peptidoglycan and PAL-TolB interactions." Journal of Bacteriology **181**(20): 6306-6311.

Bouveret, E., R. Derouiche, et al. (1995). "Peptidoglycan-associated lipoprotein-TolB interaction. A possible key to explaining the formation of contact sites between the inner and outer membranes of *Escherichia coli*." J Biol Chem **270**(19): 11071-11077.

Bouveret, E., L. Journet, et al. (2002). "Analysis of the Escherichia coli Tol-Pal and TonB systems by periplasmic production of Tol, TonB, colicin, or phage capsid soluble domains." Biochimie **84**(5-6): 413-421.

Bouveret, E., A. Rigal, et al. (1997). "The N-terminal domain of colicin E3 interacts with TolB which is involved in the colicin translocation step." Mol Microbiol **23**(5): 909-920.

Bouveret, E., A. Rigal, et al. (1998). "Distinct regions of the colicin A translocation domain are involved in the interaction with TolA and TolB proteins upon import into Escherichia coli." Mol Microbiol **27**(1): 143-157.

Bradbeer, C., P. R. Reynolds, et al. (1986). "A requirement for calcium in the transport of cobalamin across the outer membrane of Escherichia coli." The Journal of biological chemistry **261**(6): 2520-2523.

Bradley, D. E. and S. P. Howard (1992). "A new colicin that adsorbs to outer-membrane protein Tsx but is dependent on the tonB instead of the tolQ membrane transport system." J Gen Microbiol **138**(12): 2721-2724.

Braun, V. (1989). "The structurally related exbB and tolQ genes are interchangeable in conferring tonB-dependent colicin, bacteriophage, and albomycin sensitivity." Journal of Bacteriology **171**(11): 6387-6390.

Braun, V. and C. Herrmann (1993). "Evolutionary relationship of uptake systems for biopolymers in Escherichia coli: cross-complementation between the TonB-ExbB-ExbD and the TolA-TolQ-TolR proteins." Molecular Microbiology **8**(2): 261-268.

Bredin, J., V. Simonet, et al. (2003). "Colicins, spermine and cephalosporins: a competitive interaction with the OmpF eyelet." The Biochemical journal **376**(Pt 1): 245-252.

Bychkova, V. E., A. E. Dujsekina, et al. (1996). "Molten globule-like state of cytochrome c under conditions simulating those near the membrane surface." Biochemistry **35**(19): 6058-6063.

Cao, Z. and P. E. Klebba (2002). "Mechanisms of colicin binding and transport through outer membrane porins." Biochimie **84**(5-6): 399-412.

Carr, S., C. N. Penfold, et al. (2000). "The structure of TolB, an essential component of the tol-dependent translocation system, and its protein-protein interaction with the translocation domain of colicin E9." Structure **8**(1): 57-66.

Carr, S., D. Walker, et al. (2000). "Crystallization of the cytotoxic domain of a ribosome-inactivating colicin in complex with its immunity protein." **56 Pt 12**: 1630-1633.

Carr, S., D. Walker, et al. (2000). "Inhibition of a ribosome-inactivating ribonuclease: the crystal structure of the cytotoxic domain of colicin E3 in complex with its immunity protein." Structure Fold Des **8**(9): 949-960.

Carvard, D. and B. Oudega (1992). General introduction to the secretion of bacteriocins Bacteriocins, Microcins and Lantibiotics. R. James, C. Lazdunski and F. pattus, NATO ASI series. **65:** 297-305.

Cascales, E., A. Bernadac, et al. (2002). "Pal lipoprotein of Escherichia coli plays a major role in outer membrane integrity." Journal of Bacteriology **184**(3): 754-759.

Cascales, E., S. K. Buchanan, et al. (2007). "Colicin biology." Microbiol Mol Biol Rev **71**(1): 158-229.

Cascales, E., M. Gavioli, et al. (2000). "Proton motive force drives the interaction of the inner membrane TolA and outer membrane pal proteins in Escherichia coli." Molecular Microbiology **38**(4): 904-915.

Cascales, E. and R. Lloubes (2004). "Deletion analyses of the peptidoglycan-associated lipoprotein Pal reveals three independent binding sequences including a TolA box." Molecular Microbiology **51**(3): 873-885.

Cavard, D. (1992). "Colicin A and colicin E1 lysis proteins differ in their dependence on secA and secY gene products." FEBS letters **298**(1): 84-88.

Cavard, D. (1994). "Rescue by vitamin B12 of *Escherichia coli* cells treated with colicins A and E allows measurement of the kinetics of colicin binding on BtuB." FEMS Microbiol Lett **116**(1): 37-42.

Cavard, D., D. Baty, et al. (1987). "Lipoprotein nature of the colicin A lysis protein: effect of amino acid substitutions at the site of modification and processing." J Bacteriol **169**(5): 2187-2194.

Cavard, D., D. Baty, et al. (1987). "Lipoprotein nature of the colicin A lysis protein: effect of amino acid substitutions at the site of modification and processing." Journal of Bacteriology **169**(5): 2187-2194.

Cavard, D., C. Lazdunski, et al. (1989). "The acylated precursor form of the colicin A lysis protein is a natural substrate of the DegP protease." J Bacteriol **171**(11): 6316-6322.

Chak, K. F. and R. James (1984). "Localization and characterization of a gene on the ColE3-CA38 plasmid that confers immunity to colicin E8." J Gen Microbiol **130**(3): 701-710.

Chak, K. F. and R. James (1985). "Analysis of the promoters for the two immunity genes present in the ColE3-CA38 plasmid using two new promoter probe vectors." Nucleic Acids Res **13**(7): 2519-2531.

Chak, K. F. and R. James (1986). "Characterization of the ColE9-J plasmid and analysis of its genetic organization." J Gen Microbiol **132**(1): 61-70.

Chak, K. F., M. K. Safo, et al. (1996). "The crystal structure of the immunity protein of colicin E7 suggests a possible colicin-interacting surface." Proceedings of the National Academy of Sciences of the United States of America **93**(13): 6437-6442.

Chauleau, M., L. Mora, et al. (2011). "FtsH-dependent Processing of RNase Colicins D and E3 Means That Only the Cytotoxic Domains Are Imported into the Cytoplasm." The Journal of biological chemistry **286**(33): 29397-29407.

Chen, Y. R., T. Y. Yang, et al. (2011). "Delineation of the translocation of colicin E7 across the inner membrane of Escherichia coli." Archives of microbiology **193**(6): 419-428.

Cheng, Y. S., Z. Shi, et al. (2006). "High-resolution crystal structure of a truncated ColE7 translocation domain: implications for colicin transport across membranes." J Mol Biol **356**(1): 22-31.

Chimento, D. P., R. J. Kadner, et al. (2003). "The Escherichia coli outer membrane cobalamin transporter BtuB: structural analysis of calcium and substrate binding, and identification of orthologous transporters by sequence/structure conservation." Journal of molecular biology **332**(5): 999-1014.

Chimento, D. P., A. K. Mohanty, et al. (2003). "Substrate-induced transmembrane signaling in the cobalamin transporter BtuB." Nature structural biology **10**(5): 394-401.

Clavel, T., P. Germon, et al. (1998). "TolB protein of Escherichia coli K-12 interacts with the outer membrane peptidoglycan-associated proteins Pal, Lpp and OmpA." Molecular Microbiology **29**(1): 359-367.

Collins, E. S., S. B. Whittaker, et al. (2002). "Structural dynamics of the membrane translocation domain of colicin E9 and its interaction with TolB." J Mol Biol **318**(3): 787-804.

Collins, E. S., S. B. Whittaker, et al. (2002). "Structural dynamics of the membrane translocation domain of colicin E9 and its interaction with TolB." Journal of molecular biology **318**(3): 787-804.

Cooper, P. C. and R. James (1984). "Two new E colicins, E8 and E9, produced by a strain of *Escherichia coli*." J Gen Microbiol **130**(1): 209-215.

Cowan, S. W., R. M. Garavito, et al. (1995). "The structure of OmpF porin in a tetragonal crystal form." Structure **3**(10): 1041-1050.

Cramer, W. A., J. B. Heymann, et al. (1995). "Structure-function of the channel-forming colicins." Annu Rev Biophys Biomol Struct **24**: 611-641.

Cramer, W. A., J. B. Heymann, et al. (1995). "Structure-function of the channel-forming colicins." Annual review of biophysics and biomolecular structure **24**: 611-641.

Curtis, M. D., R. James, et al. (1989). "An evolutionary relationship between the ColE5-099 and the ColE9-J plasmids revealed by nucleotide sequencing." J Gen Microbiol **135**(10): 2783-2788.

Dankert, J. R., Y. Uratani, et al. (1982). "On a domain structure of colicin E1. A COOH-terminal peptide fragment active in membrane depolarization." The Journal of biological chemistry **257**(7): 3857-3863.

Davidov, Y., R. Rozen, et al. (2000). "Improved bacterial SOS promoter∷lux fusions for genotoxicity detection." Mutation research **466**(1): 97-107.

Davidson, V. L., K. R. Brunden, et al. (1985). "Acidic pH requirement for insertion of colicin E1 into artificial membrane vesicles: relevance to the mechanism of action of colicins and certain toxins." Proceedings of the National Academy of Sciences of the United States of America **82**(5): 1386-1390.

Davies, J. K. and P. Reeves (1975a). "Genetics of resistance to colicins in *Escherichia coli* K-12: cross-resistance among colicins of group A." J Bacteriol **123**(1): 102-117.

Davies, J. K. and P. Reeves (1975b). "Genetics of resistance to colicins in Escherichia coli K-12: cross-resistance among colicins of group B." Journal of Bacteriology **123**(1): 96-101.

de Zamaroczy, M., L. Mora, et al. (2001). "Cleavage of colicin D is necessary for cell killing and requires the inner membrane peptidase LepB." Molecular cell **8**(1): 159-168.

Dekker, N., J. Tommassen, et al. (1999). "Bacteriocin release protein triggers dimerization of outer membrane phospholipase A in vivo." J Bacteriol **181**(10): 3281-3283.

Deprez, C., L. Blanchard, et al. (2002). "Macromolecular import into Escherichia coli: the TolA C-terminal domain changes conformation when interacting with the colicin A toxin." Biochemistry **41**(8): 2589-2598.

Deprez, C., R. Lloubes, et al. (2005). "Solution structure of the E.coli TolA C-terminal domain reveals conformational changes upon binding to the phage g3p N-terminal domain." Journal of molecular biology **346**(4): 1047-1057.

Derouiche, R., H. Benedetti, et al. (1995). "Protein complex within Escherichia coli inner membrane. TolA N-terminal domain interacts with TolQ and TolR proteins." The Journal of biological chemistry **270**(19): 11078-11084.

Derouiche, R., H. Benedetti, et al. (1997). "Binding of colicins A and E1 to purified TolA domains " Microbiology **143**: 3185-3192.

Derouiche, R., M. Gavioli, et al. (1996). "TolA central domain interacts with Escherichia coli porins." The EMBO journal **15**(23): 6408-6415.

Derouiche, R., R. Lloubes, et al. (1999). "Circular dichroism and molecular modeling of the E. coli TolA periplasmic domains." Biospectroscopy **5**(3): 189-198.

Di Masi, D. R., J. C. White, et al. (1973). "Transport of vitamin B12 in *Escherichia coli:* common receptor sites for vitamin B12 and the E colicins on the outer membrane of the cell envelope." J Bacteriol **115**(2): 506-513.

Dorit, R. L. and M. A. Riley (2002). "When comparative information leads us astray: the receptor-binding region of colicin E9." Journal of molecular evolution **55**(3): 347-355.

Dover, L. G., L. J. Evans, et al. (2000). "Colicin pore-forming domains bind to Escherichia coli trimeric porins." Biochemistry **39**(29): 8632-8637.

Dower, W. J., J. F. Miller, et al. (1988). "High efficiency transformation of E. coli by high voltage electroporation." Nucleic Acids Res **16**(13): 6127-6145.

Dubuisson, J. F., A. Vianney, et al. (2002). "Mutational analysis of the TolA C-terminal domain of Escherichia coli and genetic evidence for an interaction between TolA and TolB." Journal of Bacteriology **184**(16): 4620-4625.

Dubuisson, J. F., A. Vianney, et al. (2002). "Mutational analysis of the TolA C-terminal domain of *Escherichia coli* and genetic evidence for an interaction between TolA and TolB." J Bacteriol **184**(16): 4620-4625.

Duche, D. (2007). "Colicin E2 is still in contact with its receptor and import machinery when its nuclease domain enters the cytoplasm." Journal of Bacteriology **189**(11): 4217-4222.

Duche, D., A. Frenkian, et al. (2006). "Release of immunity protein requires functional endonuclease colicin import machinery." J Bacteriol **188**(24): 8593-8600.

Duche, D., M. Issouf, et al. (2009). "Immunity protein protects colicin E2 from OmpT protease." Journal of biochemistry **145**(1): 95-101.

Durkacz, B. W., C. K. Kennedy, et al. (1974). "Plasmid Replication and the Induced Synthesis of Colicins E1 and E2 in *Escherichia coli*." J. Bacteriol. **117**(3): 940-946.

Ebina, Y., F. Kishi, et al. (1982). "Direct participation of lexA protein in repression of colicin E1 synthesis." J Bacteriol **150**(3): 1479-1481.

Ebina, Y., F. Kishi, et al. (1979). "Gene expression in vitro of colicin El plasmid." Nucleic Acids Res **7**(3): 639-649.

Ebina, Y. and A. Nakazawa (1983). "Cyclic AMP-dependent initiation and rho-dependent termination of colicin E1 gene transcription." J Biol Chem **258**(11): 7072-7078.

Ebina, Y., Y. Takahara, et al. (1983). "LexA protein is a repressor of the colicin E1 gene." J Biol Chem **258**(21): 13258-13261.

el Kouhen, R., H. P. Fierobe, et al. (1993). "Characterization of the receptor and translocator domains of colicin N." European journal of biochemistry / FEBS **214**(3): 635-639.

Elkins, P., A. Bunker, et al. (1997). "A mechanism for toxin insertion into membranes is suggested by the crystal structure of the channel-forming domain of colicin E1." Structure **5**(3): 443-458.

Escuyer, V. and M. Mock (1987). "DNA sequence analysis of three missense mutations affecting colicin E3 bactericidal activity." Molecular Microbiology **1**(1): 82-85.

Evans, L. J., S. Labeit, et al. (1996). "The central domain of colicin N possesses the receptor recognition site but not the binding affinity of the whole toxin." Biochemistry **35**(48): 15143-15148.

Ferguson, A. D., R. Chakraborty, et al. (2002). "Structural basis of gating by the outer membrane transporter FecA." Science **295**(5560): 1715-1719.

Fognini-Lefebvre, N., J. C. Lazzaroni, et al. (1987). "tolA, tolB and excC, three cistrons involved in the control of pleiotropic release of periplasmic proteins by Escherichia coli K12." Molecular & general genetics : MGG **209**(2): 391-395.

Fourel, D., S. Mizushima, et al. (1992). "Dynamics of the exposure of epitopes on OmpF, an outer membrane protein of Escherichia coli." European journal of biochemistry / FEBS **206**(1): 109-114.

Fredericq, P. (1957). "Colicins." Annual review of microbiology **11**: 7-22.

Garinot-Schneider, C., C. N. Penfold, et al. (1997). "Identification of residues in the putative TolA box which are essential for the toxicity of the endonuclease toxin colicin E9." Microbiology **143 (Pt 9)**: 2931-2938.

Garinot-Schneider, C., A. J. Pommer, et al. (1996). "Identification of putative active-site residues in the DNase domain of colicin E9 by random mutagenesis." J Mol Biol **260**(5): 731-742.

Germon, P., T. Clavel, et al. (1998). "Mutational analysis of the Escherichia coli K-12 TolA N-terminal region and characterization of its TolQ-interacting domain by genetic suppression." Journal of Bacteriology **180**(24): 6433-6439.

Germon, P., M. C. Ray, et al. (2001). "Energy-dependent conformational change in the TolA protein of *Escherichia coli* involves its N-terminal domain, TolQ, and TolR." J Bacteriol **183**(14): 4110-4114.

Gillor, O., J. A. Vriezen, et al. (2008). "The role of SOS boxes in enteric bacteriocin regulation." Microbiology **154**(Pt 6): 1783-1792.

Gilson, L., H. K. Mahanty, et al. (1990). "Genetic analysis of an MDR-like export system: the secretion of colicin V." The EMBO journal **9**(12): 3875-3884.

Gokce, I., E. M. Raggett, et al. (2000). "The TolA-recognition site of colicin N. ITC, SPR and stopped-flow fluorescence define a crucial 27-residue segment." Journal of molecular biology **304**(4): 621-632.

Gratia, A. (1925). "Sur un remarquable exemple d'antagonisme entre deux souches de coilbacille." Comp Rend Soc Biol **93**.

Gratia, A. F., Pierre (1946). "Diversity of antibiotic strains of Escherichia coli and the variable extent of their field of action ".

Gudmundsdottir, A., C. Bradbeer, et al. (1988). "Altered binding and transport of vitamin B12 resulting from insertion mutations in the Escherichia coli btuB gene." The Journal of biological chemistry **263**(28): 14224-14230.

Guihard, G., P. Boulanger, et al. (1994). "Colicin A and the Tol proteins involved in its translocation are preferentially located in the contact sites between the inner and outer membranes of Escherichia coli cells." The Journal of biological chemistry **269**(8): 5874-5880.

Gumbart, J., M. C. Wiener, et al. (2009). "Coupling of calcium and substrate binding through loop alignment in the outer-membrane transporter BtuB." Journal of molecular biology **393**(5): 1129-1142.

Hands, S. L., L. E. Holland, et al. (2005). "Interactions of TolB with the translocation domain of colicin E9 require an extended TolB box." J Bacteriol **187**(19): 6733-6741.

Hann, E., N. Kirkpatrick, et al. (2007). "The effect of protein complexation on the mechanical stability of Im9." Biophysical journal **92**(9): L79-81.

Hannan, J. P., S. B. Whittaker, et al. (1999). "NMR study of Ni2+ binding to the H-N-H endonuclease domain of colicin E9." Protein science : a publication of the Protein Society **8**(8): 1711-1713.

Hannan, J. P., S. B. Whittaker, et al. (2000). "NMR studies of metal ion binding to the Zn-finger-like HNH motif of colicin E9." Journal of inorganic biochemistry **79**(1-4): 365-370.

Hardy, K. G., G. G. Meynell, et al. (1973). "Two major groups of colicin factors: their evolutionary significance." Mol Gen Genet **125**(3): 217-230.

Harkness, R. E. and V. Braun (1989). "Colicin M inhibits peptidoglycan biosynthesis by interfering with lipid carrier recycling." J Biol Chem **264**(11): 6177-6182.

Henry, T., S. Pommier, et al. (2004). "Improved methods for producing outer membrane vesicles in Gram-negative bacteria." Research in microbiology **155**(6): 437-446.

Hermanson, G. T. (1996). Bioconjugate techniques.

Hilsenbeck, J. L., H. Park, et al. (2004). "Crystal structure of the cytotoxic bacterial protein colicin B at 2.5 A resolution." Mol Microbiol **51**(3): 711-720.

Housden, N. G., S. R. Loftus, et al. (2005). "Cell entry mechanism of enzymatic bacterial colicins: porin recruitment and the thermodynamics of receptor binding." Proc Natl Acad Sci U S A **102**(39): 13849-13854.

Housden, N. G., J. A. Wojdyla, et al. (2010). "Directed epitope delivery across the Escherichia coli outer membrane through the porin OmpF." Proceedings of the National Academy of Sciences of the United States of America **107**(50): 21412-21417.

Howard, S. P., D. Cavard, et al. (1991). "Phospholipase-A-independent damage caused by the colicin A lysis protein during its assembly into the inner and outer membranes of *Escherichia coli*." J Gen Microbiol **137**(1): 81-89.

Howard, S. P., C. Herrmann, et al. (2001). "In vivo synthesis of the periplasmic domain of TonB inhibits transport through the FecA and FhuA iron siderophore transporters of Escherichia coli." Journal of Bacteriology **183**(20): 5885-5895.

Hsieh, S. Y., T. P. Ko, et al. (1997). "A novel role of ImmE7 in the autoregulatory expression of the ColE7 operon and identification of possible RNase active sites in the crystal structure of dimeric ImmE7." The EMBO journal **16**(6): 1444-1454.

Isnard, M., A. Rigal, et al. (1994). "Maturation and localization of the TolB protein required for colicin import." J Bacteriol **176**(20): 6392-6396.

Jakes, K. S. and N. D. Zinder (1974). "Highly purified colicin E3 contains immunity protein." Proc Natl Acad Sci U S A **71**(9): 3380-3384.

James, R., C. Kleanthous, et al. (1996). "The biology of E colicins: paradigms and paradoxes." Microbiology **142**(Pt 7): 1569-1580.

James, R., C. N. Penfold, et al. (2002). "Killing of E. coli cells by E group nuclease colicins." Biochimie **84**(5-6): 381-389.

Jeanteur, D., T. Schirmer, et al. (1994). "Structural and functional alterations of a colicin-resistant mutant of OmpF porin from Escherichia coli." Proceedings of the National Academy of Sciences of the United States of America **91**(22): 10675-10679.

Journet, L., E. Bouveret, et al. (2001). "Import of colicins across the outer membrane of Escherichia coli involves multiple protein interactions in the periplasm." Mol Microbiol **42**(2): 331-344.

Journet, L., A. Rigal, et al. (1999). "Role of TolR N-terminal, central, and C-terminal domains in dimerization and interaction with TolA and tolQ." Journal of Bacteriology **181**(15): 4476-4484.

Kampfenkel, K. and V. Braun (1993). "Membrane topologies of the TolQ and TolR proteins of Escherichia coli: inactivation of TolQ by a missense mutation in the proposed first transmembrane segment." Journal of Bacteriology **175**(14): 4485-4491.

Kleanthous, C. (2010). "Swimming against the tide: progress and challenges in our understanding of colicin translocation." Nat Rev Microbiol **8**(12): 843-848.

Kleanthous, C., U. C. Kuhlmann, et al. (1999). "Structural and mechanistic basis of immunity toward endonuclease colicins." Nat Struct Biol **6**(3): 243-252.

Kleanthous, C. and D. Walker (2001). "Immunity proteins: enzyme inhibitors that avoid the active site." Trends Biochem Sci **26**(10): 624-631.

Knowling, S., A. I. Bartlett, et al. (2011). "Dissecting key residues in folding and stability of the bacterial immunity protein 7." Protein engineering, design & selection : PEDS **24**(6): 517-523.

Ko, T. P., C. C. Liao, et al. (1999). "The crystal structure of the DNase domain of colicin E7 in complex with its inhibitor Im7 protein." Structure **7**(1): 91-102.

Kolade, O. O., S. B. Carr, et al. (2002). "Structural aspects of the inhibition of DNase and rRNase colicins by their immunity proteins." Biochimie **84**(5-6): 439-446.

Krewulak, K. D. and H. J. Vogel (2011). "TonB or not TonB: is that the question?" Biochemistry and cell biology = Biochimie et biologie cellulaire **89**(2): 87-97.

Kuhar, I. (1999). "Transcription Regulation of the Colicin K cka Gene Reveals Induction of Colicin Synthesis by Differential Responses to Environmental Signals." J. Bacteriol. **181**(23): 7373-7380.

Kuhlmann, U. C., A. J. Pommer, et al. (2000). "Specificity in protein-protein interactions: the structural basis for dual recognition in endonuclease colicin-immunity protein complexes." J Mol Biol **301**(5): 1163-1178.

Kumar, A., E. Hajjar, et al. (2010). "Structural and dynamical properties of the porins OmpF and OmpC: insights from molecular simulations." Journal of physics. Condensed matter : an Institute of Physics journal **22**(45): 454125.

Kumar, N., P. Gammell, et al. (2008). "Differential protein expression following low temperature culture of suspension CHO-K1 cells." BMC Biotechnology **8**(1): 42.

Kurisu, G., S. D. Zakharov, et al. (2003). "The structure of BtuB with bound colicin E3 R-domain implies a translocon." Nature structural biology **10**(11): 948-954.

Laemmli, U. K. (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." Nature **227**(5259): 680-685.

Lakey, J. H., D. Duche, et al. (1993). "Fluorescence energy transfer distance measurements. The hydrophobic helical hairpin of colicin A in the membrane bound state." Journal of molecular biology **230**(3): 1055-1067.

Larsen, R. A. and K. Postle (2001). "Conserved residues Ser(16) and His(20) and their relative positioning are essential for TonB activity, cross-linking of TonB with ExbB, and the ability of TonB to respond to proton motive force." The Journal of biological chemistry **276**(11): 8111-8117.

Lau, P. C., R. W. Rowsome, et al. (1984). "Comparative nucleotide sequences encoding the immunity proteins and the carboxyl-terminal peptides of colicins E2 and E3." Nucleic Acids Res **12**(22): 8733-8745.

Law, C. J., C. N. Penfold, et al. (2003). "OmpF enhances the ability of BtuB to protect susceptible Escherichia coli cells from colicin E9 cytotoxicity." FEBS letters **545**(2-3): 127-132.

Lazdunski, C. J. (1988). "Pore-forming colicins: synthesis, extracellular release, mode of action, immunity." Biochimie **70**(9): 1291-1296.

Lazdunski, C. J., E. Bouveret, et al. (1998). "Colicin import into Escherichia coli cells." J Bacteriol **180**(19): 4993-5002.

Lazzaroni, J. C., N. Fognini-Lefebvre, et al. (1989). "Cloning of the excC and excD genes involved in the release of periplasmic proteins by Escherichia coli K12." Molecular & general genetics : MGG **218**(3): 460-464.

Lazzaroni, J. C., P. Germon, et al. (1999). "The Tol proteins of Escherichia coli and their involvement in the uptake of biomolecules and outer membrane stability." FEMS microbiology letters **177**(2): 191-197.

Lazzaroni, J. C., A. Vianney, et al. (1995). "Transmembrane alpha-helix interactions are required for the functional assembly of the Escherichia coli Tol complex." Journal of molecular biology **246**(1): 1-7.

Lei, G. S., W. J. Syu, et al. (2011). "Repression of btuB gene transcription in Escherichia coli by the GadX protein." BMC microbiology **11**(1): 33.

Levengood, S. K., W. F. Beyer, Jr., et al. (1991). "TolA: a membrane protein involved in colicin uptake contains an extended helical region." Proc Natl Acad Sci U S A **88**(14): 5939-5943.

Li, W., S. J. Hamill, et al. (1998). "Dual recognition and the role of specificity-determining residues in colicin E9 DNase-immunity protein interactions." Biochemistry **37**(34): 11771-11779.

Little, J. W., S. H. Edmiston, et al. (1980). "Cleavage of the *Escherichia coli* lexA protein by the recA protease." Proc Natl Acad Sci U S A **77**(6): 3225-3229.

Lloubes, R., D. Baly, et al. (1986). "The promoters of the genes for colicin production, release and immunity in the ColA plasmid: effects of convergent transcription and Lex A protein." Nucl. Acids Res. **14**(6): 2621-2636.

Lloubes, R., E. Cascales, et al. (2001). "The Tol-Pal proteins of the *Escherichia coli* cell envelope: an energized system required for outer membrane integrity?" Res Microbiol **152**(6): 523-529.

Lloubes, R., M. Granger-Schnarr, et al. (1988). "LexA repressor induces operator-dependent DNA bending." J Mol Biol **204**(4): 1049-1054.

Locher, K. P., B. Rees, et al. (1998). "Transmembrane signaling across the ligand-gated FhuA receptor: crystal structures of free and ferrichrome-bound states reveal allosteric changes." Cell **95**(6): 771-778.

Loftus, S. R., D. Walker, et al. (2006). "Competitive recruitment of the periplasmic translocation portal TolB by a natively disordered domain of colicin E9." Proceedings of the National Academy of Sciences of the United States of America **103**(33): 12353-12358.

Lu, F. M. and K. F. Chak (1996). "Two overlapping SOS-boxes in ColE operons are responsible for the viability of cells harboring the Col plasmid." Mol Gen Genet **251**(4): 407-411.

Lubkowski, J., F. Hennecke, et al. (1999). "Filamentous phage infection: crystal structure of g3p in complex with its coreceptor, the C-terminal domain of TolA." Structure **7**(6): 711-722.

Mark, G., P. Lawrence, et al. (1984). "Characterisation of the ColE8 plasmid, a new member of the group E colicin plasmids." Gene **29**(1-2): 145-155.

Masaki, H. and T. Ogawa (2002). "The modes of action of colicins E5 and D, and related cytotoxic tRNases." Biochimie **84**(5-6): 433-438.

Meury, J. and G. Devilliers (1999). "Impairment of cell division in tolA mutants of Escherichia coli at low and high medium osmolarities." Biology of the cell / under the auspices of the European Cell Biology Organization **91**(1): 67-75.

Mizuno, T. (1979). "A novel peptidoglycan-associated lipoprotein found in the cell envelope of Pseudomonas aeruginosa and *Escherichia coli*." J Biochem **86**(4): 991-1000.

Mora, L., N. Diaz, et al. (2005). "Import of the transfer RNase colicin D requires site-specific interaction with the energy-transducing protein TonB." Journal of Bacteriology **187**(8): 2693-2697.

Mosbahi, K., C. Lemaitre, et al. (2002). "The cytotoxic domain of colicin E9 is a channel-forming endonuclease." Nat Struct Biol **9**(6): 476-484.

Mulec, J., Z. Podlesek, et al. (2003). "A cka-gfp Transcriptional Fusion Reveals that the Colicin K Activity Gene Is Induced in Only 3 Percent of the Population." J. Bacteriol. **185**(2): 654-659.

Muller, M. M., A. Vianney, et al. (1993). "Membrane topology of the Escherichia coli TolR protein required for cell envelope integrity." Journal of Bacteriology **175**(18): 6059-6061.

Nagel de Zwaig, R. and S. E. Luria (1967). "Genetics and physiology of colicin-tolerant mutants of Escherichia coli." Journal of Bacteriology **94**(4): 1112-1123.

Nomura, M. and C. Witten (1967). "Interaction of colicins with bacterial cells. 3. Colicin-tolerant mutations in *Escherichia coli*." Journal of Bacteriology **94**(4): 1093-1111.

Nossal, N. G. and L. A. Heppel (1966). "The release of enzymes by osmotic shock from Escherichia coli in exponential phase." The Journal of biological chemistry **241**(13): 3055-3062.

Ogawa, T., K. Tomita, et al. (1999). "A cytotoxic ribonuclease targeting specific transfer RNA anticodons." Science **283**(5410): 2097-2100.

Ohno, S., Y. Ohno-Iwashita, et al. (1977). "Purification and characterization of active component and active fragment of colicin E3." J Biochem **82**(4): 1045-1053.

Osborne, M. J., A. L. Breeze, et al. (1996). "Three-dimensional solution structure and 13C nuclear magnetic resonance assignments of the colicin E9 immunity protein Im9." Biochemistry **35**(29): 9505-9512.

Osborne, M. J., R. Wallis, et al. (1997). "Identification of critical residues in the colicin E9 DNase binding region of the Im9 protein." Biochem J **323 (Pt 3)**: 823-831.

Oudega, B. (2003). Protein Secretion Pathways in Bacteria, Dordrecht: Kluyver Academic Pblisher.

Oudega, B., P. Klaasen-Boor, et al. (1975). "Mode of action of the cloacin DF13-immunity protein." Biochim Biophys Acta **392**(1): 184-195.

Ozeki, H., B. A. Stocker, et al. (1959). "Production of colicine by single bacteria." Nature **184**: 337-339.

Parker, M. W., F. Pattus, et al. (1989). "Structure of the membrane-pore-forming fragment of colicin A." Nature **337**(6202): 93-96.

Patterson, G., R. N. Day, et al. (2001). "Fluorescent protein spectra." J Cell Sci **114**(5): 837-838.

Patterson, G. H., D. W. Piston, et al. (2000). "Förster Distances between Green Fluorescent Protein Pairs." Analytical biochemistry **284**(2): 438-440.

Penfold, C. N., C. Garinot-Schneider, et al. (2000). "A 76-residue polypeptide of colicin E9 confers receptor specificity and inhibits the growth of vitamin B12-dependent *Escherichia coli* 113/3 cells." Molecular Microbiology **38**(3): 639-649.

Penfold, C. N., B. Healy, et al. (2004). "Flexibility in the receptor-binding domain of the enzymatic colicin E9 is required for toxicity against Escherichia coli cells." Journal of Bacteriology **186**(14): 4520-4527.

Pilsl, H. and V. Braun (1995). "Evidence that the immunity protein inactivates colicin 5 immediately prior to the formation of the transmembrane channel." J Bacteriol **177**(23): 6966-6972.

Pilsl, H. and V. Braun (1995). "Novel colicin 10: assignment of four domains to TonB- and TolC-dependent uptake via the Tsx receptor and to pore formation." Mol Microbiol **16**(1): 57-67.

Pilsl, H. and V. Braun (1995). "Strong function-related homology between the pore-forming colicins K and 5." J Bacteriol **177**(23): 6973-6977.

Pommer, A. J., U. C. Kuhlmann, et al. (1999). "Homing in on the role of transition metals in the HNH motif of colicin endonucleases." The Journal of biological chemistry **274**(38): 27153-27160.

Pommer, A. J., R. Wallis, et al. (1998). "Enzymological characterization of the nuclease domain from the bacterial toxin colicin E9 from Escherichia coli." The Biochemical journal **334 (Pt 2)**: 387-392.

Pommier, S., M. Gavioli, et al. (2005). "Tol-dependent macromolecule import through the Escherichia coli cell envelope requires the presence of an exposed TolA binding motif." J Bacteriol **187**(21): 7526-7534.

Pommier, S., M. Gavioli, et al. (2005). "Tol-dependent macromolecule import through the Escherichia coli cell envelope requires the presence of an exposed TolA binding motif." Journal of Bacteriology **187**(21): 7526-7534.

Pugsley, A. P. (1983). "Obligatory coupling of colicin release and lysis in mitomycin-treated Col+ Escherichia coli." J Gen Microbiol **129**(6): 1921-1928.

Pugsley, A. P. (1984). "The ins and outs of colicins. Part I: Production, and translocation across membranes." Microbiol Sci **1**(7): 168-175.

Pugsley, A. P. (1987). "Nucleotide sequencing of the structural gene for colicin N reveals homology between the catalytic, C-terminal domains of colicins A and N." Molecular Microbiology **1**(3): 317-325.

Pugsley, A. P. (1988). "The immunity and lysis genes of ColN plasmid pCHAP4." Molecular & general genetics : MGG **211**(2): 335-341.

Pugsley, A. P. and M. Schwartz (1983). "Expression of a gene in a 400-base-pair fragment of colicin plasmid ColE2-P9 is sufficient to cause host cell lysis." Journal of Bacteriology **156**(1): 109-114.

Pugsley, A. P. and M. Schwartz (1984). "Colicin E2 release: lysis, leakage or secretion? Possible role of a phospholipase." EMBO J **3**(10): 2393-2397.

Raggett, E. M., G. Bainbridge, et al. (1998). "Discovery of critical Tol A-binding residues in the bactericidal toxin colicin N: a biophysical approach." Mol Microbiol **28**(6): 1335-1343.

Ray, M. C., P. Germon, et al. (2000). "Identification by genetic suppression of Escherichia coli TolB residues important for TolB-Pal interaction." Journal of Bacteriology **182**(3): 821-824.

Rigal, A., E. Bouveret, et al. (1997). "The TolB protein interacts with the porins of Escherichia coli." Journal of Bacteriology **179**(23): 7274-7279.

Riley, M. A. (1993). "Molecular mechanisms of colicin evolution." Mol Biol Evol **10**(6): 1380-1395.

Riley, M. A. (1993). "Positive selection for colicin diversity in bacteria." Mol Biol Evol **10**(5): 1048-1059.

Riley, M. A. and D. M. Gordon (1992). "A survey of Col plasmids in natural isolates of Escherichia coli and an investigation into the stability of Col-plasmid lineages." J Gen Microbiol **138**(Pt 7): 1345-1352.

Saiki, R. K., S. Scharf, et al. (1985). "Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia." Science **230**(4732): 1350-1354.

Salles, B., J. M. Weisemann, et al. (1987). "Temporal control of colicin E1 induction." J Bacteriol **169**(11): 5028-5034.

Sambrook, J., E. F. Fritsh, et al. (2001). Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press.

Sanger, F., S. Nicklen, et al. (1977). "DNA sequencing with chain-terminating inhibitors." Proc Natl Acad Sci U S A **74**(12): 5463-5467.

Schagger, H. (2006). "Tricine-SDS-PAGE." Nature protocols **1**(1): 16-22.

Schaller, K. and M. Nomura (1976). "Colicin E2 is DNA endonuclease." Proc Natl Acad Sci U S A **73**(11): 3989-3993.

Schendel, S. L., E. M. Click, et al. (1997). "The TolA protein interacts with colicin E1 differently than with other group A colicins." J Bacteriol **179**(11): 3683-3690.

Selenko, P. and G. Wagner (2007). "Looking into live cells with in-cell NMR spectroscopy." Journal of structural biology **158**(2): 244-253.

Sharma, O., E. Yamashita, et al. (2007). "Structure of the complex of the colicin E2 R-domain and its BtuB receptor. The outer membrane colicin translocon." The Journal of biological chemistry **282**(32): 23163-23170.

Shi, Z., K. F. Chak, et al. (2005). "Identification of an essential cleavage site in ColE7 required for import and killing of cells." J Biol Chem **280**(26): 24663-24668.

Shirabe, K., Y. Ebina, et al. (1985). "Positive regulation of the colicin E1 gene by cyclic AMP and cyclic AMP receptor protein." Nucleic acids research **13**(13): 4687-4698.

Shub, D. A., H. Goodrich-Blair, et al. (1994). "Amino acid sequence motif of group I intron endonucleases is conserved in open reading frames of group II introns." Trends in biochemical sciences **19**(10): 402-404.

Smilowitz, H. (1974). "Bacteriophage f1 infection and colicin tolerance." Journal of virology **13**(1): 100-106.

Soelaiman, S., K. Jakes, et al. (2001). "Crystal structure of colicin E3: implications for cell entry and ribosome inactivation." Mol Cell **8**(5): 1053-1062.

Soong, B. W., S. Y. Hsieh, et al. (1994). "Mapping of transcriptional start sites of the cea and cei genes of the ColE7 operon." Molecular & general genetics : MGG **243**(4): 477-481.

Sui, M. J., L. C. Tsai, et al. (2002). "Metal ions and phosphate binding in the H-N-H motif: crystal structures of the nuclease domain of ColE7/Im7 in complex with a phosphate ion and different divalent metal ions." Protein science : a publication of the Protein Society **11**(12): 2947-2957.

Suit, J. L. and S. E. Luria (1988). "Expression of the kil gene of the ColE1 plasmid in Escherichia coli Kilr mutants causes release of periplasmic enzymes and of colicin without cell death." Journal of Bacteriology **170**(10): 4963-4966.

van den Bremer, E. T., W. Jiskoot, et al. (2002). "Probing metal ion binding and conformational properties of the colicin E9 endonuclease by electrospray ionization time-of-flight mass spectrometry." Protein science : a publication of the Protein Society **11**(7): 1738-1752.

van den Elzen, P. J., J. Maat, et al. (1982). "The nucleotide sequence of the bacteriocin promoters of plasmids Clo DF13 and Co1 E1: role of lexA repressor and cAMP in the regulation of promoter activity." Nucleic Acids Res **10**(6): 1913-1928.

van der Goot, F. G., N. Didat, et al. (1993). "Role of acidic lipids in the translocation and channel activity of colicins A and N in Escherichia coli cells." European journal of biochemistry / FEBS **213**(1): 217-221.

van der Goot, F. G., J. M. Gonzalez-Manas, et al. (1991). "A 'molten-globule' membrane-insertion intermediate of the pore-forming domain of colicin A." Nature **354**(6352): 408-410.

van der Wal, F. J., J. Luirink, et al. (1995). "Bacteriocin release proteins: mode of action, structure, and biotechnological application." FEMS Microbiol Rev **17**(4): 381-399.

Vankemmelbeke, M., B. Healy, et al. (2005). "Rapid Detection of Colicin E9-Induced DNA Damage Using *Escherichia coli* Cells Carrying SOS Promoter-lux Fusions." J. Bacteriol. **187**(14): 4900-4907.

Vankemmelbeke, M., Y. Zhang, et al. (2009). "Energy-dependent immunity protein release during tol-dependent nuclease colicin translocation." J Biol Chem **284**(28): 18932-18941.

Vetter, I. R., M. W. Parker, et al. (1998). "Crystal structure of a colicin N fragment suggests a model for toxicity." Structure **6**(7): 863-874.

Vianney, A., T. M. Lewin, et al. (1994). "Membrane topology and mutational analysis of the TolQ protein of Escherichia coli required for the uptake of macromolecules and cell envelope integrity." Journal of Bacteriology **176**(3): 822-829.

Walburger, A., C. Lazdunski, et al. (2002). "The Tol/Pal system function requires an interaction between the C-terminal domain of TolA and the N-terminal domain of TolB." Mol Microbiol **44**(3): 695-708.

Walker, D., L. Lancaster, et al. (2004). "Identification of the catalytic motif of the microbial ribosome inactivating cytotoxin colicin E3." Protein science : a publication of the Protein Society **13**(6): 1603-1611.

Walker, D., G. R. Moore, et al. (2003). "Thermodynamic consequences of bipartite immunity protein binding to the ribosomal ribonuclease colicin E3." Biochemistry **42**(14): 4161-4171.

Walker, D. C., T. Georgiou, et al. (2002). "Mutagenic scan of the H-N-H motif of colicin E9: implications for the mechanistic enzymology of colicins, homing enzymes and apoptotic endonucleases." Nucleic acids research **30**(14): 3225-3234.

Wallis, R., K. Y. Leung, et al. (1998). "Specificity in protein-protein recognition: conserved Im9 residues are the major determinants of stability in the colicin E9 DNase-Im9 complex." Biochemistry **37**(2): 476-485.

Wallis, R., K. Y. Leung, et al. (1995). "Protein-protein interactions in colicin E9 DNase-immunity protein complexes. 2. Cognate and noncognate interactions that span the millimolar to femtomolar affinity range." Biochemistry **34**(42): 13751-13759.

Wallis, R., G. R. Moore, et al. (1995). "Protein-protein interactions in colicin E9 DNase-immunity protein complexes. 1. Diffusion-controlled association and femtomolar binding for the cognate complex." Biochemistry **34**(42): 13743-13750.

Wallis, R., A. Reilly, et al. (1992). "In vivo and in vitro characterization of overproduced colicin E9 immunity protein." European journal of biochemistry / FEBS **207**(2): 687-695.

Webster, R. E. (1991). "The tol gene products and the import of macromolecules into Escherichia coli." Molecular Microbiology **5**(5): 1005-1011.

Wiener, M., D. Freymann, et al. (1997). "Crystal structure of colicin Ia." Nature **385**(6615): 461-464.

Wilmsen, H. U., A. P. Pugsley, et al. (1990). "Colicin N forms voltage- and pH-dependent channels in planar lipid bilayer membranes." European biophysics journal : EBJ **18**(3): 149-158.

Witkin, E. M. (1976). "Ultraviolet mutagenesis and inducible DNA repair in Escherichia coli." Bacteriological reviews **40**(4): 869-907.

Wu, H. C. and M. Tokunaga (1986). "Biogenesis of lipoproteins in bacteria." Current topics in microbiology and immunology **125**: 127-157.

Yamashita, E., M. V. Zhalnina, et al. (2008). "Crystal structures of the OmpF porin: function in a colicin translocon." The EMBO journal **27**(15): 2171-2180.

Yanisch-Perron, C., J. Vieira, et al. (1985). "Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors." Gene **33**(1): 103-119.

Yue, W. W., S. Grizot, et al. (2003). "Structural evidence for iron-free citrate and ferric citrate binding to the TonB-dependent outer membrane transporter FecA." Journal of molecular biology **332**(2): 353-368.

Zakharov, S. D. and W. A. Cramer (2002). "Colicin crystal structures: pathways and mechanisms for colicin insertion into membranes." Biochim Biophys Acta **1565**(2): 333-346.

Zakharov, S. D., V. Y. Eroukova, et al. (2004). "Colicin occlusion of OmpF and TolC channels: outer membrane translocons for colicin import." Biophysical journal **87**(6): 3901-3911.

Zakharov, S. D., M. Lindeberg, et al. (1999). "Kinetic description of structural changes linked to membrane import of the colicin E1 channel protein." Biochemistry **38**(35): 11325-11332.

Zakharov, S. D., M. Lindeberg, et al. (1998). "Membrane-bound state of the colicin E1 channel domain as an extended two-dimensional helical array." Proceedings of the National Academy of Sciences of the United States of America **95**(8): 4282-4287.

Zakharov, S. D., O. Sharma, et al. (2008). "Primary events in the colicin translocon: FRET analysis of colicin unfolding initiated by binding to BtuB and OmpF." Biochemistry **47**(48): 12802-12809.

Zakharov, S. D., M. V. Zhalnina, et al. (2006). "The colicin E3 outer membrane translocon: immunity protein release allows interaction of the cytotoxic domain with OmpF porin." Biochemistry **45**(34): 10199-10207.

Zeth, K., C. Romer, et al. (2008). "Crystal structure of colicin M, a novel phosphatase specifically imported by *Escherichia coli*." J Biol Chem **283**(37): 25324-25331.

Zhang, Y., C. Li, et al. (2010). "The crystal structure of the TolB box of colicin A in complex with TolB reveals important differences in the recruitment of the common TolB translocation portal used by group A colicins." Molecular Microbiology **75**(3): 623-636.

Zinder, N. D. (1973). "Resistance to colicins E3 and K induced by infection with bacteriophage f1." Proceedings of the National Academy of Sciences of the United States of America **70**(11): 3160-3164.

APPENDIX A

Buffers and reagents and antibiotics

M9 Salts

Na_2HPO_4	6 g/l
KH_2PO_4	3 g/l
NaCl	0.5 g/l
NH_4Cl	1 g/l

-dissolved with dH_2O. The pH was adjusted to 7.4 and the broth was autoclaved

TAE buffer (50 × stock)

242 g Tris base (Life technologies), 57.1 ml glacial acetic acid (Fisher chemicals) 100 ml 500 mM EDTA (pH 8.0) made up to 1 L with dH_2O

Agarose gel

0.8-1.0 % (w/v) agarose was prepared by dissolving 0.8-1 g of agarose powder (sigma), 2 ml 50 × TAE buffer and made up to 0.1 L with dH_2O; melt and add 5 µl ethidium bromide (10 mg ml^{-1}).

Sodium dodecyl sulfate (SDS-running buffer (10×)

(30.3 g (0.25 M) Tris base, 187.7 g (2.5 M) Glycine, 950 ml dH_2O, 10 g SDS (1 %) w/v, made up to 1 L ml with dH_2O, mix well.

Resolving gel

1.7 ml SDS-resolving buffer, 2.33 ml Acrylamide/Bis-Acrylamide (30 %), 2.88 ml dH_2O, 30 µl 10 % w/v ammonium persulfate (APS) and 30 µl Tetramethyl ethylenediamine (TEMED).

Stacking gel

1 ml SDS-stacking buffer, 0.8 ml Acrylamide/Bis-Acrylamide (30 %), 2.18 ml dH_2O, 30 µl 10 % w/v APS and 30 µl TEMED.

Phosphate buffered saline solution (PBS)

1 × PBS was prepared by dissolving 1 tablet of Phosphate buffered saline (Dulbecco, Oxoid) in 0.1 L dH_2O and autoclaving, this gives sodium chloride 0.16 mol, Potassium chloride 0.003 mol, Disodium hydrogen phosphate 0.008 mol and Potassium dihydrogen phosphate 0.001 mol with a pH value of 7.4

Semi-dry blotting buffer

1 x stock solution was prepared by dissolving 5.82 g Tris base, 2.93 g Glycine, 3.75ml 10 % SDS, 200 ml methanol and make up to 1000 ml with dH2O.

IPTG (Isopropyl-β-D-1 thiogalactopyranoside [FW 238.8])

1 M stock solution was prepared by dissolving 0.23 g of IPTG in 1 ml dH_2O, sterilized by filtration and stored at -20 °C.

DNA loading dye (10×)

10 mM Tris-HCl (pH 7.6), 0.03 % Bromophenol blue, 0.03 % xylene cyanol FF, 60 % v/v glycerol, 60 mM Ethylenediamine tetraacetic acid (EDTA)

Antibiotics

Antibiotics were purchased from Sigma-Aldrich UK, and prepared according to the manufacturer's recommendations, sterilized by filtration and stored at 4 °C.

Ampicillin (100 mg ml^{-1} stock solution prepared in dH_2O)

Chloramphenicol (34 mg ml^{-1} stock solution prepared in ethanol)

APPENDIX B

Plasmids descriptions, protein sequences, ColE9 and ColA sequence

Table 8-1: Name and Descriptions of different constructs used in this project			
Construct name	**Description**	**Vector**	**Restriction sites**
pBH29^{s-s}	ColE9 with disulfide bond in receptor binding domain	pET21A	*Nde*I/*Xho*I
pFA3	ColE9^{S-S}: Cys in DNase at position 469 with disulfide bond in receptor binding domain	pET21A	*Nde*I/*Xho*I
pFA4	Wild type ColA TA_{1-172}	pBAD/gIIIc	*Nco*I/*Xho*I
pFA5	ColA TA_{1-172} containing 7 TolB box residues of ColE9	pBAD/gIIIc	*Nco*I/*Xho*I
pFA6	Wild type ColA TA_{1-172} with alanine mutation in the TolB box at position 18 (E18A)	pBAD/gIIIc	*Nco*I/*Xho*I
pFA7	Wild type ColA TA_{1-172} with alanine mutation in the TolA box at position 58 (Y58A)	pBAD/gIIIc	*Nco*I/*Xho*I
pFA8	ColA TA_{1-172} containing 7 TolB box residues of ColE9 with alanine mutation in the TolB box at position 18 (E18A)	pBAD/gIIIc	*Nco*I/*Xho*I
pFA9	ColA TA_{1-172} containing 7 TolB box residues of ColE9 with alanine mutation in the TolA box at position 58 (Y58A)	pBAD/gIIIc	*Nco*I/*Xho*I
pFA10	Wild type ColA TA_{1-172} with alanine mutation in both the TolB box at position 18 (E18A) and the TolA box at position 58 (Y58A)	pBAD/gIIIc	*Nco*I/*Xho*I

pFA11	ColA TA_{1-172} containing 7 TolB box residues of ColE9 with alanine mutation in both the TolB box at position 18 (E18A) and the TolA box at position 58 (Y58A)	pBAD/gIIIc	*Nco*I/*Xho*I
pFA12	Wild type ColA TA_{1-107}	pBAD/gIII	*Nco*I/*Xho*I
pFA13	ColA TA_{1-107} with ColE9 TolB box	pBAD/gIII	*Nco*I/*Xho*I
pFA14	Lysostaphin targeting domain 172 amino acids	pBAD/gIII	*Nco*I/*Xho*I
pFA15	ColA TA_{52-172}	pBAD/gIII	*Nco*I/*Xho*I
pFA16	ColA TA_{1-52}	pBAD/gIII	*Nco*I/*Xho*I
pFA17	ColA TA_{1-52} containing 7 TolB box residues of ColE9	pBAD/gIII	*Nco*I/*Xho*I
pFA18	Wild type ColA TA_{1-172} (R92-R96 A mutants)	pBAD/gIII	*Nco*I/*Xho*I
pFA19	ColA TolB box::Thrombin::ColN TolA box	pBAD/gIII	*Nco*I/*Xho*I
pFA20	ColN TolA box	pBAD/gIII	*Nco*I/*Xho*I
pMV16	Im9 with Ser at position 23 and Cys at position 6	pET21a	*Nde*I/*Xho*I
pMV47	E9 DNase His575Ala	pET21a	*Nco*I/*Xho*I
pNP203	Wild type ColA TA_{1-172}	pACT2a	*Nco*I/*Xho*I
pYZ78	Wild type ColA TA_{1-172}	pET21a	*Nde*I/*Xho*I

Proteins sequences of constructed plamids: sequence prediction using DNAMAN software

All mutated amino acids are highlighted in yellow.

FA4: Total amino acid number: 172, MW=17782
Max ORF: 1-516, 172 AA, MW=17782
MAGFNYGGKGDGTGWSSERGSGPEPGGGSHGNSGGHDRGDSSNVGNESVTVM
KPGDSYNTPWGKVIINAAGQPTMNGTVMTADNSSMVPYGRGFTRVLNSLVNN
PVSPAGQNGGKSPVQTAVENYLMVQSGNLPPGYWLSNGKVMTEVREERTSGG
GGKNGNERTWTVKVPR

FA5: Total amino acid number: 172, MW=17895
Max ORF: 1-516, 172 AA, MW=17895
MAGFNYGGASDGSGWSSENNPWPEPGGGSHGNSGGHDRGDSSNVGNESVTVM
KPGDSYNTPWGKVIINAAGQPTMNGTVMTADNSSMVPYGRGFTRVLNSLVNN
PVSPAGQNGGKSPVQTAVENYLMVQSGNLPPGYWLSNGKVMTEVREERTSGG
GGKNGNERTWTVKVPR

Fa6: Total amino acid number: 172, MW=17724
Max ORF: 1-516, 172 AA, MW=17724
MAGFNYGGKGDGTGWSSARGSGPEPGGGSHGNSGGHDRGDSSNVGNESVTVM
KPGDSYNTPWGKVIINAAGQPTMNGTVMTADNSSMVPYGRGFTRVLNSLVNN
PVSPAGQNGGKSPVQTAVENYLMVQSGNLPPGYWLSNGKVMTEVREERTSGG
GGKNGNERTWTVKVPR

FA7: Total amino acid number: 172, MW=17690
Max ORF: 1-516, 172 AA, MW=17690
MAGFNYGGKGDGTGWSSERGSGPEPGGGSHGNSGGHDRGDSSNVGNESVTVM
KPGDSANTPWGKVIINAAGQPTMNGTVMTADNSSMVPYGRGFTRVLNSLVNN
PVSPAGQNGGKSPVQTAVENYLMVQSGNLPPGYWLSNGKVMTEVREERTSGG
GGKNGNERTWTVKVPR

FA8: Total amino acid number: 172, MW=17837
Max ORF: 1-516,172AA,MW=17837
MAGFNYGGASDGSGWSSANNPWPEPGGGSHGNSGGHDRGDSSNVGNESVTVM
KPGDSYNTPWGKVIINAAGQPTMNGTVMTADNSSMVPYGRGFTRVLNSLVNN
PVSPAGQNGGKSPVQTAVENYLMVQSGNLPPGYWLSNGKVMTEVREERTSGG
GGKNGNERTWTVKVPR

FA9: Total amino acid number: 172, MW=17803
Max ORF: 1-516,172AA,MW=17803
MAGFNYGGASDGSGWSSENNPWPEPGGGSHGNSGGHDRGDSSNVGNESVTVM
KPGDSANTPWGKVIINAAGQPTMNGTVMTADNSSMVPYGRGFTRVLNSLVNN
PVSPAGQNGGKSPVQTAVENYLMVQSGNLPPGYWLSNGKVMTEVREERTSGG
GGKNGNERTWTVKVPR

FA10: Total amino acid number: 172, MW=17632
Max ORF: 1-516,172AA,MW=17632
MAGFNYGGKGDGTGWSSARGSGPEPGGGSHGNSGGHDRGDSSNVGNESVTVM
KPGDSANTPWGKVIINAAGQPTMNGTVMTADNSSMVPYGRGFTRVLNSLVNN
PVSPAGQNGGKSPVQTAVENYLMVQSGNLPPGYWLSNGKVMTEVREERTSGG
GGKNGNERTWTVKVPR

Fa11: Total amino acid number: 172, MW=17745
Max ORF:1-516,172AA,MW=17745
MAGFNYGGASDGSGWSSANNPWPEPGGGSHGNSGGHDRGDSSNVGNESVTVM
KPGDSANTPWGKVIINAAGQPTMNGTVMTADNSSMVPYGRGFTRVLNSLVNN
PVSPAGQNGGKSPVQTAVENYLMVQSGNLPPGYWLSNGKVMTEVREERTSGG
GGKNGNERTWTVKVPR

FA12: Total amino acid number: 107, MW=10834
Max ORF:1-321,107AA,MW=10834
MAGFNYGGKGDGTGWSSERGSGPEPGGGSHGNSGGHDRGDSSNVGNESVTVM
KPGDSYNTPWGKVIINAAGQPTMNGTVMTADNSSMVPYGRGFTRVLNSLVNN
PVS

FA13: Total amino acid number: 107,MW=10947
Max ORF:1-321,107AA,MW=10947
MAGFNYGGASDGSGWSSENNPWPEPGGGSHGNSGGHDRGDSSNVGNESVTVM
KPGDSYNTPWGKVIINAAGQPTMNGTVMTADNSSMVPYGRGFTRVLNSLVNN
PVS

FA14: Total amino acid number:172, MW=19268
Max ORF:1-516,172AA,MW=19268
MGWKTNKYGTLYKSESASFTPNTDIITRTTGPFRSMPQSGVLKAGQTIHYDE
VMKQDGHVWVGYTGNSGQRIYLPVRTWNKSTNTLGVLWGTIKLEGWKTNKYG
TLYKSESASFTPNTDITRTTGPFRSMPQSGVLKAGQTIHYDEVMKQDGHVWV
GYTGNSGQRIYLPVR

FA15: Total amino acid number:121, MW=12882
Max ORF: 1-363,121AA,MW=12882
MEPGDSYNTPWGKVIINAAGQPTMNGTVMTADNSSMVPYGRGFTRVLNSLVN
NPVSPAGQNGGKSPVQTAVENYLMVQSGNLPPGYWLSNGKVMTEVREERTSG
GGGKNGNERTWTVKVPR

FA16: Total amino acid number: 52, MW=5032
MaxORF:1-156,52AA,MW=5032
MAGFNYGGKGDGTGWSSERGSGPEPGGGSHGNSGGHDRGDSSNVGNESVTVM

FA17: Total amino acid number: 52, MW=5145
Max ORF:1-156,52AA,MW=5145
MAGFNYGGASDGSGWSSENNPWPEPGGGSHGNSGGHDRGDSSNVGNESVTVM

FA18: Total amino acid number:172, MW=17520
Max ORF:1-516,172AA,MW=17520
MAGFNYGGKGDGTGWSSERGSGPEPGGGSHGNSGGHDRGDSSNVGNESVTVM
KPGDSYNTPWGKVIINAAGQPTMNGTVMTADNSSMVPYGAAAAAVLNSLVNN
PVSPAGQNGGKSPVQTAVENYLMVQSGNLPPGYWLSNGKVMTEVREERTSGG
GGKNGNERTWTVKVPR

FA19: Total amino acid number: 101, MW=10369
Max ORF: 1-303,101AA,MW=10369
MAGFNYGGKGDGTGWSSERGSGPEPGGGSHGNSGGHDRGDSSNVGNESVTVM
LVPRGSNRGNSNGWSWSNKPHKNDGFHSDGSYHITFHGDNNSKPKPGGN

FA20: Total amino acid number: 46, MW=5101
Max ORF: 1-138,46AA,MW=5101
MNRGNSNGWSWSNKPHKNDGFHSDGSYHITFHGDNNSKPKPGGNLE

ColE9 plasmid: complete sequence

ORIGIN

```
   1 CCATGCACAC ATCATGCTGA CCACGAGAGC TGTCGGGGAA ACGGGATTCG CAGGAAAGGT
  61 CAGGGACTGG AACGACCGGG CACTCGCCGA GACGTGGCGC GCATCATGGG CTGACCATGC
 121 GAACAGAGCG CTTGCGAACG CCGGCTACCA GGAAGAGATA GACCATCGTT CATACGAGCG
 181 TCAGGGACTG GAGAAAGCGC CGGGCCTTCA CCTCGGAAAG GCTGCCTGTG CGATGGAAAA
 241 ACGGGGCATG GAAACAGAAC GCGGTGAGCA GAACCGCCTG ATTAACAGCC TTAACCTGGA
 301 AATACAGGTT TCCCGCACAA GGCTGGCAGT CATCCAGGTA CAGGAGAACG TGCGGAAACG
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ColA gene sequence

ORIGIN

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 301 CTGGTCAATA ATCCTGTTTC GCCGGCAGGT CAGAATGGCG GGAAGTCTCC TGTTCAGACT
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1741 GATGCTTTGA ATAATGAAAT AATCCGACCT GCACTCGAGC ACCACCACCA CCACCACTGA
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APPENDIX C

Internet Addresses

1- Perkin Elmer, UK

http://las.perkinelmer.co.uk/Catalog/CategoryPage.htm?CategoryID=Microplates

2- Sigma-Aldrich UK

http://www.sigmaaldrich.com/united-kingdom.html

3- New England Biolabs

www.neb.uk.com

4- Qiagen DNA manipulation kits

http://www.qiagen.com/products/dna.aspx?WT.svl=m

5- Invitrogen

www.Invitrogen.com

6- Vector NTI programme (Invitrogen)

http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cloning/Vector-Design-Software.html

7- Superscript II reverse transcriptase (Invitrogen)

http://www.invitrogen.com/site/us/en/home/brands/superscript.html

8- Applied Biosystems

www.AppliedBiosystems.com

9- Promega-UK

http://www.promega.com/uk/

11- Sigma Genosys

http://www.sigmaaldrich.com/life-science/custom-oligos/custom-dna.html

13- GeneMark (gene prediction)

http://exon.biology.gatech.edu/heuristic_hmm2.cgi