BIOPHYSICAL INVESTIGATIONS OF THE MECHANISM OF COLICIN TRANSLOCATION

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DEDICATION

This thesis is dedicated to my Grandmother, Dorothy Rendall, a kindred spirit.

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There are so many people that I owe thanks to for so many things that I'm bound to miss someone out, but I'm going to have a bash anyway!

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CONTENTS

ABBREVIATIONS

AFM	Atomic Force Microscopy
ATP	Adenosine triphosphate
bp	DNA base pairs
BFP	Biomembrane Force Probe
BRP	Bacteriocin Release Protein
cAMP	Cyclic adenosine monophosphate
CD	Circular Dichroism
ColA	Colicin A
ColE3	Colicin E3
ColE9	Colicin E9
CRP	cAMP binding protein
Da	Dalton
DNA	Deoxyribonucleic acid
DFS	Dynamic force spectroscopy
E.coli	*Escherichia coli*
EDC	1-ethyl-(3-dimethylamino-propyl) carbodiimide
GST	Glutathione S-transferase
HSQC	Heteronuclear Single Quantum Coherence
Im3	Immunity protein for colicin E3
Im9	Immunity protein for colicin E9
IPTG	Isopropyl-β-D-thiogalactopyranoside
ITC	Isothermal Titration Calorimetry
K_a	Association affinity constant
k_{ass}	Association rate constant
kb	DNA kilobase pairs
K_d	Dissociation affinity constant
kDa	kiloDalton
k_{diss}	Dissociation rate constant
l	Litre
LB	Luria-Bertani broth
LPS	Lipopolysaccharide

MFP	Molecular force probe
mM	Millimolar
mRNA	Messenger ribonucleic acid
μM	Micromolar
N	Newton
NHS	N-hydroxysuccinimide
nM	Nanomolar
NMR	Nuclear magnetic resonance
NOESY	Nuclear Overhauser effect spectroscopy
OD	Optical density
OM	Outer membrane
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
Pal	Peptidoglycan associated lipoprotein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pmf	Proton motive force
R domain	Receptor-binding domain
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RU	Response/Resonance unit
SDM	Site-directed mutagenesis
SDS	Sodium dodecyl sulphate
SPM	Scanning Probe Microscope
SPR	Surface Plasmon Resonance
tRNA	Transfer ribonucleic acid
T domain	Translocation domain
Ve	Elution volume
Vo	Void volume

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 outer membrane and periplasm and cytotoxic action on a specific target. Colicins usually kill cells either by attacking the bacterial RNA or DNA or by forming pores in the inner membrane of the cell. Their 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).

Three residues of colicin E9 have previously been shown to be essential for an interaction with TolB. This study suggests that these residues play differing roles in the interaction with TolB. Other residues surrounding these previously identified residues are also shown to be involved in the interaction with TolB.

In order to allow cytotoxicity, the immunity protein of colicins E3 and E9 must be lost on entry of the colicin to a target cell. This work has demonstrated by Surface Plasmon Resonance and Atomic Force Microscopy that the affinity of colicins E3 and E9 for TolB is increased when the immunity protein is removed. This observation has implications for the mechanism by which the immunity protein dissociates from the colicin.

Finally this study has used Surface Plasmon Resonance to explore differences between pore-forming and enzymatic colicins in their interactions with Tol proteins. Although the pore-former colicin A interacts with TolR, TolA and TolB, the endonuclease colicins E3 and E9 were shown only to interact with TolB. This suggests that pore-forming and endonuclease colicins use the Tol system in different ways in order to translocate across the periplasm.

CHAPTER 1 - Introduction

1.1. Discovery of colicins

In 1925, the Belgian microbiologist, André Gratia, observed that certain strains of bacteria secrete proteins into the extracellular medium, killing non-producing strains (Gratia 1925). These protein antibiotics (or bacteriocins) are now known as colicins when produced by *Escherichia coli* and Shigella and are active against strains of *Escherichia coli* and closely related species. Over 20 colicins have now been identified (Pugsley 1984, Riley and Gordon 1992), ranging in size from 385 (Colicin N) to 626 (Colicin Ia) amino acids (Pugsley 1987, Wiener *et al* 1997). Four mechanisms of colicin cytotoxicity have been identified:

1. Formation of an ion-permeable channel in the inner membrane of the bacteria
2. Non-specific degradation of cellular DNA
3. Degradation of 16S rRNA or tRNA, inhibiting protein synthesis
4. Inhibition of peptidoglycan synthesis.

Colicins exert their effect through a one-hit mechanism at concentrations lower than 0.1 nM, indicating that a single colicin molecule can kill a cell (Cramer *et al* 1983).

1.2. Col plasmids

Colicins are encoded on plasmids and these colicin-encoding plasmids can be divided into two groups *ie* small (~6 kb), high-copy-number plasmids, *eg* ColE1, or large (~150 kb), conjugative plasmids, *eg* ColB (Riley and Gordon 1992). Col plasmids are found in approximately 30% of naturally occurring populations of *Escherichia coli* (Pugsley 1984, Riley and Gordon 1992) and provide host cells with the means to manipulate their environment and maximise their competitive advantage over other microbial populations. Col plasmids contain the colicin structural gene (*cxa,* where x is the specific colicin *eg caa* encodes ColA), encoding the colicin protein; and a specific immunity gene (*imm*), encoding the immunity protein, which protects host cells against the cytotoxic activity of the colicin. Except for colicin Ia, Ib, B and M, the colicin region contains a third gene, the lysis gene (*kil)*, encoding the Bacteriocin Release Protein (BRP), to allow release of the colicin from the cell (van der Wal *et al* 1995).

The arrangement of colicin operons depends on the function of the encoded colicin (see Figure 1.1, Cavard and Oudega 1992). The lysis gene for pore-forming colicins is separated from the structural gene by a large intercistronic region of approximately 550 bp, containing the gene encoding the immunity protein on the opposite strand, which is therefore transcribed in the opposite direction (Lloubes *et al* 1996). A transcription terminator (T1) is located immediately after the colicin structural gene and the immunity gene (see Figure 1.1). For nuclease colicins, however, the lysis gene is located a few base pairs away from the immunity gene, with a transcription terminator (T1) located just after the immunity gene (Cavard and Oudega 1992, Chak and James 1985, James *et al*, 1996).

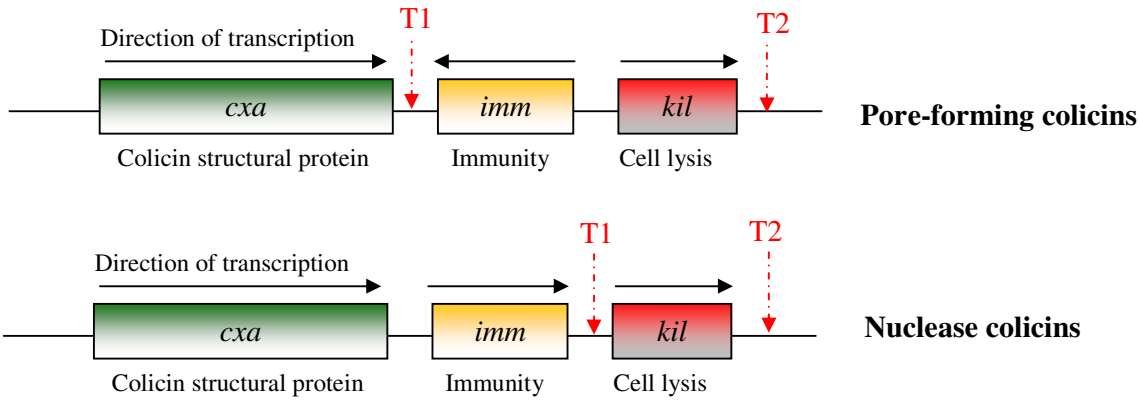


Figure 1.1 Gene organisation in colicin operons. The immunity gene is located between the colicin structural gene and the lysis gene and, for pore-forming colicins, is transcribed in the opposite direction. T1 and T2 indicate the position of transcription terminator sites.

1.3. Colicin expression

Under normal conditions, colicins are only produced at a basal level due to expression of the structural gene (*cxa*) in only a small fraction of cells (Salles *et al* 1987). The expression of colicins is induced during the SOS response (see Figure 1.2). In most cells the LexA protein, a repressor of the SOS response genes of the cell (Witkin 1976), represses transcription of *cxa* and the lysis gene (*kil*), although the immunity gene (*imm*) is continually expressed at low levels. Footprinting experiments showed that LexA binds tightly (apparent K_d = 0.4 nM) to a 40 bp sequence containing two overlapping SOS boxes between the Pribnow box and the start codon of the *cea* gene (Ebina *et* al 1983a, Lu and Chak 1996). Induction of the DNA-damage inducible SOS promoter, located proximal to *cxa*, results in cleavage of LexA by the RecA protease

(Little *et al* 1980). This allows enhanced expression of *cxa* and *kil* genes. Expression of *cea*, the gene encoding colicin E1, occurs 20-30 minutes after induction of the SOS response (Salles *et al* 1987). This lag time is not seen for other SOS response genes and could be due to the presence of two SOS boxes upstream of the start codon of the *cea* gene, allowing LexA to cooperatively bind to this regulatory region or the requirement for accumulation of an SOS-controlled factor in the cell before *cea* can be expressed (Salles *et al* 1987). The delay could allow the cells to repair low levels of DNA damage without being killed.

In addition to regulation by the SOS response, the expression of *cea* and *kil* genes is also regulated by catabolite repression, which is dependent on cAMP and the cAMP binding protein (CRP) (Ebina *et al* 1983b). Transcription of *cea* is stimulated when cAMP and CRP are present and there are two binding sites for the cAMP-CRP complex near the *cea* promoter, although only one of these sites has been shown to be involved in the control of *cea* (Shirabe *et al* 1985, Salles and Weinstock 1989). Catabolite repression increases the delay in expression of *cea* following DNA damage, suggesting a potential role of the cAMP-CRP complex in removing tightly bound LexA from the *cea* regulatory region, making the promoter more accessible to RNA polymerase (Salles *et al* 1987). Alternatively, the binding of CRP to the promoter region could affect the secondary structure of the DNA (Salles and Weinstock 1989).

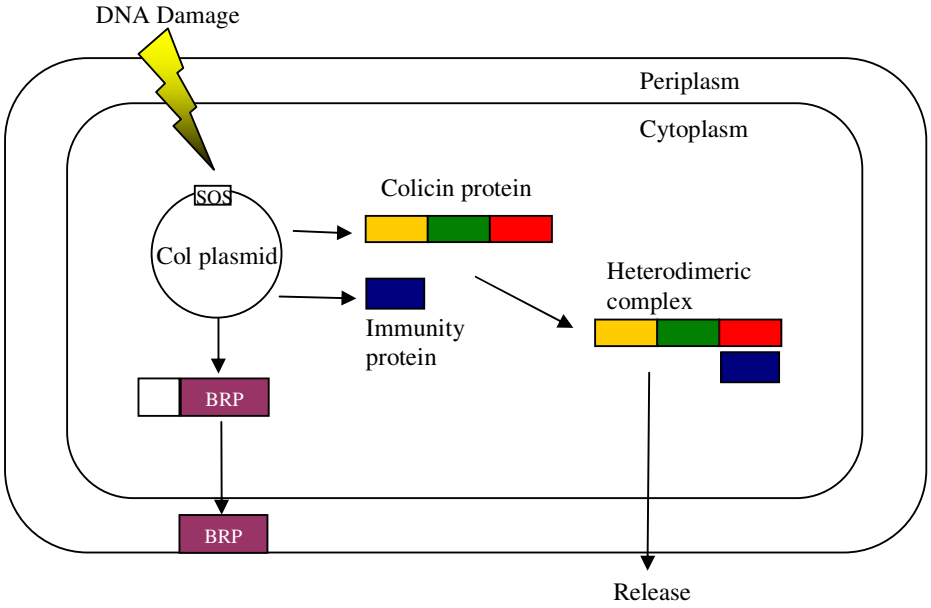


Figure 1.2 **Production and release of endonuclease colicin proteins**

1.4. Colicin Release

Colicins are unlike other groups of proteins that are released into the extracellular medium by Gram-negative bacteria for several reasons. Firstly they do not contain an N-terminal or internal signal sequence and their release relies on the expression of a single gene (encoding BRP). They are also exported late after synthesis and are non-specifically released with a subset of cytoplasmic and periplasmic proteins during a quasi-lysis state, causing cell death (Cavard and Oudega 1992, Suit and Luria, 1988).

1.4.1. Bacteriocin Release Proteins

BRPs are similar in nucleotide and amino acid sequences (Riley 1993, van der Wal 1995) and are functionally interchangeable, shown by the secretion of colicin E2 by simultaneous expression of colicin E1, E3, E7, A or D gene clusters (Pugsley and Schwarz 1983), suggesting a common mode of action for the different BRPs. Therefore, although most of the work on BRPs has been concentrated on BRPs from colicin E1, A and cloacin DF13 (a bacteriocin from *Klebsiella pneumoniae*, closely related to colicins), it is reasonable to assume that BRPs from other colicins will function in a similar way.

When the colicin operon is induced, a decrease in culture turbidity is observed, characteristic of the quasi-lysis state. This decrease in turbidity, is caused by the activity of the BRP and its stable signal peptide (van der Wal *et al* 1995).

BRPs are synthesised as precursor polypeptides, containing a signal sequence for the periplasm with 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 (Cavard *et al* 1992). The cysteine in this sequence is acylated, before the precursor protein is processed by signal peptidase II, producing a stable signal peptide and the BRP of approximately 30 amino acids. The amino group of the acylated cysteine residue of the BRP is then modified by a fatty acid residue (Cavard *et al* 1987, Cavard *et al* 1992). The mature lipoproteins, are predominantly localised in the outer membrane, with the exception of colicin N, where the BRP is localised only in the outer membrane (Pugsley, 1988).

1.4.2. Activation of outer-membrane phospholipase A

BRPs have been shown to activate phospholipase A of the outer membrane (Cavard *et al* 1987, Pugsley and Schwartz 1984) by dimerisation (Snijder *et al* 1999, Dekker *et al* 1999). Changes in phospholipid content release were measured and the major

changes were a decrease in phosphatidylethanolamine (PE) and an increase in lysophosphatidylethanolamine (lysoPE) and free fatty acid content, indicative of the action of a phospholipase (Pugsley and Schwartz 1984). Cells containing a mutation in the *pldAI* gene, encoding phospholipase A, were shown to be much less proficient at release of both colicins E1 and A and also showed no quasi-lysis (Pugsley and Schwartz 1984, Cavard *et al* 1987). Therefore, both BRP and phospholipase A seem to be necessary for efficient colicin release. However, it has been observed that in *pldA1* mutants, the BRP is degraded by the DegP protease (Cavard *et al* 1989) and therefore, the BRP may not reach sufficient levels to enable colicin release and lysis, which could mean that the lack of colicin release in these mutants is an artefact. Colicin A has also been shown to affect the integrity of the outer membrane, independently of phospholipase A (Howard *et al* 1991).

1.4.3. Mechanism of colicin release

Several mechanisms for how the BRP causes release of colicins into the extracellular medium have been suggested.

The first model proposes that activation of the phospholipase by BRP may cause changes in the lipid composition of the outer membrane to enable leakage of the colicin and other proteins into the extracellular medium. The BRP itself would therefore be involved in transferring the colicin across the inner membrane and activating phospholipase A (Pugsley and Schwartz 1984).

An alternative model has been proposed, involving the stable signal peptide produced by cleavage of the immature BRP by signal peptidase II (Luirink *et al* 1986, Luirink *et al* 1991). The signal peptide has been shown to be stable and disrupt the cytoplasmic membrane (Cavard 1997, van der Wal *et al* 1995, Luirink *et al* 1991). The BRP of cloacin DF13, with its signal peptide replaced with the signal sequence of the murein-lipoprotein, was unable to release the cloacin but still caused lysis (Luirink *et al* 1991), indicating that the signal peptide plays a significant role in the release of colicins. Thin section electron micrographs show that colicins accumulate exclusively in the cytoplasm (Cavard *et al* 1981, Luirink *et al* 1991), indicating that colicins move in a one-step process from the cytoplasm to the extracellular medium. Therefore it has been proposed that the BRP, the stable signal peptide and phospholipase A co-operate to form trans-envelope pores through which the colicin and other proteins would pass (Luirink *et al* 1991, Cavard and Oudega 1992, van der

Wal *et al* 1995). Recent work with high molecular-mass forms of colicin A in cells with a BRP gene deletion, showed that colicin A appeared to be able to reach the outer membrane without the help of BRP. Colicin A was shown to interact with phospholipase A in the outer membrane. Phospholipase A forms a 12-stranded β-barrel, which spans the membrane (Snijder *et al* 1999), and therefore it is possible that phospholipase A may facilitate transport of colicin A across the outer membrane, with the help of BRP (Cavard 2002).

Colicins Ia, Ib, B and M appear to have no lysis-like protein and as yet it is unclear how these colicins are released from the cell (Riley 1993, Thum 1988).

1.5. Immunity proteins

Cells that produce a specific colicin are immune to the action of the colicin they produce (Fredericq 1957). This immunity is conferred by an immunity protein, the product of the *imm* gene.

1.5.1. Expression of the *imm* gene

Most Col plasmids contain a single *imm* gene, under the control of constitutive promoters within the 3' end of the colicin gene (Soong *et al* 1994). However, three colicins have been identified that have more than one immunity gene. Colicins E3 and E6 contain a second *imm* gene 5' to the *kil* gene, encoding an immunity protein with sequence similarity to the colicin E8 immunity protein (Im8) (Chak and James 1984). Both immunity proteins have their own promoters, independent of the colicin structural gene (Chak and James 1985) and are therefore constitutively expressed under normal conditions. The colicin E9 plasmid also has a second *imm* gene, similar to the sequence of Im5 (James *et al* 1987).

In pore-forming colicin operons, the *imm* gene is present in the reverse orientation to the *kil* and *cxa* genes. This potentially provides several advantages (Zhang *et al* 1988). Firstly, the *imm* gene can be constitutively expressed, without the simultaneous expression of the *kil* gene, preventing the deleterious effects of expression of BRP under normal conditions and protecting the cell from any exogenous colicin produced by the same strain. Western blot analysis has demonstrated that under normal conditions the amount of Im7 is at least 20 times greater than the amount of colicin E7 and BRP (Hsieh *et al* 1997). Therefore, there is more than enough immunity protein available to protect cells from exogenous or

endogenous colicin, produced by basal level expression of the colicin operon. Secondly, the *imm* RNA inhibits transcription of the *kil* gene and therefore transcription of the *imm* gene allows transcription of the *kil* gene to be delayed whilst large amounts of colicin structural protein are produced, before protein synthesis is inhibited by the BRP. It may also allow inhibition of colicin synthesis, during periods of derepression *eg* during plasmid replication.

1.5.2. Putative RNase activity of immunity proteins

Studies with Im7 have suggested a role for the dimeric form of Im7 in controlling translational expression of the ColE7 operon by exerting a specific RNase activity (Hsieh *et al* 1997, Chang *et al* 2002). The putative active site for this RNase activity is at the interface of the two Im7 molecules. If induction of the SOS response promoter only lasts for a short time, Im7 could keep the level of translation of the BRP below toxic levels, by cleaving the RNA within the *imm* RNA, leading to uncoupling of translational expression of the *kil* gene. However, if the SOS response induction lasts for 100 minutes, the amount of dimeric Im7 would not be sufficient to cleave the significantly increased numbers of transcripts and would be unable to keep the translation of the BRP below the lethal dose, causing quasi-lysis (Chang *et al* 2002). Therefore the lag time, observed after induction of the SOS response, could reflect the time taken for the colicin mRNA to overpower the RNase activity of the dimeric immunity protein. However, analytical ultracentrifugation experiments demonstrated that Im7 only ever exists as a monomer (Dennis *et al* 1998) and therefore the functional significance of the dimeric form of Im7 observed in the crystallography is questionable.

1.5.3. Sequences of immunity genes

Immunity proteins are more variable in sequence than the *kil* and *cxa* structural genes. Many immunity proteins show no detectable sequence similarity. However, E2, E8 and E9 immunity sequences are similar at 54-97 % of their residues (Riley 1993). Immunity proteins have a high affinity for the C-terminus of the colicin protein, although the mechanism of action of the immunity protein is dependent on the cytotoxic activity of the colicin.

1.5.4. Immunity proteins for endonuclease colicins

Before release into the extracellular medium, endonuclease colicins form a complex with a specific immunity protein, which neutralises the activity of the colicin. The immunity protein must be lost on entering a target cell but how and where this occurs is as yet uncertain. Free colicin E9 has the same bactericidal activity as colicin E9 bound to Im9 (Schaller and Nomura 1976, Wallis *et al* 1992a) and it would therefore appear that the immunity protein is not required for receptor binding or translocation of colicin E9. However, removal of Im3 from colicin E3 leads to substantial loss of bactericidal activity indicating that, in addition to providing immunity to colicin E3 producing cells, Im3 may also stabilise colicin E3 on entering susceptible cells (Walker *et al* 2003).

1.5.4.1. Affinity of colicin-immunity protein interactions

The requirement for denaturation to separate the immunity protein from the endonuclease domain of a colicin, first indicated that the interaction between the two proteins must be strong (Jakes and Zinder 1974, Schaller and Nomura 1976). This was substantiated when it was shown that the equilibrium dissociation constant (K_d) for the colicin E9-Im9 complex was 9.3×10^{-17} M in conditions of low ionic strength (Wallis *et al* 1995a). This is one of the highest affinity protein-protein interactions known. The K_d for the interaction between the DNase domain of colicin E9 and Im9 was found to be 7.2×10^{-17} M, a value very similar to that for the full-length colicin and therefore it is assumed that the immunity protein only makes energetically important contacts with the DNase domain of the colicin (Wallis *et al* 1995a). This is supported by NMR studies which show a lack of chemical shift perturbation of the translocation domain when Im9 is added to colicin E9 (Collins *et al* 2002). However, the interaction between full-length colicin E3 and Im3 is stronger than the interaction between the RNase domain of colicin E3 and Im3 ($K_d = 10^{-14}$ M and 10^{-12} M respectively) (Walker *et al* 2003). The crystal structure of the full-length colicin E3-Im3 complex shows that the increased affinity of the full-length colicin E3 for Im3 is due to an interaction of Im3 with the N-terminal translocation domain of the colicin (Soelaiman *et al* 2001).

1.5.4.2. Kinetics of the colicin-immunity protein complex

Formation of the colicin E9-Im9 complex was shown, by stopped flow fluorescence, to proceed by a two-step process (Wallis *et al* 1995a). The initial rate of association (k_1) was shown to be 4 x 10^9 $M^{-1}s^{-1}$ in buffer of low ionic strength and hence the association is essentially diffusion-controlled. This rate of association is comparable to the rate of association observed for the colicin E3 rRNase domain and Im3 (10^8 $M^{-1}s^{-1}$, Walker *et al* 2003).

The first step in the association between colicin and immunity protein is highly dependent on salt concentration, consistent with the highly acidic nature of the immunity protein (pI ~4.5) and the highly basic nature of the endonuclease domain of the colicin (pI >10.5). Therefore, electrostatic interactions probably pre-orient the two proteins before collision, minimising the time for effective complex formation to occur (Wallis *et al* 1995a, Kleanthous *et al* 1998, Walker *et al* 2003, Li *et al* 2004).

The second step in the association between colicin E9 and Im9 involves a relatively slow (6.8 s^{-1}) conformational change in the DNase domain of the colicin (Wallis *et al* 1995a).

The dissociation rate constant (k_{off}) has been estimated, by radioactive exchange kinetics, to be 3.7 x 10^{-7} s^{-1}. This is inconsistent with the kinetics of colicin action, as colicins start to kill cells within minutes, and therefore the dissociation rate for the colicin-immunity protein complex must be increased by at least four orders of magnitude on entering a susceptible cell (Kleanthous *et al* 1998). The immunity protein of the RNase toxin, cloacin DF13, active against *Enterobacter cloacae*, *Klebsiella edwardsii* and *E. coli*, has been shown by immunological detection to be released into the culture medium on binding of the cloacin to its receptor (Krone *et al* 1986). Therefore it has been suggested that the magnitude of the interaction between the colicin and its immunity protein may be overcome by partial or complete unfolding of the colicin on binding to its receptor (Wallis *et al* 1995a). An alternative mechanism has been proposed for the dissociation of Im3 from colicin E3 (Walker *et al* 2003). In this mechanism receptor binding would trigger translocation of the N-terminal translocation domain of the colicin into the periplasm. The interaction between the translocation domain and TolB would cause contacts between the translocation domain of the colicin and Im3 to be lost, weakening the colicin E3-Im3 interaction and increasing the dissociation of the immunity protein from the colicin.

The association and dissociation kinetics for the colicin E2-Im2 interaction are very similar to those of the colicin E9-Im9 interaction indicating that high affinity binding is a common feature of colicin-immunity protein complexes (Li *et al* 2004).

1.5.4.3. Structures of immunity protein-colicin complexes

Determination of structures for three colicin endonuclease domains bound to their corresponding immunity proteins has provided insights into how immunity proteins protect bacteria from the cytotoxic activity of endonuclease colicins. The structures of the DNase domain of colicins E7 in complex with Im7 (at 2.3 Å resolution, Ko *et al* 1999) and E9 in complex with Im9 (at 2.05 Å and 1.7 Å resolution, Kleanthous *et al* 1999, Kühlmann *et al* 2000) have been determined as well as the structure of the RNase domain of colicin E3 in complex with Im3 (at 2.4 Å resolution, Carr *et al* 2000b). The structure of full-length colicin E3 in complex with Im3 has also been determined (Soelaiman *et al* 2001) and the structure of the RNase domain in this full-length structure is very similar to the isolated RNase domain. Structural comparison of the RNase and DNase domains reveals few similarities, the DNase domains are αβ proteins (Ko *et al* 1999, Kleanthous *et al* 1999) whereas the RNase domain is composed predominantly of β sheet (Carr *et al* 2000b, Soelaiman *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 (Osborne *et al* 1996, Chak *et al* 1996) and the rRNase immunity proteins are predominantly β sheet proteins (Li *et al* 1999). A property that is shared between the DNase and RNase complexes however, is the charge complementarity resulting from the basic nature of the endonuclease domain and the acidic nature of the immunity protein.

The colicin E9 DNase-Im9 complex shows that the complex consists of a hydrophobic core, formed mainly by aromatic residues from the DNase and Im9. Multiple hydrogen bonds and salt bridges surround this core. The structure of the DNase domain of colicin E7 in complex with Im7 show that there are 2.17 hydrogen bonds per 100 $Å^2$ formed between colicin E7 and Im7, substantially more than the average calculated for enzyme-inhibitor complexes (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 *et al* 1999).

The structure of the colicin E3 RNase-Im3 complex shows that an N-terminal helix of the RNase domain wraps around the exposed face of the four-stranded β-sheet of Im3 (Kolade *et al* 2002). There is a much greater loss of surface area on binding of the colicin E3 RNase domain to Im3 compared to binding of colicin E9 or E7 DNase domains to their respective immunity proteins (2554 $Å^2$ compared to 1575 $Å^2$ and 1473 $Å^2$ respectively). There is a high degree of surface complementarity, which may contribute to the high affinity of the interaction (Kolade *et al* 2002). This high degree of surface complementarity is indicative of a conformational change on formation of the RNase domain-immunity protein complex but so far no structure of the free RNase is available to confirm this.

1.5.4.4. Mechanism of inhibition

Unlike many enzyme inhibitors, the immunity protein does not bind directly at the active site (Kleanthous *et al* 1999, Kühlmann *et al* 2000). The structure of the DNase domain of colicin E9 in complex with Im9, shows that Im9 binds in a cleft on the DNase domain, formed by a short helix, an extended strand and two loops spanning residues 72-98, adjacent to the active site (Kühlmann *et al* 2000).

Two mechanisms of inhibition by endonuclease immunity proteins have been put forward based on structural and functional studies (Kleanthous and Walker 2001). Firstly, the immunity protein could cause conformational changes in the protein backbone of the endonuclease domain causing allosteric inhibition. Secondly, the immunity protein could sterically hinder the nucleic acid binding site.

Steric hindrance

There are now several pieces of evidence suggesting that the immunity protein hinders the nucleic acid binding site.

Docking of the active site of colicin E3 onto the cleavage site on 16S RNA, within the 30S ribosomal subunit, indicates that, although the immunity protein does not sterically hinder the approach of the endonuclease domain to the ribosome, many negatively charged residues of the immunity protein point towards the 30S rRNA backbone, resulting in strong electrostatic repulsion (Zarivach *et al* 2002).

Modelling of B-form DNA into the active site of the DNase domain of colicin E9 show that the N-terminal end of helix II and the adjoining loop of Im9 forms an "electrostatic elbow", which could hinder access of the DNA to the DNase domain by

steric and electrostatic repulsion, approximately one helical turn from the active site (Kleanthous *et al* 1999). The crystal structure of an inactive mutant of the colicin E9 DNase domain (H575A) in complex with a short single stranded DNA ligand, indicates that the immunity protein sequesters residues critical to the binding of an extended DNA substrate (Kolade *et al* 2002). Residues D499 and D502 interact with a purine ring in the DNA and Y531 forms a hydrogen bond with a phosphate group of the DNA. These residues are all involved in direct interactions with Im9 in the colicin E9 DNase-Im9 complex.

These observations are consistent with the second model of inhibition *ie* steric hindrance and this is supported by the fact that the structure of unbound colicin E9 and E7 DNase domains are very similar to the structures of the respective colicin DNase-Im complexes, indicating that the structure of the DNase domain is unaltered by the binding of the immunity protein, arguing against allosteric inhibition (Cheng *et al* 2002, Kolade *et al* 2002).

Allosteric inhibition

However, NMR has shown the existence of at least two conformational states of unbound colicin E9 that interconvert slowly (Whittaker *et al* 1998). The rate of conversion is comparable to the conformational change in the DNase domain of colicin E9 on binding of Im9 seen in stopped-flow fluorescence experiments (Wallis *et al* 1995a, van den Bremer *et al* 2004). These observations suggest that Im9 could stabilise an inactive conformation of colicin E9, acting as an allosteric inhibitor.

1.5.4.5. Specificity of immunity proteins

The structure of the DNase domain of colicin E9 bound to Im9 shows that residues 23-56, *ie* helix II and helix III, of Im9 are involved in binding to the DNase domain (Kühlmann *et al* 2000). The use of Im8 and Im9 gene fusions allowed the specificity-determining region of Im9 to be localised to residues 16-43 (Wallis *et al* 1992 b). The structure of Im9, determined by NMR shows that these residues encompass helix I and helix II of a four-helix bundle (Osborne *et al* 1996).

When helix II of Im2 is replaced with helix II of Im9, the chimeric immunity protein binds almost as tightly to the colicin E9 DNase domain as wild-type Im9, indicating that helix II is the main specificity-determinant of Im9 (Li *et al* 1997). The difference in binding affinity is due to internal packing interactions between helix I and helix II

of Im9. A similar observation was made when helix II of Im7 was replaced with the corresponding helix of Im8 (Lu *et al* 1999). The mutated Im7 then provided cells with partial immunity to colicin E8.

Five residues out of fifteen vary between helix II of Im2 and helix II of Im9. These five variable residues in Im2 were mutated to the corresponding residue in Im9 in turn and the mutants were assayed for biological activity towards colicin E2 and colicin E9 (Li *et al* 1998). Three residues in helix II of Im2 cause a change in specificity, D33, N34 and R38. N34 had also been recognised as an important specificity determinant by mutagenesis of residues in Im9 to the corresponding residue in Im8 (Wallis *et al* 1992b). When all three of these residues in Im2 are mutated to the corresponding residues in Im9, the mutant immunity protein behaves exactly the same as Im9, indicating that the specificity of Im2 and Im9 for colicin E9 is controlled by these three residues alone, with residue D33 playing the most significant role (Li *et al* 1998). Unlike Im9 where several residues appear to be contributing to specificity, only residue D33 makes a major contribution to specificity in Im2 (Li *et al* 2004). It has been speculated that this residue may form a salt bridge with a basic residue in the colicin E2 DNase domain (Li *et al* 2004).

Site directed mutagenesis has identified residues D31, D35 and E39, in the loop 1-helix II region as specificity determining residues in Im7, and D31 is the most critical of the three residues (Lu *et al* 1999). A sequence alignment shows that in Im2, Im7, Im8 and Im9, residue 31 is a negatively charged residue, which may play an important role in an electrostatic or steric interaction (Lu *et al* 1999). Loop 1 in Im7 contains an insertion of one residue to Im9 (Dennis *et al* 1998), therefore residues D35 and E39 in Im7 correspond to residues N34 and R38 in Im9. It would therefore seem that the equivalent residues in Im7 and Im9 are important in determining specificity.

1.5.4.6. Dual recognition

Alanine scanning mutagenesis and stopped flow fluorescence has shown that approximately two thirds of the colicin E9 DNase and colicin E2 DNase binding free energy is derived from five conserved residues of helix III of the immunity protein (Wallis *et al* 1998, Li *et al* 2004). Helix II residues provide approximately one third of the binding energy (Wallis *et al* 1998). Therefore, the conserved residues of helix III are the main contributors to the binding energy and the variable residues of helix II govern specificity as well as making a small contribution to binding energy (Wallis *et*

al 1998). On the basis of this data, a dual recognition model has been proposed. In this model, if helix II of the immunity protein is able to make favourable interactions *ie* with its cognate colicin, then helix III of the immunity protein interacts with the colicin to allow maximal binding. However, if the interactions of helix II with the colicin are wrong, the residues of this helix do not dock properly and the colicin-immunity protein interaction is much weaker (Kleanthous *et al* 1998). For example, if a non-cognate immunity protein binds to the colicin E9 DNase, despite the conserved residues of helix III docking onto the DNase, insertion of hydrophilic residues from helix II into inappropriate hydrophobic binding sites, could destabilise the complex (Kleanthous *et al* 1998).

The structures of Im7 and Im9 and the E9 DNase-Im9 and E7 DNase-Im7 complexes have provided a molecular rationale for the dual-recognition mechanism

Comparison of the crystal structure of Im7 with the NMR structure of Im9 (Osborne *et al* 1996, Dennis *et al* 1998, Chak *et al* 1996) shows that although both proteins adopt similar four-helix bundle structures, there are subtle structural differences, the most significant being the position of a tyrosine residue (Y56 in Im7 and Y55 in Im9) in helix III (Dennis *et al* 1998). In Im9, the residue extends into the solvent, whereas in Im7 it folds back onto the helix.

Structural superposition of the crystal structure of Im7 bound to the colicin E7 DNase domain and the structure of Im9 bound to the colicin E9 DNase domain shows that the immunity proteins are displaced relative to one another due to a 19º rigid-body rotation (Kühlmann *et al* 2000). The axis for this rotation is formed by two tyrosine residues, Y54 and Y55 in Im9 (see Figure 1.3). The rotation does not significantly alter the position of the tyrosine residues but does affect the position of helix II relative to the DNase. Conserved residues of helix III of Im9 interact with the DNase backbone via hydrogen bonds and hydrophobic interactions, whilst the rotation around the two tyrosine residues in helix III, causes different regions of helix II to be presented to the DNase so that the variable residues form different interactions: predominantly hydrophobic in the case of colicin E9 DNase-Im9 and charged in the case of colicin E7 DNase-Im7 (Kühlmann *et al* 2000).

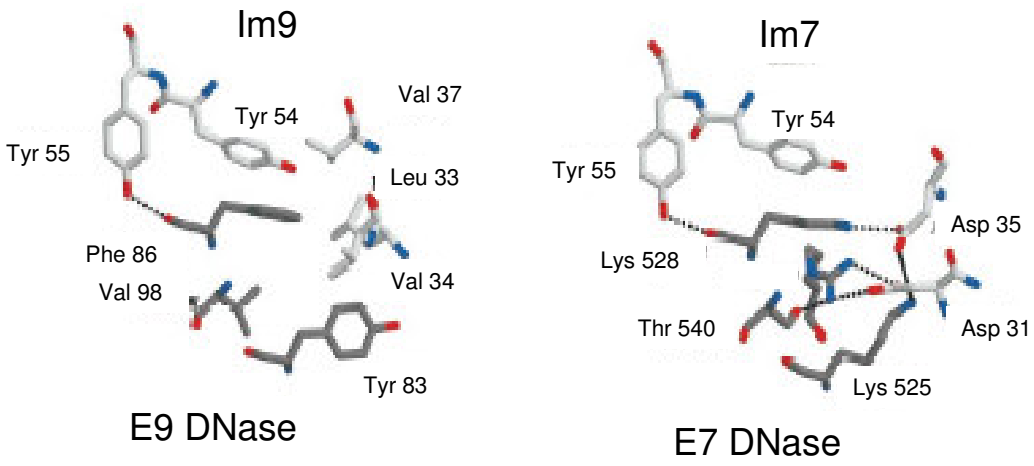


Figure 1.3 Comparison of the DNase-Im interfaces showing the relationship between the conserved tyrosine residues of the immunity proteins, Im9 (left) and Im7 (right) and specificity residues of the immunity proteins and DNase domains [from Kühlmann *et al* 2000]. Immunity protein residues are shaded light, DNase residues are shaded dark. Y54 presents a key specificity residue of the DNase to specificity residues of the immunity protein. Immunity protein amino acid residues are numbered according to the Im9 sequence.

1.5.5. Immunity proteins for pore-forming colicins

On the basis of homology, immunity proteins for pore-forming colicins have been classified into two groups: type A, including ImA, ImB, ImN and ImU and type E, including ImE1, Im5, ImK, Im10, ImIa and ImIb (Duché 2002). The immunity proteins for pore-forming colicins are 11-18 kDa in size (Lazdunski *et al* 1988). As with immunity proteins for endonuclease colicins, the immunity proteins are highly specific and only provide full protection against one pore-forming colicin (Song and Cramer 1991). However, ImA has been shown to provide cells with partial protection against colicin B, when encoded on a high-copy-number plasmid (Géli and Lazdunski 1992). Cells producing the specific immunity protein are approximately 10^5 times more resistant to the colicin compared to cells that lack the immunity protein (Cramer *et al* 1995).

1.5.5.1. Location of immunity proteins for pore-forming colicins

In contrast to immunity proteins for endonuclease colicins, immunity proteins for pore-forming colicins are not released with the colicin but are localised to the cytoplasmic membrane of producing cells. This has been shown by several methods,

including topology analysis of ImA, ImE1, ImU and Im5 fusion proteins (Song and Cramer 1991, Duché 2002), immunological detection of ImA (Géli *et al* 1988) and detergent extraction of ImE1 (Bishop *et al* 1985).

1.5.5.2. Topology of immunity proteins for pore-forming colicins

Topology analysis of ImA and ImU has indicated that these proteins have four transmembrane helices, with the N- and C- termini located in the cytoplasm, whereas ImE1 and Im5 are shorter, consisting of three transmembrane helices, with the N-terminus in the cytoplasm and the C-terminus in the periplasm (Géli *et al* 1989, Song and Cramer 1991, Pilsl and Braun 1995a, Pilsl *et al* 1998).

1.5.5.3. Mechanism of action of immunity proteins for pore-forming colicins

The transmembrane helices of immunity proteins have been shown to be important in their activity (Zhang and Cramer 193) and the hydrophobic hairpins of colicin E1 and colicin A have been shown to be important for immunity recognition (Bénédetti *et al* 1991a, Espesset *et al* 1994). Therefore, it has been proposed that the hydrophobic helices of the immunity protein and the hydrophobic hairpin of the colicin pore-forming domain interact when their transmembrane regions are exposed to each other, on insertion of the colicin into the cytoplasmic membrane, neutralising the pore-forming ability of the colicin (Zhang and Cramer 1993, Cramer *et al* 1995, Espesset *et al* 1996). Colicin E1 immunity protein has been shown to be able to interact with the pore-forming domain of the colicin upon voltage-gating, which places helix VI of the pore-forming domain in a transmembrane orientation, enabling it to interact with the transmembrane helices of ImE1 (Lindeburg and Cramer 2001).

Pore-forming immunity proteins are able to protect a cell against colicin concentrations $10^4 – 10^7$ times greater than those required to kill a non-immune cell (Cramer *et al* 1983). However, only approximately 100 immunity protein molecules are present in the inner membrane. This is possible because the diffusion rate constant in the membrane of $10^{-9} – 10^{-10}$ cm^2s^{-1} means that the probability of an interaction between the colicin and the immunity protein is high, as the immunity

protein can travel through the membrane at least 500 nm in 10 seconds (Song and Cramer 1991).

1.6. Classification of colicins

Colicins can be classified in several ways (see Table 1.1). Firstly, they can be classified according to the cell surface receptor to which they bind *eg* E colicins bind to BtuB, the vitamin B_{12} receptor. Following receptor-binding, colicins enter the periplasm by one of two routes, known as the Ton and Tol pathways, involving periplasmic and inner membrane proteins. Colicins can be split into two groups according to the translocation pathway they utilise to enter the susceptible cell. Group A are inactive against strains containing lesions in the *tolA* gene, whilst group B are inactive against bacteria carrying mutations in the *tonB* gene.

Once they have crossed the outer membrane, colicins can begin their cytotoxic activity. Several cytotoxic activities have been attributed to colicins. For example colicins A, E1 and N cause depolarisation of the cell by the formation of voltage-gated ion channels in the inner membrane. Colicin M inhibits peptidoglycan synthesis causing lysis of susceptible bacteria. Other colicins take the form of endonucleases, cleaving the bacterial DNA (colicins E2, E7, E8 and E9), ribosomal RNA (colicins E3 and E6) or transfer RNA (colicin E5).

Table 1.1 Classification of colicins (adapted from Lazzaroni *et al* 2002, Lazdunski *et al* 2000)

Colicin	*Group*	*Receptor*	*Translocation system*	*Cytotoxic activity*
A	A	BtuB	OmpF, TolABQR	Pore-forming
B	B	FepA	TonB, ExbBD	Pore-forming
D	B	FepA	TonB, ExbBD	Inhibition of protein synthesis
E1	A	BtuB	TolCAQ	Pore forming
E2, E7, E8, E9	A	BtuB	OmpF, TolABQR	DNase
E3, E4, E6	A	BtuB	OmpF, TolABQR	16s rRNase
E5	A	BtuB	OmpF, TolABQR	Anticodon tRNase
K	A	Tsx	OmpF, OmpA, TolABQR	Pore-forming
M	B	FhuA	TonB, ExbBD	Inhibition of murein & LPS synthesis
N	A	OmpF	OmpF, TolAQR	Pore-forming
U	A	OmpA	OmpF, LPS, TolABQR	Pore-forming
1a, 1b	B	Cir	TonB, ExbBD	Pore-forming
28b	A	OmpA	OmpF, LPS, TolABQR	Pore-forming
DF13	A	IutA	TolAQR	16s rRNase
Col5, Col10	B	Tsx	TolC, TonB, ExbBD	Pore-forming

1.7. Colicin structures

In general, colicins can be divided into 3 domains on the basis of function, a C-terminal cytotoxic domain, an N-terminal translocation (T) domain and a receptor-binding (R) domain.

Figure 1.4 General structure of a colicin, consisting of an N-terminal translocation (T) domain, a receptor-binding (R) domain and a C-terminal cytotoxic (C) domain.

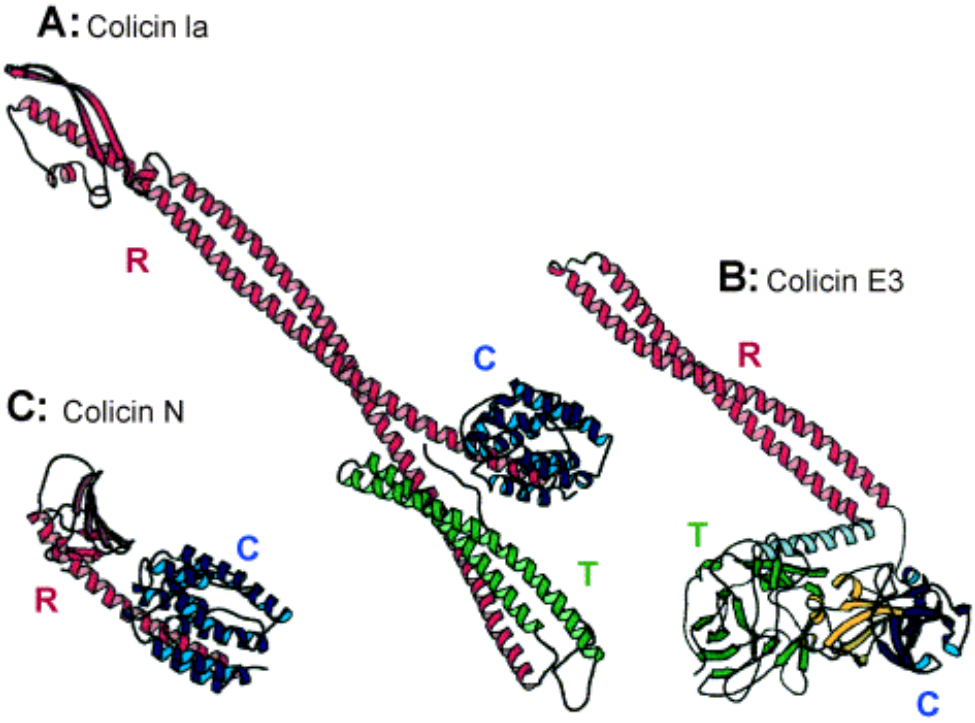


Figure 1.5 Crystal structures of colicins N, Ia and E3 (from Zakharov and Cramer 2002). The receptor binding domains of colicins Ia and E3 are formed by long helical hairpins, at the top of which sit the translocation domains and cytotoxic domains. The first 66 residues of colicin N, the first 83 residues of colicin E3 and the first 22 residues of colicin Ia are missing in the crystal structures.

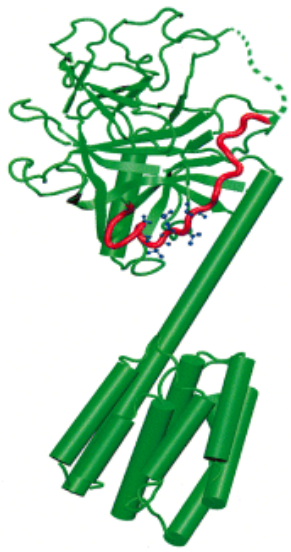


Figure 1.6 The crystal structure of colicin B (Hilsenbeck *et al* 2004). No electron density was detected for residues 1-10 and 29-43 (dotted line). In contrast to the full-length structures of other colicins, which have clearly delineated cytotoxic, translocation and receptor-binding domains, colicin B 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.

The only published structures of full-length colicins to date, shown in Figure 1.5 and Figure 1.6, are the structures of the endonuclease colicin E3 (Soelaiman *et al* 2001) and the pore-forming colicins Ia (Wiener *et al* 1997), N (Vetter *et al* 1998) and B (Hilsenbeck *et al* 2004). Generally, these structures concur with the above domain organisation, although the structural and functional definitions of the domains, at times, overlap. Colicin E1 and colicin 10 also contain a fourth domain between the translocation domain and the receptor binding domain, involved in binding to TolC (Pilsl and Braun 1995b).

1.8. Receptor-binding

The first step for a colicin entering 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 the colicin killing pathway.

1.8.1. Colicin Receptors

Several colicin receptors have been identified, including the vitamin B12 receptor, BtuB (Di Masi 1976, James *et al* 1996), the siderophore receptors, FepA and FhuA

(Ferguson and Deisenhofer 2002, Locher *et al* 1998), and the porins, Tsx (Bradley and Howard 1992, Pilsl and Braun 1995b), OmpA (Pilsl and Braun, 1995c) and OmpF (Bourdineaud *et al* 1990, Cowan *et al* 1992). It has been proposed that these receptors act as gated channels, whereby binding of the ligand opens a channel, through which the ligand may pass (Lazdunski *et al* 1998).

1.8.1.1. FepA

The structure of FepA, the receptor for colicins B and D, shows that this receptor consists of a 22-stranded β-barrel and an N-terminal plug domain (Buchanan *et al* 1999). The region involved in ligand recognition was predicted to lie in the large extracellular loops of the β-barrel domain, as complete removal of the N-terminal plug domain did not affect the ability of FepA to bind to colicins B and deletion of loops 7 or 8, rendered FepA unable to bind colicin B (Newton *et al* 1999, Scott *et al* 2001, Barnard *et al* 2001). However, recent work has shown that the genetic backgrounds used for these FepA deletions could allow interprotein complementation, such that the plug domain of the inactive chromosomally encoded FepA would insert into the empty β-barrel of the plasmid-encoded FepA (Vakharia and Postle 2002). Therefore, the work has been repeated in a strain in which the chromosomally encoded FepA does not contain the plug domain so that this complementation would not be possible. In this strain, constructs with deletions of the plug domain did not retain sensitivity to colicin B, indicating that the N-terminal plug domain is important in binding of colicins (Vakharia and Postle 2002).

The plug domain is firmly anchored to the β-barrel domain, with extensive hydrogen bonding between the two domains, indicating that a large amount of energy would be required to remove the plug from the barrel and it is proposed that instead of removal of the plug, a conformational rearrangement would allow the natural ligand, ferric enterobactin (716 Da), to pass through the channel (Buchanan *et al* 1999). However, this does not explain how the much larger colicin B (58 kDa) or colicin D (75 kDa) molecules could use this transporter to cross the outer membrane. The affinities of colicins B and D (10^{-7} M) for FepA are much weaker than the affinity of ferric enterobactin (10^{-10} M) (Payne *et al* 1997, Scott *et al* 2001). This is likely to be due to the lack of shape complementarity between the receptor-binding domain of the colicin and the binding pocket of FepA (Cao and Klebba 2002). However, as colicin-producing cells are protected from the cytotoxic action of the colicin, and they can

therefore produce the toxin in a high local concentration, and as the concentration of the natural ligand is usually low, it is likely that this difference in affinity is overcome to allow adequate binding of colicin to FepA (Cao and Klebba 2002). The biphasic kinetics for binding of ferric enterobactin and colicins to FepA, indicate that a conformational change may occur on binding (Payne *et al* 1997). Mutagenesis experiments have shown that although some residues of FepA are important in binding both ferric enterobactin and colicins, other residues are important specifically to either colicins or ferric enterobactin *eg* residues R286 and R316 are important to both colicins and ferric enterobactin but residue E319 is only important to ferric enterobactin binding and residue G549 is only important to colicin binding (Newton *et al* 1997, Cao and Klebba 2002). The larger surface area of colicins due to their greater mass than ferric enterobactin makes it likely that although the binding sites for ferric enterobactin and colicins may overlap, colicins will interact with a greater number of residues on the receptor than enterobactin (Newton *et al* 1997).

1.8.1.2. FhuA

FhuA, the receptor for colicin M, also consists of a β-barrel, which is obstructed by an N-terminal plug domain (Locher *et al* 1998). The barrel domain was suggested to be involved in binding colicin M, as complete removal of the plug domain did not affect binding of colicin M (Scott *et al* 2001) but as was the case for FepA, this was shown to be due to complementation of the empty β-barrel of the plasmid-encoded FhuA with the plug domain of the inactive chromosomally encoded FhuA (Braun *et al* 2003). In a mutant with the chromosomally encoded *fhuA* gene deleted, deletion of the plug domain resulted in the inability of FhuA to bind colicin M, indicating that the plug domain is involved in binding colicins. On binding of the ferrichrome ligand, it had been proposed that this plug could be removed from the barrel, allowing the ligand to pass through the barrel. The crystal structure of FhuA shows that the plug obstructs the entire channel lumen and tightly fits in the wall of the barrel, which is inconsistent with a complete removal of the plug upon ligand binding (Locher *et al* 1998). Removal of the plug would be unnecessary for the translocation of ferrichrome across the membrane, due to the small size (740 Da) of this ligand and it is proposed that more subtle conformational changes could allow translocation of ferrichrome. However, colicin M is a much larger (29 kDa) ligand and therefore if

this ligand were to pass through the barrel of FhuA, it is likely that the plug would have to be removed.

1.8.1.3. BtuB

The receptor used by the E colicins is BtuB, the vitamin B_{12} receptor (see Table 1.1). The mature 66 kDa protein is produced from cleavage of a 20 amino acid signal sequence (Heller and Kadner 1985). The protein is a minor component of the outer membrane, with approximately 200 copies present per cell (James *et al* 1996).
The crystal structures of wild-type BtuB, BtuB with bound calcium and BtuB with bound calcium and bound vitamin B_{12} have been determined (Chimento *et al* 2003, see Figure 1.7). The basic structure of BtuB is very similar to FepA and FhuA and consists of an N-terminal plug domain inserted in a 22-stranded β-barrel. In the presence of calcium, extracellular loops connecting β-strands 3-4 and 5-6 of the barrel domain become partially ordered and it has been suggested that calcium serves to order these loops, exposing the high-affinity substrate-binding site. This is consistent with the 50-100 fold reduction in binding affinity for vitamin B_{12} when calcium is removed from BtuB (Bradbeer *et al* 1996).
The vitamin B_{12} binding site is formed by two loops of the plug domain, and two loops connecting strands 5-6 (one of the loops ordered by the addition of calcium) and 7-8 in the barrel domain (Chimento 2003). The substrate also makes additional interactions with other loops of the barrel domain. The largest structural change that occurs, on binding to vitamin B_{12}, is the movement of Cα carbons in a loop of the plug domain by up to 6 Å. Other smaller conformational changes occur in the barrel domain (Chimento 2003).

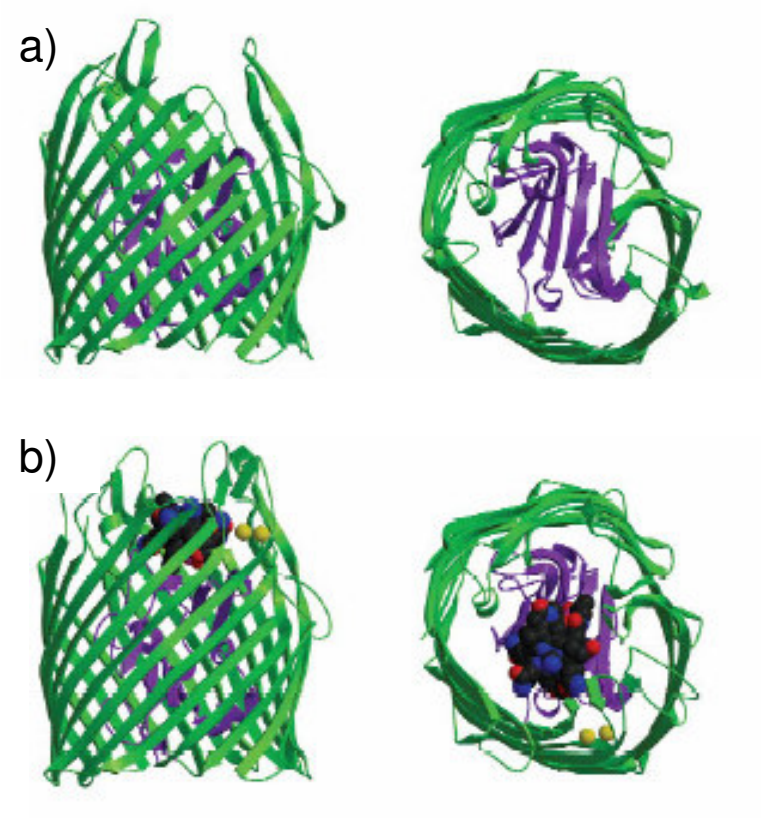


Figure 1.7 Crystal structures of BtuB a) in the absence of calcium ions and vitamin B_{12} and b) in the presence of calcium ions and vitamin B_{12} (from Chimento *et al* 2003). Structures on the left depict the molecule with extracellular loops pointing up and periplasmic loops pointing down. Structures on the right show the molecule looking down the extracellular face. The plug domain is depicted in purple and the barrel domain is coloured green. Extracellular loops that are disordered in the apo-BtuB structure (a) become fully ordered in the Ca^{2+}-vitamin B_{12}-BtuB structure (b).

1.8.2. Receptor-binding domains of colicins

Despite utilising different outer membrane receptors (see Table 1.1), the receptor-binding domains of colicins E3 and Ia are structurally similar, both consisting of a coiled coil, 160 Å in length in the case of colicin Ia and 100 Å for colicin E3 (Wiener *et al* 1997, Soelaiman *et al* 2001). The length of the coiled coil of colicin Ia was suggested to allow the colicin to span the periplasmic space, which has an approximate width of 150 Å (Wiener *et al* 1997), but the coiled coil of colicin E3 is too short to allow this. The helical regions of both colicins Ia and E3 are Alacoils, such that an alanine is present at every seventh position. The minimum receptor-binding domain of colicin E9 has been shown to consist of residues 343-418 (Penfold *et al* 2000) and forms a helical hairpin structure (Boetzel *et al* 2003). This 76-residue minimal receptor-binding domain protects cells in an *in vivo* biological protection

assay at least as efficiently as the full-length colicin (Penfold *et al* 2000). The region is identical in sequence to the corresponding region of colicin E3. NMR has shown that the minimum receptor-binding domain has slowly interchanging conformers and a flexible inter-helix loop (Boetzel *et al* 2003). It has been proposed that the flexible loop may act as a hinge to allow unwinding of the helical hairpin upon receptor-binding, allowing access of the translocation domain to the periplasm (Boetzel *et al* 2003). This is supported by the loss of activity of colicin mutants with disulphide bridges near the middle or the top of the receptor-binding domain of colicin E9 (Penfold *et al* 2004).

In contrast to the receptor-binding domains of colicins E3 and Ia, the receptor-binding domain of colicin N consists of a six-stranded antiparallel β-sheet wrapped around a 63 Å long α-helix of the pore-forming domain (Vetter *et al* 1998).

The function of residues 419-448, between the minimum receptor-binding domain and the DNase domain of colicin E9 is as yet unknown. This region has been termed the 'linker region' and forms the upper part of the coiled coil of colicin E3 (James *et al* 2002). It has been suggested that this region could bridge the periplasmic space to assist delivery of the endonuclease domain to the cytoplasm, although the region is too short (~50 Å) unless the translocation of the colicin occurs at zones of adhesion, which would bring the outer and inner membranes into closer proximity (James *et al* 2002). It has been suggested that colicins may preferentially bind to newly synthesised BtuB molecules, present at adhesion sites and radioactively labelled colicin A has also been show to associate with adhesion sites (Penfold *et al* 2000, Guihard *et al* 1994).

The receptor binding domain of colicin B is difficult to distinguish from the translocation domain as the two domains are intertwined at one end of the dumbbell structure (Hilsenbeck *et al* 2004, see Figure 1.6). Amino acids 262-282 of colicin B show significant sequence homology to the two β-strands in the receptor-binding domain of colicin Ia, indicating that these residues could be involved in receptor-binding. Amino acids 1-291 of colicin B also share significant sequence similarity with the same region in colicin D. These two colicins use the same receptor, FepA, and therefore these residues are likely to be used in receptor-binding (Hilsenbeck *et al* 2004). There is also similarity between residues 130-291 of colicins B, D, E2, E3, E6, E7 and E9 and this region was recently proposed to be the receptor-binding domain of all these colicins (Hilsenbeck *et al* 2004). Based on this sequence

similarity, it was recently suggested that residues 343-418 could be involved in binding to OmpF, rather than BtuB, supported by the fact that colicins B and D, which do not bind to OmpF, do not contain sequences homologous to residues 343-418 in colicin E9 (Hilsenbeck *et al* 2004). However this suggestion ignores substantial evidence supporting the involvement of residues 343-418 of colicin E9 in binding to BtuB, *ie* the structure of BtuB bound to the residues 313-447 of the colicin E3 receptor-binding domain (Kurisu *et al* 2003), the demonstration that a 34-residue peptide binds to BtuB with nanomolar affinity (Mohanty *et al* 2003) and the demonstration that the isolated residues 343-418 of colicin E9 compete with vitamin B_{12} for binding to BtuB (Penfold *et al* 2000).

1.8.3. Crossing the outer membrane

The mechanism by which colicins, once bound to their receptor, traverse the outer membrane, once bound to their receptor, is still unknown. Two hypotheses have been put forward in an attempt to explain the mechanism although there is currently a lack of evidence to support either hypothesis (Cao and Klebba 2002).

1.8.3.1. The "Nail" Hypothesis

This hypothesis suggests that binding of the tip of the helical hairpin of the colicin receptor-binding domain to its receptor allows the colicin to penetrate the membrane by passing through the β-barrel of the receptor. 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. Although it is possible that the hairpin of the receptor-binding domain could fit into the barrel of the receptor, the translocation and cytotoxic domains are too large to pass through any known outer membrane channels unless they are in a denatured state (Cao and Klebba 2002).

1.8.3.2. Denaturation of colicins

There is some evidence available to suggest that at least partial denaturation of colicins may occur on binding to the outer membrane receptor. Denaturation of colicin A by urea has been shown to decrease the time taken by the cytotoxic domain to depolarise the inner membrane, shown by the decreased lag time for onset of the

efflux of cytoplasmic potassium (Bourdineaud *et al* 1990, Bénédetti *et al* 1992). Binding of colicin A to bacterial cells was also shown to increase the susceptibility of site-directed disulphide bonds in the colicin to DTT, in a similar manner to denaturation of colicin A using urea, again indicating that denaturation of the colicin occurs on binding to a cell surface receptor, which may promote its entry into the cell (Duché *et al* 1994). However, disulphide bonds in the pore-forming domain of colicin A did not prevent translocation, although they did slow it down, indicating that extensive unfolding of the pore-forming domain does not occur (Duché *et al* 1994). Isothermal titration calorimetry data for the binding of colicin N to OmpF indicates that a structural rearrangement occurs on formation of the OmpF-colicin N complex (Evans *et al* 1996).

It has been suggested that proteins can become less ordered on approaching a membrane due to a local increase in pH and in the dielectric constant (Bychkova *et al* 1996). Unfolding of the colicin would also increase the length of the colicin, allowing the cytotoxic domain to reach the cytoplasmic membrane, with the receptor-binding domain still bound to its outer-membrane receptor. Addition of trypsin to cells treated with colicin A causes membrane depolarization to be halted (Bénédetti *et al* 1992). As trypsin cannot access the periplasm or cytoplasmic membrane, it would appear that part of the colicin is still accessible to the external medium even after the pore-forming domain has inserted into the cytoplasmic membrane. Bacteria treated with colicin E2 can also be rescued by trypsin after they have begun their cytotoxic activity, indicating that endonuclease colicins also span the cell envelope whilst exerting their cytotoxicity (Nose and Mizuno 1968, Beppu *et al* 1972). A colicin A mutant, with a disulphide bond connecting two helices in the pore-forming domain, disabling its pore-forming activity, has been shown to prevent the potassium efflux caused by various group A colicins, when the functional colicins are added at various times following addition of the colicin A disulphide mutant (Duché 1995). The mutant was unable to prevent the cytotoxic action of group B colicins, which use different import machinery. Despite the lack of pore-forming activity, the mutant should still be able to efficiently translocate across the outer membrane, indicating that even once the colicin mutant had been translocated; it still remained in contact with its receptor and the translocation machinery.

1.8.3.3. Translocation at a distant site

The alternative hypothesis proposes that the cytotoxic domain could translocate across the membrane at a second site, away from the initial binding site (Cao and Klebba 2002). This seems plausible for colicins E3 and Ia as their extended structures would allow binding of the cytotoxic domain away from the binding site for the receptor-binding domain. Proteins, lipids or a "membrane island" containing an unusual mix of proteins and lipid have all been implicated as possible secondary sites for translocation (Cao and Klebba 2002).

Colicin N is much shorter and therefore this restricts the identity of the entry site for the cytotoxic domain, unless colicin N does not use a secondary site and simply translocates across the outer membrane through its primary receptor, OmpF. This is possible as no other outer membrane protein has been identified that is required for translocation of colicin N. A model of binding of colicin N to its receptor, OmpF, has been proposed in which the receptor-binding domain sits like a plug above the pore of OmpF with positively charged residues in a solvent-exposed cleft of the receptor-binding domain interacting with negatively charged loops of OmpF (Vetter *et al* 1998). The pore-forming and translocation domains of colicin N are predicted 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 *et al* 1996). The translocation domain needs to reach the periplasm and three routes were put forward as to how this could occur (Vetter *et al* 1998). Firstly, the translocation domain could move along the outside of the porin barrel but the requirement for direct interactions with the lipid bilayer is energetically unfeasible. Secondly, the monomers of OmpF could destabilise allowing the translocation domain to pass through the middle of the trimer, but OmpF is very stable. Thirdly, the domain could move through the pore of one of the monomers of OmpF. The translocation domain of colicin N is absent in the crystal structure and has a glycine/ proline/ serine/ asparagine-rich sequence, indicating that it is not likely to contain defined secondary structure. The absence of regular secondary structure in the translocation domain makes it more plausible that this domain could thread through the porin. Therefore, the third possibility is the most likely and it is proposed that residues from the pore-forming domain interact with the outer rim of OmpF and the translocation domain

threads through the pore of OmpF to interact with Tol proteins in the periplasm (Vetter *et al* 1998).

1.8.4. A colicin translocon?

Recently a structure of BtuB bound to residues 313-447 of the receptor binding domain of colicin E3 (R135) has been solved at a resolution of 2.75 Å (Kurisu *et al* 2003). This structure shows that only the tip of the helical hairpin of the receptor-binding domain of colicin E3 is involved in binding to BtuB. 24 % of R135 and 6.3 % of BtuB become buried on formation of the complex (Kurisu *et al* 2003). 27 residues of R135 (between residues I369-T402), and 29 residues of BtuB (mostly at the top of the plug domain) are involved in the BtuB-R135 complex. There are small conformational changes in the plug domain of BtuB, but these cannot account for translocation of the colicin (Kurisu *et al* 2003). Competition assays show that exogenous vitamin B_{12} can protect cells against colicin cytotoxicity and therefore it was predicted that colicins would bind to the same binding site on BtuB as vitamin B_{12} (Cavard 1994). The receptor binding domain of colicin E9 has also been shown to inhibit the growth of *E.coli* 113/3 cells that are dependent on the addition of vitamin B_{12} (Penfold *et al* 2000). The crystal structure shows that the binding site for R135 on BtuB overlaps with the binding site of vitamin B_{12} by five residues but the other eight residues involved in vitamin B_{12} binding are not involved in binding R135 and overall vitamin B_{12} is more deeply buried in BtuB than R135. No calcium was detected in the BtuB-R135 structure, in contrast to the BtuB-vitamin B_{12} structure (Chimento *et al* 2003).

Binding of R135 causes ordering of loops 5-6 and 7-8 of BtuB and alters the position of loop 19-20 and 3-4. Ordering of loop 5-6 in FepA caused by binding of colicin B has also been shown by electron spin resonance spectroscopy (Jiang *et al* 1997). The first ten residues at the N-terminus and nine residues at the C-terminus of R135 are disordered in the R135-BtuB complex, leading to the Cα displacement of residues of R135, which increases with increasing distance from the hairpin tip. It is proposed that this partial unfolding of the hairpin could trigger unfolding of the rest of the colicin (Kurisu *et al* 2003).

The R135-BtuB structure prompted the proposal that instead of the colicin directly translocating through BtuB, this receptor is used to concentrate the colicin on the surface, where it can then "fish" for a secondary receptor. The translocation domain

would bind to this secondary receptor, forming a translocon (see Figure 1.8). If the cytotoxic domain or translocation domain passes through the porin, whilst the receptor-binding domain is still attached to its receptor, this indicates a potential role for the "linker region" of colicin E9 mentioned earlier, as a spacer between the receptor-binding domain and the translocation domain. A long helix of the translocation domain of colicin Ia has no obvious function and has also been suggested to act as a spacer (Wiener *et al* 1997). Colicin A mutants with residues 98-108 or 154-172 deleted are still active, and it has been suggested that these regions could act as spacers (Bouveret *et al* 1998, Journet *et al* 2001).

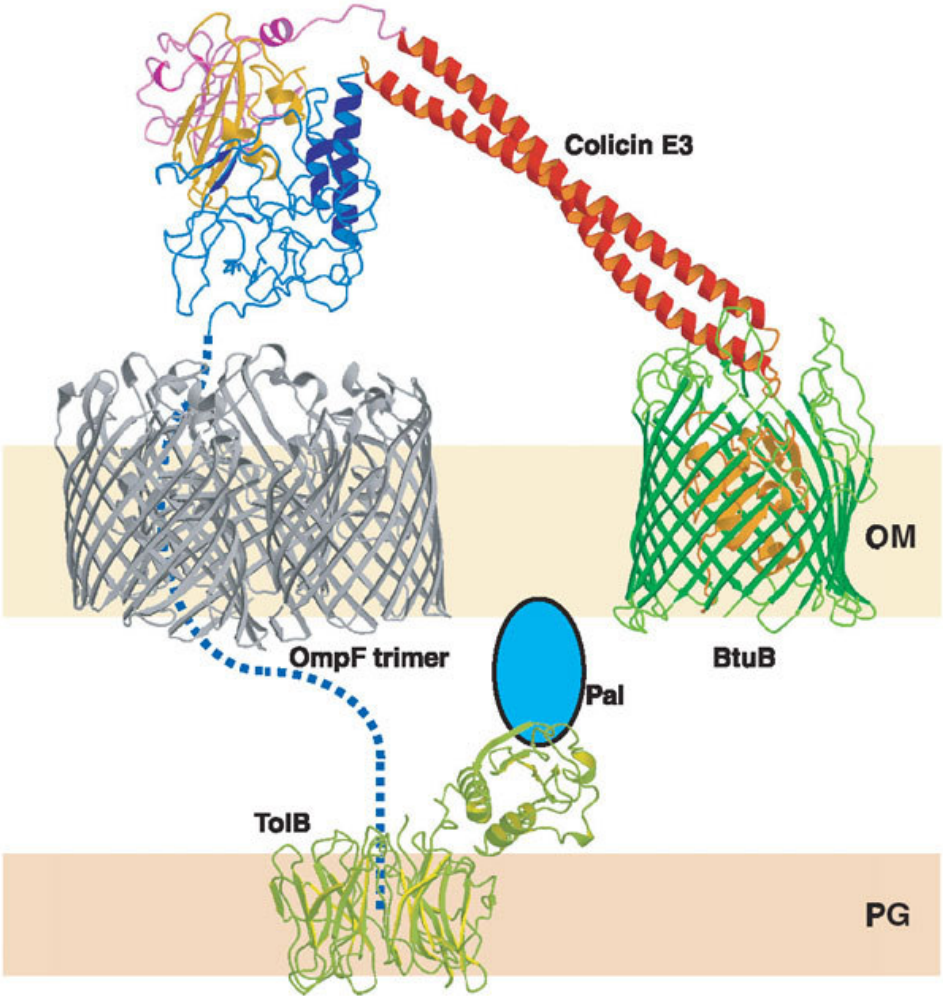


Figure 1.8 **Proposed model for the formation of a colicin translocon (Kurisu *et al* 2003). The receptor-binding domain (red) is bound to BtuB (barrel domain in green, plug domain in orange) and the positions of the translocation domain (blue), cytotoxic domain (magenta) and immunity protein (yellow) are extrapolated from the structure of the R135-BtuB complex. The model suggests that binding of the receptor-binding domain of colicin E3 to BtuB, concentrates the colicin on the cell surface. The extended coiled coil of the receptor-binding domain would then allow the colicin to scan along the cell surface for an OmpF trimer (grey) and the disordered region of the translocation domain of colicin E3 would interact with OmpF. The translocation domain would then thread through the OmpF channel where it could interact with the Tol translocation system.**

1.8.4.1. OmpF

The putative secondary receptor in the proposed colicin translocon is OmpF, as the E-colicins, except colicin E1, and colicin A have all been shown to require OmpF for translocation (Dover *et al* 2000). OmpF is a non-selective trimeric porin, each monomer consisting of a 16-stranded β-barrel with large loops between each β-strand (see Figure 1.9). The traverse loop (L3) of OmpF constricts the monomer pore diameter to 7 x 11 Å about half way down the β-barrel (Cowan *et al* 1992). The wide cross-section of the pore is sufficient for insertion of an unfolded polypeptide and the porin is present in a high density (~10^5 molecules per cell) on the cell surface (Nikaido and Vaara 1987). The N-terminal residues of the translocation domains of colicins Ia, B and E3 are unresolved in the crystal structures, implying a low degree of secondary structure and a high degree of flexibility (Wiener *et al* 1997, Soelaimain *et al* 2001, Hilsenbeck *et al* 2004). It is proposed that this disordered region of the translocation domain would thread through the OmpF pore. Due to the unfolded nature of this domain, the threading through OmpF is not predicted to require a large energy input (Kurisu *et al* 2003).

Kurisu *et al* (2003) support their hypothesis by demonstrating that OmpF channels in planar bilayers could be occluded by addition of colicin E3 (Kurisu *et al* 2003). This occlusion only occured when colicin E3 was applied to the trans-side of the bilayer, likely to be analogous to the extracellular side of the outer membrane. No occlusion occurred when colicin E1, which does not require OmpF for translocation, was added to the bilayer (Kurisu *et al* 2003).

However, the exact role of OmpF in the translocation process is still debateable. The absence of OmpF in the cell has very little effect on binding of the receptor-binding domain of colicin E9 to cells and would therefore appear not to act as a co-receptor (Penfold *et al* 2000). Pre-incubation of colicin E9 with OmpF has been shown to enhance protection to cells afforded by pre-incubation of colicin E9 with BtuB, although any interaction between OmpF and the BtuB-colicin E9 is predicted to be weak and transient, as no tertiary complex could be detected using gel filtration and chemical cross-linking (Law *et al* 2003). The pore-forming domains of colicin N and colicin B have been shown to interact with OmpF, although the formation of these complexes depends on the presence of detergent or lipopolysaccharide, and therefore the biological significance of these complexes remains questionable (Dover *et al*

2000). Cells with a G119D mutation in OmpF causing this residue of loop L3 to protrude into the lumen of the channel, dividing it into two sections of reduced diameter, are resistant to colicin N (Jeanteur *et al* 1994). As this mutation had no effects on the rest of the structure, it was concluded that colicin N normally passes through OmpF and that this mutation prevents colicin from entering the porin channel (Jeanteur *et al* 1994). However, an alternative explanation is that exposed loops, involved in binding colicin N are affected, preventing binding of colicin N to OmpF. Mutation of OmpF at residues G119 or G120 to bulkier residues has also been shown to dramatically affect the activity of colicin A, indicating that loop L3 also has a role in recognising and/or transporting colicin A (Bredin *et al* 2003). Residues D113 and D121 were also found to be important for the activity of colicins A and N. However, residue D113 was found to play a more prominent role in colicin A activity, whereas residue D121 was more important for colicin N activity. This indicates that there may be differences in the uptake of the two colicins with colicin N interacting with residues located at the top of L3 and colicin A using residues further down and on the other side of L3 (Bredin *et al* 2003).

In an attempt to discover whether colicins do translocate across the outer membrane by passing through OmpF, genetically engineered disulfide bonds were used to tether loop L3 of the porin to the barrel wall at various postions (Bainbridge *et al* 1998). This loop must undergo a conformational change to allow enough space for translocation of a polypeptide chain. Potassium efflux and fluorescence depolarisation assays were used to show that out of five disulfide pairs, only the E117C-D312C pair caused resistance to colicin N, but not to colicin A. This was predicted to be due to impaired binding of colicin N to OmpF and indicated that although small conformational changes at the tip of L3 may occur, a large conformational change in L3 to allow a polypeptide chain to pass through the pore is unlikely to occur and therefore colicins N and A do not appear to move through the OmpF pore. However, this conclusion has been questioned for several reasons (Cao and Klebba 2002). Firstly, the crystal structure of OmpF shows that the L3 loop does block the pore but in solution this loop is likely to be far more flexible. Secondly, the mutations E117C-D312C and D107C (a single mutation which affected colicin sensitivity) are beyond the reach of the colicin N binding domain as it is too large to enter the porin channels and therefore it is possible that rather than interfere with

binding of the colicin as proposed by Bainbridge *et al* (1998), these mutations may slow down the rate of colicin "threading" through the pore (Cao and Klebba 2002).

If colicins do use two different outer membrane proteins to traverse the outer membrane, the two proteins must be within a certain distance of one another, suggesting that co-localisation may occur. It has been suggested that translocation across the outer membrane could be dependent on membrane fluidity, as it is dependent on temperature (Bourdineaud *et al* 1990). This could potentially be a consequence of the requirement for OmpF and BtuB to be in close proximity in the membrane.

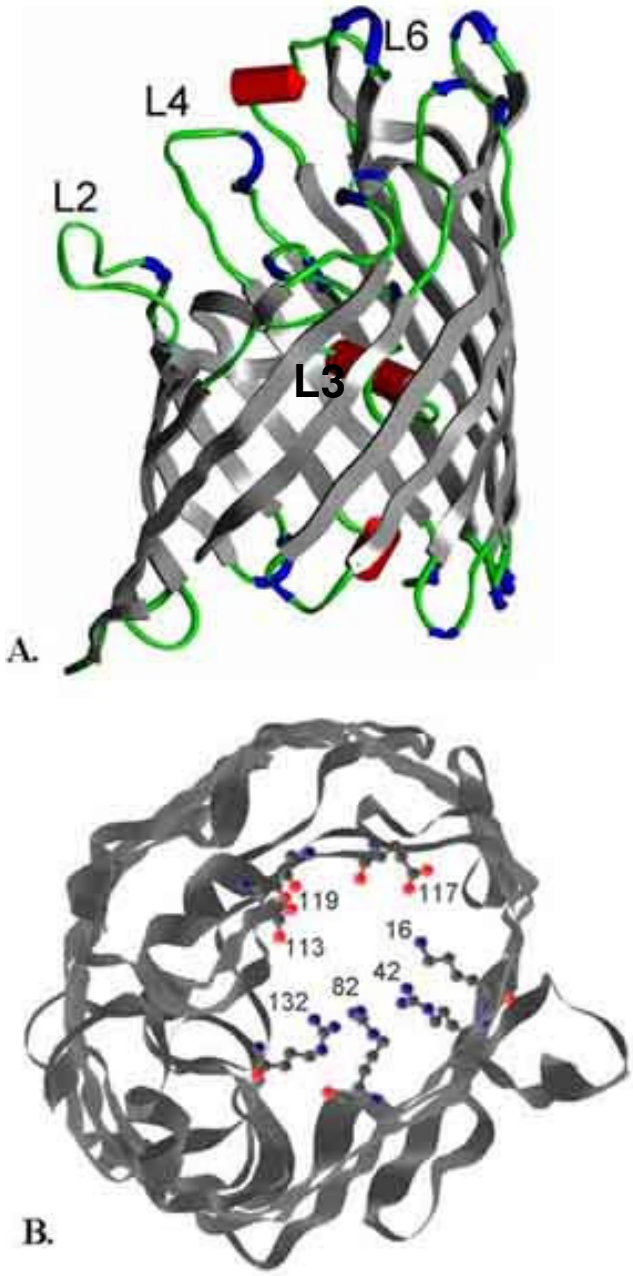


Figure 1.9 Three-dimensional structure of OmpF (from Delcour 2003)

A. A side view of an OmpF monomer; β-strands are shown as a gray ribbon, and α-helices as red cylinders; extracellular loops L2, L3, L4 and L6 are labelled.
B. A cross section of an OmpF monomer, viewed from the extracellular side. Residues labelled are K16, R42, R82, D113, E117, G119 and R132. D113, E117 and G119 form part of loop L3.

1.8.4.2. TolC

The existence of a fourth domain in colicin E1 which has been shown to bind to TolC, indicates that colicin E1, rather than using OmpF as a translocator, uses TolC. Although no direct interaction has been observed between OmpF and endonuclease colicins, a region in the translocation domain of colicin E1 has been shown to interact with TolC.

TolC is a homotrimer, comprising a 40 Å long β-barrel that spans the outer membrane and a unique 100 Å long α-helical barrel projecting into the periplasm (Koronakis *et al* 2000). Unlike other outer membrane proteins, which form one 16- or 18- stranded barrel per monomer, *eg* OmpF, in TolC, each monomer contributes 4 β-strands to form a single 12-stranded β-barrel (see Figure 1.10). This gives TolC a much greater cross-sectional area (960 Å) than other outer-membrane proteins, fifteen times that of OmpF. TolC does not contain the common loop that constricts the channel of other channel-forming proteins *ie* L3 in OmpF and does not contain a plug domain, found in iron transporters. However, the conductance of TolC remains low, probably because the diameter of the channel narrows towards the periplasmic end of the protein (Andersen *et al* 2001). TolC is known to be involved in export of large proteins (~170 kDa) from the periplasm into the extracellular medium so this narrow opening must extend to allow passage of these large proteins. This implies that TolC could allow translocation of colicin E1 across the outer membrane in the opposite direction *ie* from the extracellular medium into the periplasm (Zakharov and Cramer 2002).

If OmpF and TolC allow translocation of the colicin across the outer membrane, this contradicts the traditional view that the three separate domains of a colicin function in three separate compartments of the cell *ie* the receptor-binding domain functions at the outer membrane, the translocation domain allows translocation across the periplasm and the cytotoxic domain is involved at the cytoplasmic membrane and, for endonuclease colicins, the cytoplasm.

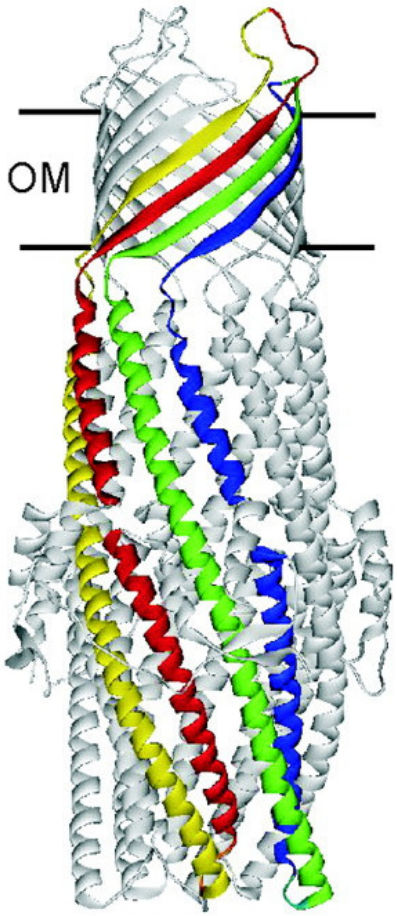


Figure 1.10 The crystal structure of TolC (from Andersen *et al* 2002). TolC is 140 Å in length and is comprised of a 40 Å long β-barrel domain spanning the outer membrane (OM) and a 100 Å long α-helical domain, which projects across the periplasmic space. One monomer is shown in colour. Each monomer contributes 4 strands to the 12-stranded β-barrel domain.

1.9. Translocation

Colicins can be divided into two groups, based on the mechanism of translocation from the outer membrane to the inner membrane (Davies and Reeves 1975a, b). Group A colicins *eg* colicins A, E1, E3, E9 (see Table 1.1) are inactive against cells containing lesions in the *tola* gene and therefore use the Tol ("Tolerant") import system, whereas group B colicins *eg* colicins B,D, Ia, M are inactive against cells carrying a *tonb* mutation and therefore use the Ton system (Nomura and Witten 1967, Webster 1991). The domain organisation is similar for Ton- and Tol-dependent colicins and some components of the two translocation systems are homologous.

Therefore it is likely that translocation of both types of colicins occurs via a similar mechanism (James *et al* 2002).

1.9.1. The Tol translocation system

The Tol system consists of five proteins, TolQ, TolR, TolA, TolB and the peptidoglycan-associated lipoprotein (Pal).

The genes involved in the Tol pathway are clustered at 17 minutes on the *E.coli* chromosome (Lazdunski *et al* 2000). They are organised in 2 operons (Vianney *et al* 1996):

i) *ybgc, tolA, tolQ* and *tolR* under the control of promoter P1

ii) *tolB, pal* and *ybgf* under the control of the strong promoter, PB and P1

ybgc and *ybgf* mutants do not cause a *tol* phenotype and the functions of YbgC and YbgF proteins are unknown (Lazdunski *et al* 1998).

The nucleotide sequences of the *tol* genes have been determined and a number of mutations in the *tol* genes have been isolated that render bacteria insensitive to certain colicins, without affecting binding of the colicins to their specific receptors (Webster 1991). Experiments with these *tol* mutations have shown that the *tol* gene products required for translocation differ for specific colicins. For example, Tol A, B, Q and R are required to translocate the enzymatic colicins, E2-E9, whereas only Tol C, A and Q are required to translocate the pore-forming colicin E1.

The protein products of *tol Q, R, A* and *B* have been identified and correspond to the sizes predicted from the DNA sequences (Webster 1991). Figure 1.11 shows the structure or predicted structures and cellular locations of proteins in the Tol system.

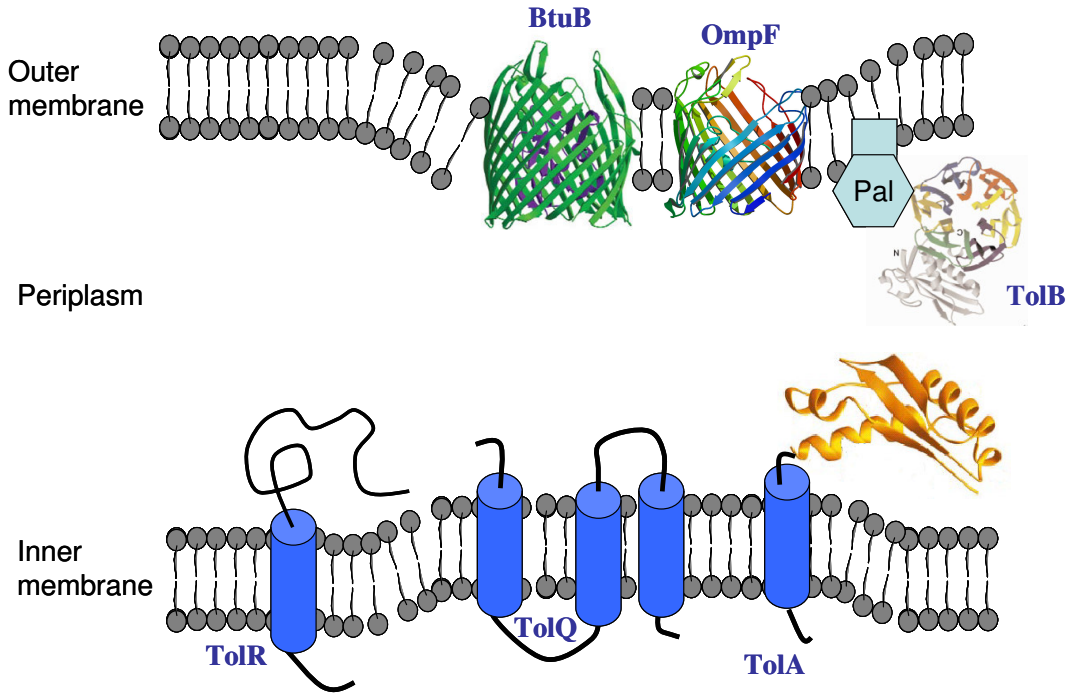


Figure 1.11 Components of the Tol/Pal translocation cellular import pathway for Group A colicins

1.9.1.1. Involvement of Tol proteins in translocation of colicins

Although the transport of colicins across the periplasm is still poorly understood, there are now many studies that show interaction of group A colicins with Tol proteins is necessary for translocation.

The first demonstration of a correlation between the translocation of group A colicins and colicin binding to a Tol protein came from analysis of *in vitro* binding of over-expressed Tol proteins to colicins by Western blotting (Bénédetti *et al* 1991b). The Tol-dependent colicins, colicins A and E1, interacted with TolA but colicin B, a Ton-dependent colicin, did not. The isolated translocation domain of colicin A was the only region shown to be involved in interacting with TolA. This was confirmed by surface plasmon resonance experiments and the interaction was shown to have a K_d of 0.2 µM. The N-terminal of colicin E1 was shown by the same technique to bind to TolA with a K_d of 0.6 µM (Derouiche *et al* 1997).

Another study reported that when cells were treated with colicin A, the amount of Tol proteins in the membrane fraction, defined as contact sites between the inner and

outer membranes, doubled (Guihard *et al* 1994). This suggested that colicins recruit Tol proteins for translocation.

The Tol-dependent colicins contain a consensus pentapeptide sequence, DG(T/S)G(S/W) close to the N-terminus, originally called the TolA box (Pilsl and Braun 1995b). Site-directed mutagenesis experiments were used to show that although mutation of the two glycine residues to alanine did not affect the biological activity of colicin E9, mutation of any of the other three residues completely abolished cytotoxic activity *in vivo*. The D35A, S37A and W39A mutants retained their ability to bind to BtuB and exhibited DNase activity *in vitro,* signifying a role for these residues in translocation (Garinot-Schneider *et al* 1997). *In vivo and in vitro* cross-linking experiments demonstrated an interaction between the N-terminal 325 residues of colicin E3 and TolB, which was abolished if the serine residue at position 37 in the putative TolA box was mutated to phenylalanine (Bouveret *et al* 1997). Therefore, although the pentapeptide sequence identified is involved in colicin-Tol interactions, it is the TolB protein with which it interacts, rather than the TolA protein, and hence it was designated the TolB box.

The interaction between the translocation domain of colicin E9 and TolB has also been demonstrated using the yeast two-hybrid system (Carr *et al* 2000a). Mutation of the TolB box residues of the T domain of colicin E9 to alanine confirmed that it was the protein interaction between the TolB box and TolB that gave rise to the positive yeast two-hybrid interaction.

The entry of a hybrid toxin, consisting of the first 372 amino acids of the attachment (gene III) protein of the bacteriophage f1 fused to the RNase domain of E3, was shown to not only be dependent on the products of *tolA, tolQ* and *tolR*, which are required for both phage f1 infection and colicin entry but also on the TolB protein, which is required for colicin E3 killing but not for phage f1 infection (Jakes *et al* 1988). This suggests that the toxin may be using the F pilus to bind to the outer membrane with pilus retraction bringing the toxin to the cell membrane but that the enzymatic portion then reaches the cytoplasm via TolB and the Tol system rather then the pathway normally used by f1.

NMR and crystal structures (Wiener *et al* 1997, Soelaiman *et al* 2001, Collins *et al* 2002) suggest that the translocation domains of colicins are relatively unstructured and it is not yet known how Tol proteins are able to specifically recognise and bind to regions of this unstructured domain.

1.9.1.2. TolB

TolB is a periplasmic protein of 408 residues, resulting from cleavage of its 21-residue signal sequence (Isnard *et al* 1994). It is mainly found in the periplasm but a small amount is also found associated with the outer membrane (Isnard *et al* 1994). By sequence homology, TolB is a member of the tryptophan-aspartate (W-D) repeat protein family. This protein family has a diverse range of cellular functions including transmembrane signalling, transcription and mRNA modification and several members of the family form multiprotein complexes, sometimes interacting through their W-D repeat regions (Neer *et al* 1994). Two different groups have determined the crystal structure of TolB to 2 Å resolution (Carr *et al* 2000a) and 1.95 Å resolution (Abergel *et al* 1999). The two structures show that the overall fold of the protein comprises a 131-residue 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 (see Figure 1.12). Each blade of the propeller consists of twisted β-sheets that are radially arranged around a central tunnel.

Interaction of TolB with colicins

β-propeller proteins usually use their central tunnel or the entrance to the tunnel to coordinate a ligand or to carry out a catalytic function that is preserved by the structural rigidity of the propellers. It has therefore been proposed that the translocation domains of colicins interact with the β-propeller domain (Carr *et al* 2000a). However, this has been questioned, as deletions and insertions in the β-propeller domain of TolB do not affect the interaction with colicins, whereas an insertion into the N-terminal domain of TolB caused a reduction in the ability of colicins to penetrate into the bacteria (Abergel *et al* 2000).
As well as interacting with colicins, TolB has been shown to interact with the peptidoglycan associated lipoprotein (Pal), Lpp and OmpA (Wallburger *et al* 2002, Bouveret *et al* 1995, see section 1.9.1.3).

TolB dimersation

Yeast two-hybrid studies and *in vitro* cross-linking experiments have indicated that TolB dimerises (Wallburger *et al* 2002, Dubuisson *et al* 2002). This dimerisation appears to occur through the N-terminal domain, as the isolated N-terminal domain

has been shown to have the capacity to dimerise and multimerise, possibly due to the adoption of different conformations, exposing different cross-linking sites. The physiological relevance of the dimerisation remains unclear as it was not detected in the determination of the crystal structure or in previous *in vitro* and *in vivo* studies (Carr *et al* 2000a, Abergel *et al* 1999, Bouveret *et al* 1995, 1998).

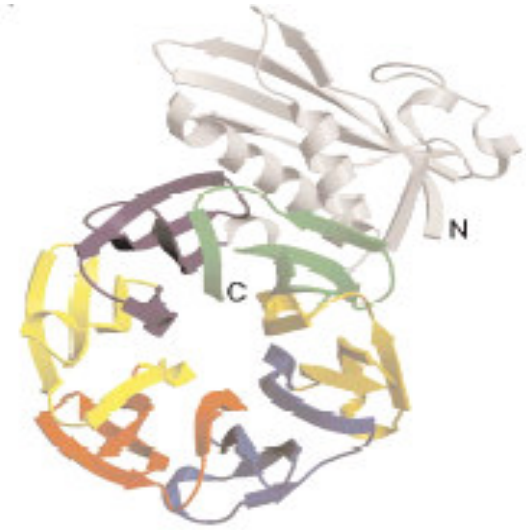


Figure 1.12 The crystal structure of TolB looking down the central barrel of the β-propeller domain. Six repeated sequence motifs produce the six propellers of the domain (Carr *et al* 2000a).

TolB box

The conserved pentapeptide sequence, DG(T/S)G(S/W), near the N-terminus of several Group A colicins, found to be important for interaction of colicins with TolB, has been designated the TolB box.

Mutation of residues D35, S37 or W39 to A in colicin E9, which abolishes cytotoxic activity of the colicin, has also been shown to abolish binding of the colicin to TolB by yeast-two hybrid screening (Carr *et al* 2000a). Mutation of either of the two glycine residues to alanine in colicin E9, had no effect on biological activity or on its interaction with TolB (Carr *et al* 2000a). Mutation to alanine of two serine residues (S34 and S40), flanking the TolB box, did not abolish biological activity, but the zones of growth inhibition caused by these mutants were hazy and slower to form than those zones formed by the wild-type colicin protein (Garinot-Schneider *et al* 1997). Mutation of S37 to F in colicin E3 also abolished binding of this colicin to TolB (Bouveret *et al* 1997).

The region of colicin A involved in binding to TolB, has been narrowed down to residues 7-20, by mutagenesis (Journet *et al* 2001). A pentapeptide sequence, very similar to that of colicin E9 *ie* DGTGW, occurs at residues 11-15. However, other residues downstream of residue 15 are predicted to be involved in binding to TolB, as a colicin A mutant with residues 16-30 deleted, does not interact with TolB (Bouveret *et al* 1998). Another colicin A mutant with residues 21-29 deleted does interact with TolB indicating that residues between 15-20 are also involved in binding to TolB (Bouveret *et al* 1998).

Colicin K has no well conserved TolB box, despite being dependent on TolB for translocation. The suggested TolB box, contains an alanine residue at the fifth position. This fifth position in colicin E9 is a tryptophan residue, which when mutated to alanine abolishes binding to TolB (Carr *et al* 2000a). This indicates that, if the TolB box for colicin K has been identified correctly, colicin K may interact with a different region of TolB, compared to other TolB-dependent colicins.

1.9.1.3. Pal

Pal is a 152 amino acid lipoprotein with a serine residue at position +2, which results in its localisation in the outer membrane via an N-terminal lipid moiety (Mizuno 1979). Residues 94 to 114 form an amphipathic helix, which contains a peptidoglycan recognition sequence that interacts with peptidoglycan (Koebnik *et al* 1995, Bouveret *et al* 1999, Cascales and Lloubes 2004).

Pal has been shown to form dimers *in vitro* and *in vivo*, probably via its C-terminal residues, independent of Tol proteins, Lpp and OmpA (Cascales *et al* 2002, Cascales and Lloubes 2004).

TolB-Pal interaction

In vivo cross-linking and immunoprecipitation experiments first indicated that TolB interacts with Pal (Bouveret *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, indicating that the β-propeller domain may be involved in binding to Pal and OmpA (Abergel *et al* 1999). Suppressor genetics also identified the C-terminal β-propeller domain of TolB as important for the interaction with Pal (Ray *et al* 2000).

Mutagenesis studies have limited the region of Pal that interacts with TolB to residues 89-104 and 126-130 (Clavel *et al* 1998). A peptide comprising residues 89-130 of Pal is able to interact with TolB and with peptidoglycan (Bouveret *et al* 1999). However, 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. Dimerisation of Pal is not required for the interaction with TolB (Cascales and Lloubes 2004).

Role of the TolB-Pal interaction in colicin translocation

Binding of colicin E3 to TolB prevents the interaction of TolB with Pal and point mutations have been identified in Pal that render cells insensitive to colicins (Bouveret *et al* 1997, Clavel *et al* 1998). Therefore, although a *pal* null mutant is equally sensitive to colicins as wild-type cells (Bouveret *et al* 1995), there is evidence to suggest that Pal is, at least indirectly, involved in colicin translocation. It has been suggested that the varying associations of Pal with TolB and peptidoglycan may play a role in the crossing of colicins across the peptidoglycan layer (Bouveret *et al* 1997). Alternatively TolB could act as a shuttle between Pal at the outer membrane and TolQRA at the inner membrane (Bouveret *et al* 1999).

Pal interaction with Lpp and OmpA

TolB and Pal can be cross-linked with outer-membrane proteins, OmpA and Lpp (Clavel *et al* 1998, Cascales *et al* 2002). The OmpA binding sequence of Pal has been localised to residues 44-61 (Cascales and Lloubes 2004). It was necessary for TolB to be associated with Pal in order for TolB to interact with these two proteins. It has therefore been suggested that TolB and Pal are part of a multiprotein complex that links the peptidoglycan to the outer membrane (Clavel *et al* 1998).

1.9.1.4. TolA

TolA is a 44 kDa membrane protein, which can be divided into three domains, although no crystal structure of the full-length protein is yet available (Webster 1991). Domain I, consisting of the N-terminal 47 amino acids, contains a 21 amino acid hydrophobic segment, anchoring the protein in the inner membrane (Webster 1991). This domain has been shown by formaldehyde crosslinking to interact with TolR and TolQ (Derouiche *et al* 1995).

Secondary structure predictions and circular dichroism measurements indicate that the central domain II is mainly α-helical (Levengood *et al* 1991, Derouiche *et al* 1999). This domain is separated from domain I by five glycine residues and from domain III by three glycine residues. Secondary structure prediction indicates that domain II lacks turns and analytical ultracentrifugation indicates that the domain is elongated (Derouiche *et al* 1999). A single helix formed by this region would be approximately 35 nm, enabling the protein to span the periplasm. However, this single helix is unlikely to form, as hydrophobic sequences in the domain would make the purified domain insoluble when exposed to hydrophilic solvents, whereas the purified domain was actually found to be soluble (Derouiche *et al* 1999). Molecular modeling of domain II, suggests that the domain could consist of a three-stranded coiled coil structure and this has been suggested to act to tether the anchoring domain I to the functional domain III (Webster *et al* 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 *et al* 1999). Domain III of TolA is a novel fold consisting of three antiparallel β strands with four helical motifs. NMR studies have shown that interaction with colicin A induces a significant structural change in domain III of TolA, involving an increase in flexibility of the domain (Deprez *et al* 2002). 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, allowing translocation of colicin A (Deprez *et al* 2002).

Interaction of TolA with colicins

TolA interacts with colicins A and E1. TolA with a deletion in domain III was unable to interact with colicins A and E1 *in vivo* (Bénédetti *et al* 1991b). *In vitro* studies with purified proteins and yeast-two hybrid experiments confirmed that it is the C-terminus of TolA that interacts with colicins A and E1 (Derouiche *et al* 1997, Wallburger *et al* 2002). A mutational analysis of the α-helical domain II of TolA, indicated that although cells containing a *tolA* gene with almost the entirety of domain II deleted, had a decreased sensitivity to colicins A, E3 and N, there was very little change in their sensitivity to colicin E1 (Schendel *et al* 1997). Colicin E1 also requires lower levels of TolA compared to other TolA-dependent colicins. It was therefore concluded that colicin E1 interacts with TolA in a different manner to other

colicins. It has been proposed that colicin E1 is able to locate domain III of TolA more easily than other colicins due to its dependence on TolC, which may be located at adhesion zones, where other Tol proteins are concentrated (Guihard *et al* 1994, Journet *et al* 1999). This is in contrast to the OmpF-dependent colicins, such as E3 and A, which may require a longer TolA molecule to bridge the periplasm, as OmpF is unlikely to be particularly concentrated at adhesion zones (Schendel *et al* 1997). This hypothesis is supported by evidence suggesting that TolA II interacts with outer membrane proteins such as the OmpF, PhoE and LamB porins, indicating that the TolA–OmpF interaction could be lost if domain II of TolA is deleted (Derouiche *et al* 1996).

TolA-TolB interaction

A strong interaction between TolB and domain III of TolA has been shown by yeast two hybrid studies and cross-linking experiments (Wallburger *et al* 2002). A suppressor mutation in the N-terminal region of TolB (D120N) was able to restore the wild-type phenotype to a *tolA* mutant, indicating that the N-terminal domain of TolB interacts with the C-terminal domain of TolA (Dubuisson *et al* 2002).

Although the N-terminal domain of TolB was sufficient to allow interaction with TolA in the yeast-two hybrid work, the C-terminal domain strengthened the interaction, possibly by stabilising the structure of the N-terminal domain (Wallburger *et al* 2002). It is therefore possible that TolB links the outer and inner membranes by simultaneously interacting with Pal and TolA.

The physiological relevance of the TolB-TolA interaction was shown by using cells with a Y345H V409E mutation in TolA, which had been shown to abolish the interaction, without affecting the interaction between TolA and colicin A (Wallburger *et al* 2002). The mutant cells presented a *tol* phenotype, with an envelope integrity defect and an increased tolerance to colicins A and E3. The mutation had no effect on sensitivity to colicin E1, which does not require TolB for translocation. This indicates that the TolB-TolA interaction is important for uptake of TolB-dependent colicins and for the maintenance of cell-envelope integrity.

TolA-Pal interaction

In vivo cross-linking and immunoprecipitation experiments have shown that the C-terminal domain III of TolA forms a complex with Pal (Cascales *et al* 2000).

Analysis of these experiments with respect to cell energisation, shows that the TolQ and TolR activate TolA in a proton motive force (pmf) dependent manner, which drives the C-terminal domain of TolA to interact with Pal (Cascales *et al* 2001). As Pal is anchored to the outer membrane and TolA to the inner membrane, the TolA-Pal interaction forms a link between the inner and outer membranes.

The region of Pal involved in binding to TolA has been localised to the 30 C-terminal residues of Pal *ie* residues 123-152 and an SYGK motif has been suggested to represent a "TolA" box in Pal (Cascales and Lloubes 2004). Similar motifs have been shown to be present in the regions of colicins A and N, involved in the colicin-TolA interaction and therefore it has been speculated that these colicins could displace the TolA-Pal interaction, disrupting membrane integrity and hence allowing colicins to move across the membrane (Cascales and Lloubes 2004).

It is not yet known whether TolA can interact with Pal and TolB at the same time.

TolA box

The region of colicin A involved in binding to TolA has been shown to lie between residues 32-107 (Bouveret *et al* 1998). The sequence of this region with other colicins only shows similarity to one other colicin, colicin K (Pilsl and Braun 1995c). Residues 50-84 of colicin A and 62-97 of colicin K have been proposed as TolA boxes (Bouveret *et al* 1998). However, colicin A with residues 85-172 deleted, does not bind to TolA (Bouveret *et al* 1998). Further mutagenesis studies have narrowed the region of colicin A involved in binding to TolA to residues 52-97 (Journet *et al* 2001).

Mutation of residues between H57 and I64 to alanine was shown by ITC and fluorescence binding assays to reduce binding of colicin N to TolA, indicating that the colicin N TolA box lies within this region (Raggett *et al* 1998). The Y62A mutation completely abolished binding of colicin N to TolA, indicating that this tyrosine residue is essential for binding. Residues W44 and W46 of colicin N were shown by an enhancement in tryptophan fluorescence to be completely buried on binding to TolA, indicating that these residues are also involved in binding to TolA, such that a region of at least 20 residues is involved in the binding of colicin N to TolA (Raggett *et al* 1998). Further mutagenesis studies have mapped the full TolA box of colicin N to residues 44-66 (Gokce *et al* 2000). All residues between D54 and F66, except S58 and S61 were found to be required for the TolA interaction. Residues 40-90 fused to

GST showed full binding to TolA in an SPR experiment, whereas residues 48-90 fused to GST did not bind to TolA, indicating that residues upstream of N40 are not required for TolA binding but that residues between N40 and N48 are involved in TolA binding (Gokce *et al* 2000). No region in other colicins has been found that is similar to the colicin N TolA box, again indicating that different group A colicins may bind to TolA in a variety of ways.

1.9.1.5. TolR

TolR is a 142-residue inner-membrane protein, which can be divided into three domains. Domain I, consisting of the N-terminal 43 residues, spans the inner membrane once (Muller *et al* 1993). Domain II is located in the periplasm and appears to be involved in homo-dimerisation (Journet *et al* 1999). The C-terminal domain III, extending from residues 117-142, has been proposed to form an amphipathic α-helix, which could interact with proteins, *eg* TolQ, TolA, in the cytoplasmic membrane (Lazzaroni *et al* 1995, Journet *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 important for TolR function (Journet *et al* 1999). Overproduction of TolRII in the bacterial periplasm, induces tolerance to colicin A, indicating that this domain is involved in interacting with colicin A (Journet *et al* 1999). *In vivo* and *in vitro* cross-linking studies have now confirmed that it is domain II of TolR that interacts with colicins A and E3 (Journet *et al* 2001).

The interaction between TolR and colicin A has only been detected using formaldehyde cross-linking. No interaction could be detected using a yeast two-hybrid screen or spectrofluorometric analysis, suggesting that the interaction between TolR and colicin A is weak and transient and may be dependent on other factors *in vivo* (Journet *et al* 2001).

TolRII cannot interact with TolA alone but the N-terminal domain of colicin A cross-linked to TolRII can interact with TolA at the same time to form a TolRII-colicin A-TolA ternary complex (Journet *et al* 2001).

TolR box

Deletion mutants in the colicin A N-terminal domain were used to localise the region of colicin A that is involved in binding to TolR (Journet *et al* 2001). Colicin A with

residues 2-9 deleted could not be cross-linked to TolR, whereas colicin A proteins with residues 2-6 or residues 15-30 deleted were able to interact with TolR. This indicates that residues 7-14 are involved in the interaction with TolR (Journet *et al* 2001). The sequence in this putative TolR box region is not well conserved with other colicins, except for the presence of several glycine residues. The region overlaps with the TolB box of colicin A (residues 11-15), consistent with the fact that all colicins requiring TolB for translocation, also require TolR. This indicates that the interaction between colicins and TolB and TolR is unlikely to be simultaneous, although the colicin-TolB and colicin-TolR interactions are probably closely linked (Journet *et al* 2001).

1.9.1.6. TolQ

TolQ consists of 230 residues and is an integral cytoplasmic membrane protein, predicted to span the membrane three times (Kampfenkel and Braun 1993). The N-terminus and a small loop are located in the periplasm and the C-terminus and a large loop, following the first transmembrane region, are situated in the cytoplasm (Vianney *et al* 1994). Mutational analysis has indicated that the transmembrane regions and parts of the cytoplasmic loop are important for TolQ activity (Kampfenkel and Braun 1993, Vianney *et al* 1994). The sequences of the predicted transmembrane regions are also highly conserved, again indicating that these regions may be important in the function of TolQ.

1.9.1.7. TolQ-TolR-TolA interactions

Biochemical and genetic studies have shown that TolR, TolQ and TolA interact together in the cytoplasmic membrane. However it is as yet unknown if these interactions are simultaneous as no trimeric TolQ-TolR-TolA complex has ever been detected (Journet *et al* 1999).

TolR-TolQ

A mutation in the third transmembrane region of TolQ induced tolerance to colicin A but not colicin E1 (Vianney *et al* 1994). TolQ is required for uptake of both of these colicins, but TolR is only required for uptake for colicin A, indicating that this mutation was affecting the interaction between TolQ and TolR. This was confirmed by the discovery of suppressor mutations in TolR that restored colicin sensitivity to

the *tolQ* mutant (Lazzaroni *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. This was confirmed with cross-linking experiments (Journet *et al* 1999). The fifth suppressor mutant was located at the C-terminus of TolR in the region predicted to consist of an amphiphilic helix. It has been proposed that this C-terminal helix of TolR interacts with TolQ and TolA in the cytoplasmic membrane (Lazzaroni *et al* 1995, Journet *et al* 1999).

A complex between a TolR dimer and TolQ has been detected, indicating that at least one molecule of the TolR dimer can interact with TolQ (Journet *et al* 1999).

TolR-TolA

Cross-linking experiments have shown that the N-terminal transmembrane domain of TolR interacts with the N-terminal transmembrane domain of TolA (Derouiche *et al* 1995). No complex between a TolR dimer and TolA could be detected in cross-linking studies (Journet *et al* 1999).

As mentioned above, it has been proposed that the C-terminal domain of TolR also plays a role in the TolR-TolA interaction, consistent with the observation that TolR domain III partially co-fractionates with membranes (Journet *et al* 1999).

TolQ-TolA

Mutation of the N-terminal region of TolA, resulted in disruption in the interaction with TolQ or TolR (Germon *et al* 1998). Suppressor mutations, which restored the activity of *tolA* mutants were found to be located in the first transmembrane region of TolQ, indicating that the TolA N-terminal domain interacts with the first TolQ transmembrane region (Germon *et al* 1998).

1.9.1.8. Ybgc and YbgF

The roles of the other two genes in the *tol-pal* operon is less clear. *ybgc* encodes a cytoplasmic protein with an unknown function (Vianney *et al* 1996). Bacteria with an insertion in the *ybgc* gene remained sensitive to colicins E1 and E3 (Sun and Webster 1987).

ybgf encodes a 29 kDa periplasmic protein but inactivation of this gene does not induce a *tol* phenotype. The protein does not interact with TolB or Pal in cross-

linking studies but has been shown to interact with TolAII-III (Vianney *et al* 1996, Wallburger *et al* 2002). The interaction between YbgF and TolA is predicted to occur through coiled coil regions of TolAII and of YbgF (Wallburger *et al* 2002).

1.9.1.9. Stoichiometry of Tol-Pal proteins

The stoichiometry of the Tol-Pal proteins is not well defined, as the expression levels have not been simultaneously measured in a given strain. It has been estimated, by using an inactive colicin A mutant in competition experiments, that there are 1000 translocation sites per cell (Duché *et al* 1995).

It is estimated from Western blotting that TolA is present at approximately 800 copies per cell and there are 2000-3000 copies of TolR (Levengood 1991, Muller *et al* 1993). This is consistent with radioactive labeling experiments, which suggest that the TolA:TolR ratio is 1:2 (Guihard *et al* 1994). These experiments also suggest that TolQ is about three times as abundant as TolR (Guihard *et al* 1994). Pal has been found to be present in large excess over the TolA protein and it is predicted that there are 10,000 to 30,000 copies of Pal per cell (Cascales *et al* 2000, Lloubès *et al* 2001). This greater amount of Pal protein per cell is consistent with the *tolb/pal* operon being under the control of the P1 promoter and the strong promoter, PB, whereas the *tola/q/r* operon is only under the control of P1.

It is not yet known whether the Tol system is dependent on formation of stable complexes of defined stoichiometry or on more subtle transient interactions between different protein partners.

A model has been proposed in which four TolQ and two TolR proteins interact with one TolA molecule in the inner membrane (Cascales *et al* 2001).

1.9.1.10. Function of the Tol system

Sensitivity of *tol* mutants to dyes and detergents and their tendency to release periplasmic proteins into the medium indicates that the membrane permeability of *tol* mutants is altered, and it has therefore been proposed that the Tol system maintains outer membrane integrity (Lazzaroni *et al* 1989). The *tol* phenotype is very similar to the *lpp* phenotype and as Lpp is covalently associated with peptidoglycan, it has been suggested that the Tol system could link the outer and inner membranes to peptidoglycan (Lloubes *et al* 2001). This is supported by the identification of the

TolA-Pal and TolB-Pal interactions and the interaction of TolB and Pal with OmpA and Lpp (Clavel *et al* 1998, Cascales *et al* 2002).

The interaction of TolA and TolB with porin trimers (Derouiche *et al* 1996, Rigal *et al* 1997, Clavel *et al* 1998, Dover *et al* 2000) suggests that these proteins could be involved in the assembly of porins. This is supported by the reduction in the amount of OmpF and LamB in *tol* mutants (Lazdunski *et al* 1998). Tol proteins could also regulate LPS translocation or assembly as *tol* mutants can have a decreased LPS content and TolA is required for the surface expression of O-antigen and LPS (Lazzaroni *et al* 1999, Gaspar *et al* 2000). The Tol-Pal proteins might help 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 *et al* 1998). The Tol-Pal apparatus assists in the transport of newly synthesized outer membrane components by bringing inner and outer membranes into close proximity via the TolA-Pal or TolA-TolB interactions (Cascales *et al* 2004). Studies showing that TolQ and TolR can transduce energy to TolA, dependent on the proton motive force, suggest that TolQRA could act as an ion potential-driven molecular motor (Cascales *et al* 2000). TolQ and TolR are homologous to the MotA and MotB components of the flagellar motor. An ion pore could be formed by the last two transmembrane helices of TolQ and the TolR transmembrane helix and possibly the C-terminal domain of TolR. By analogy with models of the MotA-MotB motor, the ion conductance would be regulated by changes in relationship between TolQ and TolR (Cascales *et al* 2001). The natural function of the ion potential created could be to export cell envelope components with colicins parasitising the system to be imported across the cell envelope.

1.9.1.11. Current model for Tol-dependent translocation of colicins

Once a colicin has bound to its receptor, the translocation domain may move through the outer membrane via a second outer membrane protein, possibly requiring unfolding of the translocation domain. As TolB associates with Pal, linking it to the outer membrane, it is likely that the colicin translocation domain first interacts with TolB. The interaction between TolB and the colicin, would displace the TolB-Pal interaction and Pal may then interact with the peptidoglycan (Lazdunski *et al* 2000).

The colicin then interacts with TolR and TolA, either separately or at the same time, possibly allowing further unfolding of the colicin polypeptide chain (Journet *et al* 2001, Lazdunski *et al* 2000). It is not yet known how these interactions could allow the C-terminal cytotoxic domain of the colicin to enter the periplasm. It has been suggested that interaction of this domain with phospholipids could induce a slow conformational change into a "molten globule" state, allowing movement of the domain across the outer membrane (Lazdunski *et al* 2000). Recent work with the colicin E9 DNase domain indicates that the structure and stability of the domain is altered on interaction of the domain with phospholipids vesicles, suggesting that a destabilising protein-lipid complex is the first step for translocation of the cytotoxic domain of colicins across the outer membrane (Mosbahi *et al* 2004).

The differing requirements for Tol proteins between colicins, indicates that a single model is insufficient for translocation of all Tol-dependent colicins. The lack of conservation of TolA and TolR boxes in different colicins also points towards several mechanisms of Tol-dependent translocation.

It has been suggested that colicin translocation occurs by Brownian ratcheting, where the movement of the colicin across the periplasm would occur by simple diffusion with interactions of the colicin with Tol proteins, preventing the colicin from moving backwards (Journet *et al* 2001). This would require each interaction between the colicin and a Tol protein to have a higher affinity than the previous interaction. This model cannot account for movement of the colicin from the outer membrane to the periplasm as the K_d for interaction of the colicin with its receptor is of nanomolar affinity, whereas the K_d for interaction with Tol proteins is of micromolar affinity (Bradbeer *et al* 1986, Journet *et al* 2001). A potential discrepancy with the model is that the affinity of the interaction between TolR and colicin A appears weaker than the affinity of the interaction between TolB and colicin A, although the affinity constant of the TolR-colicin A interaction has not yet been determined (Journet *et al* 2001). This is inconsistent with the Brownian ratcheting model if colicin A interacts with TolB first.

1.9.2. The Ton translocation system

The Ton system is composed of proteins ExbB, ExbD and TonB, which form a complex in the inner membrane (Lazdunski *et al* 1998). The *tonB* gene is located at 28 min on the *E.coli* chromosome and the *exbB* and *exbD* genes form an operon at 65 min (Lazdunski *et al* 1998). The physiological role of the Ton system is transduction of energy from the pmf of the cytoplasmic membrane into the periplasm (Larsen *et al* 2001).

1.9.2.1. Arrangement of the Ton system

TonB and ExbD are anchored by their N-terminal end to the cytoplasmic membrane and extend into the periplasm (Postle and Skare 1988, Kampfenkel and Braun 1992). ExbB is an integral cytoplasmic membrane protein with three transmembrane segments (Fischer *et al* 1989). ExbB, ExbD and TonB form a complex in the inner membrane via their transmembrane regions with ExbB at the centre of the complex (Lazdunski *et al* 1998). However, the stoichiometry may not be 1:1:1 as TonB may dimerise, and ExbB is thought to be expressed in greater quantities than ExbD and TonB (Chang *et al* 2001, Fischer *et al* 1989).

1.9.2.2. Similarities of the Ton and Tol systems

ExbB and ExbD are functionally and structurally homologous to TolQ and TolR respectively and it is thought that TolQR can complete the role of ExbBD and vice versa (Eick-Helmerich and Braun 1989, Braun and Hermann 1993). However, TolA and TonB are not interchangeable although when the N-terminal transmembrane region of TolA was exchanged with the same region of TonB, the mutated protein exhibited TonB activity, consistent with the sequence similarity observed in this region (Braun and Hermann 1993).

1.9.2.3. TonB box

The N-terminal domain of TonB interacts with outer membrane receptors (Brewer *et al* 1990).

TonB dependent receptors contain a conserved sequence at their N-terminus, termed the TonB box and consisting of five residues (D/ETXXV, Postle 1999). Disulphide and formaldehyde cross-linking has shown that the TonB boxes of BtuB, FepA and FhuA are in close proximity to TonB (Skare *et al* 1993, Moeck *et al* 1997, Cadieux

and Kadner 1999). The crystal structure of BtuB shows that the TonB box is located in the plug domain and that the TonB box shifts in position, on binding of vitamin B_{12} (Chimento *et al* 2003). This supports the hypothesis that the interaction of TonB with the TonB box is involved in the removal of the ligand from the binding site of the receptor (Cadieux *et al* 2000).

The TonB box has been identified in a number of different colicins, suggesting that Ton-dependent colicins could interact with TonB in a similar way to the Ton-dependent receptors (Wiener *et al* 1997). Mutagenesis studies have shown that colicin M requires the TonB box of FhuA as well as its own TonB box for uptake (Schöffler and Braun 1989). Therefore, the receptor TonB box-TonB interaction may induce the conformational change required for the colicin to enter the periplasm and then the colicin may interact with TonB independently.

1.9.2.4. Model for Ton-dependent translocation of colicins

Two models have been proposed to explain how TonB energy transduction can cause translocation.

The propeller model (Chang *et al* 2001) proposes that TonB remains associated with the cytoplasmic membrane with a rotation of its carboxy terminus, initiated by ExbB, ExbD and the pmf. When this propeller of TonB binds to the TonB-dependent receptor, the rotary motion causes the receptor to release its ligand into the periplasm. This model was based on the observation that the carboxy-terminal domain of TonB is a rigid, strand-exchanged dimer, likened to a propeller, but there is currently very little data to support the model (Postle and Kadner 2003).

The shuttle model (Letain and Postle 1997, see Figure 1.13) proposes that TonB starts in a complex with ExbB and ExbD in an unenergised state. ExbB and ExbD then use the cytoplasmic membrane pmf to convert TonB into an energised state. The C-terminal domain of TonB would then contact the outer membrane, whilst remaining in contact with the cytoplasmic membrane. This then causes the N-terminal domain of TonB to be released from ExbB and ExbD so it could interact with a Ton-dependent receptor in the outer membrane. This interaction would cause release of conformationally stored potential energy from TonB and release of the ligand from the receptor into the periplasm. Current evidence, including the localisation of TonB at both the outer and cytoplasmic membranes and in soluble intermediate forms, supports this shuttling model (Postle and Kadner 2003).

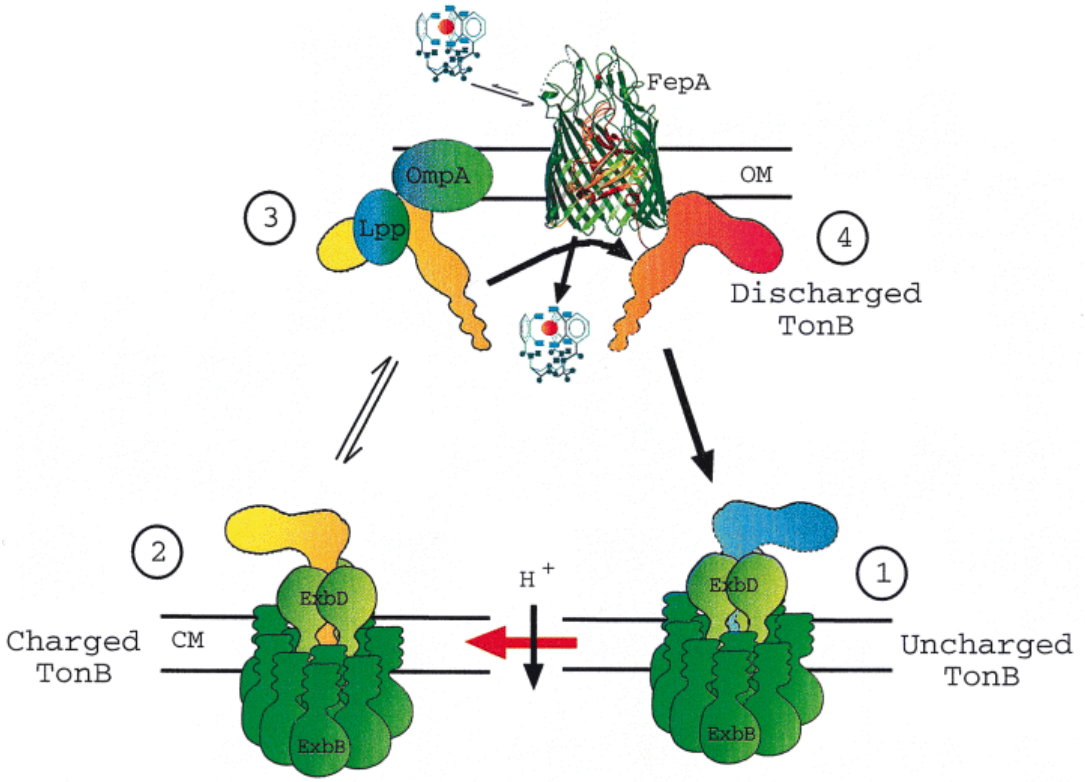


Figure 1.13 The shuttle model for translocation of a ligand across the periplasm via TonB-dependent energy transduction (from Postle and Kadner 2003). 1. TonB is associated with ExbD and ExbB at the cytoplasmic membrane. 2. ExbB, ExbD use the cyptoplasmic membrane pmf to energise TonB. 3. TonB associates with the outer membrane, initially with non-transporter proteins such as Lpp. The binding of ligand to FepA induces a conformational change allowing the receptor to interact with TonB causing a release of conformationally stored potential energy and release of ligand into the periplasm. 4. The energetically uncharged TonB then shuttles back to the cytoplasmic membrane.

It is as yet unknown whether colicins could use a similar mode of translocation to the natural ligands of Ton-dependent receptors. A model for the translocation of colicin Ia has been proposed, based on the crystal structure of this colicin (Wiener *et al* 1997). In this model, the TonB box in the translocation domain of the colicin competes with the TonB box of Cir, the Ton-dependent receptor for colicin Ia, for binding to TonB. The displacement of TonB from the receptor, allows colicin Ia to move across the outer membrane, into the periplasm (see Figure 1.14).

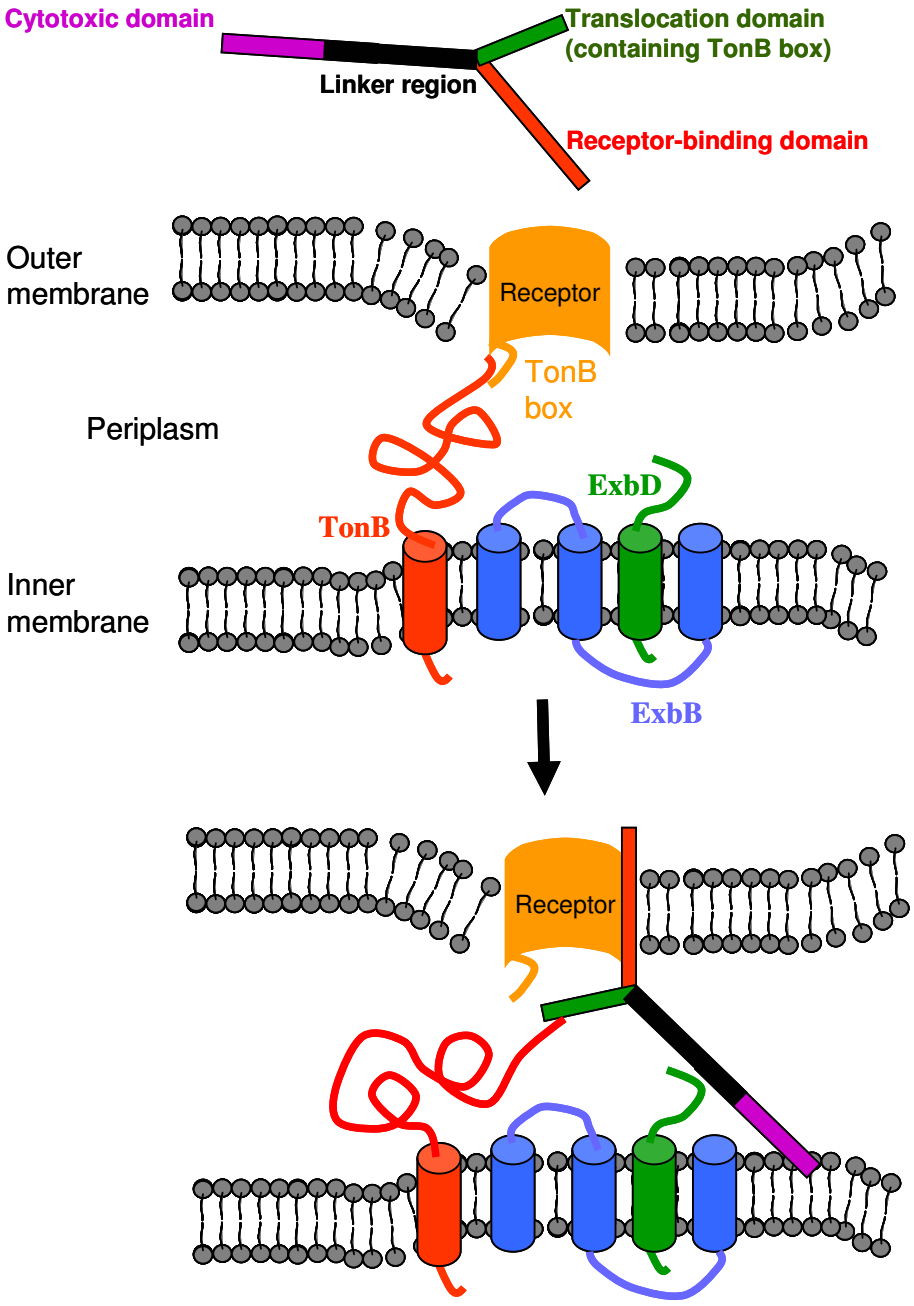


Figure 1.14 **Proposed mechanism for translocation of Ton-dependent colicins (adapted from Wiener *et al* 1997). On binding of the receptor-binding domain of the colicin to the Ton-dependent receptor, the TonB box of the colicin competes with the TonB box of the receptor for binding to TonB. This would then allow the cytotoxic portion of the colicin to enter the periplasm and move towards the cytoplasmic membrane.**

1.9.3. Translocation of endonuclease colicins across the cytoplasmic membrane

As the DNA and RNA targets for endonuclease colicins are located in the cytoplasm, endonuclease colicins must not only traverse the outer membrane and periplasm, but must also translocate across the cytoplasmic membrane.

1.9.3.1. Proteolytic processing

Only the cytotoxic domain of the colicin needs to enter the cytoplasm and it has therefore been suggested that this domain is cleaved from the rest of the colicin (de Zamaroczy *et al* 2001). The peptidase, LepB, has been proposed to perform this cleavage for colicin D, as incubation of colicin D with cell extracts containing overexpressed LepB, results in the production of a 10 kDa fragment corresponding to the RNase domain (de Zamaroczy *et al* 2001). This cleavage is prevented by the presence of ImD. However, purified LepB is not sufficient to cleave the colicin, suggesting that other proteins are involved. LepB had no effect on other endonuclease colicins, indicating that this effect is specific for colicin D (de Zamaroczy *et al* 2001).

The production of a fragment of colicin E3, corresponding to the translocation and receptor binding domains has also been detected, indicating that colicin E3 is also susceptible to proteolytic cleavage, but the smaller fragment, corresponding to the RNase domain, has not yet been identified (de Zamaroczy and Buckingham 2002). This production of the fragment was not dependent on LepB indicating that another peptidase is responsible for the cleavage. The proposed cleavage site is located at the C-terminus of the receptor-binding domain of colicin E3 (residues 424-446) and this region is conserved in all nuclease colicins but not in pore-forming colicins (de Zamaroczy and Buckingham 2002).

Processing of the nuclease domain of colicin E7 has also been reported (Liao *et al* 2001). Target cells, endogenously expressing his-tagged Im7 and treated with a colicin E7-Im7 complex were found to contain a polypeptide, slightly larger than the DNase domain of colicin E7, which was able to bind Im7. It has been proposed that the difference in size is because the putative cleavage site is upstream of the "minimal" DNase domain (de Zamaroczy and Buckingham 2002). However, the processing step took eight hours *in vitro* and one and a half hours *in vivo,* and required

a much higher concentration of colicin than the concentration needed for killing of sensitive cells (Liao *et al* 2001). Therefore the biological significance of these results remains uncertain.

1.9.3.2. Formation of channels

The cytotoxic domain of colicin E9 shows channel-forming activity in planar lipid bilayers (Mosbahi *et al* 2002). This channel-forming activity is abolished by the presence of Im9. The channels formed are different to those formed by pore-forming colicins as they are not voltage gated and have much shorter lifetimes (Mosbahi *et al* 2002). Unlike pore-forming colicins, the formation of channels in the inner membrane by the DNase colicins does not directly cause cell death, as a colicin E9 DNase active site mutant (H575A) is not cytotoxic despite being able to form channels (Mosbahi *et al* 2002). The DNase domains of colicins E2, E7 and E8 also showed channel-forming activity, but the channels varied in size and gating behaviour (Mosbahi *et al* 2002). Therefore, the formation of channels appears to be the mechanism by which DNase colicins traverse the inner membrane.
No channel-forming activity could be detected for RNase colicins, indicating that their mechanism of translocation across the inner membrane may differ to that of the DNase colicins (Mosbahi *et al* 2002).

1.10. Cytotoxicity

1.10.1. Pore-formers

The cytotoxicity of pore-forming colicins, results from the formation of ion-permeable channels formed in the inner membrane of the susceptible cell, by the C-terminal pore-forming domain of the colicin (Dankert *et al* 1982). Several models have been proposed to explain the formation of the channel, but none are readily accepted (Lakey *et al* 1993, van der Goot 1991, Zakharov and Cramer 2002).
The initial interaction of the cytotoxic domain with the membrane is predicted to be electrostatic, as pore-forming colicins require an acidic pH for binding to artifical membrane vesicles (Davidson *et al* 1995, Zakharov and Cramer 2002). A group of positive charges on the surface of the domain is predicted to orient the domain on the membrane surface (Elkins *et al* 1997). Once the cytotoxic domain has bound to the membrane, it then unfolds, possibly via a molten globule intermediate, allowing

extension of the helices and insertion into the membrane (van der Goot *et al* 1991, Cramer *et al* 1995, Zakharov and Cramer 2002). The requirement for partial unfolding of the pore-forming domain, as it moves 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 (Schendel and Cramer 1995).

1.10.1.1. Structures of pore-forming domains

The structures of the pore-forming domains of colicins A (Parker *et al* 1989), E1 (Elkins *et al* 1997), N (Vetter *et al* 1998), B (Hilsenbeck *et al* 2004) and Ia (Wiener *et al* 1997) show that they consist of a bundle of eight amphipathic helices burying two hydrophobic helices. The average length of the helices is 13 residues, but approximately 17-20 residues are required to span the membrane bilayer (Elkins *et al* 1997, Zakharov and Cramer 2002). However, the α-helical content of colicin E1 increases by ~25 % on pore formation, which may cause a sufficient extension to allow the helices to span the membrane (Elkins *et al* 1997, Zakharov *et al* 1998). The two longest helices (the hydrophobic helices, 8 and 9) form a hydrophobic helical hairpin at the core of the domain, which is predicted to provide a membrane anchor after the initial interaction of the protein with the membrane bilayer (Zakharov and Cramer 2002).

1.10.1.2. Nature of the channel

The cytotoxic domain of the pore-forming colicin remains tightly bound to the membrane once it has inserted into the membrane (K_d ~ 2-3 nM, Heymann *et al* 1996). From the permeability of the channel formed by colicin E1 to various probes, the diameter of the channel formed by colicin E1 has been estimated to be 9 Å at the narrowest part (Bullock *et al* 1992). However, if the elongated shape of these probes is taken into account, the channel could be as small as 4-5 Å (Cramer *et al* 1995). The size of the channel formed varies according to the colicin.

The channel activity of colicin A has been measured by analysing the kinetics of potassium efflux induced by the colicin (Bourdineaud *et al* 1990). The channels formed *in vivo* were shown to be voltage and pH dependent. After addition of colicin, the membrane potential drops to -85mV and the channel closes if the potential increases above this value (Bourdineaud *et al* 1990). The channel will re-open again if the cell regains polarisation (Bourdineaud *et al* 1990). The gating voltage, *ie* the

voltage at which 50 % of channels are activated, varies for different colicins (Bénédetti and Géli 1996). The gating voltage for full-length colicins is pH dependent but the gating voltage for the pore-forming domain is not pH dependent. This indicates that regions other than the pore-forming domain regulate channel behaviour (Bénédetti and Géli 1996).

The channel allows movement of monovalent ions (Na^+, K^+ and Cl^-) with a single channel conductance of > 10^6 ions $channel^{-1}$ $second^{-1}$, which is sufficient to allow a single colicin pore to depolarise the membrane (Bénédetti and Géli 1996, Bullock *et al* 1983). The depolarisation causes inhibition of active transport and depletion of intracellular ATP, potassium and phosphate, leading to cell death (Elkins *et al* 1997).

1.10.2. Endonucleases

1.10.2.1. DNases

The DNase colicins E2, E7, E8 and E9 cleave the host chromosomal DNA. They share a high sequence identity in their receptor binding and translocation domains but are only ~70-80 % identical in their DNase domains. X-ray crystallography and activity assays have localized the DNase domain of colicin E9 to residues 449-582 (Kleanthous *et al* 1999, Pommer *et al* 1998).

Structures of DNase domains

Structures of the DNase domains of colicins E7 and E9 with their respective immunity proteins have recently been determined (Ko et al 1999, Kleanthous et al 1999). The crystal structure for the E9 DNase-Im9 complex at 2.05Å resolution shows the DNase domain consists of a central core of β-sheet surrounded by α-helices (Kleanthous *et al* 1999). The C-terminal 32 amino acid residues show sequence identity to the HNH family of homing endonucleases (Shub *et al* 1994). The HNH motif resembles a distorted zinc finger and forms the core of the DNase active site (Pommer *et al* 1999). This is supported by site-directed mutagenesis experiments, which were used to identify putative active site residues in the DNase domain of colicin E9 (Garinot-Schneider *et al* 1996). Three single site mutations were identified which completely destroyed the toxic action of the colicin, R544A, E548A and H575A. All three residues are highly conserved amongst the DNase colicins suggesting that these residues are involved either in maintaining the overall fold or in

the mechanism of the enzyme (Kleanthous *et al* 1999). A nickel ion is bound in the crystal structure, and NMR has confirmed that this ion is coordinated by three histidine side chains and a phosphate molecule (Hannan *et al* 2000).

The crystal structure of the colicin E7-Im7 complex at 2.3 Å resolution shows the DNase domain is a novel αβ protein with a Zn^{2+} ion bound to 3 histidine residues and a water molecule and contains a zinc finger motif (Ko *et al* 1999). The three catalytically important residues identified by site-directed mutagenesis of colicin E9 are conserved in colicin E7 and are located near the Zn^{2+} ion.

Colicin E9 has been shown to share sequence homology with CAD enzymes *ie* DNases involved in degradation of chromatin during the terminal stages of apoptosis (Walker *et al* 2002). The similarity is supported by mutagenesis experiments. H263 in mouse CAD, equivalent to H103 in the colicin E9 DNase domain, is predicted to activate the hydrolytic water molecule. H308 of mouse CAD, equivalent to H127 in the colicin E9 DNase, is suggested to be the Mg^{2+} binding residue. The CAD enzymological properties are analogous to those of the colicin E9 DNase and therefore it is speculated that colicin DNases and CADs cleave DNA by the same mechanism, which is an interesting possibility given that both enzymes are involved in the degradation of chromosomal DNA, leading to cell death (Walker *et al* 2002).

Specificity of colicin E9 DNase

The colicin E9 DNase has been shown to preferentially nick double-stranded DNA at thymine bases, producing 3'-hydroxy and 5'-phosphate termini and it is suggested that a DNase monomer binds to the phosphate backbone of each strand (Pommer *et al* 2001). The DNase does not cleave mononucleotide phosphoryl esters or dinucleotide substrates. The DNase also cleaves single-stranded DNA, but the presence of a transition metal is required (Pommer *et al* 2001).

The metal ion

The role of the metal ion bound to the DNase domain is still debateable and it has been suggested that it may play a catalytic or structural role.

The metal-bound water molecule may serve as a nucleophilic hydroxide ion that attacks the phosphate atom (Ko *et al* 1999). A cleft near the three catalytically important residues, formed by a central β-sheet and the α-helices layered next to it, is the proposed binding site for the DNA substrate (Ko *et al* 1999). Work with the

DNase domain of colicin E7 has shown that although zinc ions are not required for binding of the domain to DNA, the metal ion is required for hydrolysis of DNA, further suggesting that the metal ion is involved in DNA hydrolysis (Ku *et al* 2002). Ordinarily the metal ion in the zinc finger motif would be tetrahedrally coordinated with four protein ligands. However, in the DNase domain of colicins E9 and E7, the fourth ligand is either a phosphate molecule or a water molecule respectively. This could reduce the affinity of the protein for the metal ion, allowing removal of the metal ion at some point during translocation to allow partial unfolding (Pommer *et al* 1999). Therefore, the metal ion could play another role in stabilising the DNase domain in the extracellular environment.

Although zinc or nickel have been identified in the crystal structures of colicin E7 and E9, enzymological characterisation of the colicin E9 DNase domain showed that it can use a number of metal ions to cleave DNA, the identity of which governs whether the enzyme produces single or double strand breaks (Pommer *et al* 1998).

Although zinc can be used for hydrolysis of DNA by colicin E7, zinc does not support hydrolysis of any DNA substrate by colicin E9 (Pommer *et al* 2001). The physiological metal ion for colicin E9 has been shown to be magnesium, which allows the formation of double-strand breaks in the DNA (Walker *et al* 2002).

1.10.2.2. RNases

The cytotoxic domains of the RNase colicins display a greater sequence conservation (~80-90%) than that for DNase colicins.

Colicin E3, E4, E5 and E6 kill cells by inactivating the protein biosynthetic machinery (Nomura and Witten 1967).

rRNases

Colicin E3 is a 58 kDa ribonuclease that cleaves 16S ribosomal RNA (rRNA) at the 49th phosphodiester bond from the 3' end (Bowman *et al* 1971). This bond is at a critical position near where interactions of the A site tRNA and the mRNA on the ribosome occur (Cate *et al* 1999). The crystal structure of the cytotoxic domain of colicin E3 in complex with its immunity protein has been solved to 2.4Å resolution revealing a highly twisted central antiparallel β-sheet elaborated with a short N-terminal helix (Carr *et al* 2000b, Soelaiman *et al* 2001). This fold is significantly different to that of other ribonucleases. Sequence and structural analysis combined

with molecular modelling and mutagenesis has revealed a putative active site within the toxin containing a His-Glu catalytic pair (Soelaiman *et al* 2001, Walker *et al* 2004).

Docking of the crystal structure of the RNase domain of colicin E3 onto the crystal structure of the 30S ribosomal subunit, has lead to the proposal of a catalytic mechanism with residues E517, H513 and D510 making up the catalytic triad (Zarivach *et al* 2002). Residue R545 is likely to stabilise the negatively charged penta-coordinated cyclic phosphate atom transition state. Alanine mutagenesis confirmed the importance of residue H513 and E517 as the acid-base pair in catalysis (Walker *et al* 2004).

A comparison of the sequences of the C-terminal domains of colicins E4 and E6 with colicin E3 shows that colicins E4 and E6 are E3 homologs and also cleave the 16S rRNA. However, the C-terminal region of E5 exhibits no sequence similarity to colicin E3.

tRNases

Examination of colicin E5 activity *in vitro* and *in vivo* demonstrated that the target of colicin E5 is not ribosomes, as in the case of colicin E3, but tRNA for tyrosine, histidine, asparagine and aspartate. These tRNAs all contain the guanine analog, queuine, at the wobble position of each anticodon, although the sequence with an unmodified guanine instead of queuine is also sensitive to the colicin (Ogawa *et al* 1999, Masaki and Ogawa 2002). Colicin E5 hydrolyses these tRNAs on the 3' side of this nucleotide. Colicin D has also been shown to act as a tRNase, cleaving arginine tRNAs (Masaki and Ogawa 2002). The tRNase activity of colicin D has been localised to the C-terminal 91 residues (de Zamaroczy *et al* 2001).

1.10.3. Inhibition of peptidoglycan biosynthesis

Colicin M is unique amongst colicins, as it causes lysis of cells. It has been shown to inhibit synthesis of peptidoglycan and LPS by inhibiting the bactoprenyl-P carrier lipid, interfering with the recycling of bactoprenyl phosphate from bactoprenyl pyrophosphate (Harkness and Braun 1989).

1.11. Project Aims

The broad aim of this research project is to investigate the mechanism by which the cytotoxic domain of colicins is delivered to its cellular target. Colicins range between 30 and 90 kDa in size and in order to reach their cytotoxic target, they must traverse the formidable barriers of the outer membrane and periplasm, and, in the case of endonuclease colicins, the inner membrane.

In order to achieve this unique event in prokaryotic biology, colicins have parasitised multiprotein systems used by cells to perform important biological functions such as porins (*eg* OmpF, OmpA), siderophore receptors (*eg* FhuA, FepA), the vitamin B12 receptor, BtuB, and the Ton and Tol systems. Therefore, colicins provide an excellent model to study the transport of polypeptides into cells.

A full understanding of this process requires knowledge of binding of the colicin to its outer-membrane receptor and interactions between the colicin and proteins of the Ton or Tol systems, in the periplasm, involved in translocation.

This thesis concentrates on characterisation of the interactions between Group A colicins and proteins of the Tol system. To achieve this, the techniques of surface plasmon resonance and atomic force microscopy have been employed.

The best characterised of the colicin-Tol protein interactions is the interaction between endonuclease colicins and TolB and the initial part of this study concentrates on this interaction.

The study is then extended to investigate differences between pore-forming and endonuclease colicins in their interactions with three Tol proteins, TolB, TolR and TolA.

CHAPTER 2 – Materials and Methods

2.1. Bacterial Strains and Media

Bacterial cultures were grown in LB broth or on LB agar plates. Selection of recombinant clones was on media supplemented with ampicillin or kanamycin (100 μg ml $^{-1}$ final concentration). Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1mM to induce expression of T7 RNA polymerase in λ (DE3) lysogenic cultures. Strains used are listed in Table 2.1.

Bacterial strains were stored in 20 % glycerol at -20 ºC.

Table 2.1 List of strains used throughout this thesis, with their relevant genotypes and source.

E.COLI STRAIN	RELEVANT GENOTYPES	SOURCE
DH5α	LacZDM15 recA1	Invitrogen
ER2566	LacZ::T7 λ (DE3)	New England Biolabs
JM83	Δ(lac-proAB), Φ90dlacZΔM15	
BL21 (DE3)	araB::T7	Invitrogen

2.2. Plasmids and Plasmid Isolation

pET21a, pET21d and pET30c (Studier and Moffat 1986; Novagen, see Figure 2.1 and Figure 2.2) were used for cloning and expression of target genes. Expression was under the control of strong bacteriophage T7 transcription and translation signals. Reverse PCR primers were designed to remove the stop codon of the target gene during cloning to allow incorporation of a six histidine (His_6) tag at the C-terminus of the encoded protein. This tag enables rapid purification of the protein using nickel affinity chromatography (Garinot-Schneider *et al* 1996).

Colicin E9 mutants were produced using pNP69 (Christopher Penfold) as a template. pNP69 is a pET21a-based plasmid, based on pCS4 (Garinot-Schneider *et al* 1997), expressing colicin E9 and its immunity protein mutated to contain the following restriction sites in the colicin E9 gene: *EcoRV* 490-95, *SacI* 988-93, *Nco*I 342-347. The colicin E9 and immunity protein genes were cloned into the vector using *Nde*I and *Xho*I restriction sites. The sequence of pNP69 is shown in Figure 2.3.

Plasmid extractions were completed using the Wizard SV Miniprep kit (Promega, WI) as directed by the manufacturer's instructions.

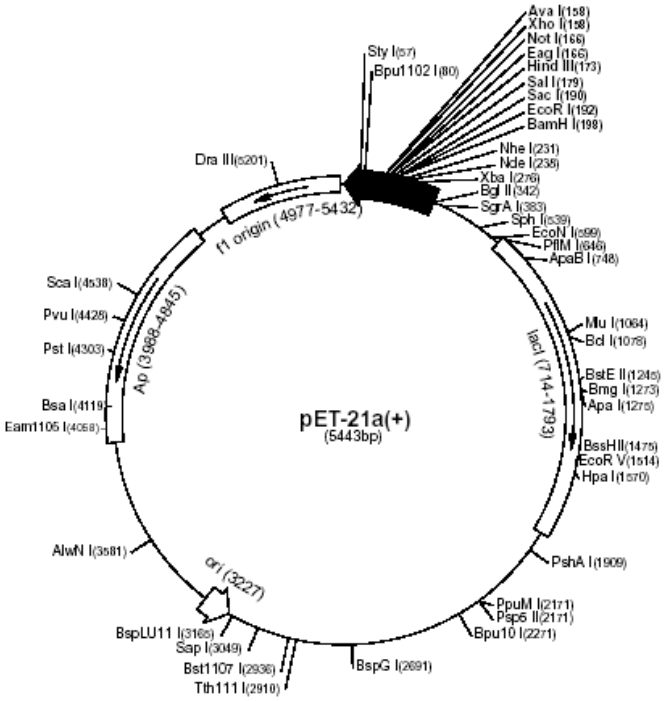


Figure 2.1 The Vector Map of pET21a (Novagen). pET21d differs slightly from pET21a. pET21d is 5440 bp as 2 bp have been removed after the *Bam*HI site (position 198). Additionally, the *Nde*I site of pET21a is replaced with an *Nco*I site in pET21d resulting in a net 1 bp loss at position 238.

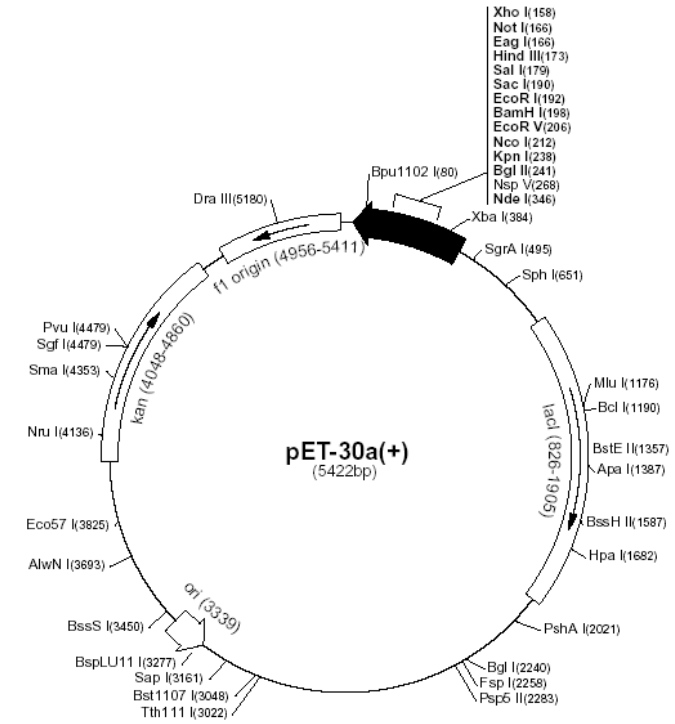


Figure 2.2 The Vector Map of pET30a (Novagen). pET30c differs from pET30a as 1 bp has been added after the *Bam*HI site (position 198). This removes the C-terminal his-tag.

```
M  S  G  G  D  G  R  G  H  N  T  G  A  H  S  T  S  G  N  I
atgagcggtggggatggacgcggccataacacgggcgcgcatagcacaagtggtaacatt

N  G  G  P  T  G  I  G  V  S  G  G  A  S  D  G  S  G  W  S
aatggtggcccgaccgggattggtgtaagtggtggtgcttctgatggttcaggatggagt

S  E  N  N  P  W  G  G  G  S  G  S  G  I  H  W  G  G  G  S
tcggaaaataacccgtggggtggtggttccggtagcggcattcactggggaggtggctcc

G  R  G  N  G  G  G  N  G  N  S  G  G  G  S  G  T  G  G  N
ggtcgtggtaatggcgggggtaatggcaattccggtggtggctcgggaacaggcggtaat

L  S  A  V  A  A  P  V  A  F  G  F  P  A  L  S  T  P  G  A
ttgtcagcagtagctgcgccagtggcatttggttttccggctctttccactccaggagct

G  G  L  A  V  S  I  S  A  S  E  L  S  A  A  I  A  G  I  I
ggcggtctggctgtcagtatttctgcaagcgaattatcggcagctattgctggtattatt

A  K  L  K  K  V  N  L  K  F  T  P  F  G  V  V  L  S  S  L
gctaaattaaaaaaagtaaatcttaaattcactccttttggggttgtcttatcttcatta

I  P  S  E  I  A  K  D  D  P  N  M  M  S  K  I  V  T  S  L
attccgtcggaaatagcgaaagatgaccccaatatgatgtcaaagattgtgacgtcatta

P  A  D  D  I  T  E  S  P  V  S  S  L  P  L  D  K  A  T  V
cccgcagatgatatcactgaatcacctgtcagttcattacctctcgataaggcaacagta
         EcoRV
D  N  V  N  V  R  V  V  D  V  K  D  E  R  Q  N  I  S  V  V
aacgtaaatgttcgtgttgttgatgatgtaaaagacgaacgacagaatatttcggttgtt

S  G  V  P  M  S  V  P  V  V  D  A  K  P  T  E  R  P  G  V
tcaggtgttccgatgagtgttccggtggttgatgcaaaacctaccgaacgtccaggtgtt

F  T  A  S  I  P  G  A  P  V  L  N  I  S  V  N  D  S  T  P
tttacggcatcaattccaggtgcacctgttctgaatatttcagttaatgacagtacgcca

A  V  Q  T  L  S  P  G  V  T  N  N  T  D  K  D  V  R  P  A
gcagtacagacattaagcccaggtgttacaaataatactgataaggatgttcgcccggca

G  F  T  Q  G  G  N  T  R  D  A  V  I  R  F  P  K  D  S  G
ggatttactcagggtggtaataccagggatgcagttattcgattcccgaaggacagcggt

H  N  A  V  Y  V  S  V  S  D  V  L  S  P  D  Q  V  K  Q  R
cataatgccgtatatgtttcagtgagtgatgttcttagtcctgaccaggtaaaacaacgt

Q  D  E  E  N  R  R  Q  Q  E  W  D  A  T  H  P  V  E  A  A
caggatgaagaaaatcgccgtcagcaggaatgggatgctacgcatccggttgaagcggct

E  R  N  Y  E  R  A  R  A  E  L  N  Q  A  N  E  D  V  A  R
gagcgaaattatgaacgcgcgcgtgcagagctcaatcaggcaaatgaagatgttgccaga
                              SacI
N  Q  E  R  Q  A  K  A  V  Q  V  Y  N  S  R  K  S  E  L  D
aatcaggagcgacaggctaaagctgttcaggtttataattcgcgtaaaagcgaacttgat

A  A  N  K  T  L  A  D  A  I  A  E  I  K  Q  F  N  R  F  A
gcagcgaataaaactcttgctgatgcaatagctgaaataaaacaatttaatcgatttgcc

H  D  P  M  A  G  G  H  R  M  W  Q  M  A  G  L  K  A  Q  R
catgacccaatggctggcggtcacagaatgtggcaaatggccgggcttaaagctcagcgg
```

```
A  Q  T  D  V  N  N  K  Q  A  A  F  D  A  A  A  K  E  K  S
gcgcagacggatgtaaataataagcaggctgcatttgatgctgctgcaaaagagaagtca

D  A  D  A  A  L  S  A  A  Q  E  R  R  K  Q  K  E  N  K  E
gatgctgatgctgcattaagtgccgcgcaggagcgccgcaaacagaaggaaaataaagaa

K  D  A  K  D  K  L  P  M  E  S  K  R  N  K  P  G  K  A  T
aaggacgctaaggataaattagccatggagagtaaacggaataagccagggaaggcgaca
                        NcoI
G  K  G  K  P  V  G  D  K  W  L  D  D  A  G  K  D  S  G  A
ggtaaaggtaaaccagttggtgataaatggctggatgatgcaggtaaagattcaggagcg

P  I  P  D  R  I  A  D  K  L  R  D  K  E  F  K  S  F  D  D
ccaattccagatcgcattgctgataagttgcgtgataaagaatttaaaagcttcgacgat

F  R  K  A  V  W  E  E  V  S  K  D  P  E  L  S  K  N  L  N
tttcggaaggctgtatgggaagaggtgtcgaaagatcctgagcttagtaaaaatttaaac

P  S  N  K  S  S  V  S  K  G  Y  S  P  F  T  P  K  N  Q  Q
Ccaagcaataagtctagtgtttcaaaaggttattctccgtttactccaaagaatcaacag

V  G  G  R  K V  Y  E  L  H  H   D  K  P  I  S  Q  G  G  E
Gtcggagggagaaaagtctatgaacttcatcatgacaagccaattagtcaaggtggtgag

V  Y  D  M  D  N  I  R  V  T  T  P  K  R  H  I  D  I  H  R
Gtttatgacatggataatatccgagtgactacacctaagcgacatatcgatattcaccga

G  K  -   M  E  L  K  H  S  I  S  D  Y  T  E  A  E  F  L
Ggtaagtaaaatggaactgaagcatagcattagtgattatacagaagctgaattttta

Q  L  V  T  T  I  C  N  A  D  T  S  S  E  E  E  L  V  K  L
caacttgtaacaacaatttgtaatgcggacacttccagtgaagaagaactggttaaattg

V  T  H  F  E  E  M  T  E  H  P  S  G  S  D  L  I  Y  Y  P
gttacacactttgaggaaatgactgagcaccctagtggtagtgatttaatatattaccca

K  E  G  D  D  D  S  P  S  G  I  V  N  T  V  K  Q  W  R  A
aaagaaggtgatgatgactcaccttcaggtattgtaaacacagtaaaacaatggcgagcc

A  N  G  K  S  G  F  K  Q  G  L  E  H  H  H  H  H  H
gctaacggtaagtcaggatttaaacagggcctcgagcaccaccaccaccaccac
                                  XhoI
```

Figure 2.3 The sequence of colicin E9 and Im9 DNA and protein from pNP69. The nucleotide sequence is shown in black lower case with restriction sites indicated in green. The amino acid translation is shown in blue for colicin E9 and red for Im9. – indicates a stop codon. The TolB box is highlighted in purple.

2.3. Plasmid Restriction, Ligation, Transformation and Electrophoresis

Endonuclease digestion of plasmid DNA, ligation of DNA fragments, transformation of competent *E. coli* cells and agarose gel electrophoresis were completed as detailed by Sambrook *et al* (1989).

2.4. Extraction of DNA from Agarose Gels

Extraction of DNA from agarose gels was completed using the QIAEX II kit (Qiagen) as directed by the manufacturer's instructions.

2.5. Polymerase Chain Reaction (PCR)

PCR was completed using an Eppendorf Mastercycler Personal. PWO polymerase (Roche, Penzberg, Germany) was used in all reactions.

Standard PCR reactions consisted of 30 cycles of 92 ºC for 30 seconds, 55 ºC for 30 seconds, 72 ºC for 30 seconds.

Second stage PCR reactions, used for site-directed mutagenesis, consisted of 30 cycles of 92 ºC for 30 seconds, 62 ºC for 60 seconds, 72 ºC for 60 seconds.

Details of primers used are shown in Table 2.2.

Primer ID	Sequence (5' – 3')	Use
SR1	GTTATTTTCCGCACTCCATCC	Mutates S41 to A in ColE9
SR2	CCACGGGTTAGCTTCCGAACT	Mutates N43 to A in ColE9
SR3	CCACGGGGCATTTTCCGAACT	Mutates N44 to A in ColE9
H52	GCTCGAGGCCCTGTTTAAATCCTG	Inserts an *Xho*I site instead of the stop codon of Im9
T7 promoter	TAATACGACTCACTATAGGG	Cloning & sequencing from pET vectors
T7 terminator	CTAGTTATTGCTCAGCGGTGG	Cloning & sequencing from pET vectors
DW22	ACCATGGAACTGAAGCATAGC	Inserts an *Nco*I site instead of the start codon of Im9
W78	GCTCGAGCCAATCACCATCACGATAAT	Replaces the stop codon of Im3 with an *Xho*I site
Z4	GCATATGAGCGGTGGCGATGGACGCGGC	Introduces an *Nde*I site at the start of ColE3

Table 2.2 List of sequences and uses of primers

2.6. Site-Directed Mutagenesis

SDM was completed in a two-step PCR method (Sarkar and Sommer 1990). A mutagenic primer was designed with a minimum of 6 complementary bases either side of the mutation. This was used with the T_7 promoter primer to complete the first stage PCR reaction using the standard PCR protocol. The first stage PCR product was then purified from an agarose gel (Section 2.4) and used in a second stage PCR reaction with primer H52.

2.7. DNA Sequencing

DNA sequencing reactions were completed using a BigDye Terminator kit (PE Applied Biosystems) in an Eppendorf Mastercycler Personal. The reaction involved 25 cycles of 96 ºC for 10 seconds, 50 ºC for 5 seconds and 60 ºC for four minutes. The DNA was then ethanol precipitated and reactions were run on a Prism 310 Genetic Analyzer (ABI) by sequencing technician, Sue Bainbridge, of the Division of Immunology, University of Nottingham.

2.8. Protein expression

4 ml of an overnight LB broth culture of *E.coli* BL21 cells or ER2566 cells, transformed with the relevant plasmid, in LB broth, was used to inoculate 400 ml LB broth. When an OD_{600nm} of 0.6 was reached, the cells were induced by addition of IPTG to 1 mM. After two hours of induction the cells were harvested by centrifugation at 8000 g for 10 minutes in a Beckman Avanti J-20 centrifuge.

2.9. Colicin Production Stab Test

An LB agar plate supplemented with 100 μg ml^{-1} ampicillin was inoculated with *E.coli* BL21 cells, transformed with the test and control plasmids. The cultures were incubated at 37 ºC for 16 hours. The cells were lysed by exposure of the plate to chloroform for ten minutes. 100 μl of an overnight *E.coli* JM83 culture transformed with pET21a was added to 5 ml 0.7 % w/v molten non-nutrient agar. The agar was then poured over the plate and allowed to set. The plate was incubated overnight at 37 ºC. Cells producing active colicin produce a clear zone, where growth of the JM83 culture is inhibited.

2.10. Colicin Production Spot Test

5ml of 0.7% w/v molten non-nutrient agar was inoculated with 100 µl *E.coli* JM83 (pET21a) and was poured onto an LB agar plate supplemented with 100 µg ml^{-1} ampicillin. The purified colicin proteins were serially diluted in potassium phosphate buffer and 2 µl of the diluted proteins were spotted onto the agar and allowed to dry. The plate was incubated for 16 hours at 37 ºC. The minimum concentration in a 10-fold dilution series that produced a clear zone in the sensitive lawn, termed the titre, was determined.

2.11. Luminescence Reporter Assay

This assay makes use of an SOS-inducible chromosomal *lux* operon to detect DNA damage induced by colicin E9 in reporter cells (Mireille Vankemmelbeke unpublished results, Davidov *et al* 2000). All assays were performed in a microtitre plate luminometer (Lucy 1, Anthos Labtech, Salzburg, Austria) at 37 ºC. The luminometer, plates and media were pre-warmed to 37 ºC, to prevent induction of a stress response due to cooling. An overnight culture of *E.coli* DPD1718, containing a fusion of the *E.coli recA* promoter region to the *Photorhabdus luminescens luxCDABE* reporter integrated into the *lacZ* locus of *E.coli* DPD1692, was diluted 1:50 and grown for three hours at 37 ºC in the presence of 30 µg ml^{-1} chloramphenicol, until reaching an OD of 0.3-0.4. The cells were then diluted 1:2 in 100 µl total volume in black 96-well optical bottom microtitre plates (Nunc, www.nuncbrand.com) and 4 µl of the wild-type or mutant colicin was added. Induction of luminescence was followed over a period of 90 minutes, with readings taken every 600 seconds. Cell density was also monitored by measuring OD at 492 nm.

The ratio of relative luminescence units (RLU) over OD_{492nm} values were used to calculate gamma values at a time point of 50 minutes, using Equation 2-1.

$$\gamma = \frac{L_{sample} - L_{control}}{L_{control}}$$

Equation 2-1 Equation to calculate gamma values, where L = RLU/OD_{492nm}

2.12. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was completed using a mini gel system (Atto). Stacking (6 %) and resolving (12 or 16 %) gels were prepared as described in Sambrook *et al* 1989. Protein samples were loaded onto a gel after 1 minute denaturation at 100 ^{o}C in an equal volume of 2 x loading buffer (100 mM Tris-HCl, pH 6.8, 4 % (w/v) SDS, 20 % (w/v) glycerol and 0.2 % (w/v) bromophenol blue dye). Protein molecular weight standards (New England Biolabs) were loaded alongside protein samples to enable estimation of molecular weights.

Gels were stained using Coomassie blue stain (0.2 % bromophenol blue dye, 45 % methanol, 10 % acetic acid) and destained in 40 % methanol, 10 % acetic acid.

2.13. Silver Staining of SDS-PAGE gels

Silver staining of SDS-PAGE gels was used when enhanced sensitivity of staining was required (Morrissey 1981). The gel was washed twice in 50 % methanol, 10 % acetic acid for 15 minutes and once in 10 % ethanol, 5 % glacial acetic acid for six minutes. The gel was then rinsed in distilled water and washed twice in distilled water for nine minutes. Sensitisation of the gel was achieved by agitation of the gel in 500 ml 20mg l^{-1} $Na_2S_2O_4$ in distilled water for nine minutes. This solution was then poured off and the gel was incubated on a shaker in 0.1 % $AgNO_3$ in distilled water with 150 µl 37 % (v/v) formaldehyde for nine minutes. Excess $AgNO_3$ was rinsed away with distilled water and 200 ml image developer (200µl 37 % formaldehyde (v/v) in 200 ml 3 % sodium carbonate) mixed with 200 µl 10 g/l sodium thiosulphate was added to the gel. The gel was incubated in this solution until the desired staining intensity was achieved. The developer solution was then poured off and 80 ml stop solution (50 g Tris, 25 ml glacial acetic acid in 1 l distilled water) was added.

2.14. Cell Lysis and Protein Purification

2.14.1. Colicin E9 and colicin E3 wild-type and mutant proteins in complex with immunity proteins

Cells were lysed by treatment with 5 ml Bugbuster (Novagen) at room temperature for 30 minutes. The cell lysate was then centrifuged at 20,000 g for 10 minutes at 4

ºC in a Sigma 3k30 centrifuge. The supernatant was filtered using a 0.2 µM sterile syringe filter (Millipore).

Proteins were purified using HiTrap chelating HP columns (Amersham Biosciences) connected to a BiologicLP HPLC machine (BioRad). Columns were equilibrated with 1 x PBS + 0.5 M NaCl, pH 7.4, charged with 50 mM $NiSO_4$ and washed with 1 x PBS + 0.5 M NaCl, pH 7.4. The filtered supernatant was then applied to the column and the column was then washed again with 1 x PBS + 0.5 M NaCl, pH 7.4 and pure protein was eluted using an increasing gradient of imidazole (1- 100% 1 M imidazole in 1 x PBS + 0.5 M NaCl, pH 7.4). Proteins were dialysed in 5 litres potassium phosphate buffer (50 mM K_2HPO_4, 50 mM KH_2PO_4, pH 7) at 4 ºC for a minimum of 16 hours.

2.14.2. Free colicin E9 and free colicin E3 (without immunity proteins)

Cells were lysed by treatment with 5 ml Bugbuster (Novagen) at room temperature for 30 minutes. The cell lysate was then centrifuged at 20,000 g for 10 minutes at 4 ºC in a Sigma 3k30 centrifuge. The supernatant was filtered using a 0.2 µM syringe filter (Millipore) and applied to a Hi-Trap column, equilibrated with 20 mM Tris-HCl pH 7.9, 5 mM imidazole, 0.5 M NaCl and charged with Nickel. After application of the supernatant the column was washed with the Tris buffer. Free colicin was then eluted using Tris-HCl pH 7.9, 5 mM imidazole, 0.5 M NaCl, 6 M Guanidine-HCl, with the polyhistidine tagged immunity protein remaining attached to the column. The column was then washed again with the Tris buffer and the immunity protein was eluted using a gradient of imidazole (0-100 % 1 M imidazole in 20 mM Tris-HCl pH 7.9, 5 mM imidazole, 0.5 M NaCl).

Proteins were dialysed in 5 litres potassium phosphate buffer (50 mM K_2HPO_4, 50 mM KH_2PO_4, pH 7.4) for 16 hours at 4 ºC. The buffer was then replaced with fresh potassium phosphate buffer and the proteins were dialysed for a further 8 hours.

2.14.3. TolA proteins and Im9

Cell pellets were resuspended in 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). The cells were lysed using sonication for 40 minutes (Soniprep 150 MSE, 20 secs on/off). The sonicated sample was centrifuged at 15000 rpm for 30

minutes in a Beckman Avanti J-20 centrifuge. Centrifugation was repeated with the supernatant produced. The supernatant was filtered using a 0.2 μM sterile syringe filter (Millipore) and applied to a Hi-trap column charged with nickel and equilibrated with 1 x PBS + 0.5 M NaCl pH 7.4. Pure protein was eluted using an increasing 1M imidazole (in 1 x PBS + 0.5 M NaCl, pH 7.4) gradient ranging from 0-100 %. Proteins were dialysed in 5 litres potassium phosphate buffer (50 mM K_2HPO_4, 50 mM KH_2PO_4, pH 7.4) at 4 ºC for a minimum of 16 hours.

2.15. Determination of protein concentrations

Protein concentrations were calculated using Beer's Law:

$$Abs_{280} = \varepsilon \times \text{path length} \times \text{concentration}$$

Equation 2-2 Beer's law

Where:

$$\varepsilon = \frac{[\text{number of tyrosine residues} \times 1210) + [\text{number of tryptophan residues} \times 5500]}{\text{molecular mass of protein}}$$

Equation 2-3 Equation used to calculate molar extinction coefficients (ε)

Table 2.3 Table listing the molecular masses and molar extinction coefficients of purified proteins used in this thesis.

Protein	Molecular mass (Da)	Number of Tyrosine residues	Number of Tryptophan residues	Molar extinction coefficient ($cm^{-1}/mgml^{-1}$)
WT colicin E9/Im9	71150	9	8	**0.77**
Free colicin E9	61587	6	7	**0.74**
T_{61}-DNase colicin E9/Im9 construct	30873	6	6	**1.30**
Free T_{61}-DNase colicin E9 construct	21291	3	5	**1.46**
T & R domains of colicin E9	46520	3	5	**0.67**
WT colicin E3/Im3	67849	13	11	**1.12**
Free colicin E3	57963	9	8	**0.95**
Colicin A (1-172)	17846	5	4	**1.57**
Im9	9582	3	1	**0.95**
Im3	9904	4	3	**2.15**
TolB	45956	13	8	**1.30**
TolA (III)	12181	4	0	**0.40**
TolR (II/III)	10725	2	0	**0.23**

2.16. Kunitz Assay

This assay is used to test for DNase activity (Garinot-Schneider *et al* 1996). The basis for the assay is the hyperchromic effect, whereby double-stranded DNA absorbs less UV light than single-stranded DNA. Colicin E9 cleaves DNA into short pieces, which then degrades to single-stranded DNA and free nucleotides, causing an increase in absorbance at 260 nm.

The assay was performed as described by Garinot-Schneider *et al* 1996. 50 µg calf thymus DNA was suspended in 50 mM Hepes.NaOH [pH 7.5], 20 mM $MgCl_2$ in a total volume of 1 ml. 10 µg of test colicin (with Im9 removed) was added and the hyperchromic effect monitored at Abs_{260nm} for 10 minutes using a Biomate 3 spectrophotometer (Thermospectronic) at 25 ºC.

2.17. DNase assay

This assay is a qualitative test for DNase activity.

pUC18 DNA was linearised with *Nde*I. 700-800 ng of colicin E9 (with Im9 removed) in 15 µl Tris-HCl buffer (50 mM Tris-HCl [pH 8], 10 mM $MgSO_4$) was incubated for 30 minutes at 25 ºC. 125 ng linearised pUC18 was then added to the colicin E9 and this was incubated at 37 ºC for one and a half hours. The DNA was then loaded onto a 1 % agarose gel and electrophoresed against untreated linearised pUC18 as a control. Disappearance of the pUC18 DNA band demonstrates degradation of the DNA by the colicin E9.

2.18. Gel filtration

2.18.1. An introduction to gel filtration

Gel filtration chromatography separates proteins on the basis of size. A gel filtration column consists of two phases: a mobile phase, consisting of buffer carrying the solute, *ie* the proteins and a stationary phase, consisting of porous beads with a well-defined range of pore sizes. Proteins small enough to fit inside all the pores in the beads can be "included" inside the beads as well as having access to the mobile phase between beads and elute last in a gel filtration separation. Proteins that are too large to fit inside any of the pores are "excluded" and, as they only have access to the mobile phase between the beads, they elute first in the "void volume" (Vo). Proteins of intermediate size can fit inside some but not all of the pores in the beads and

therefore these proteins will then elute between the large, "excluded" and small, "included" proteins. The volume at which the intermediate proteins elute is called the elution volume (Ve) and depends on the partition of the molecule between the Vo and the volume inside the beads (Vs), which is proportional to the distribution coefficient (K) *ie* Ve = Vo + KVs.

Both molecular weight and three dimensional shape can contribute to the degree of retention. The technique can be used for purification of proteins, desalting of proteins, determination of molecular weight of a pure protein against standard protein size markers and to detect formation of complexes between proteins.

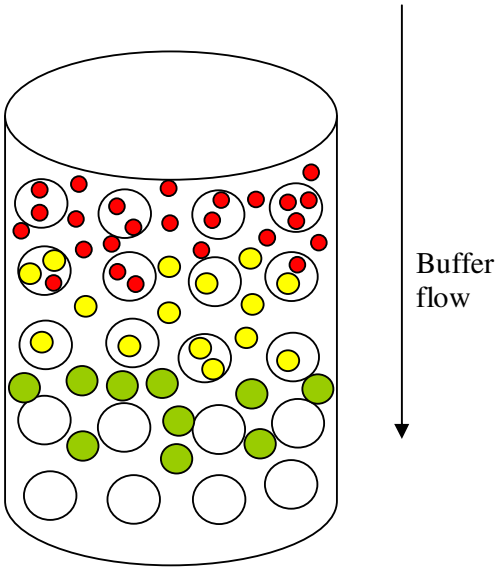


Figure 2.4 Principle of gel filtration chromatography. Red spheres denote "included" particles, yellow spheres denote partially "included" particles and green spheres denote "excluded" particles

(adapted from http://www.olemiss.edu/depts/chemistry/courses/chem472_01/Expt9.pdf, accessed August 2004).

2.18.2. Gel filtration methodology

A Superdex 75 10/300 GL column with an optimum separation range of 3000 – 70000 Da (Amersham Biosciences), attached to an ÄKTA Explorer FPLC system, was equilibrated with two column volumes (24 ml x 2) of 50 mM potassium phosphate buffer, pH 7.4 + 0.1 M NaCl (0.2 µm filtered and degassed with helium) at a flow rate of 0.8 ml min^{-1}.

100 µl sample protein was then loaded onto the column at a flow rate of 0.5 ml min^{-1}. The sample volume was kept as low as possible to enhance resolution. The change in absorbance of the eluate at 280 nm with time was recorded.

2.19. Atomic Force Microscopy

2.19.1. An introduction to AFM

The atomic force microscope (AFM), a member of the scanning probe microscope (SPM) family, was developed by Binnig and coworkers in 1986 (Binnig *et al* 1986). AFM is now an established technique for obtaining high resolution images of the surfaces of biological molecules (Dufrene 2004, Dorn *et al* 1999) and has also be used to measure inter- and intra- molecular forces in several biological systems (Rief *et al* 1997a, Rief *et al* 1997b, Allen *et al* 1997, Ellis *et al* 1999, Willemsen *et al* 2000, Zhang *et al* 2002).

2.19.2. AFM instrumentation

The main features of an AFM are illustrated in Figure 2.5. The first crucial part of the AFM is the sharp, micro-fabricated tip which actually 'feels' the surface. The diameter of the tip, usually around 5-20 nm, is the key determinant of the resolving power of the microscope (Thundat *et al* 1992).

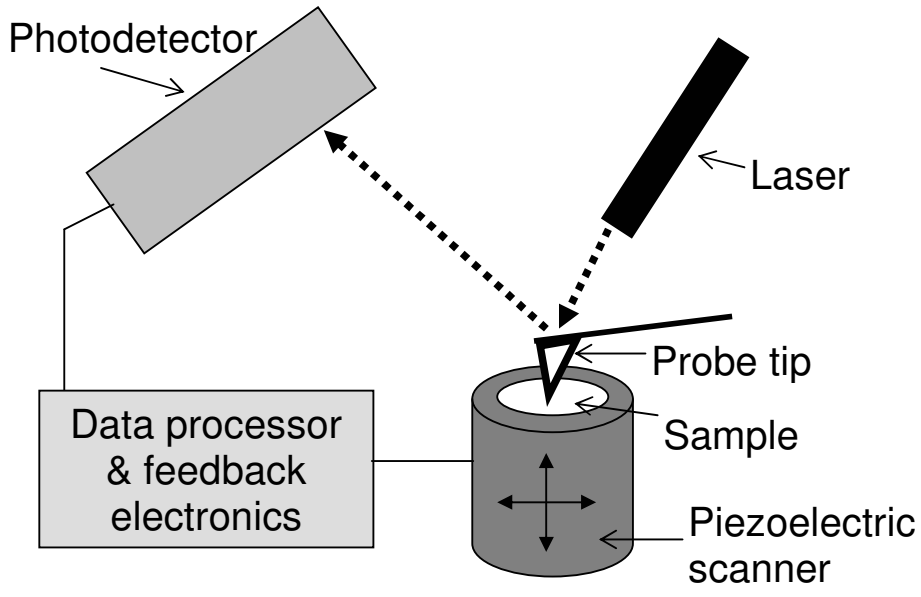


Figure 2.5 **Schematic showing the main features of the atomic force microscope**

Attached to the tip is a cantilevered spring which allows the tip to move up and down as it tracks the surface topography. The tip-cantilever assembly is usually made of silicon and silicon nitride due to its hard, wear-resistant properties and its capacity to be etched during microfabrication. The cantilever has a very low spring constant, enabling the AFM to control the force between the tip and the sample with precision. The deflection of the tip is a measure of the forces between the tip atoms and the specimen atoms (usually in the pN to nN range). Forces sensed by the cantilever are then transduced to generate molecular images.

The second key feature of an AFM is the scanning mechanism, which is based around a piezoelectric transducer. The transducer places the tip in close proximity to the sample surface. In Figure 2.5, the scanner is placed below the sample and controls the motion of the sample on the nanoscale in three orthogonal directions, x, y and z. The scanner can also be placed above the sample so that the piezo controls the motion of the tip rather than the sample. The sample is raster scanned in the x-y direction in order to build up an image of the selected area of the sample, whilst the z channel moves the sample towards and away from the tip.

The final aspect of the instrument is the translation of the deflection force into a detectable signal. This can be achieved in several ways, but the most common method is the optical lever system. A laser beam is focused directly onto the end of the cantilever and reflected onto a position-sensitive segmented photodiode detector. As the cantilever deflects, in response to the sample topography, the angle of the reflected laser beam changes so that the laser spot falling on the photodiode moves, producing changes in intensity in each of its quadrants. The optical lever system amplifies the deflection signal up to 1000 fold so that deflections of less than 1 nm can be measured (Lal and John 1994). Using an appropriate feedback mechanism, the deflection of the cantilever caused by the changes in force between the tip and sample can be kept constant or left to respond freely to the sensed forces.

2.19.3. Imaging using AFM

There are three main imaging modes available and the imaging mode used will often depend on the properties of the sample being investigated.

1. Contact mode

In contact mode, images can be generated in two ways (Morris *et al* 1999). In the constant force mode, the feedback loop changes the height of the sample by adjusting

the voltage applied to the z portion of the xyz piezoelectric scanner. The amount of change corresponds to the height of the sample at each point in the x-y plane. A three-dimensional image can then be generated by combining the information from the three coordinates.

In the alternative constant height mode, the feedback loop is open so that the cantilever undergoes a deflection proportional to the change in the forces between tip and sample and the z component of the xyz piezoelectric scanner is not actively adjusted. The image is then constructed from the deflection information.

2. Tapping mode

In many studies of biological materials, a weak binding between the sample and substrate is desirable to preserve the activity and function of the sample (Möller *et al* 1999). In an attempt to reduce destructive lateral forces that can occur in contact mode and the contact time between the tip and the sample, tapping mode AFM has been developed (Hansma *et al* 1994). In tapping mode AFM a relatively stiff cantilever is forced to oscillate above the sample at, or close to, its resonant frequency, so that it effectively intermittently taps the sample surface as it scans. Changes in the oscillation amplitude or phase, due to the sample topography, are used as a signal for image construction.

The main advantage of tapping mode AFM is that it reduces the tip-sample contact time and the lateral forces resulting from frictional interactions which can often damage and displace soft, weakly immobilised samples. However, the penalty of this decreased tip-sample contact time is the slight reduction in resolution, as the average tip-sample separation is larger in tapping mode than in contact mode.

3. Non-contact mode

In this mode the oscillating cantilever never actually touches the sample surface but remains a few nanometres above it (Morris *et al* 1999). The system monitors the oscillation amplitude of the cantilever, which is affected by the van der Waals attractive forces between the tip and the sample. A feedback circuit moves the scanner up and down to keep the oscillation amplitude constant. The motion of the scanner can then be used to form an image of the sample surface.

As there is no contact with the sample, the forces exerted on it in non-contact mode AFM are extremely low, and therefore this mode is generally used for very soft or elastic samples that are unsuitable for more conventional imaging. The resolution, however, is lower than that for both contact mode and tapping mode AFM.

The AFM has been used to produce high resolution images of biomolecules such as proteins, DNA (Hansma *et al*., 1992) and cells (Hoh and Schoenenberger 1994, Dufrêne 2004). Real time molecular resolution images of biomolecular interactions have also been achieved (Neish *et al* 2002).

2.19.4. Force measurements using AFM

In addition to topographical imaging, the AFM can also record the amount of force felt by the cantilever as the probe tip is brought close to a sample surface and then pulled away. This allows measurement of attractive or repulsive forces between the probe tip and the sample surface, elucidating local chemical and mechanical properties including adhesive and elastic properties, and bond rupture lengths.
AFM force measurements are made by recording the deflection of the free end of the AFM cantilever as the fixed end is extended towards and then retracted away from the sample. Force measurements are generally performed in liquid environments in order to eliminate capillary forces which would otherwise mask the biomolecular interaction of interest (Weisenhorn *et al* 1989).

A typical force curve obtained from these force measurement experiments is shown in Figure 2.6 which plots force against the distance of the cantilever from the surface. The force sensed by the AFM tip can be determined using Hooke's Law (Equation 2-4).

$$F = -kd$$

Equation 2-4 Hooke's Law, where F is force (N), k is the spring constant of the cantilever (N/m) and d is the displacement of the cantilever (nm).

The spring constants of commercially available AFM cantilevers can range from 0.01 – 1.0 Nm^{-1}. However, the estimated manufacturer's spring constants can vary significantly from experimentally derived stiffness (Cleveland *et al* 1993), and it is therefore necessary to calibrate the spring constant of each cantilever. There are several methods available for calibration of the spring constant but in this thesis, a thermal excitation technique was used. This technique involves positioning the probe at a distance away from the sample (where the probe is not affected by long-range forces) so that the motion experienced is purely due to thermal fluctuations. The spring force constant, k, can then be determined from a measurement of the mean-square spring displacement, using Equation 2-5.

$$k = \frac{k_\beta T}{q^2}$$

Equation 2-5 Equation for measurement of the mean square spring displacement where k is the spring force constant, k_β is Boltzmann's constant, T is temperature (K) and q is the displacement of the oscillator (spring) (m) (Hutter and Bechhoefer, 1993).

The sensitivity of the cantilever to forces in the picoNewton range has been exploited to measure forces between and within individual biomolecules. Lee *et al* (1994) were the first to demonstrate the ability of AFM to measure discrete, biologically specific rupture forces between molecular complexes. They investigated the biotin-streptavidin interaction, as it is one of the strongest non-covalent interactions in nature. The rupture force required to break the interaction between biotin on the tip and streptavidin on the surface was measured. The specificity of this interaction was demonstrated by blocking the remaining streptavidin binding sites with free biotin, after which no rupture forces were measured.

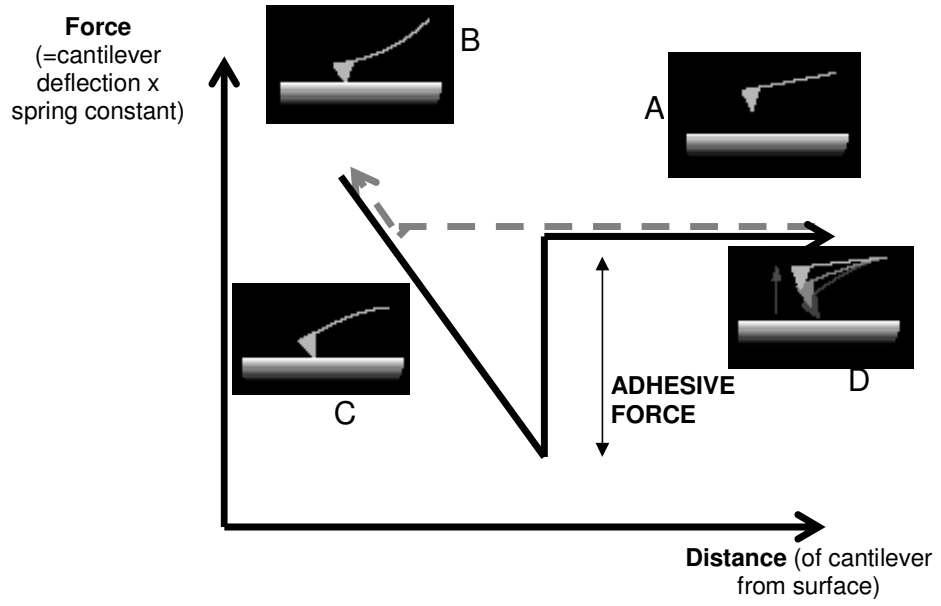


Figure 2.6 A typical force vs distance curve obtained from force measurement experiments. The cantilever starts at a point above the surface (A) and is gradually brought closer to the surface at which point, the tip may jump into contact if it feels sufficient force (predominantly due to van der Waals forces) from the sample. Once the tip is in contact with the surface, cantilever deflection increases (B) as the fixed end of the cantilever is brought closer to the sample due to overlapping of the electron orbitals of the atoms of the tip and sample (Born repulsion). Once the cantilever reaches a predefined deflection level, the cantilever is then withdrawn from the surface but at this point the cantilever may stick to the surface due the adhesive interactions between the tip and the sample (C). The adhesive interactions may be non-specific (capillary or electrostatic) or specific between the molecule on the tip (eg a receptor) and the sample surface (eg a ligand). The cantilever's spring force is eventually enough to overcome the adhesion and the cantilever can then return to its original position (D). The point at which the cantilever comes free from the surface can be used to measure the rupture force required to break the adhesion.

Several investigators reproduced and extended the streptavidin-biotin interaction experiments (Florin *et al* 1994) and this has been followed by the measurement of intra- or inter-molecular rupture forces of other biomolecules with lower affinities, including specific antibody-antigen interactions (Allen *et al* 1997), membrane receptor-ligand pairs (Dammer *et al* 1995) and flexible molecules such as titin (Marszalek *et al* 1999).

2.19.5. Dynamic force spectroscopy

Dynamic force spectroscopy can provide information about the molecular dynamics of protein-protein interactions, allowing estimation of affinity and rate constants as well as the position of energy barriers (Hinterdorfer *et al* 2002).

In 1978, Bell proposed that the application of a mechanical force to a ligand-receptor bond reduces the activation energy and hence accelerates the dissociation of the bond. His model also predicted that the unbinding force should increase with the logarithm of the rate at which an external mechanical force, *ie* the loading rate, is applied towards unbinding of the complex.

Bonds formed between complementary biomolecules have limited lifetimes and will spontaneously dissociate under zero force when the bond acquires sufficient energy (through Brownian excitation) to overcome the intermolecular potential of the dissociation landscape (Evans and Williams 2002). However the retraction of the AFM tip from the sample surface can cause bonds to rupture at a faster rate than the natural lifetime of the bonds (Evans and Williams 2002). When this occurs, the bonds demonstrate dynamic strength and force will be required to separate the complex. Over time the bond strength is reduced by thermal activation. The off rate under applied force (kdiss [f]) for a single bond can be described by Equation 2-6.

$$\text{kdiss}[f] = \text{kdiss} \exp\left\{\frac{f}{f_\beta}\right\}$$

Equation 2-6 Equation to determine the off rate under applied force (kdiss [f]) for an interaction, where kdiss is the natural rate of dissociation for the interaction, f is applied force and f_β is the force scale.

The force scale (f_β) is defined in Equation 2-7.

$$f_\beta = \frac{k_B T}{x_\beta}$$

Equation 2-7 Definition of the force scale (f_β), where k_B is the Boltzmann constant, T is temperature and x_β is the distance to the energy barrier to rupture.

Application of an external force distorts the energy landscape, causing energy barriers to be lowered, displaced inward and narrowed, altering the kinetics (Evans and Williams 2002). This distortion of the energy landscape increases the probability that the barrier will be overcome exponentially, and hence the probability that the bond will survive decreases exponentially. Therefore, in order to overcome a single energy barrier along the unbinding coordinate a linear decrease in applied force is required with a logarithmic decrease in the rate of loading.

$$F^* = f_\beta[\ln(\text{loading rate}) - \ln(f_\beta\, k_{diss})]$$

Equation 2-8 Equation describing the linear dependence of the most probable rupture force (F*) on the ln of the loading rate. f_β is the force scale and kdiss is the rate of dissociation.

From Equation 2-7 and Equation 2-8, a plot of peak rupture force against ln loading rate can be used to determine f_β (*ie* the gradient), the distance to the energy barrier (*ie* $x_\beta = k_B T / f_\beta$) and k_{diss} (x-intercept/gradient). This plot is termed a dynamic force spectrum (DFS).

These theoretical predictions have been confirmed experimentally using the AFM and the complementary approach of the biomembrane force probe (BFP), with an increase in loading rate causing an increase in rupture force between individual complexes of streptavidin/biotin (Merkel *et al* 1999, Yuan *et al* 2000).

Merkel *et al* (1999) showed that the energy landscapes for the streptavidin-biotin and the avidin-biotin interactions comprise multiple energy barriers by plotting rupture force against the log of the loading rate, *ie* dynamic force spectra, and observing that two linear regimes existed in the spectrum for the streptavidin-biotin interaction, suggesting that dissociation of the complex proceeds via an intermediate state (see Figure 2.7). Three regimes exist for the avidin-biotin interaction. DFS analysis has also been used to investigate the energy landscapes of dissociation of several other biological complexes including antibody-antigen interactions (Strigl *et al* 1999) and protein-carbohydrate interactions (Dettmann *et al* 2000).

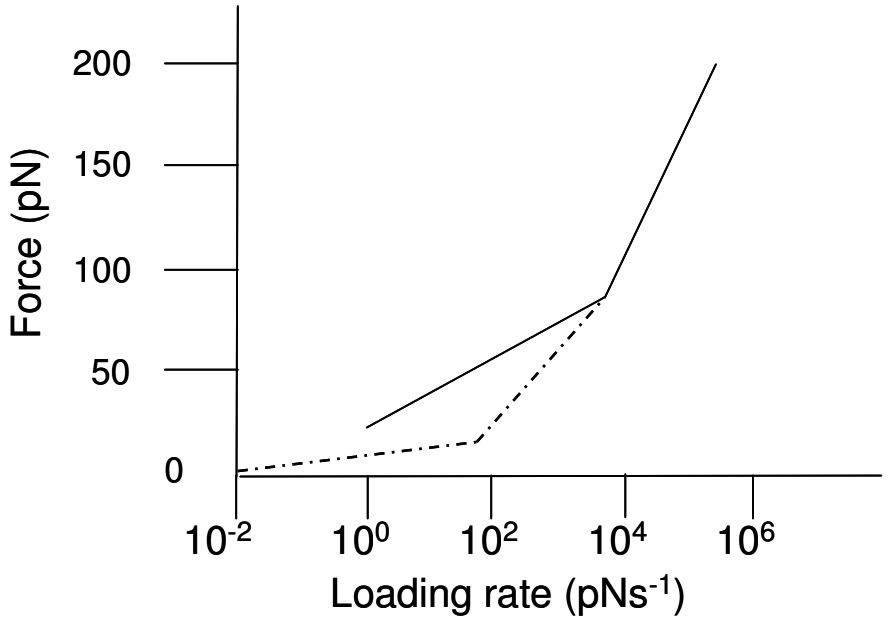


Figure 2.7 Dynamic force spectra for streptavidin-biotin and avidin-biotin. The streptavidin-biotin bond exhibits two distinct regimes mapping the position of two different energy barriers along the dissociation pathway (solid). The avidin-biotin bond exhibits three linear regimes indicating that the dissociation pathway involves three energy barriers (dashed). Adapted from Merkel *et al* (1999).

2.19.6. Sample preparation for AFM

TolB was covalently immobilised onto polished silicon wafers using a method modified from Vinckier *et al* (1995) (as previously described by Allen *et al* 1997). See Figure 2.8 for the reaction pathway. Silicon wafers were immersed in a 30% v/v solution of hydrogen peroxide (Sigma) in sulphuric acid (piranha solution) for 15 minutes at room temperature. The cleaned silicon wafers were rinsed thoroughly with deionised water and dried under a flow of nitrogen. The silicon substrates were then oxidised in 10^{-2} mbar oxygen plasma for one minute at 100 W using a RF Plasma Barrel Etcher PT7100 (BIO-RAD Polaron Division) and then incubated in 4 % v/v 3-aminopropyldimethylethoxysilane (3-APDES, Fluorochem) in toluene for two hours at room temperature, producing NH_2 groups at the substrate surface. The silanised substrates were rinsed in propanol, methanol and distilled water by sonication (Decon FS Minor, Decon Ultrasonics Ltd., UK) for one minute respectively to remove any loosely bound silane. The amino functionalised substrates were then activated by incubation in a 10 % v/v solution of glutaraldehyde (Grade II, 25 % aqueous solution, Sigma) in potassium phosphate buffer (50 mM, pH 7.4) for one hour at 2-8 ºC. The substrates were rinsed thoroughly in deionised water and incubated overnight in a 0.1

mg/ml solution of TolB. The aldehyde groups covalently couple to the side chain ε-amino groups of lysine residues in the protein. Figure 2.9 shows that lysine residues are distributed throughout the TolB protein. Prior to AFM analysis, the samples were rinsed thoroughly with deionised water to remove any non-covalently bound protein. In order to confirm that the modification of surface chemistry was successful and to optimise the concentration of TolB used, images of the silicon surfaces after the above procedure had been performed were obtained using a DI Multimode AFM (Digital Instruments, CA) in tapping mode with oxidised sharpened silicon nitride tips (Digital Instruments).

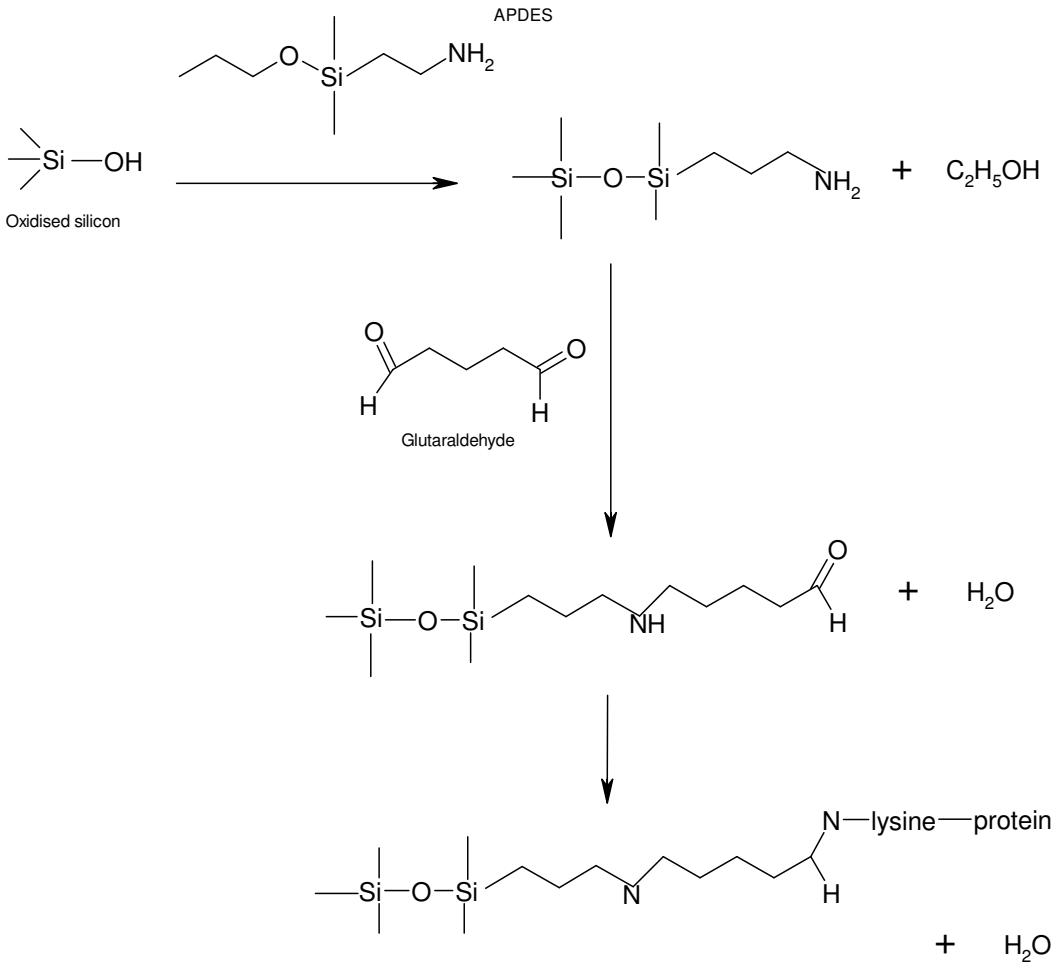


Figure 2.8 **The reaction pathway for covalent immobilisation of TolB to a silanised silicon surface via glutaraldehyde cross-linking**

MKQALRVAFGFLILWASVLHAEVRIVIDSGVDSGRPIGVVPFQWAGPGAAPEDIG
GIVAADLRNSGKFNPLDRARLPQQPGSAQEVQPAAWSALGIDAVVVGQVTPNPD
GSYNVAYQLVDTGGAPGTVLAQNSYKVNKQWLRYAGHTASDEVFEKLTGIKGA
FRTRIAYVVQTNGGQFPYELRVSDYDGYNQFVVHRSPQPLMSPAWSPDGSKLAY
VTFESGRSALVIQTLANGAVRQVASFPRHNGAPAFSPDGSKLAFALSKTGSLNLY
VMDLASGQIRQVTDGRSNNTEPTWFPDSQNLAFTSDQAGRPQVYKVNINGGAPQ
RITWEGSQNQDADVSSDGKFMVMVSSNGGQQHIAKQDLATGGVQVLSSTFLDE
TPSLAPNGTMVIYSSSQGMGSVLNLVSTDGRFKARLPATDGQVKFPAWSPYL

Figure 2.9 **Protein sequence of TolB with lysine residues highlighted in red**

2.19.7. Imaging with AFM

A TolB-functionalised silicon wafer was attached to a magnetic stub using double-sided sticky tape and mounted onto a magnet located on the sample stage of the AFM instrument. Topographic images of the samples were obtained in air using a DI Multimode AFM with a Nanoscope IIIa controller (Digital Instruments) in tapping mode using an "E" type scanner. Unfunctionalised, oxidised sharpened silicon nitride tips (Digital Instruments) with a cantilever length of 125 μm and a resonant frequency of 307-375 kHz were used.

2.19.8. AFM tip functionalisation

In order to achieve single molecular interactions between colicin E9 and TolB, colicin E9 was attached to the tip via the flexible polymer linker, carboxymethylamylose. The presence of a flexible linker is designed to reduce steric hindrance by spacing the colicin E9 protein away from the tip surface, allowing the colicin to freely diffuse around the tip and form the correct orientation for binding to TolB (Hinterdorfer *et al* 2002). In addition the linker provides a means of distinguishing between specific and non-specific intermolecular interactions. When the molecule of interest is attached directly to the tip, it is often difficult to distinguish between intermolecular interactions specific to complex formation and the non-specific interactions between the tip and sample. The addition of the flexible polymer to the tip produces a nonlinear, parabolic-like stretch characteristic in the AFM retract cycle to the point of bond rupture for a specific interaction between the molecule attached to the linker and the sample surface, whereas non-specific interactions are identified by a linear extension of the contact region.

The following method was used to immobilise colicin E9 on the tip via a carboxymethylamylose linker.

V-shaped cantilevers with silicon nitride probes (Veeco, CA) were oxidised in 10^{-2} mbar oxygen plasma at 10 W for 30 seconds using a RF Plasma Barrel Etcher (BIO-RAD Polaron Division). The probes were then incubated in 4 % 3-APDES (Fluorochem) in toluene for two hours at room temperature, producing NH_2 groups on the tip surface. In order to remove any loosely bound silane, the probes were rinsed in propanol, methanol and deionised water for one minute respectively. The amino-functionalised tips were then incubated in 1 ml sodium phosphate buffer solution (20

mM, pH 7) containing 10 mg carboxymethylamylose (Sigma), 10 mg (1-ethyl-3-dimethylamino-propyl) carbodiimide (EDC, Sigma) and 1 mg N-hydroxysuccinimide (NHS, Avocado Research Chemicals Ltd). EDC is a water-soluble derivative of carbodiimide which catalyses the formation of amide bonds between carboxylic acids (on the carboxymethylamylose) and amines (on the tip) by activating carboxyl to form an O-urea derivative (see Figure 2.10). NHS is used to assist the carbodiimide coupling by forming a more stable reactive ester that further reacts with the amine function to yield the amide bond. The tips were then washed in potassium phosphate buffer (50 mM, pH 7.4) and incubated overnight in a 0.1 mg/ml colicin E9 protein solution. The protein binds to the tip via the carboxymethylamylose linker.

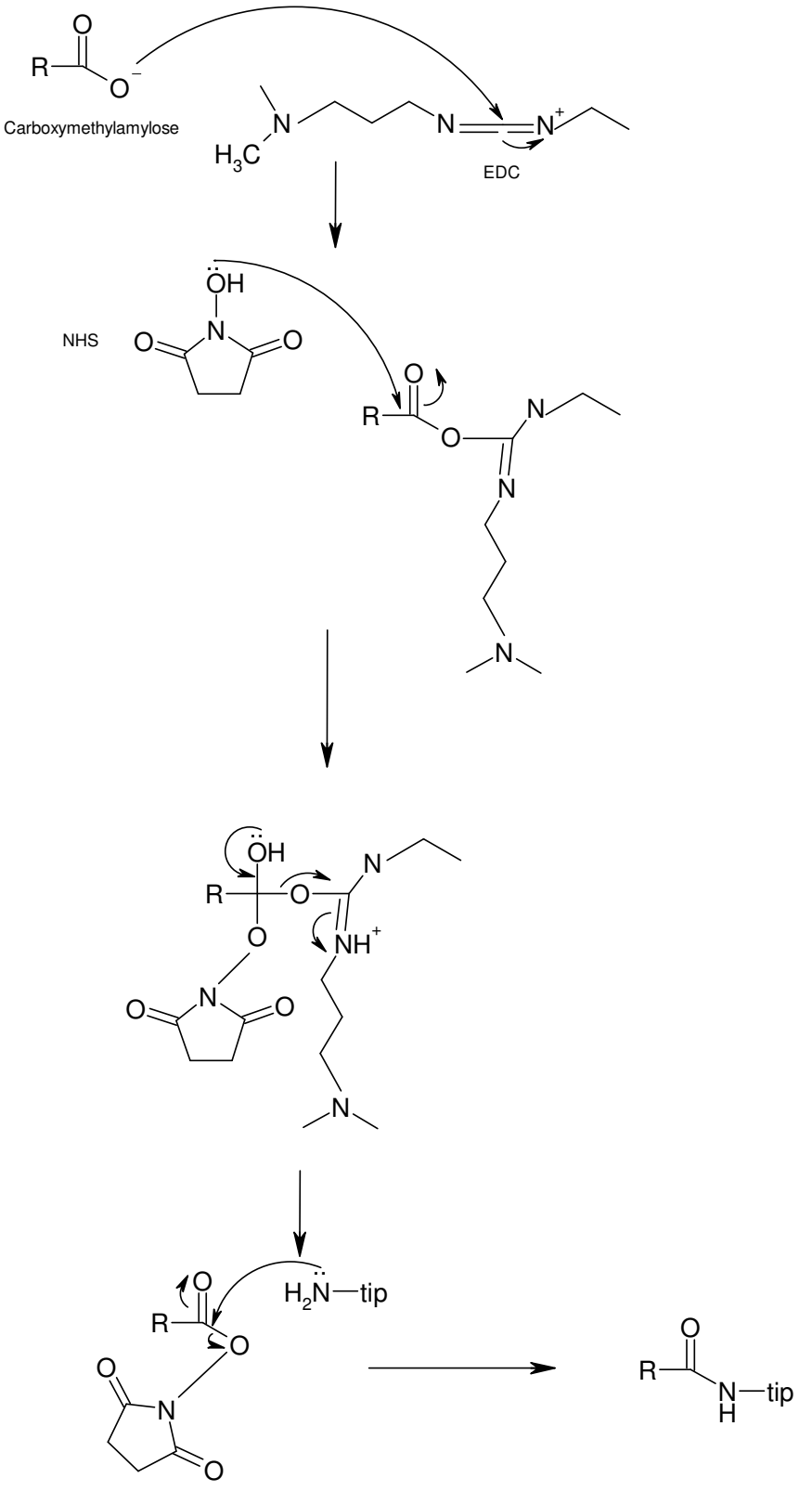


Figure 2.10 Mechanism for EDC/NHS carbodiimide coupling of the carboxymethylamylose polymer linker to an AFM silicon nitride tip

2.19.9. AFM force measurement studies

A TolB-functionalised silicon wafer was attached to a glass microscope slide using double-sided sticky tape. Approach-retract 'force-distance' curves were measured between the colicin-functionalised AFM tip and the TolB-functionalised silicon wafer in potassium phosphate buffer (50 mM, pH 7.4) at tip retraction rates of 10 μm s^{-1}, 1 μm s^{-1}, 0.5 μm s^{-1} and 0.1 μm s^{-1} using a Molecular Force Probe instrument (MFP, Asylum Research, CA).

To confirm the presence of specific TolB-colicin interactions, binding sites on TolB were blocked by incubation in a 1 mg ml^{-1} solution of colicin E9 in 50 mM potassium phosphate buffer, pH 7.4 at room temperature for a period of one hour. Force measurements were then recorded between the colicin functionalised tip and the blocked surface.

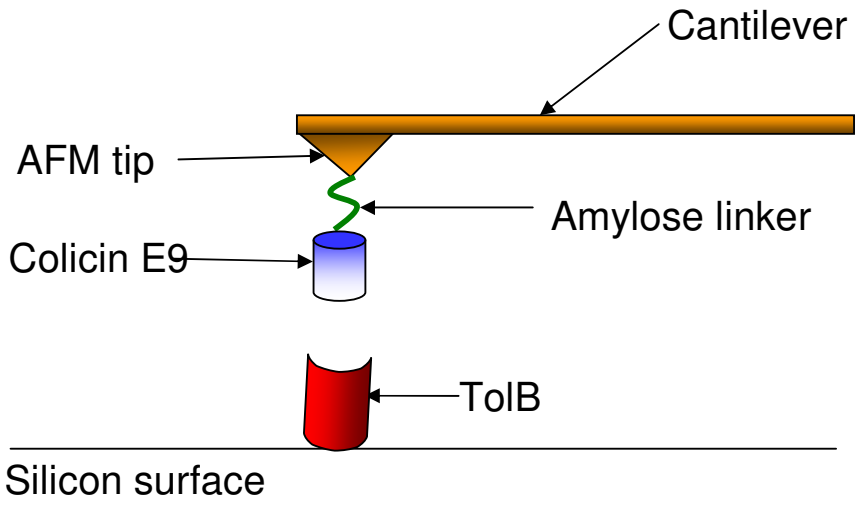


Figure 2.11 Schematic illustrating the experimental set-up for force measurement between colicin E9 and TolB

Raw force data displays the cantilever deflection signal (nA) versus distance of z-piezo movement (nm). Plots were converted to force (nN) versus probe-sample distance (nm) using the cantilever spring constant which was calibrated using the thermal fluctuation method. Rupture forces indicative of specific interactions were plotted as force versus frequency histograms. The mode values from these histograms were then plotted against log of the tip retraction rate.

2.20. Surface Plasmon Resonance

2.20.1. An introduction to the SPR technique

Surface plasmon resonance is a phenomenon which occurs when monochromatic, plane polarised light is reflected off thin metal films. A fraction of the light energy at a specific incident angle above the critical angle can interact with delocalised electrons in the metal film causing a reduction in reflected light intensity at a specific angle. The incident angle at which this happens, called the SPR angle, is dependent on the refractive index of the metal film and its immediate environment.

The phenomenon of SPR was first observed by Turbadar (Turbadar 1959). However, the first commercial SPR biosensor only became available in 1990. Since then, these biosensors have been used in a wide variety of fields.

There are several SPR-based systems, but the most widely used is the BIAcore system (BIAcore AB, Jonsson *et al* 1991). Figure 2.12 shows the set-up of a typical SPR BIAcore instrument. The glass sensor chip is coated in a thin layer of gold and a ligand can be immobilised to this gold surface. A flow channel allows exposure of the surface-bound ligand to analyte molecules and if an interaction occurs between the analyte and the ligand, the change in mass causes a change in the local refractive index of the metal film, affecting the SPR angle and in turn affecting the angle of reflected light with reduced intensity. An optical detection unit measures the angle of reflected light with minimum intensity. SPR response values are measured in resonance units (RUs), where one RU corresponds to a change in 0.0001 ° in the angle of the intensity minimum (www.biacore.com/technology/spr_technology.lasso, accessed June 2004). For most proteins this change corresponds to a change in concentration of about 1 pg/mm^2 on the sensor surface, although the exact conversion factor between RU and surface concentration depends on the properties of the sensor surface and the interacting molecules.

The response in RU is measured as a function of time over the course of the interaction, producing a sensorgram (see Figure 2.13). The maximum response produced at saturation of all the ligand sites due to binding of analyte molecules, is termed RUmax. The RUmax can be determined using Equation 2-9, as the amount of ligand bound is known.

$$\frac{\text{RMM of analyte (kDa)}}{\text{RMM of ligand (kDa)}} = \frac{\text{Amount of analyte bound (RU)}}{\text{Amount of ligand bound (RU)}}$$

Equation 2-9 **Equation describing the relationship between SPR response and relative molecular masses (RMM) of the ligand and analyte. The maximum response expected if all ligand sites are saturated by analyte is termed RUmax and can be calculated from this equation.**

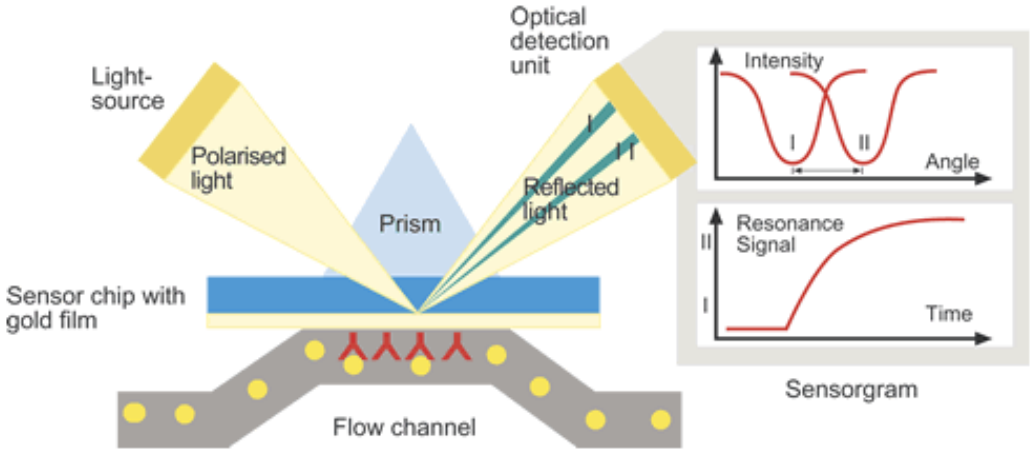


Figure 2.12 **(from www.biacore.com, accessed June 2004). A schematic of the set-up of a typical SPR instrument. The ligand, shown by a red inverted Y is attached to the thin gold layer of the sensor chip. Plane polarised light is directed at the gold film and due to SPR there is a decrease in reflected light intensity at angle 1. When the ligand is exposed to analyte molecules shown in yellow circles, the change in mass at the surface due to the interaction between the ligand and the analyte causes a local refractive index change at the sensor surface, causing a change in the angle reflected light with reduced intensity from angle 1 to angle 2.**

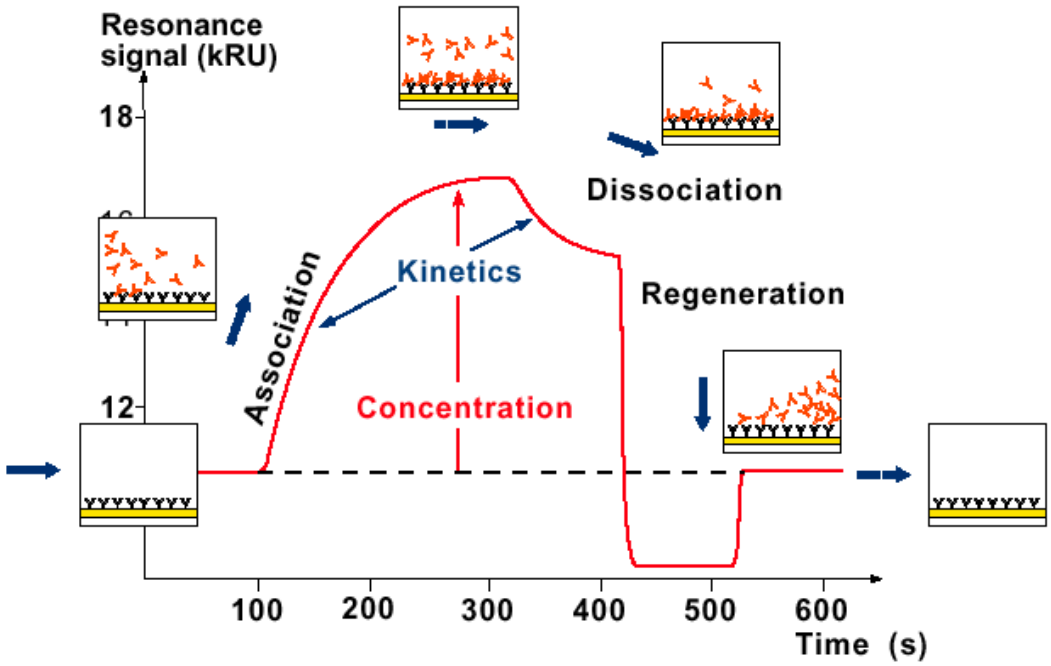


Figure 2.13 (from www.biacore.com, accessed June 2004). A typical SPR sensorgram, which measures response in resonance units (RUs) against time. Starting on the left hand side, analyte molecules are injected across ligand molecules immobilised to a gold-coated sensor chip. Due to association between the analyte and the ligand, an increase in response is seen. The analyte is then replaced with a continuous flow of buffer and a decrease in response is seen as the analyte dissociates from the ligand. Finally the sensor surface is regenerated by removing any remaining analyte molecules at the surface, restoring the response to the original level and the sensor chip can then be re-used. The sensorgram can be used to provide quantitative information on specificity, kinetics and affinity of binding.

2.20.2. Sensor chips

The sensor chips purchased from BIAcore AB, consist of a glass surface, coated with a thin layer (~50 nm) of gold. A range of specialised surfaces are available, allowing the immobilisation of a variety of molecules.

The most widely used sensor surface, consists of the gold surface modified with a carboxymethylated dextran matrix, 100-200 nm thick (Stenberg *et al* 1991, http://users.path.ox.ac.uk/~vdmerwe/internal/spr.pdf, accessed December 2002). This dextran matrix can be derivatised to allow a variety of immobilisation chemistries. The dextran layer provides a hydrophilic environment to preserve biomolecules in a native state. It also increases the volume of the surface increasing the potential level of ligand immobilisation and hence the sensitivity of the instrument.

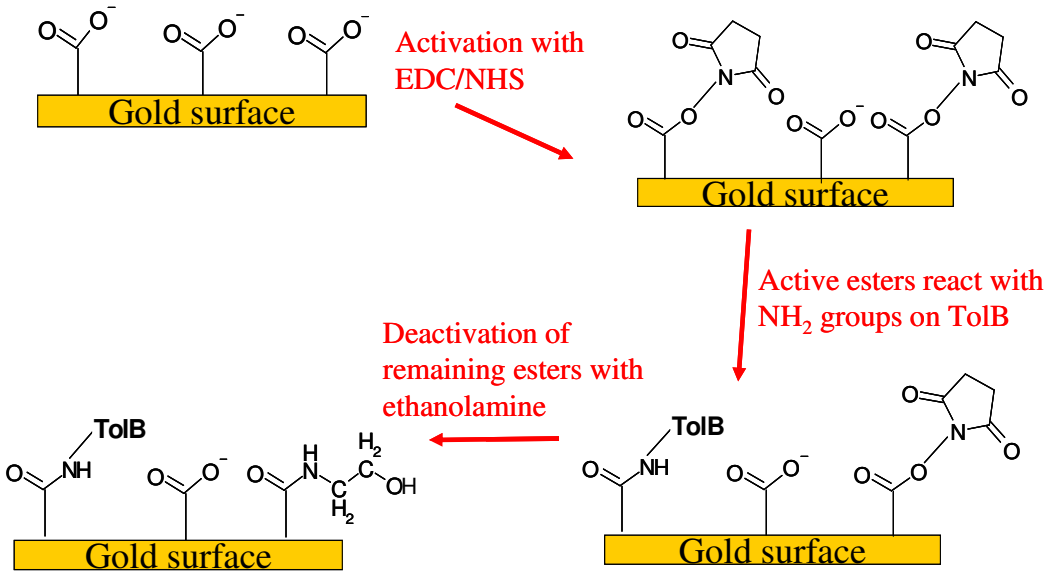


Figure 2.14 **Amine coupling TolB protein to the surface of a CM5 chip (Biacore AB).**

2.20.3. Amine coupling

A widely applied immobilisation procedure of the carboxymethylated dextran matrix activates the carboxyl groups of the matrix using a mixture of EDC and NHS, via the same chemistry shown in Figure 2.10, to form esters that can react with amine groups on proteins, resulting in covalent immobilisation of the protein to the surface (see Figure 2.14). The protein must be diluted in buffer at a pH lower than the pI value of the protein to allow electrostatic attraction of the protein to the negatively charged matrix. Caution must be taken when using this method because the low pH used has the potential to denature the protein and therefore the activity of the protein should be measured after coupling. Another disadvantage of this method is that the protein is randomly orientated on the sensor chip surface due to coupling of different lysine residues to the matrix, which may introduce sub-populations of accessibilities and reactivities (O'Shannessy *et al* 1992)

A variety of other immobilisation methods have been described (O'Shannessy *et al* 1992), for example, coupling by thiol/disulfide exchange [Cunningham and Wells 1993, O'Shannessy *et al* 1992). Other surfaces have also become available from BIAcore AB including surfaces functionalised with NTA, for capture of ligands via metal chelation; streptavidin, for capture of biotinylated ligands; and flat, hydrophobic surfaces to allow deposition of liposomes on the surface.

2.20.4. Microfluidics

Biacore systems are equipped with a microfluidics system that allows analyte to pass over the sensor surface in a continuous, pulse-free and controlled flow (www.biacore.com technology brochure). This allows the concentration at the sensor surface to remain constant.

2.20.5. Reference cell

The microfludics system allows at least two channels to be measured simultaneously. This allows comparison of binding between a derivatised surface and a blank reference surface. Binding to the reference surface can be automatically subtracted from the binding measured on the derivatised surface, so that the response recorded is due to specific binding of the analyte to the sample surface.

2.20.6. Regeneration

Following an analyte injection, remaining analyte bound to the ligand must be removed from the sensor surface, with the ligand remaining intact, before another analyte injection is performed *ie* the surface must be regenerated. The choice of regeneration conditions is dictated by the nature of the ligand-analyte interaction, the stability of the ligand and the type of ligand immobilisation employed. For example for weak interactions, an increased speed of buffer flow may remove remaining analyte. Other conditions that can be used are high or low pH (eg 10 mM glycine pH 2, 50 mM NaOH), high salt (eg 1-2 M NaCl) or detergent (eg SDS). The most frequent method used is injection of 10 mM glycine pH 1.5-2.5. This pH change causes most proteins to partially unfold and become positively charged. The protein binding sites will therefore repel each other, moving the ligand and analyte further apart. Regeneration conditions must be optimised to ensure that the ligand remains intact and fully functional after the regeneration step.

2.20.7. Applications

SPR can be used in a number of ways to characterise biomolecular interactions. SPR can provide quantitative information on affinity, kinetics, specificity and the effect of different conditions *eg* buffers, co-factors, temperature *etc* on complex formation.

2.20.7.1. Affinity

Affinity is generally expressed as either the association constant (Ka), expressed in units of M^{-1} or the dissociation constant (K_d), which is the inverse of K_a and hence has units of M.

For the interaction A + B ↔ AB,

$$Ka = \frac{[AB]}{[A]\,[B]}$$

K_a and K_d can be measured directly by equilibrium binding analysis or calculated from the rate of association (k_{ass}) and rate of dissociation (k_{diss}).

Affinity constants can be measured by using equilibrium binding analysis on the BIAcore instrument. This involves injection of a series of analyte concentrations and measurement of the response at equilibrium. In most cases, the data produced can then be fitted to the Langmuir binding model to derive affinity constants (see Equation 2-10). The model assumes that the analyte is monovalent and homogeneous, that the ligand is homogeneous and that all events are independent.

$$Req = \frac{[A] * Rmax}{[A] + Kd}$$

Equation 2-10 The Langmuir binding isotherm, where Req is the response measured at equilibrium and Rmax is the maximum response at complete saturation of immobilised binding sites

A Scatchard plot of the same data, obtained by plotting Req/[A] against Req, can be used to visualise the extent to which the data fit the model (BIAevaluation Software Handbook). A linear Scatchard plot is consistent with the data fitting the model. However, Scatchard plots alone should not be used to estimate affinity constants because they place inappropriate weighting on the data obtained with the lower concentrations of analyte, which are generally the least reliable (http://users.path.ox.ac.uk/~vdmerwe/internal/spr.pdf, accessed December 2002).

2.20.7.2. Kinetic analysis

The real-time binding data collected using SPR allows the rate constants for an interaction *ie* k_{ass} and k_{diss} to be determined.

SPR measures the kinetics of binding to surface-immobilised ligand under conditions of continuous flow with the concentration of free analyte, **[A],** kept constant by continuous supply of new sample. The concentration of complex on the surface, **[AB],** is given by the response above baseline.

Although the concentration of free ligand on the surface **[B]** is not measured directly, the total ligand concentration can be expressed in resonance units as the maximum analyte binding capacity *ie* Rmax and the concentration of free ligand is then Rmax – [AB].

$$\frac{d[AB]}{dt} = kass[A][B] - kdiss[AB]$$

Equation 2-11 General equation for binding kinetics

After the sample has passed over the surface, analyte that dissociates from the complex is carried away by the buffer flow, so that the free analyte concentration is zero. The rate of dissociation is then described by Equation 2-12.

$$\frac{[AB]}{dt} = - kdiss[AB]$$

Equation 2-12

Therefore, the rates of association and dissociation can be determined from Equation 2-11 and Equation 2-12.

There are important limitations to kinetic analysis using SPR:

1. Mass transport

This effect can occur with high ligand densities where the rate at which the surface binds the analyte exceeds the rate at which the analyte can be delivered to the surface. Therefore the measured association rate (k_{ass}) is slower than the true k_{ass}. Analyte is transported to the surface by diffusion and convection. Increasing the flow rate can increase convection transport and diffusion limitations can be reduced by decreasing the amount of bound ligand and reducing the volume of the sample cell.

2. Rebinding of analyte

If, following dissociation of analyte, the analyte rebinds to unoccupied ligand before diffusing away from the surface, the measured dissociation rate (k_{diss}) will be slower than the true k_{diss}. Rebinding can be reduced by increasing the flow rate, thus increasing convection transport to remove the analyte at a faster rate and by decreasing the level of ligand immobilisation.

These limitations mean that it is difficult to measure k_{ass} values faster than approximately 10^6 M^{-1} s^{-1} and k_{diss} values slower than 10^{-5} s^{-1} or faster than 1 s^{-1} (http://users.path.ox.ac.uk/~vdmerwe/internal/spr.pdf, accessed December 2002).

2.20.8. SPR Methodology

SPR was performed using BIAcore J and BIAcore X instruments from BIAcore AB (Uppsala, Sweden), operating BIAcore J and BIAcore X control software respectively. Operation and maintenance procedures were carried out as detailed in the BIAcore J and BIAcore X instrument handbooks, using BIAcore AB certified products. HBS-EP running buffer (10mM Hepes [pH 7.4], 150 mM NaCl, 3 mM EDTA, 0.005% [v/v] surfactant P20), purchased from BIAcore AB, was used throughout. All buffers and solutions used were filtered and degassed using 0.2 µm sterile syringe filters (Millipore) or Steritop sterile bottle-top filters (Millipore). Solutions filtered by syringe filter were degassed by centrifugation.

2.20.8.1. Immobilisation of proteins on CM5 sensor chips

The ligand was immobilised to the matrix of a newly docked CM5 sensor chip, pre-equilibrated in HBS-EP running buffer (BIAcore AB) via amine coupling. Medium flow rate (~ 35 µl min-1) was used in the BIAcore J instrument and a 10 µl min^{-1} flow rate was used in the BIAcore X instrument throughout ligand immobilisation. The carboxymethylated matrix in both flow cells was activated using a mixture of 0.1 M ethyl-N-(3-diethylaminopropyl) carbodiimide (EDS, BIAcore AB) and 0.4 M N-hydroxysuccinimide (NHS, BIAcore AB). The protein sample was diluted in coupling buffer (BIAcore AB, 10 mM sodium acetate, pH 4.5) and injected in single channel mode across flow cell 2 until the required amount of protein was immobilised on the surface. Remaining active esters in both flow cells were then deactivated using 1M ethanolamine hydrochloride, pH 8.5.

The final level of immobilisation was determined after two washes with HBS-EP buffer. A response of 1000 RU equates to 1 ng of protein attached to the chip (www.biacore.com/technology/spr_technology.lasso).

2.20.8.2. SPR binding analysis

Measurement of binding of proteins to the immobilised protein on the CM5 sensor chip was completed at 25 ºC with HBS-EP running buffer using medium flow rate (BIAcore J) or 30 µl min^{-1} (BIAcore X). Proteins to be used as analytes were diluted in HBS-EP buffer to the required concentration and a two minute injection of the diluted protein was performed. The surface was then regenerated using a two minute injection of 10 mM glycine (pH 1.8). The experiment was repeated twice for each concentration of protein.

2.20.8.3. SPR data analysis

Data from the BIAcore J instrument were analysed using BIAviewer Software (BIAcore AB).

All data used for kinetic analysis were obtained using the BIAcore X instrument. Kinetic analysis for binding of analyte to the immobilised ligand was achieved using BIAevaluation software version 3.1. Global analysis was performed employing the Langmuir 1:1 binding isotherm where very rapid changes in response are accounted for as refractive index changes.

CHAPTER 3 – Effect of mutations in and around the TolB box of colicin E9 on binding to TolB

3.1. Introduction

3.1.1. The TolB box of colicin E9

The interaction of colicin E9 with TolB has been demonstrated using the yeast-two hybrid system (Carr *et al* 2000a). The region of the translocation domain of colicin E9 involved in interacting with TolB was localised to the "TolB box" sequence (DGSGW), from residues 35 to 39 (Carr *et al* 2000a). Mutation of residues D35, S37 and W39 to alanine in this region abolished the cytotoxic activity of the colicin and it was shown, by yeast two hybrid experiments, that this was due to the loss of interaction with TolB (Garinot-Schneider *et al* 1997, Carr *et al* 2000a). Mutation of residues S34 and S40 to alanine also caused a reduction in activity in a stab test but these mutants were not investigated further (Garinot-Schneider *et al* 1997).
An interaction between the N-terminal domain of colicin E3 and TolB has also been demonstrated by cross-linking studies and this interaction is abolished if the N-terminal domain of colicin E3 contains the S37F mutation (Bouveret *et al* 1997).

3.1.2. Structure of the TolB box of colicin E9

3.1.2.1. X-ray structures

The crystal structures of two *tol*-dependent colicins, colicin N and colicin E3, have now been determined. No electron density for the translocation domain (the first 66 residues) is seen in the 3.1 Å structure of colicin N, despite the domain being present in the initial crystal form, indicating that this region is flexible and unstructured. This lack of structure in the translocation domain is consistent with CD and fluorescence data and the glycine/proline/serine/asparagine-rich composition of the domain (Evans *et al* 1996, Raggett *et al* 1998). Residues 84-315 of the translocation domain of colicin E3 adopt a jellyroll structure, with three β-sheets flanked by two α-helices (Soelaiman *et al* 2001). However, no electron density was detected for the first 83 residues, indicating that the N-terminal region of the translocation domain is probably disordered, consistent with the high glycine content (43 %) within the region.

3.1.2.2. NMR

Sharp signals in the ^{1}H NMR spectra of full-length colicin E9 and the N-terminal 299 and 349 residues have indicated that the translocation domain of colicin E9 is flexible (Collins *et al* 2002). The narrow ^{1}H chemical shift dispersion in the ^{1}H-^{15}N HSQC spectrum of colicin E9 is also a sign that the protein contains unstructured regions. The observation that this spectrum is significantly different from the spectra produced from the isolated DNase domain or receptor-binding domains, indicates that the translocation domain forms the unstructured region (Collins *et al* 2002). However, although the consensus chemical shift index for the N-terminal region of colicin E9 indicates that much of the region lacks secondary structure elements, differences in chemical shifts between colicin E9 and those of random coil values suggest that some residues *eg* S30 and D35 in the N-terminal region are not in random coil conformations (Collins *et al* 2002). Relaxation measurements indicate that residues W39 to N44 and L81 to A83 of colicin E9 have more restricted mobility than the rest of the region (Collins *et al* 2002). The restricted mobility of residue W39 suggests that the TolB box could be more structured than other regions of the translocation domain, although the mobility of D35 does not differ from that seen for the rest of the region and the resonances for residues 36-38 have not yet been assigned (Collins *et al* 2002).

Differing intensities of the resonances observed for residues S40, S41 and E42 in the NMR spectrum indicate that these residues exist in different conformational states, despite the homogeneity of the protein samples, confirmed with SDS-PAGE and chromatography (Collins *et al* 2002). Collins *et al* (2002) suggest that these residues could be involved in side-chain/side-chain interactions, leading to the formation of clusters of interacting residues, with one cluster being involved in the interaction with TolB.

Therefore, colicins appear to be amongst a growing number of recognised proteins with intrinsically disordered domains, involved in macromolecular interactions, which adopt folded structures upon binding to their biological targets (Dyson and Wright 2002). The lack of structure can be functionally advantageous, for example allowing the protein to bind in different conformations to several different targets in a specific order (Wright and Dyson 1999).

3.1.3. Extension of the TolB box

The ^{1}H-^{15}N HSQC spectrum of colicin E9 in the presence of excess, unlabelled TolB shows that backbone resonances of residues 33 to 44 of colicin E9, including the TolB box, are perturbed on binding to TolB (Collins *et al* 2002). It was therefore suggested that the region for binding to TolB should be extended from the recognised pentapeptide sequence, DGSGW, to twelve amino acids, although it could not be ruled out that the perturbations in resonances could be due to conformational changes in the region flanking the TolB box resulting from the interaction of the TolB box region with TolB, rather than a direct interaction between TolB and these residues. The backbone resonances of N10, H14, H55 and W56 were also affected by addition of TolB (Collins *et al* 2002). However, mutation of residue W56 to alanine, located in a region of colicin E9 which showed homology to colicin K, did not affect the activity of the colicin in a stab test (Garinot-Schneider *et al* 1997) and constructs lacking residues 54-164 of colicin E9 have been shown to interact with TolB in yeast two hybrid experiments (Carr *et al* 2000a).

Recently an alanine mutation at residue 42 has been shown to be abolish activity of colicin E9 and this mutant protein does not interact with TolB in SPR experiments (Holland, 2003).

The three residues after the TolB box pentapeptide sequence of colicins E2-E9 and A are conserved (see Figure 3.1), providing further evidence that residues directly downstream of the TolB box may also be involved in the interaction with TolB (Bouveret *et al* 1998). Colicin E1, which does not require TolB for translocation, contains a pentapeptide sequence homologous to the TolB box but the region immediately following this TolB box is not homologous to the endonuclease colicins, supporting the possibility that residues downstream of the TolB box are involved in the colicin-TolB interaction. Although the TolB box of colicin A is predicted to lie between residues 11 and 15, a colicin A mutant with residues 16-30 deleted does not interact with TolB, whilst a mutant with residues 21-29 deleted does interact with TolB, indicating that residues between 15 and 20 could also be involved in the interaction of colicin A with TolB (Bouveret *et al* 1998). However, no mutational data has been published to confirm the role of any of these six residues in binding to TolB.

Colicin E9	34	SDGSGWSSENNPW	46
Colicin E7	34	SDGSGWSSENNPW	46
Colicin E3	34	SDGSGWSSENNPW	46
Colicin E2	34	SDGSGWSSENNPW	46
Colicin E1	29	PDGSGSGGGGGKG	41
Colicin A	10	GDGTGWSSERGSG	22
Colicin K	16	MGGTGANLNQQGG	28
Colicin N	25	TSGAGSNGSASSN	37

Figure 3.1 Alignment of known and predicted TolB boxes (black) and flanking residues (red) of TolB-dependent colicins (adapted from Garinot-Schneider 1997).

3.1.4. TolB interaction with colicin E9

Although the region of colicin E9 involved in interacting with TolB has been localised to the TolB box, the region of TolB involved in interacting with colicin E9 is less well-defined. As discussed in Chapter 1, the crystal structure of TolB shows that the protein consists of an N-terminal mixed αβ domain and a C-terminal β-propeller domain (Carr *et al* 2000a, Abergel *et al* 1999).

β-propeller proteins usually use their central tunnel or the entrance to the tunnel to coordinate a ligand or to carry out a catalytic function that is preserved by the structural rigidity of the propellers. It was therefore proposed that the translocation domains of colicins interact with the β-propeller domain of TolB (Carr *et al* 2000a). N-terminal and C-terminal deletions have been made in the *tolb* gene and the effect of the deletions on the TolB protein on binding to colicin E9 has been investigated using the yeast-two hybrid technique (Carr *et al* 2000a). Deletion of the first 119 residues of TolB had no effect on the interaction with the translocation domain of colicin E9. However, deletion of residues 1-203, 205-431 or 304-431 abolished the TolB-colicin E9 interaction (Carr *et al* 2000a). This suggests that the region of TolB involved in the interaction with colicin E9 is located between residues 119 and 431. As this region encompasses the β-propeller domain, it has been suggested that it is the β-propeller domain of TolB that is involved in the interaction with colicin E9 (Carr *et al* 2000a).

However, Abergel *et al* (1999) suggest that it is the N-terminal domain of TolB that is involved in interacting with colicins. The A227V mutation in the β-propeller domain

of TolB does not affect the interaction with colicin, whereas insertion of two residues (SS) after residue P87 affected the ability of the colicin to enter cells (Abergel *et al* 1999). An *E.coli tolb* mutant strain, when transformed with a cloned *tolb* mutant gene, lacking most of blades V and VI of the β-propeller domain, was shown to be sensitive to colicins A and E1 (Bénédetti *et al* 1991b).

Yeast-two hybrid experiments have shown that the translocation domain of colicin A does not interact directly with either the isolated β-propeller domain or N-terminal domain, in contrast to the yeast-two hybrid result for colicin E9, which detected an interaction between residues 120-413 of TolB and colicin E9 (Wallburger *et al* 2002, Carr *et al* 2000a). It is suggested by Wallburger *et al* (2002) that the discrepancy could be due to differing translocation mechanisms between the pore-forming colicin A and the endonuclease colicin E9.

3.1.5. Aims of this chapter

The work described in this chapter further characterises the role of the residues in the recognised pentapeptide TolB box. The technique of surface plasmon resonance has been used to directly compare binding of TolB to wild-type colicin E9/Im9 and to colicin E9/Im9 mutants with an alanine mutation in the TolB box. The SPR technique has also been used to investigate the effect of mutating the three TolB box residues, known to abolish biological activity and TolB binding, to residues other than alanine. Additionally, this chapter further investigates the hypothesis that the region of the translocation domain of colicin E9, involved in interaction with TolB, is longer than the pentapeptide sequence initially proposed.

3.2. Results

3.2.1. Preparation of Colicin E9 and colicin E9 TolB box mutants

Wild-type colicin E9 and colicin E9 with mutations in and around the TolB box were expressed from the appropriate plasmid in *E.coli* host strains as shown in Table 1, using 1mM IPTG as the inducer.

Table 3.1 List of pET21a-based plasmids used for expressing mutated colicin E9 proteins and the cell line used for expression.

Mutation	Plasmid	*E.coli* host strain
Wild-type	pNP69	ER2566
S34A	pNP82	BL21 (DE3)
D35A	pNP56	ER2566 pLysS
G36A	pNP55	BL21 (DE3)
S37A	pCS9	ER2566 pLysS
G38A	pNP321	ER2566
W39A	pNP57	ER2566 pLysS
S40A	pNP84	BL21 (DE3)
S41A	pSR15	BL21 (DE3)
E42A	pLH28	BL21 (DE3)
N43A	pSR16	BL21 (DE3)
N44A	pSR17	BL21 (DE3)

The proteins were purified from harvested cells using nickel-affinity chromatography as detailed in Chapter 2. The purified fractions were dialysed in 50mM potassium phosphate buffer, pH 7.4 and were run on a 12 % SDS-PAGE gel to check for purity (see Figure 3.2).

Where appropriate, the mutants were purified free from Im9, using the method set out in Chapter 2.

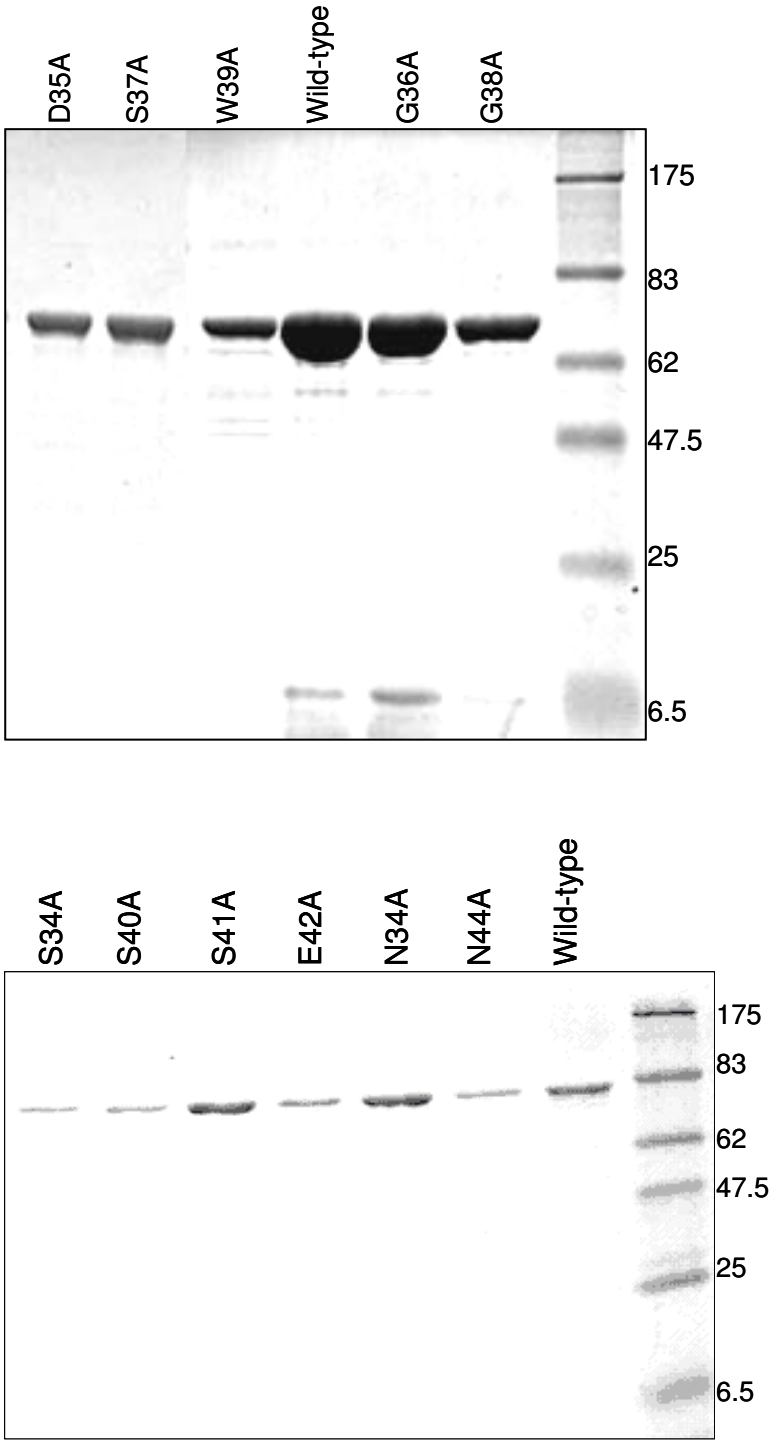


Figure 3.2 12 % SDS-PAGE gels showing purified wild-type colicin E9 and purified colicin E9 mutant proteins. The band around 10 kDa seen in some lanes corresponds to the immunity protein.

3.2.2. Preparation of TolB

pRJ376, a pET21a-based plasmid containing the *tolb* gene, was used to transform *E.coli* BL21 (DE3) cells. Expression of the TolB protein was induced with 1mM IPTG in a large scale (400 ml) culture. The cells were lysed using sonication and the TolB protein was purified using nickel-affinity chromatography as detailed in Chapter 2. The protein was dialysed in 50 mM potassium phosphate buffer, pH 7.4 and run on a gel to check for purity (see Figure 3.3).

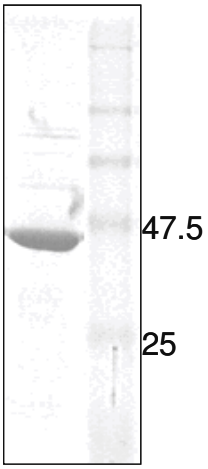


Figure 3.3 **12 % SDS-PAGE gel showing purified TolB protein, after dialysis.**

3.2.3. SPR

Preliminary SPR experiments were carried out using a BIAcore J instrument, requiring manual injection of analyte. The accuracy of the injection means that the BIAcore J is not suitable for measurement of kinetic parameters but can be used to rank mutants in terms of their ability to bind to TolB.

3.2.3.1. Immobilisation of TolB onto a CM5 chip

The BIAcore J has two flow cells, allowing derivitisation of one cell with TolB, with the other cell acting as a blank reference surface. Both flow cells were activated using EDC/NHS as described in Chapter 2. TolB (at a concentration of 1 μM) was then coupled to flow cell 1 only. Remaining active esters on both flow cells were deactivated using ethanolamine. The final level of TolB immobilised was ~3000 RU (see section 2.20.1).

3.2.3.2. Binding of wild-type colicin E9/Im9 to TolB

Two minute injections (70 µl) of 2 µM wild-type colicin E9/Im9 were performed across both flow cells. The sensorgram produced from the underivatised flow cell 2 was subtracted from the sensorgram produced from flow cell 1, derivatised with TolB protein. This produces a corrected sensorgram, removing the response produced by non-specific interactions between the analyte and the sensor surface (see Figure 3.4). Sensorgrams shown throughout this thesis are corrected for non-specific binding in this way.

Injection of 2 µM colicin E9/Im9 produced a maximum response of 1705 ± 23 RU (~ 36 % of RUmax, see section 2.20.1), demonstrating that an interaction between TolB and colicin E9/Im9 occurs on the sensor chip.

3.2.4. Binding of TolB box mutants to TolB

3.2.4.1. Colicin E9 D35A, S37A and W39A mutants

Injection of 2 µM of the D35A, S37A and W39A mutant colicin E9 proteins across the TolB chip produced sensorgrams shown in Figure 3.4.

The sensorgrams in Figure 3.4 show that the D35A, S37A, W39A mutations all drastically reduce binding of colicin E9 to TolB, producing mean maximum responses of 290 ± 10 RU, 104 ± 7 RU and 48 ± 5 RU respectively (n=3).

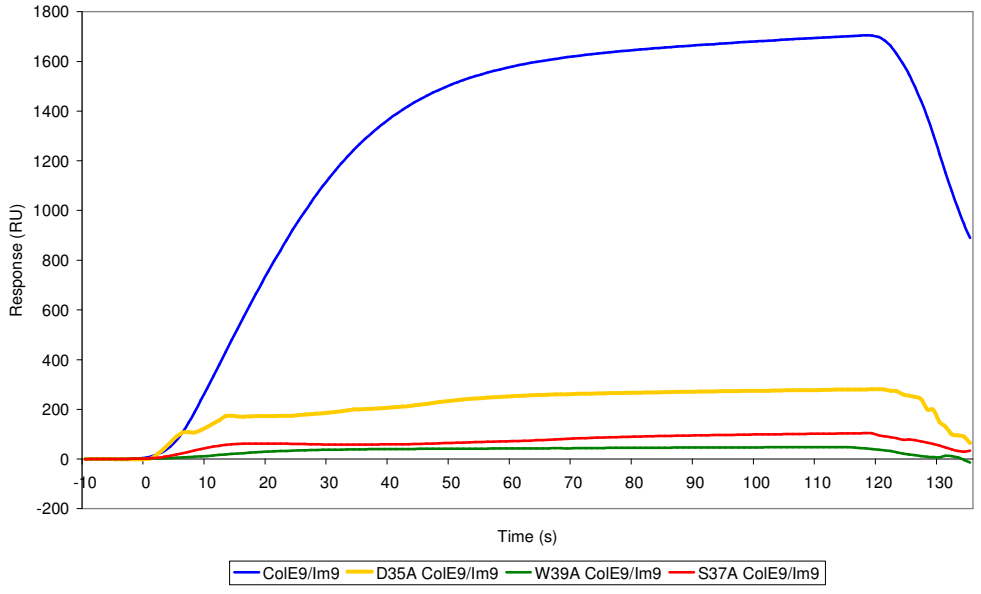


Figure 3.4 SPR sensorgrams showing reduction in binding to TolB caused by D35A, S37A and W39A mutations in colicin E9

The D35A, S37A and W39A mutations in colicin E9 were expected to reduce binding of the colicin to TolB, as these mutations abolish the activity of the colicin and have been shown by yeast-two hybrid studies to abolish the TolB-colicin E9 interaction (Carr *et al* 2000a).

3.2.4.2. G36A and G38A colicin E9 mutants

The G36A and G38A mutations do not inactivate the biological activity of the colicin in a stab test and were shown by yeast-two hybrid studies to retain their interaction with TolB (Carr *et al* 2000a). Therefore, binding of these mutants to TolB, comparable to wild type, was expected. However, as can be seen from Figure 3.5, both mutants showed significantly reduced binding to TolB compared to wild-type colicin E9 at a concentration of 2 µM.

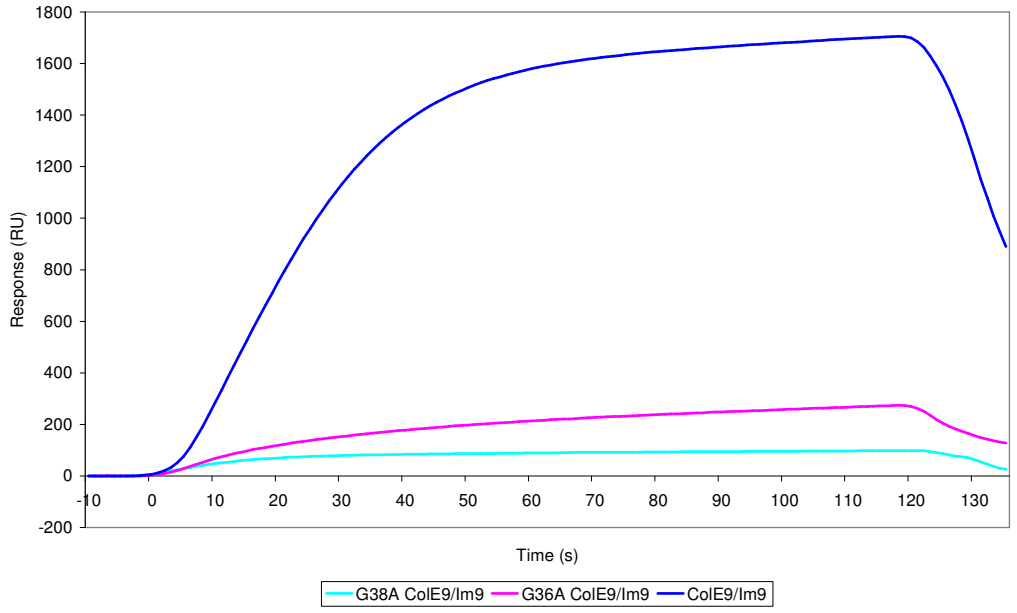


Figure 3.5 SPR sensorgrams showing reduced binding to TolB of 2 µM G36A and G38A colicin E9 mutants compared to 2 µM wild-type colicin E9.

One difference between these SPR experiments and the yeast-two hybrid experiments is that in the latter experiments colicin E9 was used in the absence of the immunity protein, whereas in this work the colicin mutant proteins had been purified in complex with their immunity proteins. Therefore, the immunity protein was removed from

both the G36A and G38A colicin E9 mutant proteins by denaturation on a Hi-trap column using 6 M guanidine, as detailed in Chapter 2.

The binding of the free colicin E9 mutant proteins to TolB was then investigated and the resulting sensorgrams can be seen in Figure 3.6. Although the G36A colicin E9 mutant binds poorly to TolB when Im9 is present, binding of the mutant to TolB when Im9 has been removed, is much greater, although the shape of the sensorgram produced is altered, suggesting that the mutation does have some effect on the binding site for TolB. The binding is also enhanced compared to the binding of wild-type colicin E9/Im9.

The response due to binding of the G38A colicin E9 mutant to TolB is very low for both the free colicin E9 protein and for the colicin E9/Im9 complex. This contradicts results from yeast two hybrid data indicating that G38A colicin E9 does interact with TolB. For a positive result in a yeast two hybrid screen, it is generally accepted that an interaction with a K_d in the micromolar range is required, yet even at 10 μM the G38A colicin E9 mutant showed very poor binding to TolB.

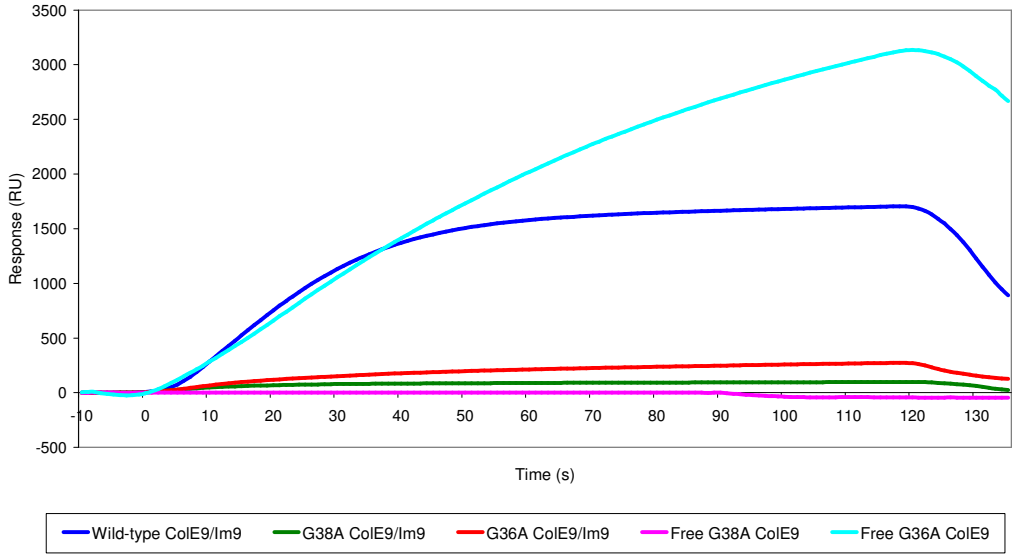
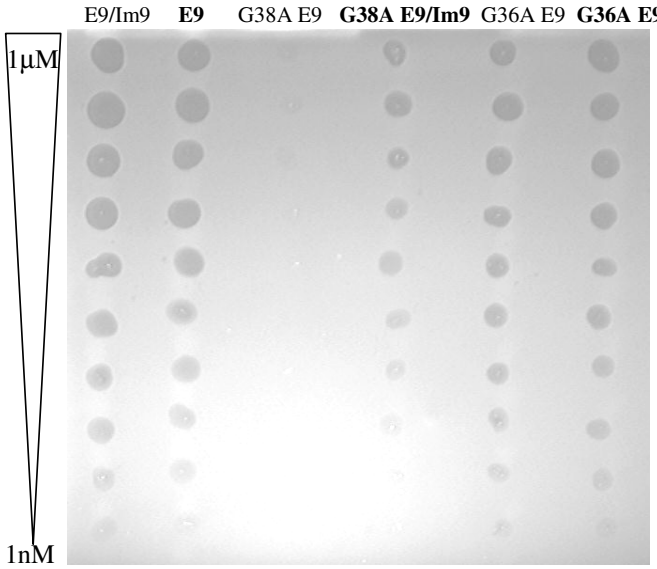


Figure 3.6 SPR sensorgrams showing the binding of G36A and G38A colicin E9 mutant proteins to TolB, in the presence and absence of Im9, compared to the binding of wild-type colicin E9/Im9.

A spot test was performed to check that G36A and G38A ColE9 proteins were active both with and without their immunity protein (see Figure 3.7).



Protein	Titre (nM)
E9/Im9	30
E9	30
G38A E9	inactive
G38A E9/Im9	250
G36A E9	60
G36A E9/Im9	60

Figure 3.7 Results of a spot test to establish the activity of G36A and G38A ColE9 mutant proteins in the presence and absence of Im9 compared to wild type ColE9/Im9.

The spot test indicates that the G36A ColE9 protein is active both with and without the immunity protein, although it is slightly less active than wild-type colicin E9. G38A is only active when Im9 is present and even then, activity is reduced compared to wild type. A further spot test using higher concentrations of protein (see Figure 3.8), indicated that the G38A mutant in the absence of Im9 was active at concentrations above 1 μM, but confirmed that the activity was much lower than the activity of wild-type colicin E9.

The colicin E9 gene in pNP321, from which G38A ColE9/Im9 was expressed, was re-sequenced to confirm that there were no additional mutations that may be affecting TolB binding.

A Kunitz assay (see section 2.16) was also performed to check that the G38A mutant was intact. This assay measures the increase in absorbance at 260nm due to DNA degradation. Figure 3.9 shows that the rate of DNA degradation by free G38A colicin E9 is comparable to that for free wild-type colicin E9, indicating that the DNase domain of the mutant is intact.

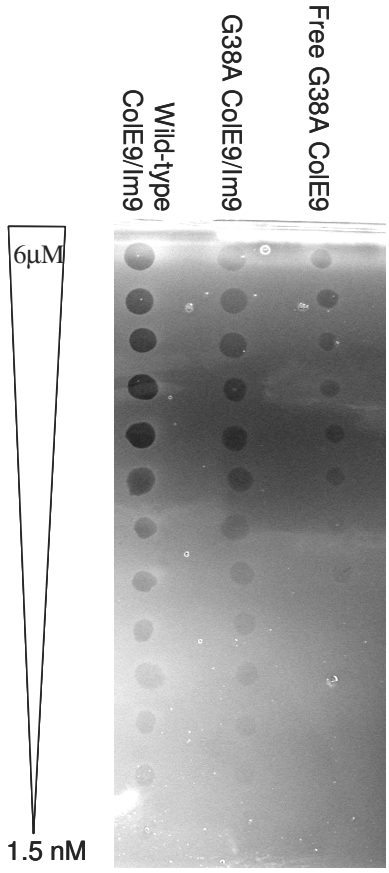


Figure 3.8 **Results of a spot test to establish the activity of G38A colicin E9 protein with and without Im9. Activity can be detected at 6 µM in the presence and absence of immunity protein. The mutant is more active in the presence of the immunity protein but still less active than wild-type colicin E9.**

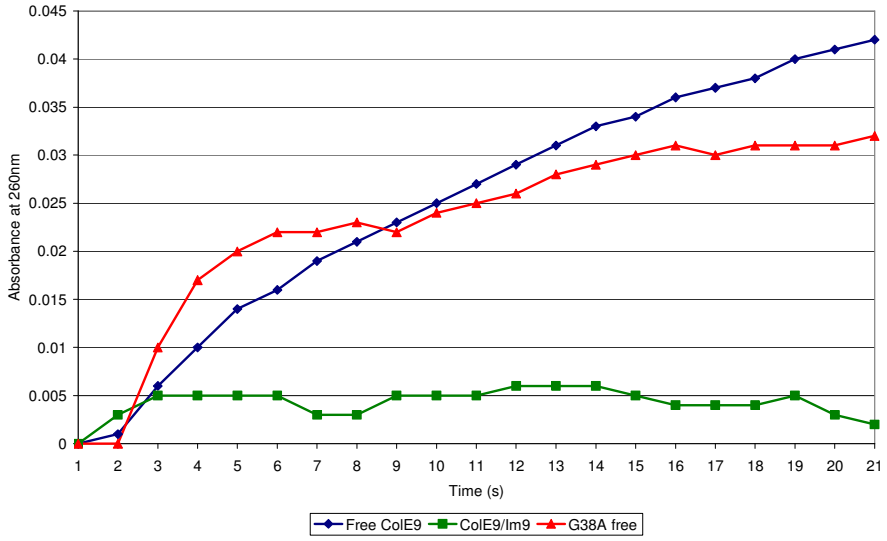


Figure 3.9 **Results of a Kunitz assay, comparing the DNase activity of wild-type colicin E9 to that of the G38A colicin E9 mutant. Results for wild-type colicin E9/Im9 are also shown as a control, the presence of the immunity protein preventing DNA degradation. Lamp fluctuations of 0.001 AU occur over the course of the experiment, accounting for fluctuations in the traces.**

The change in binding characteristics when free G36A ColE9 binds to TolB shown by the shape of the SPR sensorgram does indicate that there may be a conformational change when residue 36 is mutated, which could cause the reduction in activity seen in the spot test. The yeast-two hybrid technique only shows whether the TolB box mutants bind or do not bind to TolB, whereas these SPR experiments have highlighted a difference in binding of the G36A colicin E9 mutant to TolB compared to wild-type colicin E9

The reduced activity of the G38A mutant in the spot test is consistent with the reduced binding to TolB, seen in the SPR experiment. However, this is inconsistent with the yeast-two hybrid data, which suggested that the G38A mutation did not affect the binding of colicin E9 to TolB.

3.2.5. Kinetics of binding of ColE9/Im9 to TolB

SPR allows the direct measurement of the kinetic parameters that describe an interaction in real time. The mathematical fitting of a plot of refractive index change with time to theoretical plots for model systems of bimolecular interactions allows calculation of kinetic parameters that describe the interaction.

The BIAcore X instrument was used for all kinetic analysis presented in this thesis. This instrument allows continuous injection to maintain sample concentration and has fast buffer/sample exchange minimising sample dispersion, ensuring accurate rate constant determination (BIAcoreX Product Information).

3.2.5.1. Immobilisation of TolB on a CM5 sensor chip

In order to limit mass transfer effects (see Chapter 2), the lowest feasible level of ligand immobilisation should be used along with a fast flow-rate for analyte injection. For kinetic measurements, the maximum specific SPR response (RUmax) should be approximately 100 RU (BIAcore Advisor Kinetics CD). Equation 3-1 can be used to calculate the amount of ligand immobilisation required for a given binding capacity.

$$\frac{\text{RMM of analyte (kDa)}}{\text{RMM of ligand (kDa)}} = \frac{\text{Amount of analyte bound (RU)}}{\text{Amount of ligand bound (RU)}}$$

Equation 3-1 Equation describing the relationship between SPR response and relative molecular masses (RMM) of the ligand and analyte.

Using Equation 3-1, the target level of immobilisation for the ligand *ie* TolB on the sensor chip was calculated to be ~ 160 RU, assuming a stoichiometry of 1:1 binding. Therefore 50nM TolB was injected across flow cell 2 of an EDC/NHS activated CM5 chip with a contact time of 3 minutes at a flow rate of 10 µl/min. This produced a level of immobilisation of 300 RU, giving an Rmax of 187.5. Although this level of ligand immobilisation gives a RUmax greater than 100 RU, the percentage occupancy of TolB binding sites is likely to be less than 100 %, and therefore this level of ligand immobilisation is satisfactory. This level of immobilisation also allows the responses measured for partial TolB occupancy, observed with lower concentrations of colicin E9, to be used for kinetic analysis, by ensuring the response elicited will be above the level of background noise.

3.2.5.2. Kinetic analysis of binding of colicin E9/Im9 to TolB

2 minute injections of 2, 5, 10, 15 and 20 µM ColE9/Im9 were performed across the 300 RU of immobilised TolB on the CM5 chip at a flow rate of 30 µl/min. After each analyte injection, the sensor chip surface was regenerated using a one minute pulse of 10 mM glycine, pH 1.8. Following regeneration, an injection of the same concentration of colicin E9/Im9 produced a similar response as before, indicating that the TolB protein on the surface remained intact.

Global analysis using BIAevaluation software 3.1 was used to fit corrected SPR responses to the theoretical 1:1 Langmuir binding model (see Figure 3.10).

Table 3.2 lists the kinetic data obtained from fitting of the sensorgrams to this model, together with the residual plot comparing the experimental data to the theoretical fit. For a perfect fit, the residuals should be scattered around zero and reflect the noise of the signal (in the order of +/- 2 RU, BIAevaluation software handbook 4.1). However, constraining the parameters in a fit to have the same value for a set of curves can produce a larger scatter. The K_d for the interaction of colicin E9/Im9 with TolB is 13.8 ± 0.26 µM.

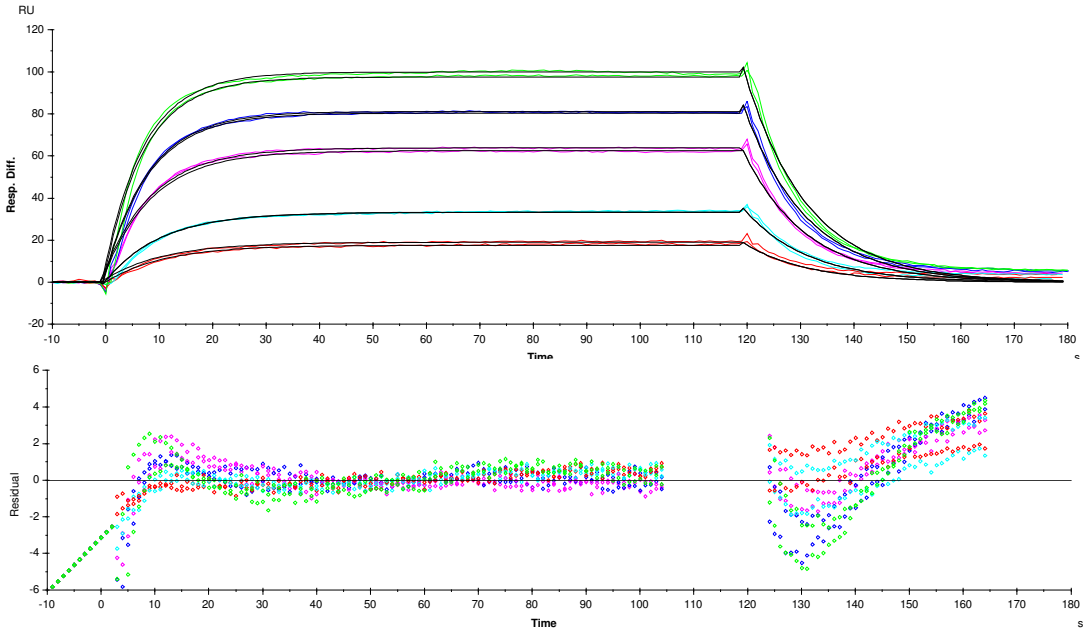


Figure 3.10 Corrected sensorgrams and residual plots for titration of 2 µM (red), 5 µM (cyan), 10 µM (magenta), 15µM (blue) and 20 µM (green) ColE9/Im9 against TolB at 25°C are shown. The fitted kinetic data for binding are superimposed onto each sensorgram in black. Residual plots highlight deviation of the experimental data from the theoretical fit, the deviations being largest at the start and end points of the injections.

Table 3.2 Kinetic data obtained from fitting of sensorgrams produced for binding of ColE9/Im9 to TolB to the Langmuir 1:1 binding model. Errors for K_a and K_d were calculated by combining the standard errors on k_{ass} and k_{diss} in quadrature.

kass (1/Ms)	kdiss (1/s)	Rmax (RU)	Ka (1/M)	Kd (µM)	Chi2
6130 ± 109	0.0847 ± 0.000574	286	72300 ± 1374	13.8 ± 0.26	2.06

Statistical parameters

Statistical parameters, which give indications as to how good the fit is, are calculated as part of the BIAevaluation fitting process. Firstly a Chi squared value is given. The Chi squared test is a standard statistical measure of the closeness of fit of the experimental response to the theoretical model, related to the sum of squared residuals averaged over the number of data points. However, as SPR sensorgrams have a large number of data points, the Chi squared value is not always a very sensitive measure of the closeness of fit. It is generally accepted that a Chi squared value of 2 or less indicates a good fit (BIAevaluation manual). The Chi squared value of 2.06 for these data suggests that the data closely fit the binding model.

The second statistical parameter reported by the BIAevaluation software is the standard error. This is a measure of how much the closeness of fit is affected by

variations in the parameter value. A small standard error indicates that changes in the parameter's value have a significant effect on the fitting: in other words, confidence in the value is high. The standard error for K_d can be calculated from combining the standard errors for k_{ass} and k_{diss} in quadrature. The standard error for K_d for these data is 0.26, a low value, compared to the value of K_d of 13.8, giving good confidence in the K_d value.

T-values can be obtained by dividing the parameter's value by the standard error, and thus provide a kind of normalised inverse standard error value. High T-values (typically above 10) indicate confidence in the parameter's value. The T-value for k_{ass} is 56, the T-value for k_{diss} is 148, and the T-value for K_a and K_d is 53, giving good confidence in the values of these parameters.

Scatchard analysis

A Scatchard plot of the data, Max RU/[ColE9/Im9] against Max RU, which can be used to visualise the extent to which the data fit the model, is shown in Figure 3.11. The R^2 value of 0.97 for a linear fit to the Scatchard plot indicates that the data fit the model well.

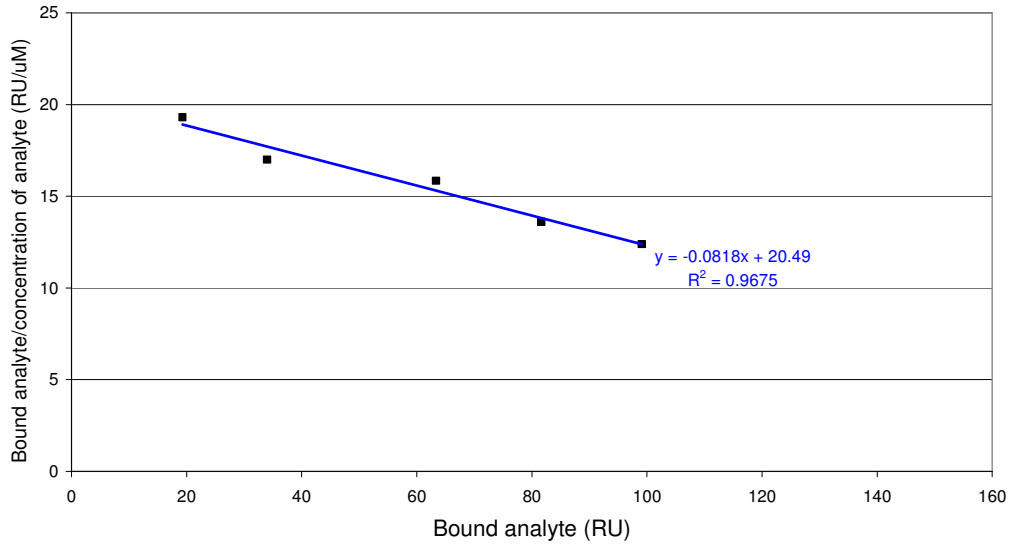


Figure 3.11 Scatchard plot for binding of colicin E9/Im9 to TolB. The K_d value can be obtained by calculating the inverse of the gradient *ie* K_d = 12.2 µM.

Mass transport

Under the flow conditions in SPR, the rate of mass transport of analyte to the surface varies with the cube root of the flow rate (BIAcore Advisor Kinetics CD). In contrast, the rate of analyte binding to ligand is independent of the flow rate. Comparison of the observed binding rates at different flow rates can thus reveal whether the binding is limited by mass transport processes. To investigate mass transport limitations of analysing the binding of colicin E9/Im9 to TolB, injections of 15 µM colicin E9/Im9 were performed at three different flow rates, 5 µl/min, 15 µl/min and 30 µl/min. The three sensorgrams produced (see Figure 3.12) show the same slope, indicating that mass transport limitations are negligible and thus the measured k_{ass} should be close to the true k_{ass}.

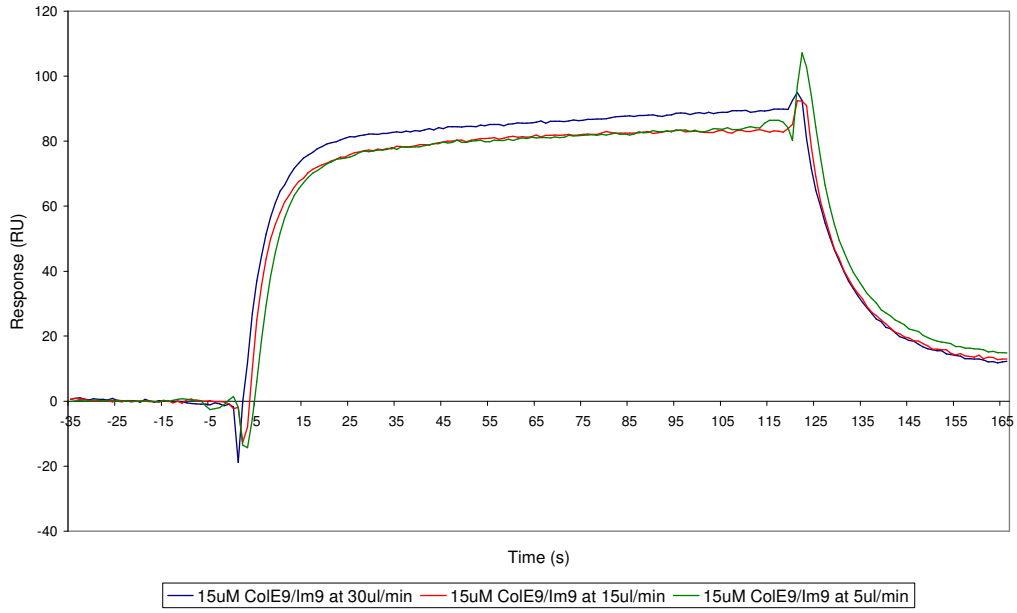


Figure 3.12 Sensorgrams recorded for injections of 15 µM ColE9/Im9 at three different flow rates in a test for mass transport effects. If mass transport was a limiting factor, the slopes of the sensorgrams would be different at different flow rates. However, as the slopes of the three sensorgrams are the same, it can be concluded that mass transport limitations are negligible.

3.2.5.3. Kinetic analysis of binding of G36A mutant to TolB

The same method used above, for determining kinetic parameters for binding of wild-type colicin E9/Im9 to TolB (see section 3.2.5.2), was used to determine kinetic parameters for binding of the G36A colicin E9/Im9 mutant to TolB. Global analysis

using BIAevaluation software 3.1 was used to fit corrected SPR responses to the theoretical 1:1 Langmuir binding model (see Figure 3.13).

Higher concentrations of protein were used to investigate the kinetics of binding of G36A colicin E9/Im9, compared to those used for the wild-type colicin E9/Im9, due to the decreased binding seen for this mutant in the preliminary experiments.

Table 3.3 lists the kinetic data obtained from fitting of the sensorgrams produced for binding of G36A colicin E9/Im9 to this model. The K_d for the interaction of colicin E9/Im9 with TolB is 45.1 ± 1.1 μM.

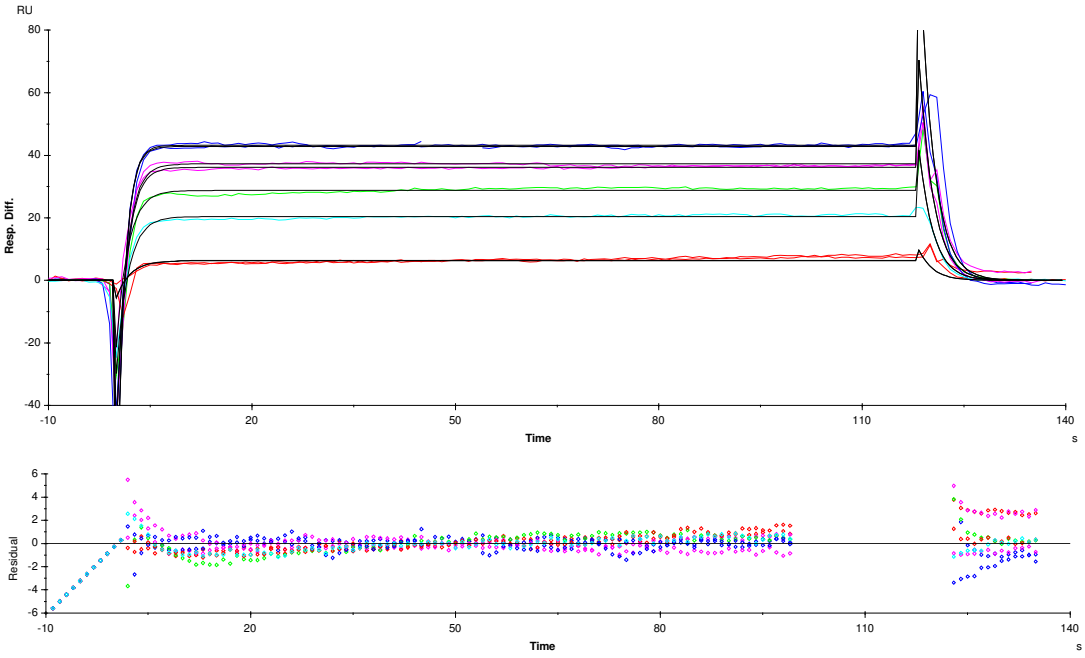


Figure 3.13 Corrected sensorgrams and residual plots for titration of 2 μM (red), 5 μM (cyan), 10 μM (green), 20 μM (magenta) and 40 μM (blue) G36A ColE9/Im9 against TolB at 25°C are shown. The fitted kinetic data for binding are superimposed onto each sensorgram in black. Residual plots highlight deviation of the experimental data from the theoretical fit, the deviations being largest at the start and end points of the injections.

Table 3.3 Kinetic data obtained from fitting of sensorgrams produced for binding of G36A ColE9/Im9 to TolB to the Langmuir 1:1 binding model, errors for K_a and K_d were calculated by combining the standard errors on k_{ass} and k_{diss} in quadrature.

kass (1/Ms)	kdiss (1/s)	Ka (1/M)	Kd (μM)	Chi2
10700 ± 437	0.481 ± 0.0119	22200 ± 550.6	45.1 ± 1.1	0.935

Sensorgrams were also recorded for injections of varying concentrations of free G36A colicin E9 across the TolB chip. The sensorgrams for binding of the free G36A

colicin E9 to TolB produced a poor fit to the Langmuir 1:1 binding model. Therefore, fitting was attempted to several other models, with the best fit obtained to a two state conformational change model. This model is appropriate when the complex AB formed on the sensor surface undergoes a conformational change to a different form, AB* (see Equation 3-2).

$$A + B \leftrightarrows AB \leftrightarrows AB^*$$

Equation 3-2 Equation for a two-state reaction, involving a conformational change from AB to AB*.

Kinetic parameters can be determined using the following equations:

$$\frac{dB}{dt} = -(kass1\,[A]\,[B] - kdiss1\,[AB])$$

$$\frac{dAB}{dt} = -(kass1\,[A]\,[B] - kdiss1\,[AB]) - (kass2\,[AB] - kdiss2\,[AB^*])$$

$$\frac{dAB^*}{dt} = -(kass2\,[AB] - kdiss2\,[AB^*])$$

where k_{ass1} is the association rate constant for formation of AB, k_{diss1} is the dissociation rate constant for complex AB, k_{ass2} is the rate constant for conversion of AB to AB* and k_{diss2} is the rate constant for conversion of AB* to AB.
For simplicity, the model assumes that the transition from AB to AB* and back can only occur when A and B are bound to each other *ie* the modified complex AB* cannot dissociate directly into A+B without first going through the state AB.

Figure 3.14 shows the sensorgrams produced for binding of free G36A colicin E9 to TolB, with the fit to the two-state conformational change model superimposed in black. Table 3.4 shows the kinetic parameters calculated from fitting the data to the two-state conformational change model. Although the Chi squared value is higher than normal, the T-values for the kinetic parameters are all above 10, giving good confidence in the values. The affinity constant (K, *ie* $[k_{diss1}/k_{ass1}] \times [k_{diss2}/k_{ass2}]$) for the formation of the complex, including the conformational change, is 1.24 ± 0.12 μM.

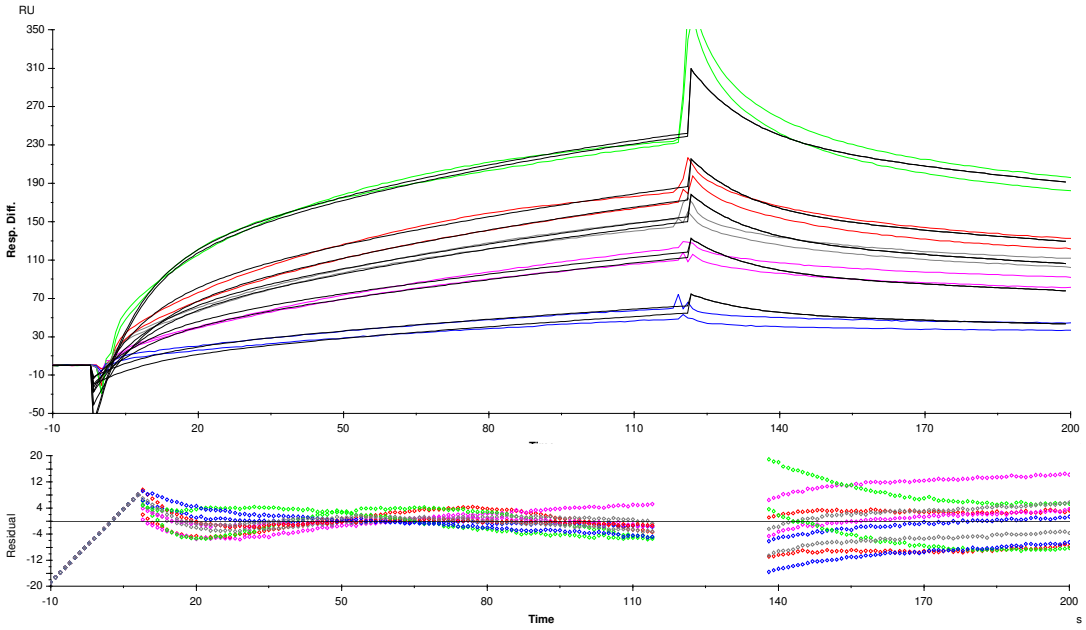


Figure 3.14 Corrected sensorgrams and residual plots for titration of 0.5 µM (blue), 1 µM (magenta), 2 µM (grey), 4 µM (red) and 6 µM (green) free G36A ColE9 against TolB at 25°C are shown. The fitted kinetic data for binding are superimposed onto each sensorgram in black. Residual plots highlight deviation of the experimental data from the theoretical fit.

Table 3.4 Kinetic data obtained from fitting of sensorgrams produced for binding of free G36A ColE9 to TolB to a two-state conformational change model. The kinetic parameters are k_{ass1} (association rate constant for formation of AB), k_{diss1} (dissociation rate constant for complex AB), k_{ass2} (rate constant for conversion of AB to AB*) and k_{diss2} (rate constant for conversion of AB* to AB).

kass1 (1/Ms)	kdiss1 (1/s)	kass2 (1/s)	kdiss2 (1/s)	K (1/M)	Chi2
11100 ± 337	0.0657 ± 0.0027	0.0161 ± 0.000585	0.0034 ± 0.000127	806000	25.7

3.2.6. TolB box revertant mutants

Although the TolB box region of colicin E9 has been localised to residues D35 - W39 (Carr *et al* 2000a, Garinot-Schneider *et al* 1997), there is no published information available as to the role of these residues in the interaction with TolB. Mutation of D35, S37 and W39 residues in colicin E9 to alanine, and mutation of S37 to F in colicin E3 abolishes the biological activity of the colicin and the interaction of the colicin with TolB (Bouveret *et al* 1997, Carr *et al* 2000a). However, it is unknown whether other residues could be tolerated at positions in the TolB box, without compromising the activity of the colicin or binding to TolB.

Therefore, mutagenic primers were used to randomly mutate a biologically inactive TolB box alanine mutant to any residue and those clones with restored activity in a stab test were sequenced (Holland 2003). ColE9/Im9 proteins were purified from the

clones with restored activity and colicin titres of these proteins were determined using a spot test (Holland 2003). This mutagenic analysis was performed for residues 35, 37 and 39.

For residue 35, three clones were found to have restored activity. For two of these clones, the residue had reverted back from alanine to the wild-type residue *ie* aspartate. The other clone contained a serine at position 35. The activity of the purified A35D and A35S proteins was shown to be the same as wild-type in a spot test (Holland 2003).

For residue 37, 18 clones had restored activity and were found to contain either a valine, threonine or serine (wild-type) residue at position 37. Spot tests with purified colicin E9 proteins from these clones, showed that the mutant proteins had similar activity to wild-type colicin E9 (Holland 2003).

For residue 39, 21 clones had restored activity and were found to contain either a tryptophan (wild-type), phenylalanine or tyrosine residue at position 39. Spot tests with the purified proteins from these clones, showed that the A39Y mutation reduced activity considerably, producing only a hazy zone in a spot test. The activities of A39F and A39W mutants were shown to be slightly lower than that of wild-type colicin E9.

3.2.6.1. Affinity of TolB box revertant mutants for TolB

In order to relate the titres of the revertant mutants to the affinity of the mutants for TolB, the affinities of binding of four of the mutants with restored activity *ie* A35S, A37T, A37V and A39Y were determined by SPR.

Kinetic parameters for the interaction between the mutant colicin E9/Im9 proteins and TolB were obtained in the same way as described above for wild-type colicin E9/Im9. Two minute injections of 2, 5, 10, 15 and 20 μM of the mutants were performed across immobilised TolB on a CM5 chip at a flow rate of 30 μl/min. Global analysis using BIAevaluation software 3.1 was used to fit corrected SPR responses to the theoretical 1:1 Langmuir binding model. K_d values for the mutants are shown in Table 3.5. Chi squared values were less than or equal to 2 and Scatchard plots were linear with R^2 values >0.9 (data not shown).

Table 3.5 Table showing comparison of K_d values for binding of TolB box revertant mutants to TolB, determined by SPR, to the titre for the mutants, determined by a spot test (Holland 2003).

Mutation	Kd (μM)	Titre (nM)
Wild-type	13.8 ± 0.3	10
D35S	12 ± 0.2	10
S37T	24.3 ± 1.2	14.5
S37V	12.2 ± 0.2	10
W39Y	Low affinity	Hazy zone

Table 3.5 shows that a serine residue at position 35 has very little effect on the K_d for binding to TolB, consistent with the identical titre seen in the spot test. Similarly the affinity of the A37V mutant for TolB is very similar to the affinity of wild-type colicin E9/Im9 for TolB, consistent with the identical titres for mutant and wild-type. A threonine residue at position 37 significantly decreases the affinity of the colicin for TolB and this is reflected in the slight increase in titre.

It was not possible to get kinetic data for the binding of the A39Y mutant to TolB, as the level of binding was so low. The reduced affinity for TolB of this mutant is consistent with the hazy zone seen in the spot test, indicating that the low affinity of the mutant for TolB, substantially reduces its activity.

3.2.7. Extension of the TolB box

The perturbation of the backbone resonances of residues 33 to 44 of colicin E9, in the ^{1}H-^{15}N HSQC spectrum of colicin E9 in the presence of excess, unlabelled TolB are perturbed on binding to TolB (Collins *et al* 2002) has lead to the suggestion that the region for binding to TolB should include residues A33, S34, S40, S42, E42, N43 and N44, in addition to the recognised TolB box pentapeptide sequence, from residues 35-39 (DGSGW). Residue E42 has already been shown by SPR to be involved in binding to TolB (Holland 2003) and therefore this section investigates the effect of S34A, S40A, S41A, N43A and N44A mutations on colicin E9 activity and TolB binding.

3.2.7.1. Alanine mutagenesis of residues S41, N43 and N44

Mutation of residues S41, N43 and N44 was achieved using a 2-step PCR method (Sarkar and Sommer 1990). In the first stage pNP69 (see section 2.2) was used as the template along with T7 promoter primer and a mutagenic primer *ie* SR1, 2 or 3. The megaprimer produced in the first stage was then used along with primer H52 with pNP69 as the template in the second stage.

The second stage PCR product was restricted with *Nde*I and *Xho*I and cloned into pET21a and used to transform DH5α cells. The purified plasmids (pSR15, 16 and 17) from the DH5α cells were sequenced to confirm the presence of the required mutation and then used to transform *E.coli* BL21 cells. IPTG was used to induce expression of the mutated colicin E9 proteins in a 400ml culture of these BL21 cells. Cells were harvested and lysed and the mutant protein was purified using nickel-affinity chromatography as described in Chapter 2.

3.2.7.2. Stab tests to check activity of mutants

E.coli BL21 cells expressing a S34A, S40A, S41A, N43A or N44A colicin E9 mutant were used in a stab test to test the activity of the mutant colicins (see Table 3.1 for appropriate plasmids). Figure 3.15 shows that mutation of residues S34, S40 and N44 to alanine, abolishes activity of the colicin, whereas the colicin E9 S41A and N43A mutants retain activity. A slight hazy zone develops for the S34A mutant after incubation at 25 ºC for 24 hours but no hazy zone was observed for the S40A mutant, in contrast to the findings of Garinot-Schneider *et al* (1997).

3.2.7.3. Spot tests to check activity of mutants

The activity of purified S34A, S40A, S41A, E42A, N43A and N44A colicin E9 mutant proteins was tested in a large plate spot test against wild-type colicin E9. Figure 3.16 shows that, consistent with the stab test, S34A and S40A mutant proteins are inactive along with the E42A mutant, whereas S41A and N43A proteins have comparable activity to wild-type colicin E9. The N44A mutant produces a hazy zone at 30 μM but no zone at concentrations of 5 μM or less, indicating that this mutant has residual activity.

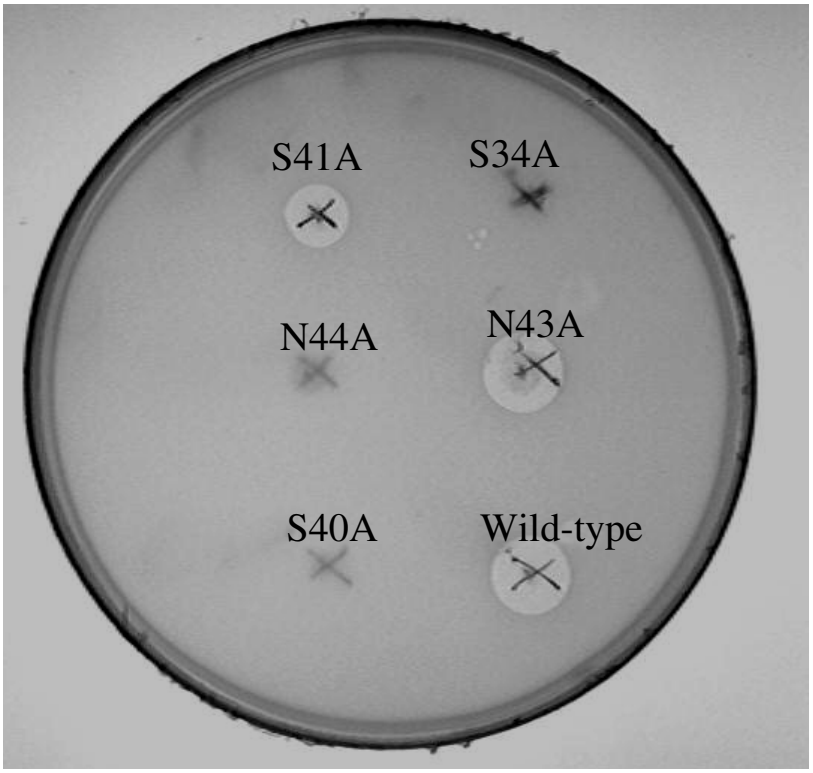


Figure 3.15 Results of a stab test performed with *E.coli* BL21 cells transformed with plasmids encoding S34A, S40A, S41A, N43A or N44A mutant colicin E9, compared to results with cells transformed with wild-type colicin E9

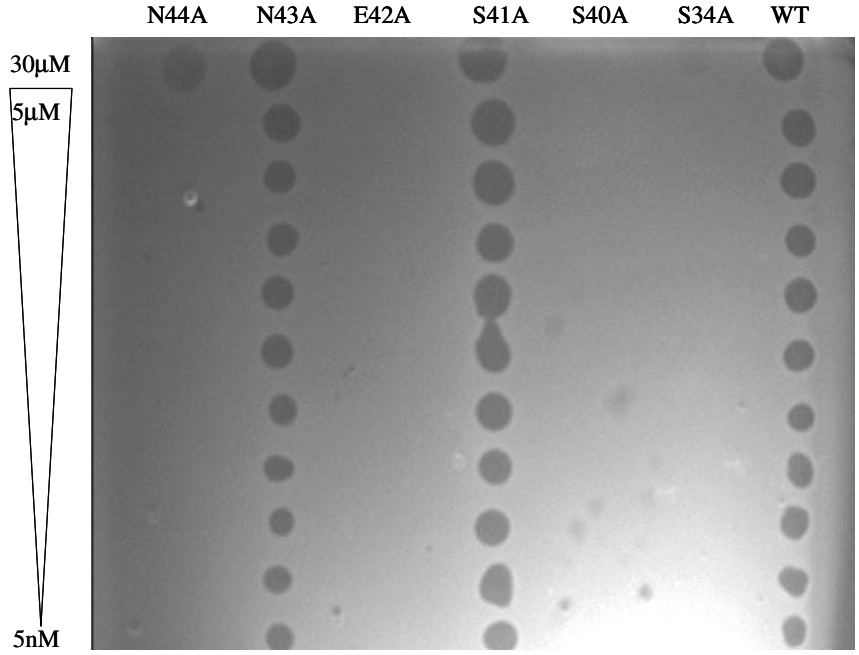


Figure 3.16 Results of a large plate spot test with purified S34A, S40A, S41A, E42A, N43A, N44A colicin E9 mutants and wild-type colicin E9.

3.2.7.4. Reporter assay to compare activity of mutants

In order to obtain a more precise comparison of the activity of the purified mutant proteins, a luminescence reporter assay was performed, which uses an SOS-inducible chromosomal *lux* operon to detect DNA damage induced by colicin E9 (Vankemmelbeke, unpublished data; Davidov *et al* 2000).

4 µl of S34A, S40A, S41A, E42A, N43A, N44A and H575A mutant colicin E9/Im9 proteins and wild-type colicin E9/Im9, at a concentration of 10 nM, were added to 100 µl aliquots of *E.coli* DPD1718 cells, giving a final concentration of ~0.4 nM colicin. The inactive colicin E9 H575A DNase mutant was used as a control. Four replicates for each mutant and the wild-type colicin were performed.

The mean gamma value was calculated for each mutant and the wild-type at the 50 minute time point (see Chapter 2, Equation 3.1). Data for each mutant are displayed in Figure 3.17 as the percentage of the mean wild-type gamma value and as expected, in addition to the control H575A mutant, the TolB box mutants (D35A, S37A and W39A) showed no activity. The S34A, S40A and E42A were also inactive, consistent with the stab and spot tests. The N44A mutant shows 3 % activity compared to wild-type, consistent with the hazy zone seen for a concentration of 30 µM of the mutant in the spot test. The S41A mutant has 26 % of wild-type activity, whilst the N43A mutant has 86 % of wild-type activity. These differences in activity of the S41A and N43A mutants compared to wild-type were not detected in the spot test.

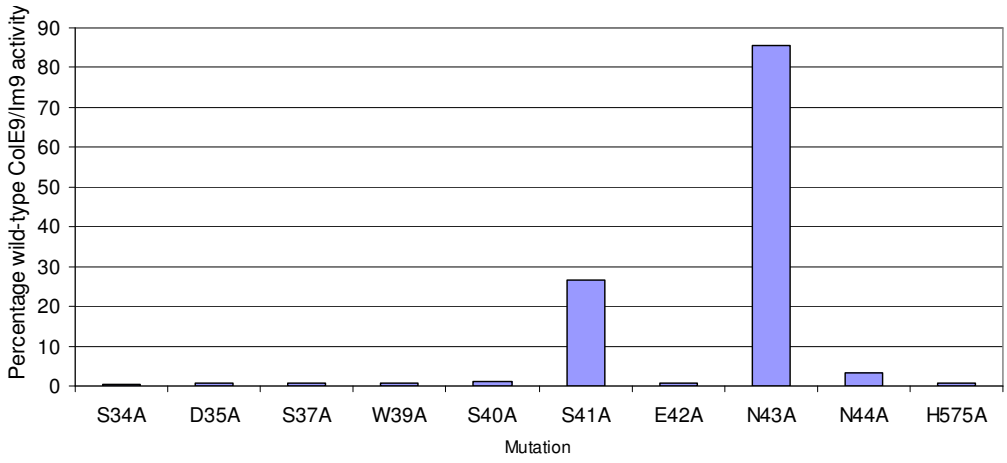


Figure 3.17 Results from a reporter assay comparing the activity of TolB box mutants and mutants surrounding the TolB box to wild-type colicin E9/Im9. The inactive H575A colicin E9/Im9 mutant was used as a control.

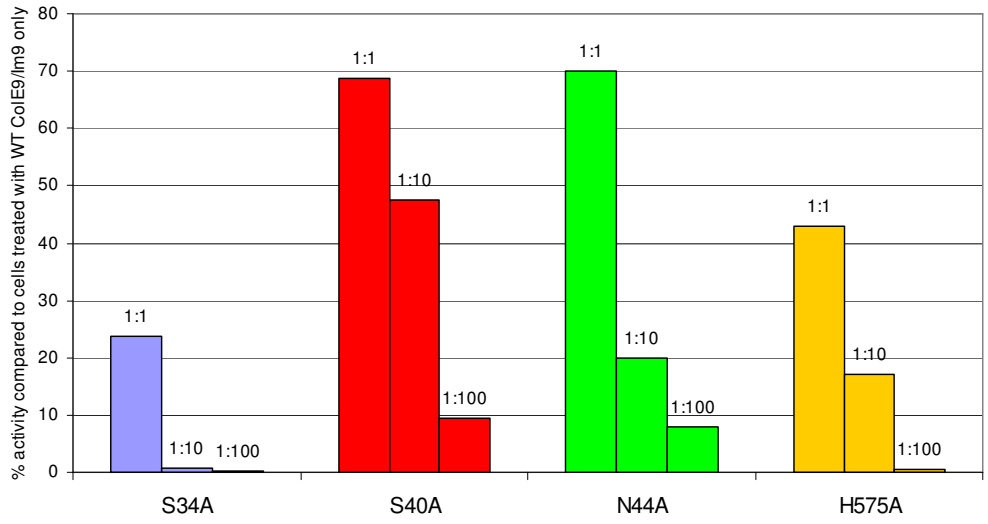


Figure 3.18 Results from a competition assay using DPD1718 reporter cells. Addition of the mutant colicins in 1:1, 1:10 and 1:100 ratios to wild-type colicin, protects the reporter cells from DNA damage caused by the wild-type colicin.

3.2.7.5. Competition assay to test receptor-binding of mutants

In order to check that the S34A, S40A and N44A mutants were intact proteins and that the mutation was not affecting the receptor-binding domain, a competition assay was performed on these mutants using the luminescence reporter system described above. This assay tests for the ability of the mutants to bind to the BtuB receptor of sensitive *E.coli* cells, thus protecting the cells from DNase damage by wild-type colicin E9.

The mutant colicin was added to cells at concentrations of 0.4 nM, 4 nM and 40 nM, in addition to 0.4 nM wild-type colicin E9. The inactive H575A DNase mutant was used as a control. Four replicates for each mutant were performed.

The mean gamma value was calculated for each concentration of mutant at the 50 minute time point (see Chapter 2, Equation 3.1). The data for each mutant are displayed in Figure 3.18 as a percentage of the mean gamma value for cells treated with 0.4 nM wild-type colicin E9 only. Figure 3.18 shows that when the S34A, S40A or N44A mutants are added to the reporter cells in a ratio of 100:1 to wild-type colicin E9, the percentage DNA damage compared to cells treated only with wild-type colicin E9, is less than 10% for all three mutants. The protection shown is similar to that shown by the control H575A mutant. This indicates that all three of these mutants are able to protect the cells from DNA damage by the wild-type colicin E9, indicating that the mutants have intact receptor-binding domains, capable of binding to BtuB. Therefore the loss of activity of the S34A, S40A and N44A mutants is not due to an inability to bind to the primary receptor, BtuB.

3.2.7.6. Kunitz assay to test DNase activity of mutants

In order to check that the loss of activity of the S34A, S40A and N44A mutants was not due to an inability to cleave DNA, a Kunitz assay was performed on the mutants. As the DNase activity of colicin E9 is inhibited by the presence of Im9, the immunity protein was removed from the mutant colicins, before the assay was carried out, by denaturation on a Hi-trap column using 6 M guanidine hydrochloride, as described in Chapter 2.

Figure 3.19 shows that all three mutants are able to degrade DNA to a similar extent to wild-type colicin E9. Therefore the loss of activity of these mutants seen in the stab test, spot test and reporter assay is not due to an inability to cleave DNA.

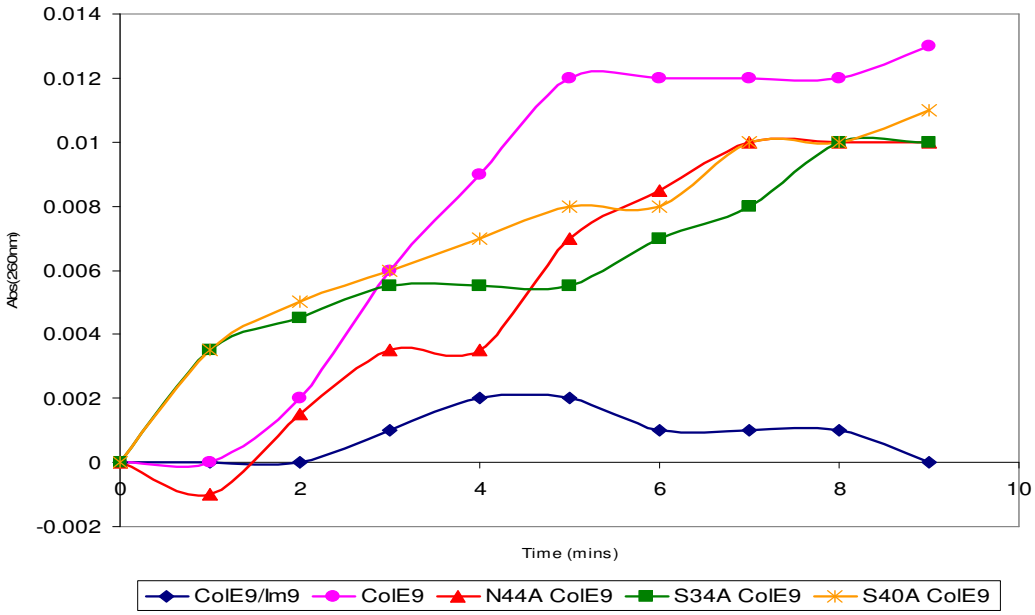


Figure 3.19 Results of a Kunitz assay performed with the free S34A, S40A and N44A colicin E9 mutants and free wild-type colicin E9. The increase in absorbance due to the mutant colicins is similar to that for wild-type colicin E9. The presence of Im9 prevents cleavage of DNA and therefore there is very little increase in absorbance due to colicin E9/Im9 (blue). Lamp fluctuations of the order of 0.001 occur over the time scale of this experiment, accounting for fluctuations in the traces.

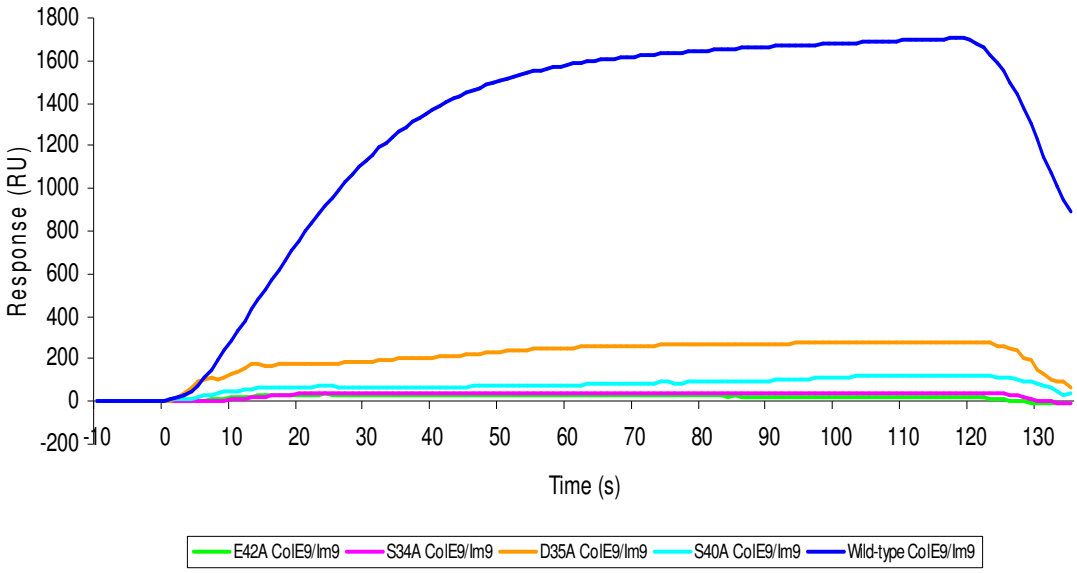


Figure 3.20 SPR sensorgrams showing reduction in binding to TolB caused by S34A, S40A and E42A mutations in colicin E9, compared to the reduction in binding caused by the D35A mutation.

3.2.7.7. SPR to test TolB binding of mutants

The binding of the colicin E9 proteins with mutations around the TolB box to TolB was measured using SPR.

Injections of 2 μM of the S34A and S40A mutants were performed across ~3000 RU TolB immobilised on a CM5 chip. Figure 3.20 shows that the binding of S34A and S40A mutants was drastically reduced compared to wild-type. The sensorgram for the D35A mutant is also shown as a reference point and demonstrates that an alanine mutation at residue 34 or residue 40 reduces binding of the colicin to TolB at least as much as a mutation in the recognised pentapeptide TolB box region.

The S41A and N43A mutants were also shown to bind to TolB, a result that was expected due to their biological activity (data not shown). However, in order to compare the affinity of these mutants for TolB to the affinity of wild-type colicin E9/Im9 for TolB, SPR was used to obtain Kd values for the binding of these mutants to TolB.

Figure 3.21 shows the corrected sensorgrams and residual plots for binding of the colicin E9 S41A mutant protein to TolB. Table 3.6 shows that the K_d for binding of

this mutant to TolB is 92.3 ± 2.5 μM, demonstrating that the affinity of the S41A mutant for TolB is significantly reduced compared to the affinity of wild-type colicin E9/Im9 for TolB. This is consistent with the reduced activity (26%) of the mutant seen in the reporter assay (see section 3.2.7.4). It can therefore be concluded that mutation of residue S41 to A, reduces biological activity of colicin E9/Im9 due to a reduction in affinity of this mutant for TolB.

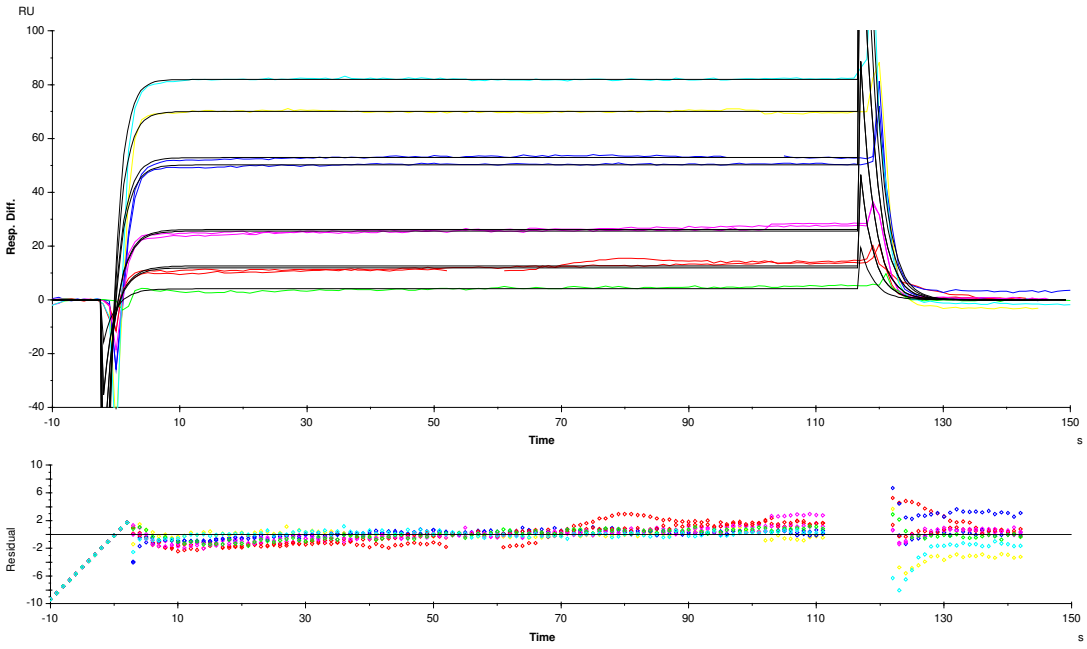


Figure 3.21 Corrected sensorgrams and residual plots for titration of 2 μM (green), 5 μM (red), 10 μM (magenta), 20 μM (blue), 30 μM (yellow) and 40 μM (cyan) S41A ColE9/Im9 against TolB at 25°C are shown. The fitted kinetic data for binding are superimposed onto each sensorgram in black. Residual plots highlight deviation of the experimental data from the theoretical fit.

Table 3.6 Kinetic data obtained from fitting of sensorgrams produced for binding of S41A ColE9/Im9 to TolB to the Langmuir 1:1 binding model, errors for K_a and K_d were calculated by combining the standard errors on k_{ass} and k_{diss} in quadrature.

kass (1/Ms)	kdiss (1/s)	Ka (1/M)	Kd (μM)	Chi2
5380 ± 140	0.496 ± .00438	10800 ± 297	92.3 ± 2.54	1.7

Figure 3.22 shows the corrected sensorgrams and residual plot for the binding of the N43A mutant to TolB. Table 3.7 shows that the K_d for binding of the N43A mutant protein to TolB is 51.5 ± 1.5 μM, indicating that the affinity of this mutant for TolB is also reduced compared to the affinity of wild-type colicin E9/Im9 for TolB, although

to a lesser extent than the S41A mutant. This is consistent with the activity of 85% of wild-type recorded in the reporter assay for the N43A mutant (see section 3.2.7.4). Therefore, it can be concluded that mutation of the N43 residue to A reduces the biological activity of ColE9/Im9 due to a reduction in affinity for TolB.

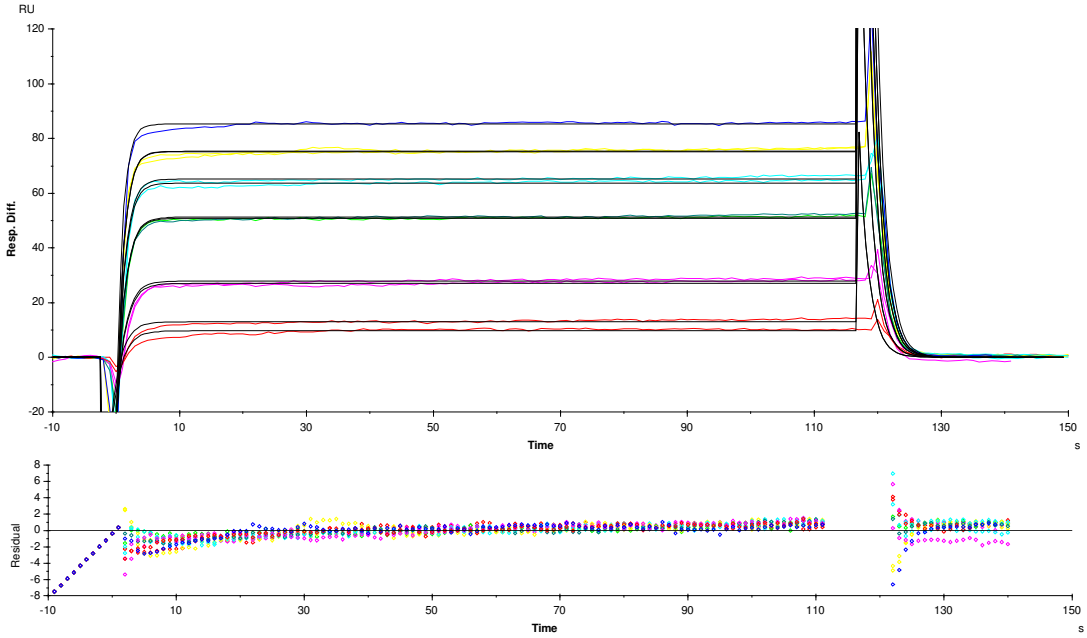


Figure 3.22 Corrected sensorgrams and residual plots for titration of 2 µM (red), 5 µM (magenta), 10 µM (green), 20 µM (cyan), 30 µM (yellow) and 40 µM (blue) N43A ColE9/Im9 against TolB at 25°C are shown. The fitted kinetic data for binding are superimposed onto each sensorgram in black. Residual plots highlight deviation of the experimental data from the theoretical fit.

Table 3.7 Kinetic data obtained from fitting of sensorgrams produced for binding of N43A ColE9/Im9 to TolB to the Langmuir 1:1 binding model, errors for K_a and K_d were calculated by adding the standard errors on k_{ass} and k_{diss} in quadrature.

kass (1/Ms)	kdiss (1/s)	Ka (1/M)	Kd (µM)	Chi2
12600 ± 309	0.649 ± 0.0097	19400 ± 556.7	51.5 ± 1.48	0.864

The binding of the N44A colicin mutant protein to TolB was also investigated. Figure 3.23 shows the corrected sensorgrams and residual plot for binding of the N44A mutant protein to TolB. Table 3.8 shows that the mutant binds to TolB with a K_d of 19.4 ± 0.2 µM. This value is very similar to the K_d value seen for the binding of wild-type colicin E9/Im9 to TolB *ie* 13.8 µM (see section 3.2.5.2). This indicates that the mutation of residue N44 to A in colicin E9/Im9 has very little effect on the binding of the colicin to TolB.

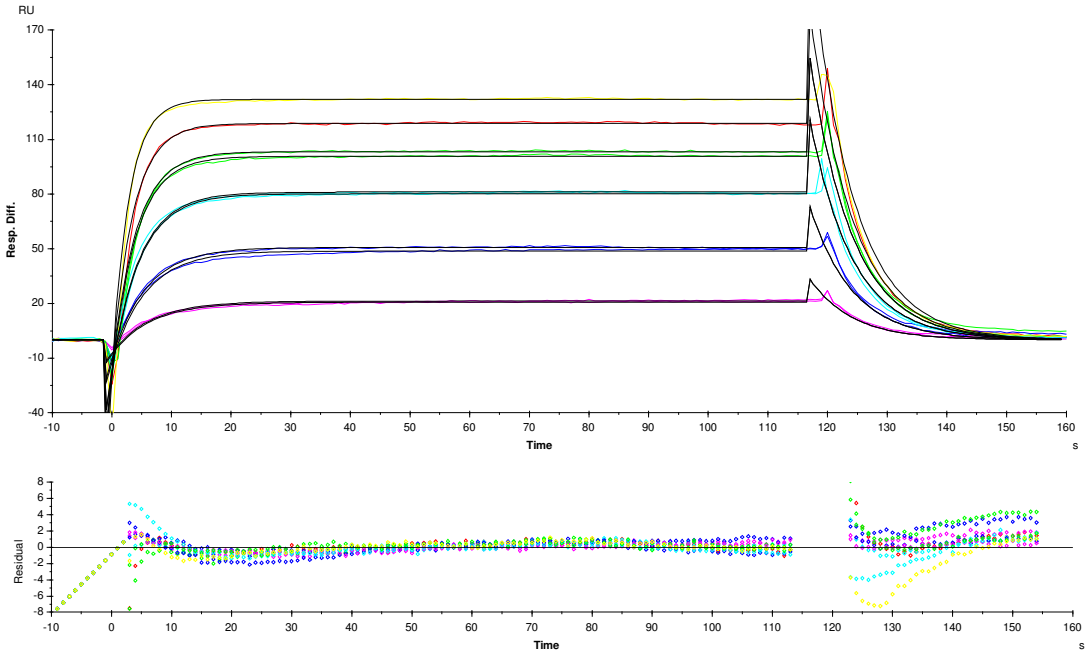


Figure 3.23 Corrected sensorgrams and residual plots for titration of 2 µM (magenta), 5 µM (blue), 10 µM (cyan), 15 µM (green) 20 µM (red) and 30 µM (yellow) N44A ColE9/Im9 against TolB at 25°C are shown. The fitted kinetic data for binding are superimposed onto each sensorgram in black. Residual plots highlight deviation of the experimental data from the theoretical fit.

Table 3.8 Kinetic data obtained from fitting of sensorgrams produced for binding of N44A ColE9/Im9 to TolB to the Langmuir 1:1 binding model, errors for K_a and K_d were calculated by combining the standard errors on k_{ass} and k_{diss} in quadrature.

kass (1/Ms)	kdiss (1/s)	Ka (1/M)	Kd (µM)	Chi2
7210 ± 69.3	0.14 ± 0.0009	51600 ± 587.6	19.4 ± 0.22	1.58

The lack of effect of the N44A mutation on binding to TolB is a surprising result considering the reduction in activity to 5% of wild-type activity observed for this mutant in the reporter assay. The ability of the mutant to bind to TolB as well as the ability to bind to BtuB and degrade DNA shown by SPR, a competition assay and a Kunitz assay respectively, indicates that the colicin E9 N44A mutation must be affecting another part of the translocation pathway, rather than the interaction between the colicin and TolB.

Further sequence analysis showed that pSR17, the plasmid from which the N44A mutant was expressed, contained a second mutation Y561C, in the DNase domain. Although the Kunitz assay showed that the colicin E9 N44A mutant had comparable

DNase activity to wild-type colicin E9/Im9, a further assay was used to confirm the DNase activity of the N44A mutant.

3.2.7.8. DNase assay with N44A mutant

This assay assesses the ability of the N44A mutant colicin E9 to hydrolyse linear DNA (see Chapter 2). Figure 3.24 shows that, in the absence of Im9, the degradation of DNA by the N44A mutant is equivalent to the DNA degradation by wild-type colicin E9. This result confirms that the N44A mutation does not affect the DNase domain and that the second mutation in pSR17 is unlikely to affect the activity of the mutant colicin E9.

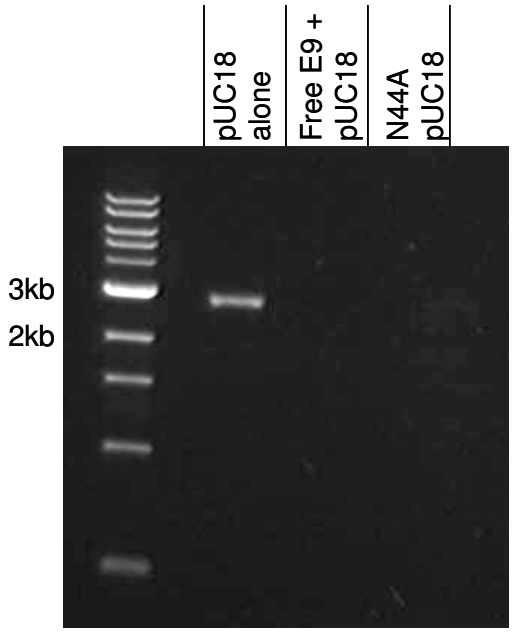


Figure 3.24 Results from a pUC18 DNase assay showing that N44A colicin E9 is able to fully degrade DNA, with equivalent efficiency to wild-type colicin E9. Due the inhibition of DNase activity by Im9, this was removed from the wild-type and mutant colicin before the assay.

3.3. Discussion

This study has used the technique of SPR as a rapid and sensitive method for quantitatively comparing the affinity of colicin E9 mutants for TolB. It has revealed some interesting results concerning the role of residues in the traditional TolB box and the involvement of residues surrounding this box in the interaction with TolB.

3.3.1. Affinity for binding of wild-type colicin E9

SPR was used to determine the kinetics for binding of wild-type colicin E9/Im9 to TolB. Prior to this study, the only published kinetic data on the interaction of a colicin with TolB was that for the interaction of the pore-forming colicin A with TolB. Isothermal titration calorimetry produced a K_d value of 10.4 ± 2.6 μM for the binding of colicin A to TolB (Gokce *et al* 2000). In this study SPR was used to determine the kinetics of binding of wild-type colicin E9/Im9 to TolB. SPR determined a K_d value for the colicin E9/Im9-TolB interaction of 13.8 ± 0.26 μM. This K_d value determined for the binding of colicin E9/Im9 to TolB is satisfyingly similar to the value determined for binding of colicin A to TolB.

3.3.2. Pentapeptide TolB box of colicin E9

3.3.2.1. Colicin E9 D35A, S37A, W39A mutants

As expected, from their loss of activity and yeast-two hybrid data, the D35A, S37A and W39A colicin E9/Im9 mutants showed very low binding to TolB in SPR analysis. This confirms that the D35, S37 and W39 residues are crucial for binding of colicin E9 to TolB.

3.3.2.2. Colicin E9 G36A, G38A mutants

In contrast to yeast-two hybrid data, the binding of the G36A and G38A mutants to TolB was also reduced in SPR experiments. Due to the use of these colicin mutants in the absence of the immunity protein in the yeast-two hybrid experiments, the immunity protein was removed from the G36A and G38A mutants and the binding to TolB was re-measured by SPR.

G36A

Although the binding of the colicin E9 G36A mutant to TolB was reduced compared to wild-type colicin E9/Im9, when the immunity protein was removed from the G36A mutant, enhanced binding to TolB was observed, compared to binding of G36A colicin E9/Im9 and binding of wild-type colicin E9/Im9.

The shape of the sensorgram for binding of free G36A colicin E9 to TolB was altered compared to the binding of wild-type colicin E9/Im9 to TolB, indicating that either the G36A mutation or the removal of Im9, or a mixture of the two, was affecting the colicin E9 binding site for TolB.

The kinetic parameters for binding of the G36A colicin E9/Im9 mutant were then determined. Due to the decreased binding of the G36A mutant in the presence of Im9 observed in the preliminary experiment, compared to wild-type colicin E9/Im9, higher concentrations of this mutant were injected across the TolB chip. Fitting of the sensorgrams produced to the Langmuir 1:1 binding model, produced a K_d of 45.1 ± 1.1 μM. This K_d value confirms that the affinity of G36A colicin E9/Im9 for TolB is lower than the affinity of wild-type colicin E9/Im9 for TolB. Therefore, the sensitivity of the SPR technique has permitted the detection of a difference between the affinity of wild-type colicin E9/Im9 and G36A colicin E9/Im9 mutant for TolB. The reduction in affinity of G36A colicin E9/Im9 for TolB is consistent with the reduction in activity seen in the spot test for this mutant. Previously it has been reported that the activity of the G36A colicin E9 mutant is the same as the activity of wild-type colicin E9 (Garinot-Schneider *et al* 1997) but it should be noted that this observation is only based on a relatively insensitive stab test and no spot test with the purified mutant protein has previously been performed.

In order to investigate the effect of the immunity protein on the G36A mutant, SPR was used in an attempt to determine the kinetic parameters for the binding of the free G36A colicin E9 mutant to TolB. However, fitting of the sensorgrams to the Langmuir 1:1 model produced a very poor fit, with a high Chi squared value and low T-values. Therefore fitting to other, more complex, models was attempted. The best fit was to a two-state conformational change model, handling a situation in which the ligand and analyte form a complex on the sensor surface and the complex then

undergoes a conformational change. The affinity constant (K, *ie* [k_{diss1}/k_{ass1}] x [k_{diss2}/k_{ass2}]) for the formation of the complex, including the conformational change, is 1.24 ± 0.12 μM. Therefore the affinity of the colicin E9 G36A mutant protein for TolB is greater in the absence of Im9 than in the presence of Im9.

It is possible that the reduction in affinity of the colicin E9 G36A mutant for TolB when Im9 is present is due to the immunity protein preventing the conformational change, required to form a stable complex between the colicin and TolB. The effect of the immunity protein on binding of colicins to TolB, is investigated further in Chapter 4.

G38A

The affinity of G38A colicin E9 for TolB was shown to be low in the presence and absence of Im9. This is consistent with the reduced activity of this mutant observed in the spot tests. The activity of G38A colicin E9 mutant protein in the presence of Im9 was significantly reduced compared to wild-type colicin E9. Although the activity of wild-type colicin E9 in the spot test was not affected by the presence or absence of Im9, consistent with previous observations (Schaller and Nomura 1976, Wallis *et al* 1992a), the activity of the G38A mutant protein was reduced even further in the absence of Im9. Previous data regarding the activity of the G38A mutant has been obtained using the stab test, with the expressed colicin protein attached to Im9 (Garinot-Schneider *et al* 1997). No information has been published on the activity of the purified mutant protein or on the comparison of the activity of the mutant in the presence or absence of the immunity protein.

The ability of the free G38A mutant to degrade DNA to a similar extent to wild-type colicin E9, shown by the Kunitz assay, indicates that there is no gross defect in the three-dimensional structure of the protein. It is possible that the immunity protein stabilises the G38A mutant, prior to cell entry. A stabilising role has been proposed for Im3, as the activity of free colicin E3 is significantly reduced compared to the activity of colicin E3/Im3 (Walker *et al* 2003). Although Im9 does not affect the activity of wild-type colicin E9, it is possible that the G38A mutation destabilises the colicin, introducing a requirement for the stabilising effect of the immunity protein.

Due to the reduced binding to TolB of the G38A mutant in the presence and absence of Im9, it was not possible to determine kinetic parameters for the interaction. At concentrations of 10 μM G38A colicin E9/Im9, a small response is detected due to

binding of the mutant to TolB. Therefore, although the affinity of the G38A mutant for TolB appears to be much lower than wild-type, the affinity may be high enough to enable detection of an interaction in the yeast-two hybrid screen. It is generally accepted that an interaction with a $K_d < 1\mu M$ is required for a positive result in a yeast-two hybrid screen (Estojak *et al* 1995). However the SPR data obtained for the affinity of the G36A mutant for TolB show that the K_d is much greater than 1 μM and yet this protein still produces a positive result in the yeast-two hybrid screen. Therefore, it would appear that the yeast-two hybrid screen used may have the ability to detect interactions with a K_d greater than 1 μM. The yeast-two hybrid screen used the translocation domain of colicin E9 fused to the binding domain of GAL-4. The use of full-length colicin E9 mutants in the SPR study should produce more accurate information about the *in vivo* interaction between colicin E9 and TolB, particularly if regions other than the translocation domain affect binding to TolB. The absence of the receptor-binding domain, immunity protein and DNase domain in the yeast-two hybrid screen may increase the affinity of the colicin for TolB (see Chapter 4), with the binding domain of GAL-4 stabilising the conformation of the colicin domains, which may explain the discrepancy between the yeast-two hybrid and SPR results.

Role of glycine residues in the TolB box

This study is the first time that an effect on binding to TolB, of mutating glycine residues 36 and 38 in colicin E9 to alanine, has been observed. However, as this study also presents the first quantitative method to compare the affinities of colicin E9 mutants for TolB, this is perhaps less surprising. The yeast-two hybrid technique is only able to provide a positive or negative result according to whether an interaction occurs or not between two proteins, whereas the SPR technique is able to quantitatively compare affinities of mutated colicin proteins for TolB.

It is interesting to note that when the proposed and known TolB box sequences of TolB-dependent colicins are compared, it is the two glycine residues, at positions 36 and 38 of colicin E9, which are most conserved (see Figure 3.1). The N-terminal region of the translocation domain of colicin E9 is very rich in glycine residues, with the first 80 residues containing 34 glycine residues, corresponding to a glycine content of 43%. Some of these glycine residues are also clustered into triple glycine repeats (Soelaiman *et al* 2001). As discussed earlier (see section 3.1.2) the N-

terminal regions of the translocation domain of colicin E3 and N are flexible and the high glycine content contributes to this flexibility. As extensive unfolding is likely to occur during translocation of the colicin translocation domain across the outer membrane, it is reasonable to assume that the translocation domain is a flexible peptide when it becomes exposed to TolB. A parallel scenario is seen for the TonB box of Ton-dependent receptors, which changes from a helical to an extended conformation upon binding of the ligand to the receptor (Locher *et al* 1998, Vakharia and Postle 2002). Therefore it has been suggested that interaction of Ton or Tol domains with other proteins requires a flexible peptide (Anderluh *et al* 2003). Mutation of a glycine residue to an alanine residue, has the potential to reduce flexibility, as addition of the methyl group causes more steric hindrance than a hydrogen atom (Stryer 1995), and it is therefore possible that mutation of glycine residues 36 and 38 in colicin E9/Im9 to alanine reduces the flexibility of the TolB box region, reducing the ability of the colicin to bind to TolB.

Steady-state and time resolved fluorescence resonance energy transfer (FRET) and NMR have demonstrated that the translocation domain of colicin N undergoes gross conformational changes on binding to TolA (Anderluh *et al* 2003). CD, fluorescence and SPR have recently shown that the translocation domain of colicin N acquires cooperatively folded secondary and tertiary structure in the colicin N-TolA complex (Anderluh *et al* 2004).

The broadening of resonances in the ^{1}H-^{15}N HSQC NMR spectrum of the translocation domain of colicin E9 bound to TolB, indicates that the backbone segmental motions of residues 40-44 could be reduced due to binding to TolB (Collins *et al* 2002). This suggests that this region becomes less flexible on binding TolB, potentially due to the formation of structure, in a similar manner to that seen for binding of colicin N to TolA, although the structure induced would be localised to the TolB box region, as not all resonances in the N-terminal region of colicin E9 are perturbed on addition of TolB (Collins *et al* 2002). The structure induced in colicin E9 by binding to TolB, could allow the binding of colicin E9 to other proteins of the Tol/Pal system such as TolA and TolR (see Chapter 5 for further discussion regarding interactions of colicin E9 with Tol proteins).

It would be interesting to investigate the effect of mutating glycine residues 36 and 38 to a residue which would reduce the flexibility of the backbone further, such as

proline, to supply further information on the role of these glycine residues in providing flexibility of the TolB box region, required for binding of colicin E9 to TolB.

3.3.3. Revertant mutants

The lack of published information on the role of the residues in the pentapeptide TolB box, led to mutation of residues 35, 37 and 39 from alanine to any other residue. Stab tests and DNA sequencing were used to identify residues that can restore colicin activity (Holland 2003). In this study, SPR has been used to investigate the direct effect on binding to TolB of replacing the wild-type residue with a residue that restores colicin activity.

3.3.3.1. Residue 35

The random mutagenesis experiment showed that apart from the wild-type residue (aspartate), serine could also be tolerated at position 35. Mutation of residue 35 from alanine to serine restored the level of activity in a spot test to a comparable level to wild-type. SPR kinetic analysis has shown that the K_d for binding of the A35S colicin E9 mutant to TolB is 12 μM, a comparable value to the K_d for binding of wild-type colicin E9/Im9 to TolB (13.8 μM). Therefore the SPR data confirm that a serine residue at position 35 of colicin E9/Im9 does not affect the ability of the colicin to bind to TolB.

The wild-type residue at position 35 is aspartate, a polar, acidic amino acid (see Figure 3.25). Serine is also a polar residue but is uncharged and smaller than aspartate (see Figure 3.25). Both aspartate and serine residues are able to form hydrogen bonds.

Asparagine or threonine residues were not tolerated at position 35 (Holland 2003). In asparagine and threonine, the partial negative charge of the OH group is less accessible than in serine and aspartate. Therefore it has been proposed that it is the OH group that is important for the function of residue 35, with the orientation and the accessibility of the OH group being important to form a hydrogen bond, either with another residue of colicin E9 or with TolB (Holland 2003).

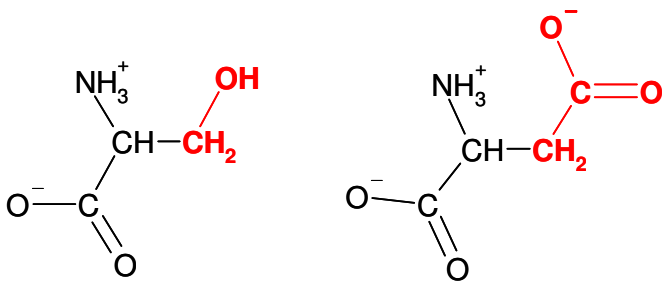


Figure 3.25 The structures of serine (left) and aspartate (right). Aspartate has a pKa of 4.5 and therefore in physiological systems does not carry the hydrogen of the carboxylic acid group.

3.3.3.2. Residue 37

Replacing the alanine at position 37 with serine (wild-type residue), valine or threonine restored the activity of the colicin (Holland 2003). The presence of a threonine residue at position 37 slightly reduced the activity of the colicin in a spot test, compared to wild-type. The slightly increased K_d for the binding of the A37T mutant to TolB (24.3 ± 1.2 μM), compared to the value for wild-type colicin E9/Im9, is consistent with this reduction in activity. Threonine is the residue found at the equivalent position of the TolB box of colicin A and this residue is the only difference between the TolB box of colicin A and colicin E9 (see Figure 3.1). It is therefore interesting that the presence of this residue, at least in colicin E9, reduces the affinity of the colicin for TolB. Chapter 5 of this thesis compares the affinity of colicin A and colicin E9 for TolB and shows that the affinity of colicin A for TolB is reduced compared to the affinity of colicin E9 for TolB. It is therefore tempting to speculate that the reason for this reduced affinity is the presence of a threonine residue instead of a serine residue in the TolB box of colicin A (see Chapter 5 for further discussion). The A37V mutant had the same activity as wild-type in a spot test, consistent with a K_d for binding of the mutant to TolB of 12.2 ± 0.2 μM, a value very similar to the K_d for wild-type colicin E9/Im9. The lack of a hydroxyl group on the valine side chain (see Figure 3.26) suggests that the hydroxyl group of serine and threonine is not important for function of the residue at position 37. The substitution of two non-hydrogen groups at the C-β carbon in both valine and threonine, which restricts mobility of the protein backbone, also suggests that flexibility of the backbone is not important for the function of the residue at position 37 (Holland 2003).

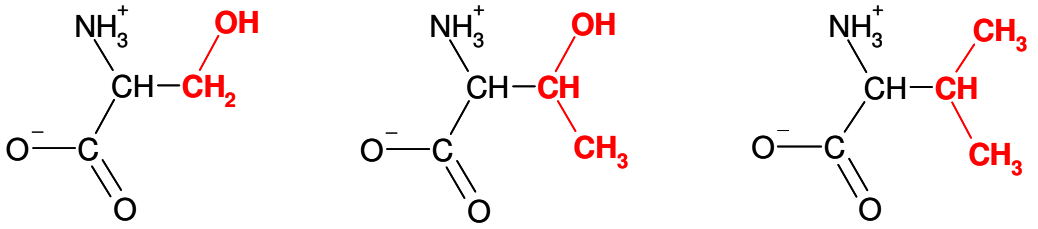


Figure 3.26 **Structures of serine (left), threonine (middle) and valine (right).**

3.3.3.3. Residue 39

The residues at position 39 that restored activity were the aromatic residues, tryptophan (wild-type residue), phenylalanine and, to some extent, tyrosine. The low affinity of the A39Y mutant for TolB seen in the SPR experiment is consistent with the reduced activity of this mutant in a spot test.

Although tryptophan and phenylalanine are hydrophobic residues and are therefore generally found buried within proteins, tyrosine is only partially hydrophobic with a polar hydroxyl group (see Figure 3.27). This reactive polar hydroxyl group may hinder the interaction of the colicin with TolB.

As the only common feature of tryptophan, phenylalanine and tyrosine is the aromatic ring, it has been proposed that residue 39 may be involved in an interaction, involving stacking of π orbitals of adjacent aromatic rings (Holland 2003). Residue 39 of colicin E9 may interact directly with two proline rings encircling the central channel of the β-propeller of TolB or with aromatic residues on the surface of TolB such as W208, F252 and W296 that are conserved in TolB proteins from different bacteria (Holland 2003). However, mutagenic analysis of residues in TolB is required to investigate the role of these proline and aromatic residues in TolB.

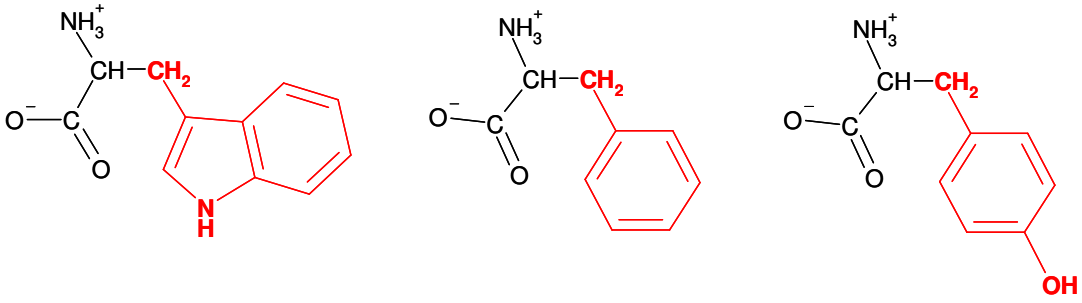


Figure 3.27 **Structures of tryptophan (left), phenylalanine (middle) and tyrosine (right).**

3.3.4. TolB box extension

Following initial analysis of the activity of the S34A and S40 colicin E9 mutants using a stab test (Garinot-Schneider *et al* 1997), this study further investigated the effect of the S34A and S40A mutations on the activity of colicin E9 in stab tests and spot tests and has shown that these mutations abolish the activity of the colicin. The S34A and S40A mutant proteins were shown to have comparable receptor-binding and DNase activity to wild-type colicin E9. SPR with TolB immobilised on a sensor chip has shown that the reason for the loss of activity of these mutants is their failure to bind to TolB. This indicates that the TolB box should be extended to include at least one residue upstream and one residue downstream of the pentapeptide TolB box. The loss of activity of the E42A mutant due to loss of TolB binding (Holland 2003) is consistent with this proposal and indeed suggests that the TolB box should be extended further, at least as far as residue 42.

Mutation of residue S41A significantly reduces the activity of colicin E9. SPR has shown that the reason for this reduction in activity is a decreased ability of this mutant to bind to TolB, with a reduced affinity of the S41A mutant for TolB (K_d = 92.3 ± 2.5 μM) compared to the affinity of wild-type colicin E9/Im9 for TolB (K_d = 13.8 ± 0.3 μM).

Mutation of residue N43A also partially reduces the activity of colicin E9 and SPR has shown that this reduction in activity is due to a decreased affinity of the mutant for TolB compared to wild-type colicin E9/Im9 (K_d = 51.5 ± 1.5 μM vs K_d = 13.8 ± 0.3 μM).

This indicates that residues S41 and N43 are involved in binding to TolB, but that they may increase the affinity of the interaction indirectly, as mutation of these residues does not completely abolish TolB binding. It is possible that the side chains of residues S41 and N43 are involved in interacting with side chains of other residues in the TolB box region, creating a "structural unit" and hence increasing the affinity of colicin E9 for TolB. This is consistent with the suggestion by Collins *et al* (2002) that the side-chains of residues S40, S41 and E42 interact with each other, based on the identification of two resonances for these residues in the ^{1}H-^{15}N HSQC NMR spectrum. It would be interesting to perform a similar random mutagenesis analysis to that performed on residues 35, 37 and 39, on residues S40 – N43 to investigate the involvement of these residues in side-chain interactions.

Mutation of residue N44A abolishes activity of colicin E9, shown in a stab test and a spot test, and drastically reduces DNase activity, identified by the reporter assay. The N44A mutant colicin E9 has been shown to be intact in its receptor-binding domain and in its DNase domain. The affinity of the N44A mutant for TolB has been shown by SPR to be comparable to the affinity of wild-type colicin E9/Im9 for TolB (19.4 ± 0.2 μM vs 13.8 ± 0.3 μM). This shows that the drastic reduction in activity of this mutant is not due to a loss of binding to TolB and therefore indicates that the C-terminal boundary of the TolB box may be formed by this residue.

The lack of effect on binding of colicin E9/Im9 to TolB caused by the N44A mutation, along with the demonstration that the receptor-binding and DNase domains of the mutant are intact, indicates that residue N44 is involved in some other aspect of translocation of colicin E9. The residue is conserved between the endonuclease colicins but is not conserved in pore-forming colicins, raising the possibility that the residue is involved in part of the translocation mechanism of endonuclease colicins that differs from that for pore-forming colicins.

However, residues 43 - 45 (NNP) of colicin E9 are homologous to residues 103 – 105 of colicin A, suggesting the possibility that these residues are important in another aspect of translocation. The residues of colicin A lie just outside the region predicted to be involved in interacting with TolA.

3.3.5. Use of alanine mutagenesis

The use of site-directed mutagenesis is now widespread, providing information about the role of side-chain functional groups in protein structure, folding and protein-ligand interactions. However, a general concern is that replacement of the wild-type residue with another residue can result in a structural change in the protein, rendering the protein inactive, without specifically affecting the protein-ligand interaction. Although this is possible for the mutations made in and around the TolB box, the lack of structure in this domain makes it unlikely. In addition, wild-type residues have been mutated to alanine, which should not increase the flexibility of the protein, although as we have seen with the G36A and G38A mutations, a decrease in flexibility has the potential to alter the interaction with TolB (Morrison and Weiss 2001). The introduction of alanine upstream and downstream of the TolB box has no effect on the activity of the protein, indicating that alanine residues can be tolerated in

the region of interest unless a specific side chain is required (Garinot-Schneider *et al* 1997, Christopher Penfold, personal communication).

3.4. Summary

The work in this chapter provides further insight into the interaction between TolB and colicin E9. Kinetic parameters for the interaction have been determined, providing a value for the K_d for the interaction of 13.8 μM.

The data presented here confirm previous observations that residues D35, S37 and W39 are required for binding of colicin E9 to TolB. These residues appear to be required for different reasons. The data supports the suggestion that the tryptophan residue at position 39 could form stacking interactions with aromatic residues in TolB. The aspartate residue at position 35 may be involved in hydrogen bonding with another residue in colicin E9 or a residue in TolB, whilst the serine at position 37 is likely to be required to maintain local structure of the TolB box.

In contrast to previous data, glycine residues at positions 36 and 38 have also been shown to be important for binding of colicin E9 to TolB. These residues are likely to be important in maintaining flexibility of the TolB box.

Data presented in this chapter also suggest that TolB box should be extended to include residues S34, S40, S41, E42 and N43. The apparent lack of involvement of residue N44 in binding to TolB implies that this may be the C-terminal boundary of the TolB box, although mutagenic analysis of residues further downstream of residue 44 is required to confirm this. The N-terminal boundary of the TolB box may be formed by residue 34 but again further mutagenic analysis upstream of this residue is required to establish the N-terminus of the TolB box.

CHAPTER 4 – Effect of immunity proteins on binding of colicin E9 and colicin E3 to TolB

4.1. Introduction

4.1.1. Affinities of colicin E9 and E3 for Im9 and Im3.

The K_d for the interaction of Im9 with the colicin E9 DNase domain of 9.3 x 10^{-17} M, makes the interaction one of the highest affinity protein-protein interactions in nature (Wallis *et al* 1995a). The high affinity of the interaction is implied in the requirement for denaturation of the colicin E9/Im9 complex in order to separate the immunity protein from the colicin.

The affinity of the immunity protein for the DNase domain of colicin E9 is very similar to the affinity of the immunity protein for full-length colicin E9 (Wallis *et al* 1995). Therefore, it has been assumed that the only region of colicin E9 that makes energetically important contacts with Im9 is the DNase domain.

However, the affinity of full-length colicin E3 for Im3 is greater than the affinity of the RNase domain for Im3 (Walker *et al* 2003). The increased affinity of the full-length colicin for Im3 has been attributed to interactions between Im3 and the translocation domain of colicin E3, based on the crystal structure of the colicin E3/Im3 complex (Soelaiman *et al* 2001).

4.1.2. Non-cognate immunity proteins

Although each immunity protein is specific for its cognate colicin, interactions have been detected between colicins and non-cognate immunity proteins (Wallis *et al* 1995b). Comparison of the *in vivo* and *in vitro* cross-reactivities of colicin E9 with its cognate immunity protein, Im9, and the non-cognate immunity proteins, Im2, Im7 and Im8 (from DNase colicins) has shown that the affinities of the protein-protein interactions are spread over a wide range (Wallis *et al* 1995b). The order of cross-reactivity *in vivo,* determined by the level of protection provided by transformation of *E.coli* cells with a vector containing a cloned immunity gene, was Im9 >> Im2 > Im8 with Im7 providing no protection (Wallis *et al* 1995b). The *in vitro* binding affinities of each immunity protein for the DNase domain of colicin E9 were also determined and the K_d values for Im2, Im8 and Im7 were 10^{-8} M, 10^{-6} M and 10^{-4} M respectively,

corresponding to the *in vivo* cross-reactivity data. The difference in K_d was shown to be due to increased dissociaton rates of the non-cognate immunity proteins (Wallis *et al* 1995b). Therefore, although non-cognate immunity proteins were able to bind to the colicin, they were not able to make specific interactions and rapidly dissociated from the colicin (Kleanthous *et al* 1998).

The K_d for binding of an immunity protein to a colicin must be less than 10^{-10} M in order for the immunity protein to provide complete biological protection and complexes with K_d values of 10^{-6} M or greater provide no biological protection (Li *et al* 2004).

4.1.3. Effect of Im9 on the translocation domain of colicin E9

The addition of Im9 did not perturb the chemical shifts or the T_2 relaxation times of the peaks in the ^{1}H-^{15}N HSQC spectrum of colicin E9 (Collins *et al* 2002). This indicates that the translocation region of colicin E9 is not affected by binding of Im9 to the colicin.

4.1.4. Effect of receptor-binding and cytotoxic domains on the translocation domain of colicins

4.1.4.1. Binding of the translocation domain of colicin N to TolA

Isothermal titration calorimetry has been used to determine a K_d for binding of colicin N to TolA of 18 μM (Raggett *et al* 1998). However, the K_d for binding of the isolated colicin N translocation domain to TolA was 1 μM and the K_d for binding of the isolated translocation and receptor-binding domains was 8μM. Therefore, the affinity of colicin N for TolA is reduced in the presence of the pore-forming and receptor-binding domains. No binding of the isolated pore-forming and receptor-binding domains to TolA was detected.

4.1.4.2. Binding of colicin A to TolA and TolB

Although colicin A requires both TolA and TolB for translocation, no interaction of colicin A with TolA was detected by isothermal titration calorimetry (Gokce *et al* 2000). However, binding of the isolated translocation domain of colicin A to TolA has been detected by SPR (Derouiche *et al* 1997). Gokce *et al* (2000) suggest that

this may be due to the TolA site of colicin A being masked by the rest of the protein in the full-length colicin A, in a similar way to the TolA site of colicin N (see above). The observation that colicin A translocation can be accelerated by unfolding the colicin with urea, also suggests that the sites for binding to Tol proteins, are partially occluded by the rest of the protein in the native state (Bénédetti *et al* 1992).

4.1.4.3. Structure of colicin Ia

The structure of colicin Ia, indicates that the TonB box is located at the end of an extended N-terminal peptide which interacts along one surface of the antiparallel helical sheet formed by the translocation domain (Wiener *et al* 1997). This peptide is buried within the structure of the colicin, at the top of the receptor-binding domain, sandwiched between the cytotoxic and translocation domains. The lack of electron density for the first 83 residues of colicin E3 prevents identification of the position of the TolB box of this colicin. However, the similarity of the colicin Ia and colicin E3 structures suggest that the TolB box could potentially be buried between the translocation and cytotoxic domains of colicin E3.

4.1.5. Effect of Im9 on binding of G36A colicin E9 to TolB

The effect of Im9 on binding of the G36A colicin E9 mutant to TolB has been described in the previous chapter of this thesis. Removal of the immunity protein from the G36A mutant, appeared to increase the affinity of the mutant for TolB. Although the sensorgrams produced for the binding of G36A colicin E9/Im9 to TolB fitted to the simple 1:1 Langmuir binding model, the sensorgrams produced for the binding of the free G36A colicin E9 mutant were an altered shape and did not fit to simple models. The sensorgrams did fit to a two-state conformational change model, indicating that removal of the immunity protein affects the conformation of the translocation domain, affecting the binding of the TolB box to TolB.

4.1.6. Aims of Chapter

The intriguing effect of the immunity protein on binding of the G36A colicin E9 mutant to TolB, reported in the previous chapter, has led to further investigation of the effect of the immunity protein on binding of colicin E9 to TolB using dynamic force spectroscopy and surface plasmon resonance. Unlike colicin E9, the structure of the colicin E3/Im3 complex is known and Im3 has been shown to interact with the

translocation domain as well as the translocation domain. Therefore the effect of Im3 on binding of colicin E3 to TolB is also investigated.

The reason for the effect of the immunity protein on binding of these colicins to TolB is also explored.

4.2. Results

4.2.1. Purification and verification of free colicin E9

The presence of a polyhistidine tag at the end of Im9 allows the purification of colicin E9, free from Im9, by denaturation of the colicin E9/Im9 complex on a Ni-affinity column using 6 M guanidine hydrochloride. This results in the elution of free colicin E9 with Im9 remaining bound to the column. Following denaturation, colicin E9 was refolded by extensive dialysis against potassium phosphate buffer pH 7.4. Renaturation of free colicin E9 by dialysis has previously been shown to be effective by fluorescence spectroscopy and by the demonstration that the dialysed colicin regains full biological activity (Wallis *et al* 1992a).

To ensure that the purification procedure had removed all immunity protein from the colicin E9 protein, the protein was run on an SDS-PAGE gel against the colicin E9/Im9 complex. The gel was silver stained to increase the detection sensitivity, compared to staining with Coomassie blue (see Chapter 2). Figure 4.1 shows that the band around 10 kDa, corresponding to Im9, is absent from the lane containing free colicin E9, confirming that the colicin is free from immunity protein.

4.2.2. Purification of Im9

Following denaturation of the colicin E9/Im9 complex and elution of free colicin E9, Im9 can be eluted with imidazole. However, Figure 4.2 shows that the eluted Im9 is not completely pure and is contaminated with at least one other protein. Therefore Im9 was cloned separately into pET21d to enable production of pure Im9 protein. PCR was used to amplify the Im9 gene from pNP57 (a pET21a clone containing colicin E9 with a W39A mutation and Im9) using primers H52 and DW22 (see Chapter 2). The PCR product was restricted with *Xho*I and *Nco*I and then ligated with pET21d, restricted with the same restriction enzymes. The ligation product was then used to transform *E.coli* DH5α cells. The purified plasmid (pSR10) from the DH5α cells was sequenced to confirm that the plasmid encoded wild-type Im9 and then used to transform *E.coli* ER2566 cells. IPTG was used to induce expression of the Im9 protein in a large scale culture of the ER2566 cells (see Figure 4.3). Cells were harvested by centrifugation and the mutant protein was purified using nickel affinity chromatography as described in Chapter 2. The purified protein was dialysed in 50

mM potassium phosphate buffer, pH 7.4 and run on an SDS-PAGE gel to check for purity.

As can be seen from Figure 4.4, pure protein has been produced by the nickel affinity chromatography, but the band on the gel corresponds to an apparent size of around 6 kDa compared to the expected 10 kDa, although it is the same size as the Im9 band in the ColE9/Im9 complex sample on the same gel.

To ensure that the purified Im9 protein was biological active, a Kunitz assay was performed to test whether the purified Im9 could protect DNA from being degraded by wild-type colicin E9. The DNase activity of free colicin E9, colicin E9/Im9 (purified complex) and free colicin E9 pre-incubated with purified Im9 was measured. Figure 4.5 shows that the purified Im9 prevents colicin E9 degrading DNA, as the absorbance trace for the free colicin E9 with purified Im9 added is very similar to the absorbance trace for colicin E9/Im9 purified as a complex.

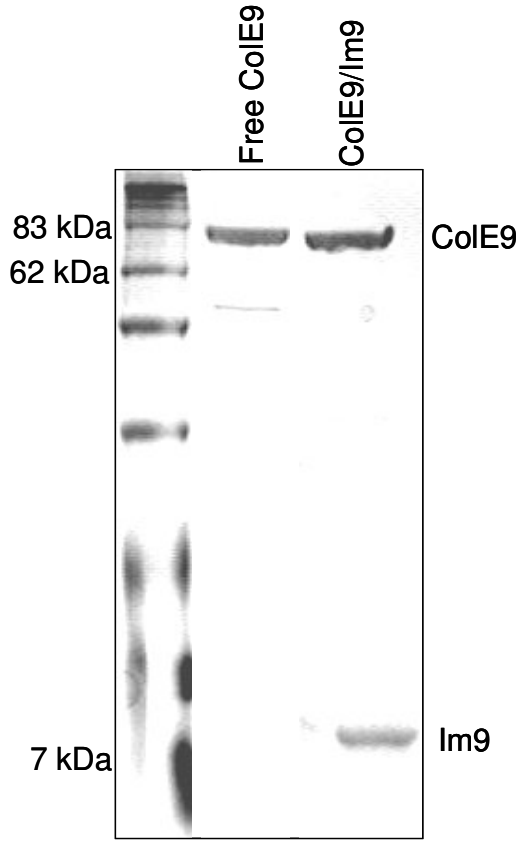


Figure 4.1 Silver-stained 16 % SDS-PAGE gel comparing free ColE9 with no band at 10 kDa to ColE9/Im9 complex with a clear band at 10 kDa.

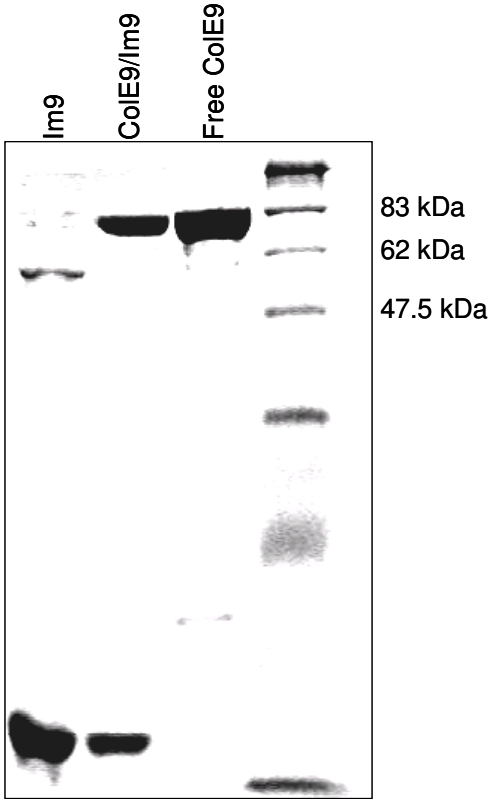


Figure 4.2 Coomassie blue stained 12 % SDS-PAGE gel showing Im9 produced from denaturation of the colicin E9/Im9 on a Hi-trap column and then elution with imidazole. The gel shows that the Im9 produced is contaminated with at least one protein.

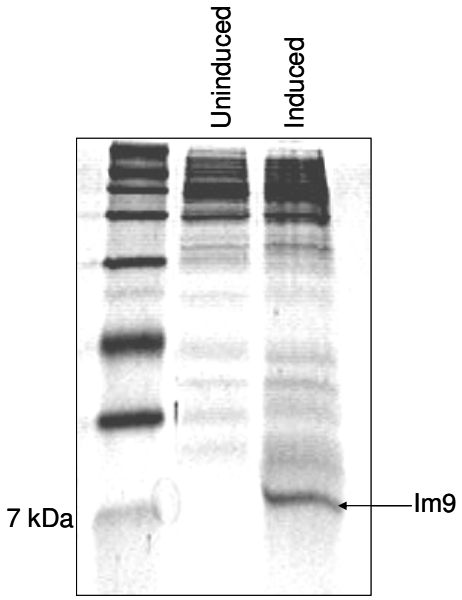


Figure 4.3 16 % Coomassie-stained SDS-PAGE gel showing the expression of Im9 protein from pSR10.

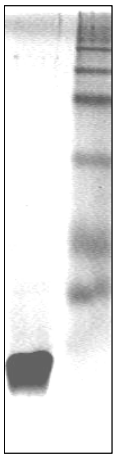


Figure 4.4 **16 % Coomassie-stained SDS-PAGE gel showing purified Im9. The protein runs at an apparent molecular weight of approximately 6 kDa, smaller than its predicted molecular weight of 10 kDa.**

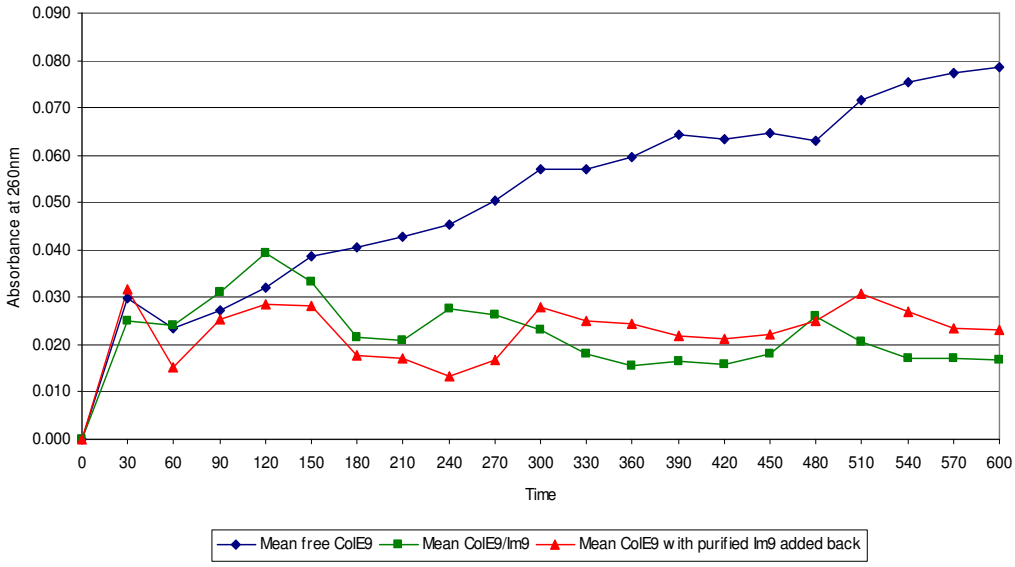


Figure 4.5 **Results from a Kunitz assay measuring DNA degradation activity of free colicin E9, colicin E9/Im9 and free colicin E9 with purified Im9 added back.**

4.2.3. Purification of T_{61}-DNase

The colicin E9 fusion protein, T_{61}-DNase, consisting of the first 61 residues of the translocation domain of colicin E9 fused to the DNase domain of colicin E9, was expressed from BL21 (DE3) cells transformed with plasmid pNP330, a pET21a-based plasmid encoding the T_{61}-DNase/Im9 construct. The T_{61}-DNase protein was purified in complex with Im9, using the same method set out for full-length colicin E9/Im9.

The construct was also purified free from Im9, using the denaturation method set out in Chapter 2. Figure 4.6 shows the purified proteins run on a 12 % gel, following dialysis in 50 mM potassium phosphate buffer, pH 7.4.

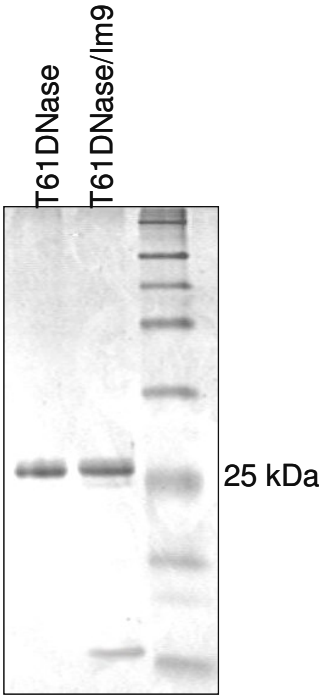


Figure 4.6 12 % SDS-PAGE gel showing purified T_{61}-DNase/Im9 and free T_{61}-DNase proteins. The calculated molecular weight of the T_{61}-DNase is 20 kDa but the apparent molecular weight on a gel is slightly higher.

4.2.4. Purification of Colicin E3

Colicin E3/Im3 had been cloned in plasmid pRJ382 (a pET21a-based vector containing full-length colicin E3 and Im3), but an *Xho*I site had been introduced after the stop codon of Im3 so that the sequence lacked a his-tag, preventing purification of the protein complex via nickel affinity chromatography. Therefore, primer W78, which replaces the stop codon of Im3 with an *Xho*I site, was used with primer Z4, which puts an *Nde*I site at the start of ColE3, in a PCR reaction with pRJ382 as a template. The resulting PCR product was then restricted with *Nde*I and *Xho*I and ligated into pET21a restricted with the same restriction enzymes. The ligated product was used to transform *E.coli* DH5α cells. The colicin E3 and Im3 genes encoded by the purified plasmid (pSR14) obtained from the DH5α cells was sequenced to ensure no mutations had been introduced and pSR14 was then used to transform BL21 pLysS cells. IPTG was used to induce expression of ColE3/Im3 in the transformed BL21

cells and crude cell lysate samples, taken before and after induction, were run on a 12 % SDS-PAGE gel. Figure 4.7 shows that a protein of the expected size is produced after induction. Cells were harvested and lysed and the colicin E3/Im3 protein was then purified using nickel affinity chromatography. The purified protein was dialysed in 50 mM potassium phosphate buffer, pH 7.4 and run on a gel to check for purity (see Figure 4.8). Colicin E3 was also purified without Im3 using the method detailed in Chapter 2. The free protein was run on an SDS-PAGE gel and silver-stained to check for the absence of Im3 (see Figure 4.8). The Im3 protein, separated from colicin E3 by denaturation, appeared pure on an SDS-PAGE gel after dialysis (see Figure 4.8) and was therefore considered suitable for use in SPR analysis.

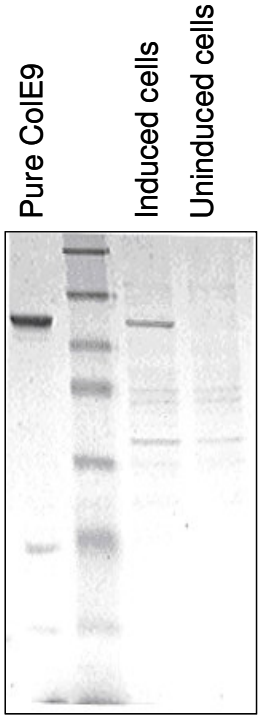


Figure 4.7 12 % SDS-PAGE gel showing crude cell lysate samples from BL21 DE3 cells transformed with pSR14, before and after induction with IPTG. Colicin E9 was also run alongside these samples to check that the induced protein was of the appropriate size. A strong band can be seen in the induced sample, corresponding to the size of colicin E9, indicating that expression of the colicin E3 protein has been achieved.

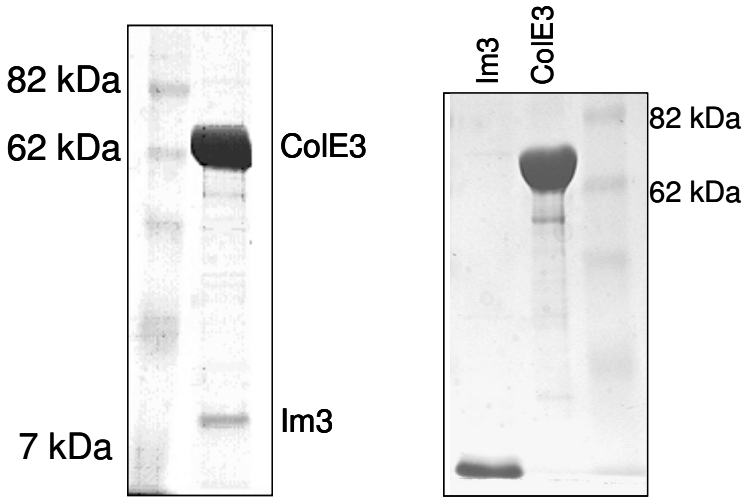


Figure 4.8 12 % SDS-PAGE gels showing purified colicin E3/Im3, Im3 and free colicin E3. The right-hand gel shows that the band corresponding to Im3 is absent from free colicin E3 and that the Im3 protein is free from contaminants.

4.2.5. Effect of Im9 on binding of colicin E9 to TolB

4.2.5.1. Preliminary SPR experiment

A preliminary SPR experiment, using the BIAcore J instrument, involved the injection of 2 μM free colicin E9 across TolB, immobilised to a CM5 sensor chip. The response produced was compared to the response produced when 2 μM colicin E9/Im9 was injected across the TolB chip.

Figure 4.9 shows that the response recorded for the binding of free colicin E9 to TolB was increased compared to the response recorded for the binding of the colicin E9/Im9 complex to TolB. As the SPR response is directly proportional to changes in mass, the differing relative molecular masses of colicin E9 and colicin E9/Im9 must be taken into account. The sensorgrams shown are therefore corrected for mass differences by dividing the response by the mass.

As a further test for the effect of Im9 on binding of colicin E9 to TolB, purified Im9 was added back to colicin E9 purified free from Im9, and this was injected across the TolB chip. Figure 4.9 shows that when Im9 is added back to free colicin E9, the response recorded is almost exactly the same as the response recorded for colicin E9 purified in complex with Im9.

Therefore Im9 reduces the binding of wild-type colicin E9 to TolB in a similar way to the G36A colicin E9 mutant.

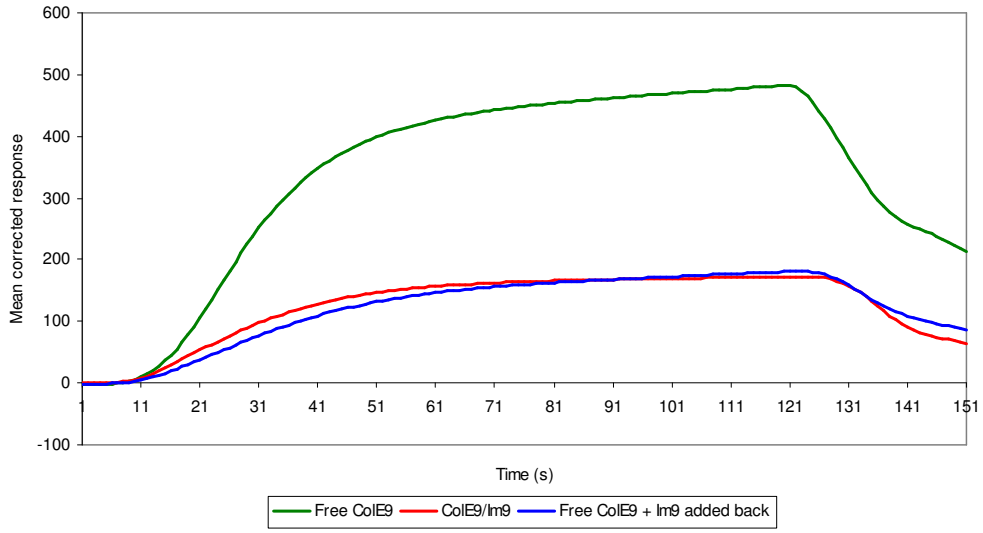


Figure 4.9 Sensorgrams (corrected for mass differences) produced from injection of colicin E9 in the absence of Im9, colicin E9 purified in complex with Im9 and colicin E9 pre-incubated with purified Im9 across a TolB chip. A clear increase in binding can be seen for free colicin E9 compared to colicin E9/Im9 and to colicin E9, pre-incubated with Im9.

4.2.5.2. SPR kinetic analysis

In order to compare the affinities of binding of colicin E9 with and without Im9 to TolB, the BIAcore X instrument was used to determine the kinetic parameters for binding of free colicin E9 to TolB. The same method described in Chapter 3 for colicin E9/Im9 was used. Injections of 0.25 μM to 4 μM free colicin E9 were performed across the same TolB chip as had been used for the colicin E9/Im9 complex. The sensorgrams produced were fitted to the Langmuir 1:1 binding model (see Figure 4.10). The kinetic parameters produced are shown in Table 4.1. Scatchard analysis (see Figure 4.11), T-values and Chi squared values (see Table 4.1) are consistent with a good fit of the data to the model. This is in contrast to the sensorgrams produced for the free G36A colicin E9 mutant, which did not fit the Langmuir 1:1 model (see Chapter 3).

The K_d for binding of free colicin E9 to TolB is 1.17 ± 0.03 μM. This value is an order of magnitude lower than the value for binding of colicin E9/Im9 to TolB (13.8 ± 0.3 μM), confirming that the affinity of colicin E9 for TolB is reduced when the colicin is complexed with the immunity protein.

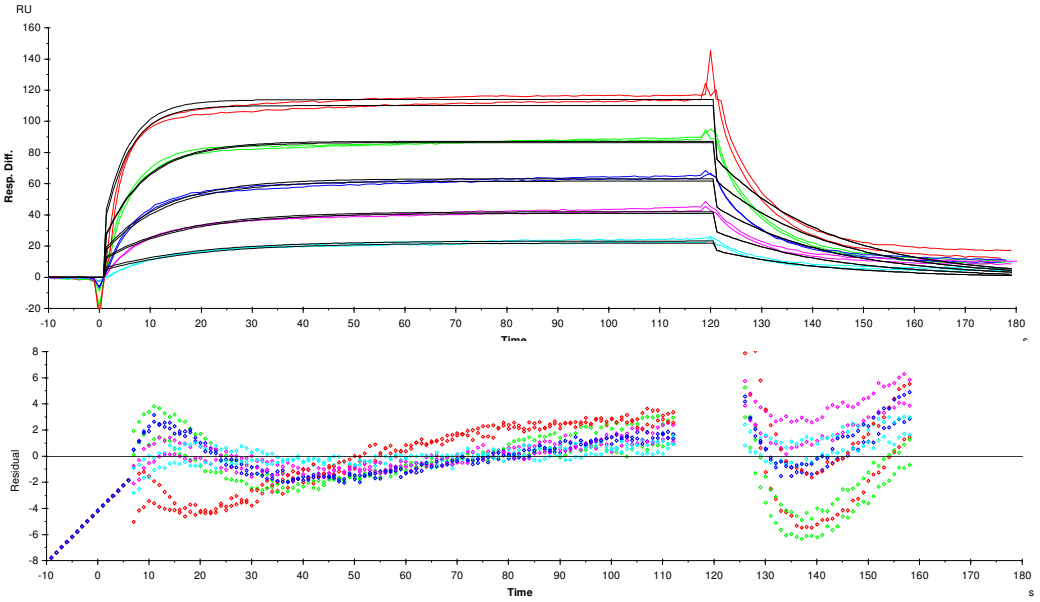


Figure 4.10 **Corrected sensorgrams and residual plots for titration of 0.25 µM (cyan), 0.5 µM (magenta), 1 µM (blue), 2 µM (green) and 4 µM (red) free colicin E9 against TolB at 25°C are shown. The fitted kinetic data for binding are superimposed onto each sensorgram in black. Residual plots highlight deviation of the experimental data from the theoretical fit.**

Table 4.1 **Kinetic data obtained from fitting of sensorgrams produced for binding of free colicin E9 to TolB to the Langmuir 1:1 binding model. Errors for K_a and K_d were calculated by combining the standard errors on k_{ass} and k_{diss} in quadrature.**

kass (1/Ms)	kdiss (1/s)	Ka (1/M)	Kd (µM)	Chi2
38500 ± 671	0.0451± 0.000558	854000 ± 18250	1.17 ± 0.025	4.13

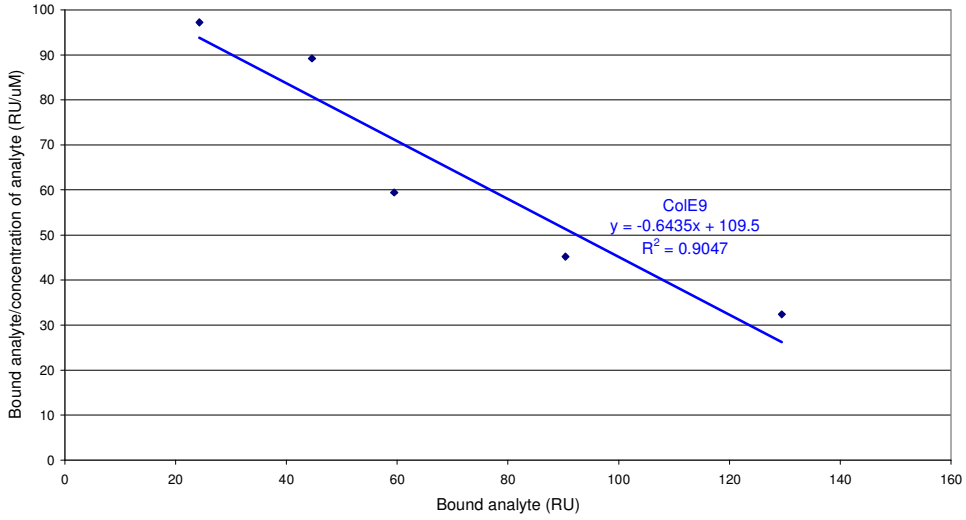


Figure 4.11 **Scatchard plot of data for binding of free colicin E9 to TolB. An R squared value of 0.9 is produced for a linear fit to the data indicating that the data are a good fit to the 1:1 Langmuir binding model**

4.2.5.3. Effect of non-cognate immunity proteins

The effect of non-cognate immunity proteins on binding of colicin E9 to TolB, was used as a test for the specificity of the effect of Im9 in the reduction of binding of colicin E9 to TolB. The non-cognate immunity proteins have differing but lower affinities for colicin E9.

A two-minute injection of 2 μM free colicin E9 across a CM5 chip, to which TolB had been immobilised, was performed. Two-minute injections were then performed of 2 μM (final concentration) free colicin E9, pre-incubated with 2 μM (final concentration) of either the cognate immunity protein, Im9 or one of the non-cognate immunity proteins, Im2, Im7 or Im8 (a gift from Daniel Walker, University of York). Each injection was repeated three times and the mean corrected response was recorded.

Figure 4.12 shows that pre-incubating free colicin E9 with the cognate immunity protein, Im9, significantly reduces the response, consistent with data presented earlier in the chapter. Im2, with the closest affinity for colicin E9 to Im9, reduces the response significantly but causes less reduction in response than Im9. Im8 also reduces the response, but less so than Im2 and Im9. Im7 causes the least reduction in response, consistent with this immunity protein having the lowest affinity for colicin E9.

The order for the reduction in response caused by the cognate and non-cognate immunity proteins follows the same pattern as the *in vitro* and *in vivo* immunity protein affinity for the colicin E9 DNase domain (Wallis *et al* 1995) *ie* Im9>Im2>Im8>Im7. Therefore, this provides further evidence that the effect of the immunity protein in reducing binding of colicin E9 to TolB is a specific effect, dependent on the affinity of the immunity protein for the DNase domain.

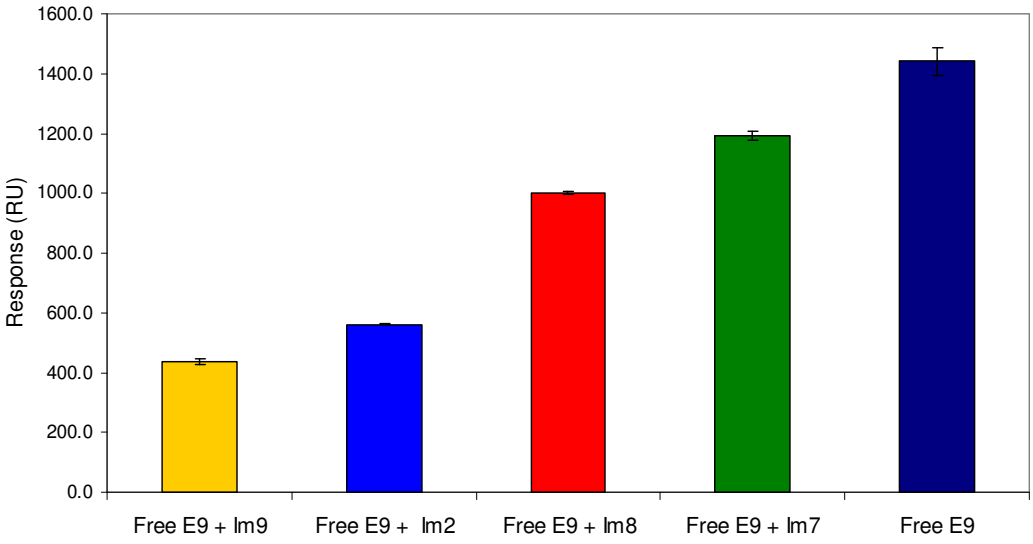


Figure 4.12 Bar chart comparing the mean responses for a 2 minute injection across TolB, immobilised to a CM5 sensor chip, for free colicin E9 and free colicin E9 pre-incubated with Im9, Im2, Im8 and Im7.

4.2.5.4. Effect of Im3 on binding of colicin E3 to TolB

Although no crystal structure is available for colicin E9/Im9 or colicin E9, the crystal structure of the RNase colicin E3/Im3 complex shows that 83% of the Im3 surface area is buried in the complex and that the translocation domain forms 38% of the buried surface area (Soelaiman *et al* 2001). Therefore, this section investigates the effect of Im3 on binding of colicin E3 to TolB.

The same method for SPR kinetic analysis of the colicin E3 – TolB interaction in the presence and absence of Im3 was used as described in Chapter 3 for colicin E9/Im9. Sensorgrams produced for injection of 2 - 15 μM colicin E3/Im3 across the TolB chip are shown in Figure 4.13, with the fit to the Langmuir 1:1 binding model superimposed in black. The kinetic parameters for the interaction are shown in Table 4.2. Scatchard analysis (see Figure 4.15), T-values and Chi-squared values (see Table 4.2) all show that the data fit well to the binding model.

The K_d for the interaction between colicin E3/Im3 and TolB is 7.07 ± 0.13 μM, a slightly lower value than obtained for the binding of colicin E9/Im9 to TolB (13.8 μM), indicating that the affinity of colicin E3 for TolB is enhanced compared to the affinity of colicin E9 for TolB.

Sensorgrams produced for injection of 0.1 – 2 μM free colicin E3 across the TolB chip are shown in Figure 4.14, with the fit to the Langmuir 1:1 binding model superimposed in black. The kinetic parameters for the interaction are shown in Table 4.2. Scatchard analysis (see Figure 4.15), T-values and Chi-squared values (see Table 4.2) all show that the data fit well to the binding model. The K_d for the interaction between free colicin E3 and TolB is 0.92 ± 0.02 μM, a very similar value to the K_d for the interaction between free colicin E9 and TolB (1.2 μM). .

Therefore, the affinity of colicin E3 for TolB is increased in the absence of Im3, in a similar manner to the increase in affinity already seen for free colicin E9.

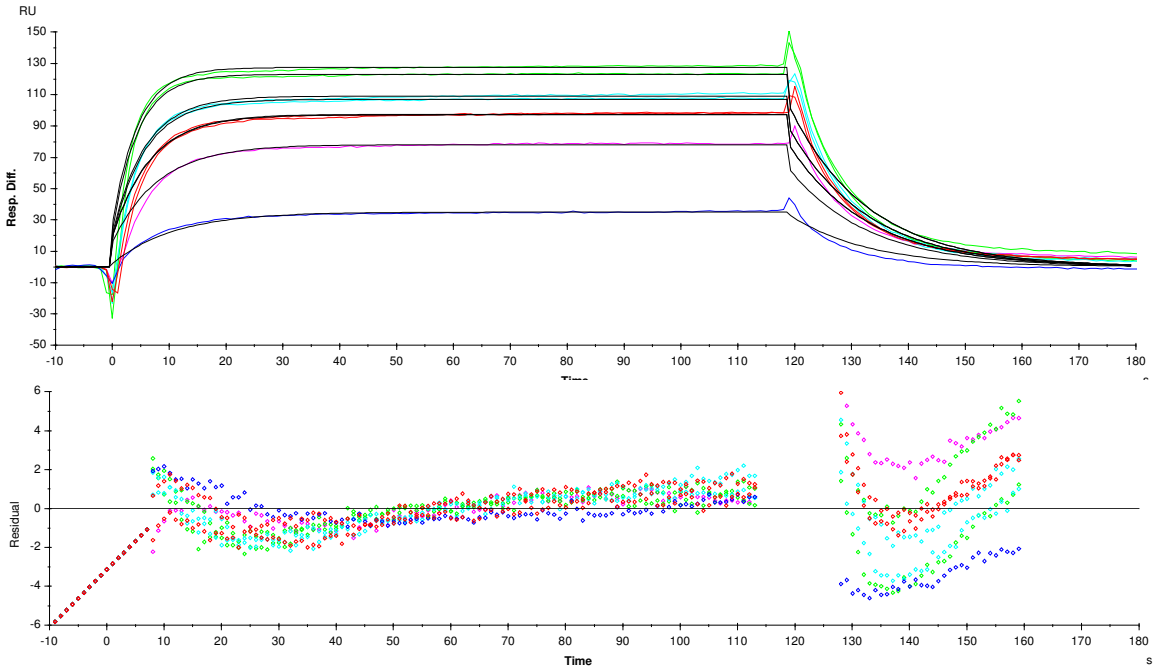


Figure 4.13 Corrected sensorgrams and residual plots for titration of 2 μM (blue), 5 μM (magenta), 7.5 μM (red), 10 μM (cyan) and 15 μM (green) colicin E3/Im3 against TolB at 25°C are shown. The fitted kinetic data for binding are superimposed onto each sensorgram in black. Residual plots highlight deviation of the experimental data from the theoretical fit.

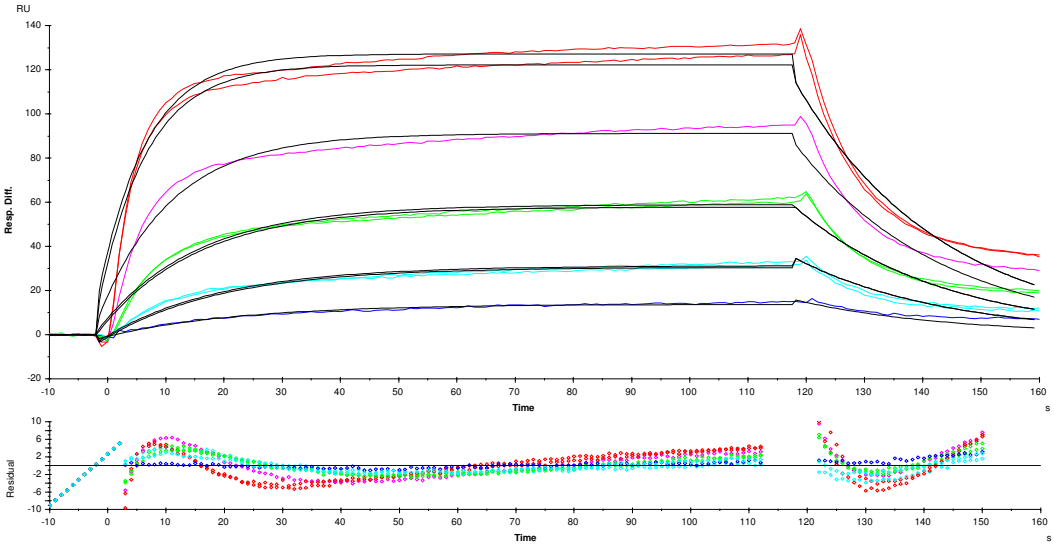


Figure 4.14 **Corrected sensorgrams and residual plots for titration of 0.1 µM (blue), 0.25 µM (cyan), 0.5 µM (green), 1 µM (magenta) and 2 µM (red) free colicin E3 against TolB at 25°C are shown. The fitted kinetic data for binding are superimposed onto each sensorgram in black. Residual plots highlight deviation of the experimental data from the theoretical fit.**

Table 4.2 **Kinetic data obtained from fitting of sensorgrams produced for binding of colicin E3/Im3 and free colicin E3 to TolB to the Langmuir 1:1 binding model. Errors for K_a and K_d were calculated by combining the standard errors on k_{ass} and k_{diss} in quadrature.**

	kass (1/Ms)	kdiss (1/s)	Ka (1/M)	Kd (µM)	Chi2
Colicin E3/Im3	10200 ± 152	0.0719 ± 0.000698	141000 ± 2496	7.07 ± 0.13	2.17
Free colicin E3	40900 ± 578	0.0395 ± 0.000458	1030000 ± 22660	0.92 ± 0.02	5.97

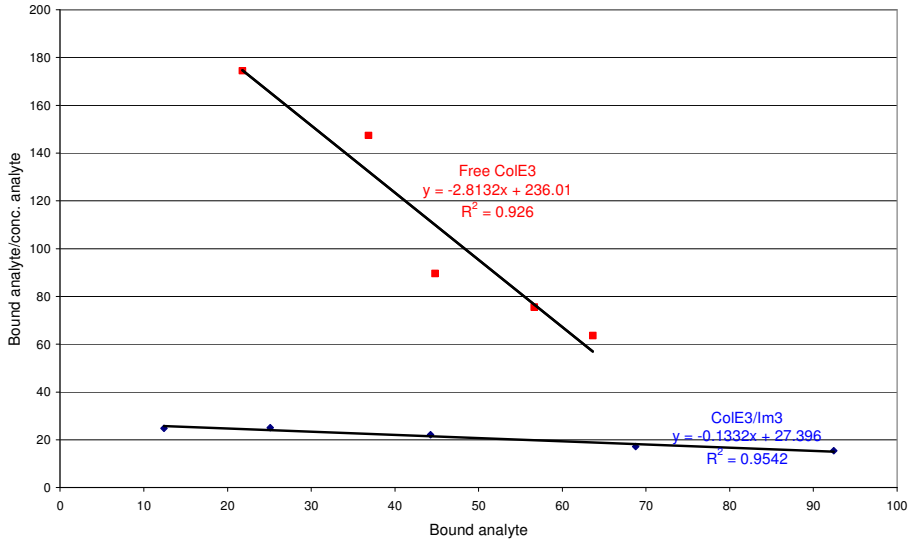


Figure 4.15 **Scatchard plot for data for binding of free colicin E3 and colicin E3/Im3 to TolB. The R squared values of >0.9, produced for a linear fit to the data, indicate that the data are a good fit to the 1:1 Langmuir binding model. The increased gradient for free colicin E3 compared to the colicin E3/Im3 complex, is a direct indication that the affinity of the free colicin is increased. K_d values calculated from the gradients are 7.5 µM for colicin E3/Im3 and 0.4 µM for colicin E3.**

4.2.5.5. Effect of Im9 on binding of T_{61}-DNase domain of colicin E9

One explanation for the effect of the immunity protein on binding of colicin E3 and E9 to TolB could be that binding of the immunity protein to the RNase or DNase domain of the colicin respectively, induces a conformational change that is transmitted to the translocation domain, via the receptor-binding domain, causing a reduction in affinity of the colicin for TolB. The receptor-binding domain forms a helical hairpin, located between the translocation and DNase domains, providing scope for feeding through a conformational change from the DNase domain to the translocation domain.

In order to test this hypothesis, the effect of Im9 on a truncated colicin E9 construct, consisting of residues 1-61 of the translocation domain linked to the DNase domain of colicin E9 via a thrombin cleavage site (T_{61}-DNase) has been investigated. If the theory were correct, the immunity protein should have no effect on the binding of this fusion protein to TolB because the receptor binding domain is not present.

Figure 4.16 shows the mean maximum corrected responses recorded for injection of 2 μM free colicin E9, colicin E9/Im9, free T_{61}-DNase and T_{61}-DNase/Im9. The binding of T_{61}-DNase to TolB is significantly reduced in the presence of Im9. This suggests the reduction in binding of colicin E9 to TolB due to Im9 is not due to a conformational change in the translocation domain fed through from the DNase domain via the receptor-binding domain, as the receptor binding domain is not present in the T_{61}-DNase construct.

It should be noted that the binding of the T_{61}-DNase construct to TolB is enhanced compared to the binding of colicin E9 to TolB. This indicates that the TolB box is perhaps more accessible in the T_{61}-DNase construct than in full-length colicin E9.

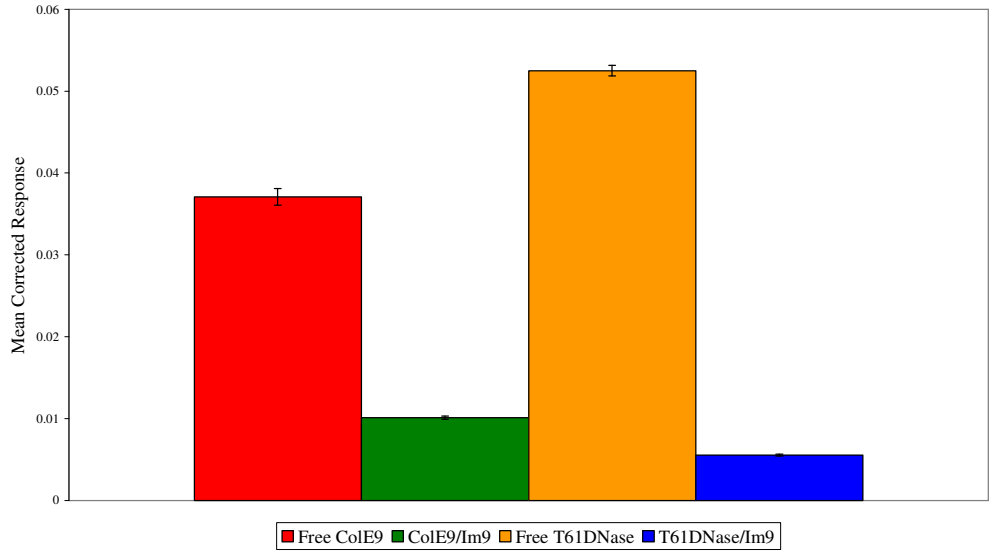


Figure 4.16 Bar chart comparing the mean response (corrected for mass differences) due to binding to TolB of free colicin E9, colicin E9/Im9, free T_{61}-DNase and T_{61}-DNase/Im9.

4.2.5.6. Effect of Im9 on binding of colicin E9 T & R domains to TolB

Another possible reason for the effect of the immunity protein on binding of colicin E9 and E3 to TolB is that the colicin interacts with TolB via a second site in the DNase domain. Binding of Im9 to the DNase domain would thus obscure the second site and reduce the affinity of the colicin E9-TolB interaction.

To test this hypothesis, the binding of a truncated colicin E9, consisting of the translocation (T) and receptor-binding (R) domains (a gift from Mireille Vankemmelbeke, University of Nottingham), to TolB was investigated.

Sensorgrams produced from injection of 0.5 – 4 μM T & R domains, in the absence of Im9 and pre-incubated with equimolar amounts of Im9, across a TolB chip are shown in Figure 4.17 and Figure 4.18 respectively. Kinetic parameters produced from fitting of the sensorgrams to the 1:1 Langmuir binding model are shown in Table 4.3.

Scatchard analysis indicates that the data produced here do not fit the model as well as the data produced for full-length colicin E9. However the R squared values for the linear fit are still within reasonable limits and the T values for kinetic parameters and Chi squared values for fitting are consistent with a good fit to the model.

The K_d for binding of T & R domains of colicin E9 to TolB in the absence of Im9 is 1.66 ± 0.03 μM and the K_d for binding of T & R domains of colicin E9 to TolB in the presence of Im9 is 2.96 ± 0.03 μM. Therefore the affinity of the isolated T and R

domains of colicin E9 for TolB is very similar to the affinity of the full-length colicin E9 for TolB (1.66 vs 1.17 μM), making the hypothesis that a site in the DNase domain of colicin E9 is involved in binding to TolB highly unlikely. The immunity protein appears to have less effect on the affinity of the T and R domains of colicin E9 for TolB compared to the effect on the affinity of full-length colicin E9 for TolB.

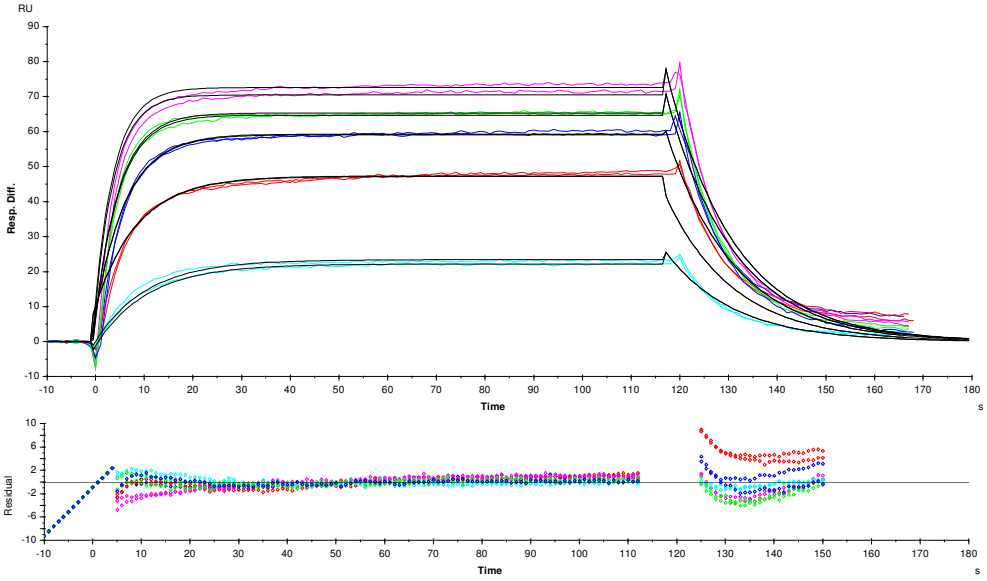


Figure 4.17 Corrected sensorgrams and residual plots for titration of 0.5 μM (cyan), 1 μM (red), 2μM (blue), 3 μM (green) and 4 μM (magenta) T and R domains of colicin E9 against TolB at 25°C are shown. The fitted kinetic data for binding are superimposed onto each sensorgram in black. Residual plots highlight deviation of the experimental data from the theoretical fit.

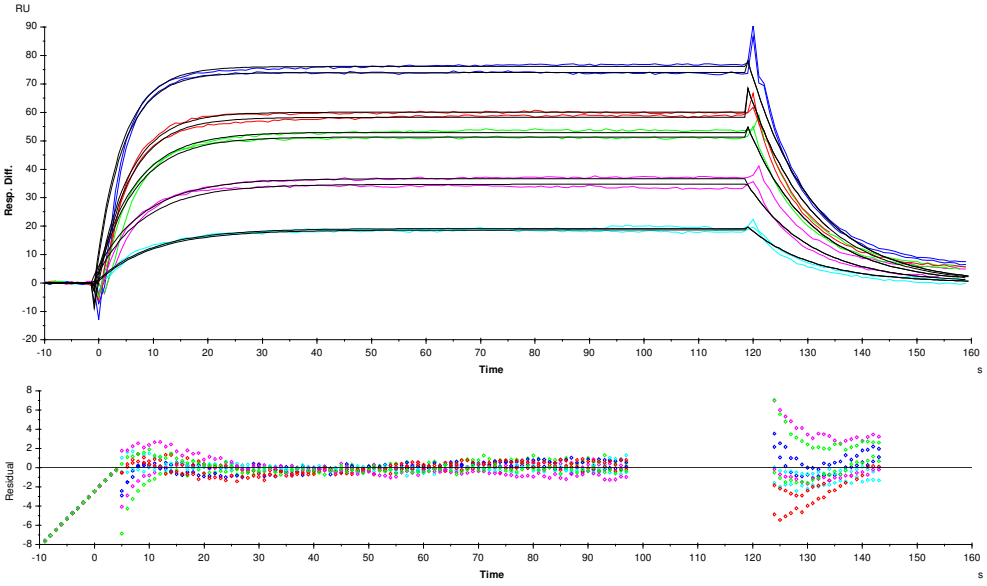


Figure 4.18 Corrected sensorgrams and residual plots for titration of 0.5 μM (cyan), 1 μM (magenta), 2μM (green), 3 μM (red) and 4 μM (blue) T and R domains of colicin E9 + Im9 against TolB at 25°C are shown. The fitted kinetic data for binding are superimposed onto each sensorgram in black. Residual plots highlight deviation of the experimental data from the theoretical fit.

Table 4.3 **Kinetic data obtained from fitting of sensorgrams produced for binding of the T&R domains of colicin E9, in the presence and absence of Im9, to TolB to the Langmuir 1:1 binding model. Errors for K_a and K_d were calculated by combining the standard errors on k_{ass} and k_{diss} in quadrature.**

	kass (1/Ms)	kdiss (1/s)	Ka (1/M)	Kd (μM)	Chi2
Free T&R ColE9	43600 ± 692	0.0724 ± 0.000844	602000 ± 10716	1.66 ± 0.03	2.01
T & R ColE9 + Im9	28800 ± 0.623	0.0853 ± 0.000823	338000 ± 3261	2.96 ± 0.03	1.25

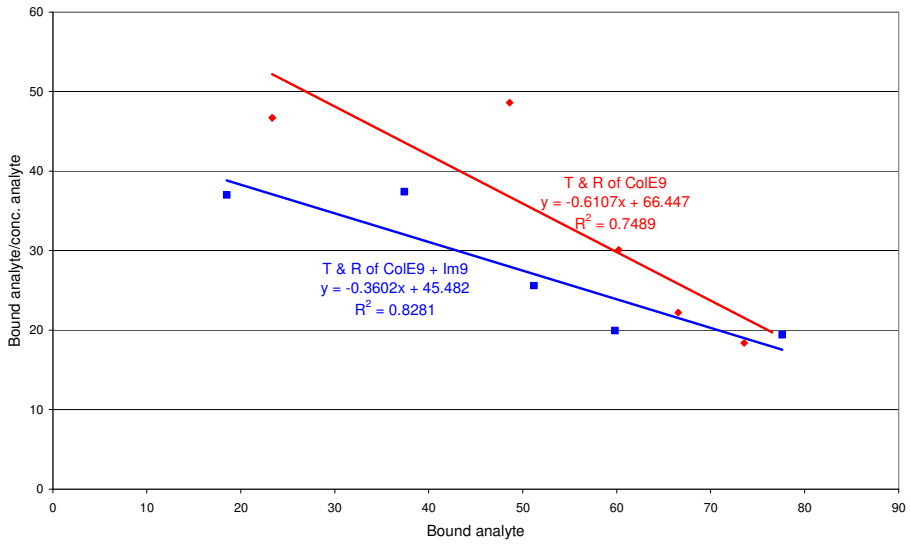


Figure 4.19 **Scatchard plot for data for binding of the T & R domains of colicin E9 in the presence and absence of Im9 to TolB. The R squared values produced for a linear fit to the data are lower than seen previously, but the T-values for kinetic parameters and the Chi squared value for the fitting, indicate that the data are a good fit to the 1:1 Langmuir binding model.**

4.2.6. Binding of Im9 to T & R domains

In order to investigate whether Im9 was able to directly bind to the translocation domain of colicin E9, in the absence of the DNase domain, the T&R domains of colicin E9 were amine coupled to a CM5 chip. A final response due to T&R domains of 839 RU was produced, giving an Rmax for Im9 of 180 RU.

Concentrations of 1-20 μM of Im9 were injected across the chip but no response was recorded indicating that Im9 does not bind to the translocation domain of colicin E9 or if it does, it binds with a lower affinity than can be detected here, *ie* the K_d must be much higher than 20 μM. It is possible that Im9 has to bind to the DNase domain of colicin E9 with high affinity first, which then stabilises the lower affinity interaction with the T domain.

The activity of the T & R domains on the chip was checked by ensuring that TolB could bind. A response of 339 was produced on injection of 2 µM TolB (RUmax = 812) and therefore the T & R construct on the chip was assumed to be intact.
To check for an effect of Im9 on binding of TolB to T & R domains, the response for injection of 2, 5 and 10 µM TolB across the chip was compared to the response due to injection of 2, 5 and 10 µM TolB, pre-incubated for 5 minutes with equimolar amounts of Im9 before injection. Figure 4.20 shows that no difference in response was recorded when TolB was pre-incubated with Im9 before injection across the chip, supporting evidence from the SPR kinetic data that Im9 does not reduce binding of TolB to the T & R domains of colicin E9. The data also show that Im9 has to be bound to colicin E9 to exert its effect, further supporting the specificity of the reduction in binding.

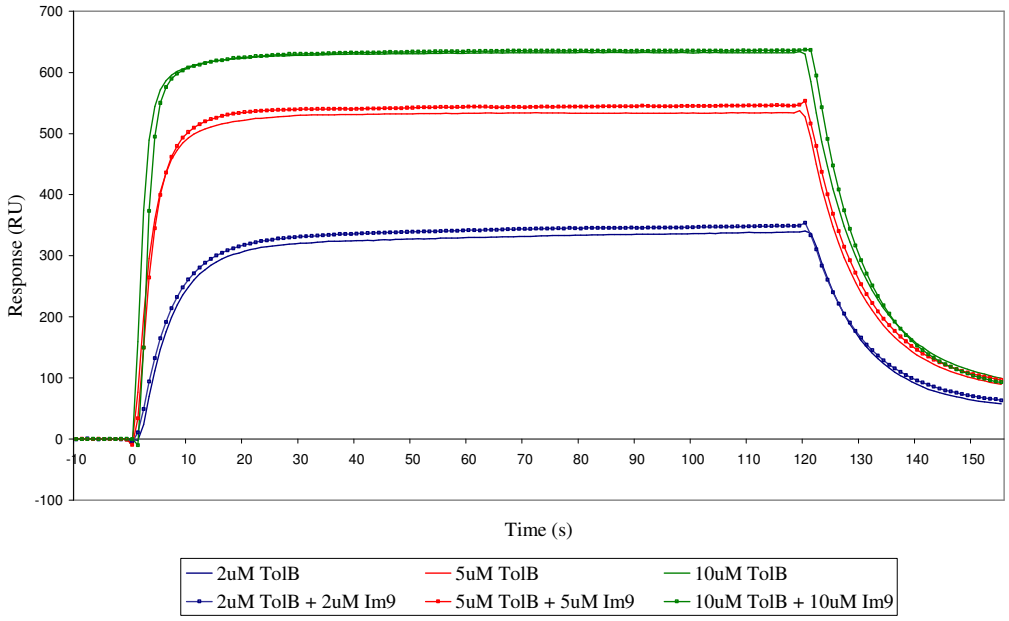


Figure 4.20 Sensorgrams produced when 2, 5 and 10 µM concentrations of TolB were injected across the T and R domains of colicin E9, immobilised to a CM5 sensor chip, in the presence and absence of equimolar amounts of Im9. No difference in binding is detected when Im9 is present compared to when Im9 is absent.

4.2.7. AFM to investigate effect of Im9 on binding of colicin E9 to TolB

AFM was used as an alternative method to investigate the effect of Im9 on the binding of colicin E9 to TolB. This method has the advantage that it measures protein interactions at the single-molecule level and, as outlined in Chapter 2, is able to explore the energy barriers of the interaction that are difficult to investigate using near-equilibrium kinetics.

4.2.7.1. AFM imaging

A monolayer coverage of protein on the sample surface is required for force measurement experiments to minimise non-specific interactions, which can occur between the tip and underlying surface. Therefore, in order to determine a suitable concentration of TolB to use for the force measurement studies, a DI Multimode AFM with a Nanoscope III controller (Digital Instruments), operated in tapping mode, was used to obtain AFM images of varying concentrations of TolB protein, covalently immobilised to silicon surfaces.

Figure 4.21 shows typical AFM height images when 100 μg/ml and 10 μg/ml TolB was immobilised to silicon surfaces. The approximate diameter of each globular feature is 15-20 nm. The crystal structures of TolB (Abergel *et al* 1999, Carr *et al* 2000a) indicate that TolB has a diameter of approximately 5nm. However, the geometry of the tip can produce broadening of features and therefore, when this is taken into account the features observed in the images can be attributed to individual TolB molecules.

The uniformity of the globular features in the image of 100 μg/ml TolB shows that at 100 μg/ml TolB, a monolayer is formed and therefore this concentration was used for the force-measurement studies.

4.2.7.2. Force measurement studies

An AFM tip was functionalised with either free colicin E9 or the colicin E9/Im9 complex. Force measurements were recorded between the colicin E9 attached to the AFM tip and TolB covalently immobilised to a silicon surface at tip retraction rates of 0.1, 1, 5 and 10 μm/s using a Molecular Force Probe instrument (MFP, Asylum Research). At least 1000 force-distance curves were recorded at each tip retraction

rate. Example force-distance curves obtained at 1 μm/s for the interaction between colicin E9 and TolB are shown in Figure 4.22.

The probability of observing a specific rupture event, characterised by a non-linear stretching in the retract curve (see Figure 4.22), was approximately 10% in all experiments. This is consistent with Poisson analysis for single molecular interactions, which suggests that when the probability of observing a rupture event is below 10%, approximately 95% of rupture events can be attributed to single molecular dissociation (Williams and Evans 2002).

In order to verify that the pull-off events observed were specific to the interaction between colicin E9 and TolB, excess colicin E9 was added into the buffer towards the end of the experiment. This reduced the probability of observing a specific event to ~ 2 %, indicating that the interaction between the colicin E9 on the tip and TolB was blocked by the presence of colicin E9 in the buffer and hence the pull-off events observed were due to specific interactions between colicin E9 and TolB.

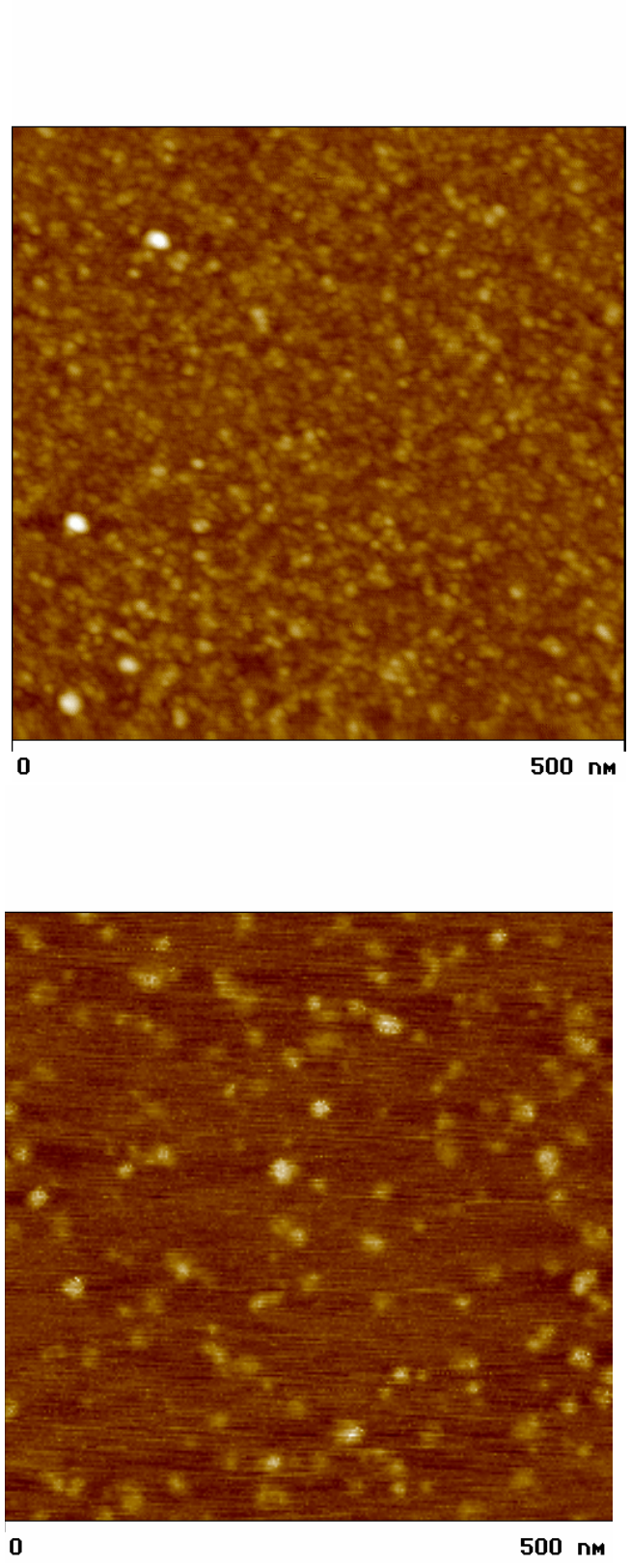


Figure 4.21 Typical AFM height images of silicon substrate functionalised with 100 µg/ml TolB (top) and 10 µg/ml TolB (bottom). Image sizes are 500nm x 500nm and the Z-range is 5nm.

A distribution of rupture forces was obtained for the specific interactions at each rate of tip retraction. Therefore, rupture forces indicative of specific interactions were plotted as force vs frequency histograms (see Figure 4.23 and Figure 4.24). The mode value for each histogram, which represents the most probable rupture force, was calculated and is shown above each histogram, along with the mean rupture force and standard deviation. For a simple bond the shape of the histogram can be predicted (Evans and Williams 2002), and the distribution should contain very few forces with values more than 1.2 times the mode. Higher forces and the subsequently wider force distributions are indicative of the measurement of multiple interactions. Therefore a model force distribution is calculated by removing all data more than 1.2 times the mode, the mode of this distribution is used to estimate a force scale ($f\beta$, from the curvature) and a relative zero-force dissocation rate (k_{off} or k_{diss}). These data are then used to predict a distribution for the event (assuming a single barrier), which is overlaid onto each histogram in red. The mode of the model histogram (reported in brackets) should be similar to the mode calculated for the experimental data unless the system is not a simple bond, or there are lots of multiple interactions or non-specific interactions included in the data.

Mode values for the rupture forces recorded for the colicin E9-TolB interaction and the colicin E9/Im9-TolB interaction were plotted as a function of the logarithm of the tip retraction rate (see Figure 4.25). The plots show that each interaction produces a single linear regime, with a decrease in retraction rate producing a decrease in rupture force (R squared values > 0.9). This relationship is consistent with previous studies, measuring biotin-streptavidin interactions, antibody-antigen interactions and protein-carbohydrate interactions (Yuan *et al* 2000, Strigl *et al* 1999, Dettmann *et al* 2000) and with theoretical models (see Chapter 2).

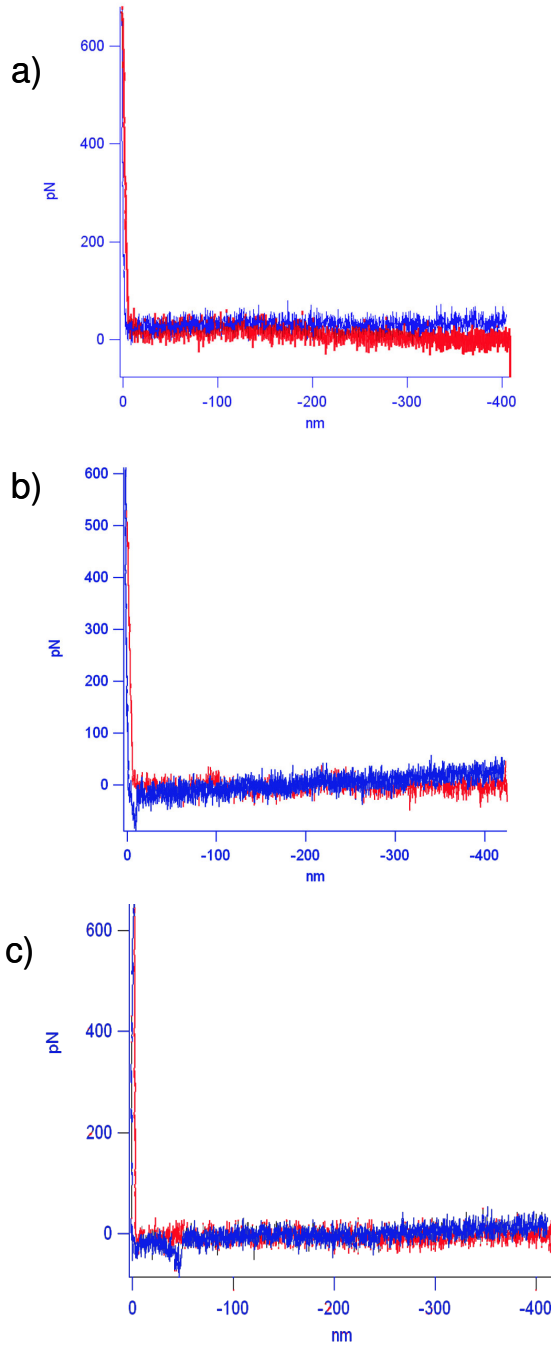


Figure 4.22 Typical force-distance curves with approach traces shown in red and retract traces shown in blue. a) Curve produced when no interaction occurs. b) Curve produced when a non-specific interaction occurs, characterised by the continuation of the gradient of the contact region of the retract curve before a sharp pull-off. c) Curve produced when a specific interaction occurs between colicin E9 on the tip and TolB on the surface. Specificity is characterised by the non-linear stretching seen in the retract curve, before detachment of the tip from the surface.

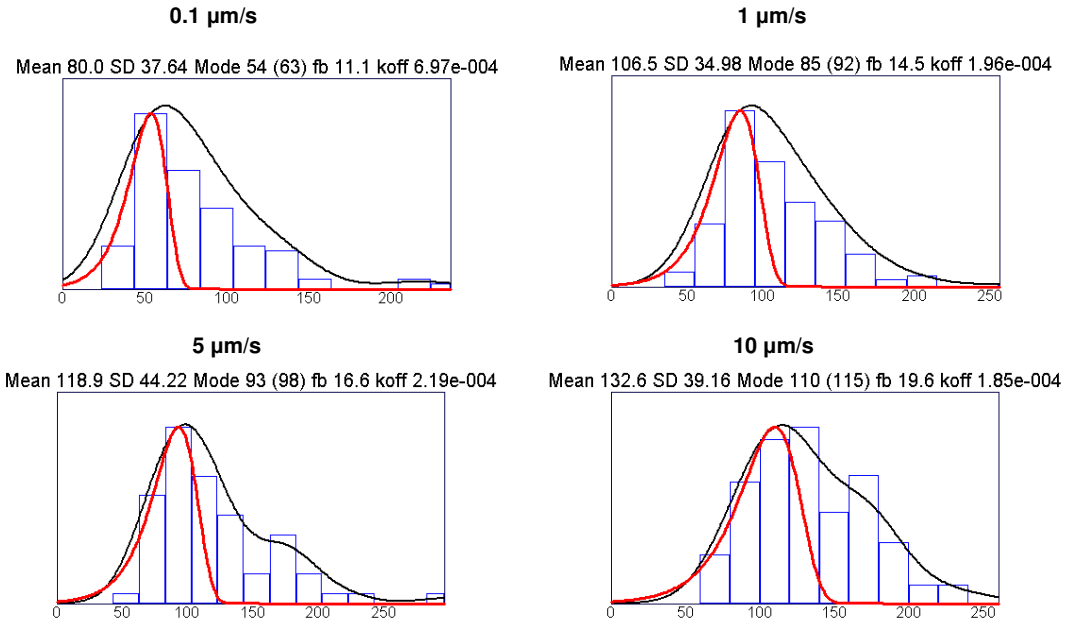


Figure 4.23 Histograms produced for colicin E9/Im9 at tip retraction rates of 0.1, 1, 5 and 10 µm/s. Blue bars show the force vs frequency data binned to the noise level, with the highest column centered on the predicted mode value. The black trace shows the noise-filtered data. Forces from 0 up to 1.2 times the mode value are then used to determine a new histogram and the mode of these is used to determine a force-scale (fb, from the curvature) and relative off-rate (koff). These data are then used to predict a distribution for the event (assuming a single barrier), shown in red.

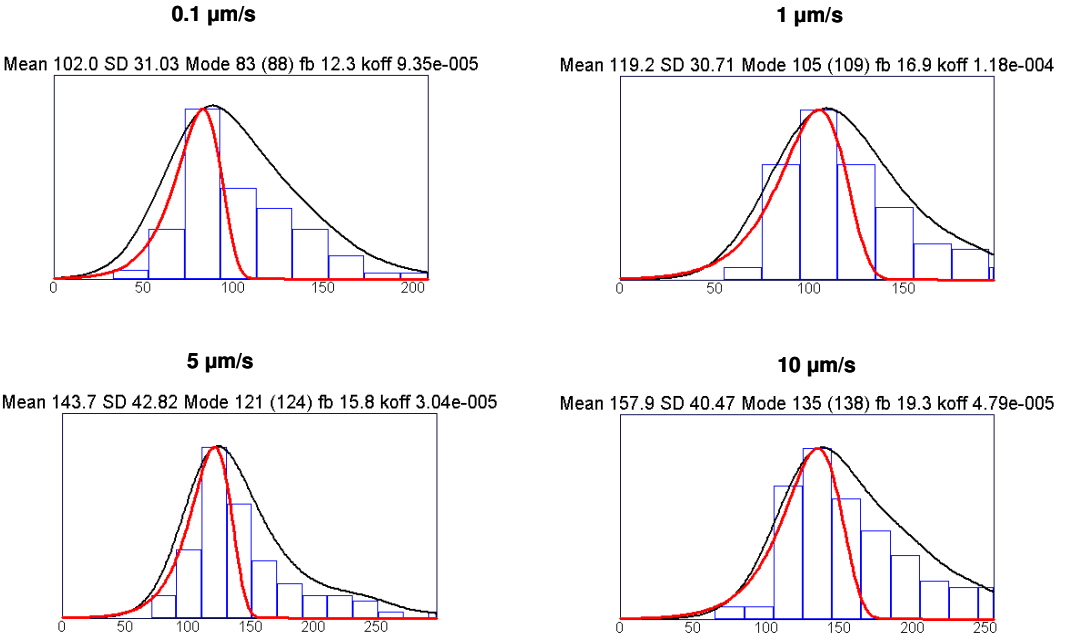


Figure 4.24 Histograms produced for free colicin E9 at tip retraction rates of 0.1, 1, 5 and 10 µm/s.

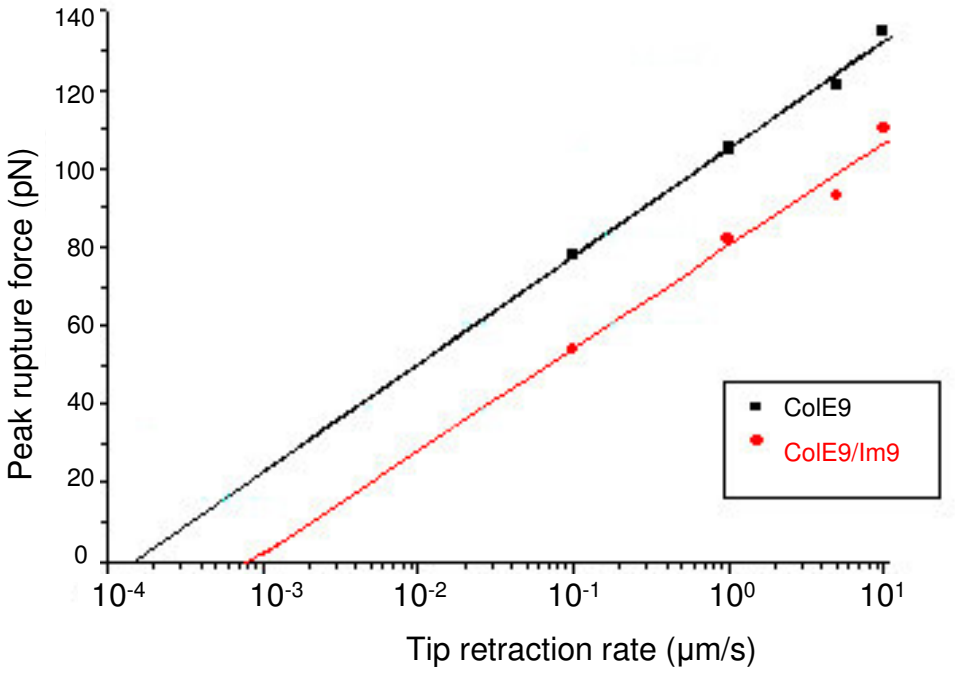


Figure 4.25 Dynamic force spectra for the interaction between TolB and colicin E9 in the presence and absence of Im9. A single linear gradient can be seen for both interactions. The linear regime is extrapolated to y=0, in order to obtain the x-intercept. The x-intercept (in log units) is -3.83 ± 0.27 for the colicin E9-TolB interaction and -3.07 ± 0.38 for the colicin E9/Im9-TolB interaction.

Ideally for dynamic force spectroscopy, the most probable rupture forces are plotted against the loading rate (tip retraction rate x spring constant of the system). However, in this study the dynamic and mechanical properties of the carboxymethylamylose linker, used to attach the colicin E9 to the tip, are unknown. Therefore the exact rate of loading cannot be determined. An assumption can be made that at the retract velocities used, the polymer linker is stretched to its asymptotic regime, where it will become stiff, the loading rate should be linearly related to the tip retract velocity. Therefore the calculated gradient or force scale should be a reasonable estimate of the force scale of the barrier measured in this study.

The force scale for the colicin E9/Im9-TolB interaction is 26.2 ± 3.1 pN. The force scale for the colicin E9-TolB interaction is 27.5 ± 1.9 pN. The maintenance of a comparable force scale for the two interactions implies that the energy barrier to dissociation is the same for both interactions. If this is the case, then the decrease in peak rupture forces measured for the colicin E9/Im9-TolB interaction, compared to those measured for the colicin E9-TolB interaction, indicates a decrease in the energy

barrier height and hence a decrease in affinity for the colicin E9/Im9-TolB interaction. From the equation $f_\beta = k_B T/x_\beta$, the position of the energy barrier from the bound state can be calculated as approximately 1.4 Å for the colicin E9-TolB interaction and 1.6 Å for the colicin E9/Im9-TolB interaction.

In order to determine the k_{diss} at zero force for the probed barrier, from Equation 4-1, the x-intercept, expressed as a loading rate, is required.

$$F^* = f_\beta[\ln(\text{loading rate}) - \ln(f_\beta\, kdiss)]$$

Equation 4-1

The polymer linker employed, adds a second timescale to the interaction required to extend the polymer and apply force. This timescale is dependent on the contour and persistence length of the polymer linker, values that are as yet undetermined for carboxymethylamylose (Williams and Evans 2002). Therefore, it is not possible to determine an accurate k_{diss} for the interaction between colicin E9 and TolB or colicin E9/Im9 and TolB.

However, as the gradients of the linear regimes plotted for the two interactions are very similar, a direct comparison of the x-intercept values allows a comparison of the k_{diss} values for the two interactions. The x-intercept for the colicin E9/Im9-TolB interaction is 8.5×10^{-4} μm/s and the x-intercept for the colicin E9-TolB interaction is 1.5×10^{-4} μm/s. Therefore, it can be concluded that the k_{diss} for the colicin E9/Im9-TolB interaction is greater than the k_{diss} for the colicin E9-TolB interaction.

From the equation, $K_d = k_{diss}/k_{ass}$, an increased k_{diss} for the colicin E9/Im9-TolB interaction compared to the k_{diss} for the colicin E9-TolB interaction is consistent with the SPR data, which shows that the K_d for the colicin E9/Im9-TolB interaction is greater than the K_d for the colicin E9-TolB interaction.

4.2.8. Trypsin cleavage of ColE9 and ColE9/Im9

The increase in affinity of colicin E9 for TolB, in the absence of Im9, suggests that the translocation domain could become more accessible for binding to TolB, which may require a conformational change.

As a test for a change in conformation in free colicin E9, compared to the colicin E9/Im9 complex, a trypsin digest was performed on colicin E9 in the presence and absence of Im9. A change in conformation in free colicin E9, could cause the

exposure of further trypsin cleavage sites, known to occur in colicin E9, increasing the number of fragments produced by digestion with trypsin.

The free colicin E9 and colicin E9/Im9 proteins were dialysed overnight in 50mM Tris-HCl, pH 8.5. A colicin/trypsin (Electrophoresis grade, Promega) ratio of 20 was used. 10% acetonitrile (v/v) was added to solubilise hydrophobic peptides generated during the digest. The colicin/trypsin solution was incubated at 37°C overnight. 5µl glacial acetic acid was added to stop the reaction and the digests were then run on an SDS-PAGE gel against the undigested proteins. The digested samples were then run on an SDS-PAGE gel against the undigested proteins and the gel was silver stained. Wallis *et al* (1994) showed that a trypsin digest of colicin E9/Im9 produces a 37 kDa fragment and a 27 kDa fragment on a gel filtration column with bands at 32 kDa, 16 kDa and 12 kDa seen on an SDS-PAGE gel.

A band of approximately 32kDa can be seen on the gel in Figure 4.26 for digests of both free colicin E9 and colicin E9/Im9. The two smaller bands of 16 and 12 kDa cannot be detected on this SDS-PAGE gel. However, the gel indicates that free colicin E9 is no more susceptible to protease cleavage than the colicin E9/Im9 complex, and therefore this experiment supports NMR data suggesting that there is no conformational change in the T domain of colicin E9 on binding of Im9.

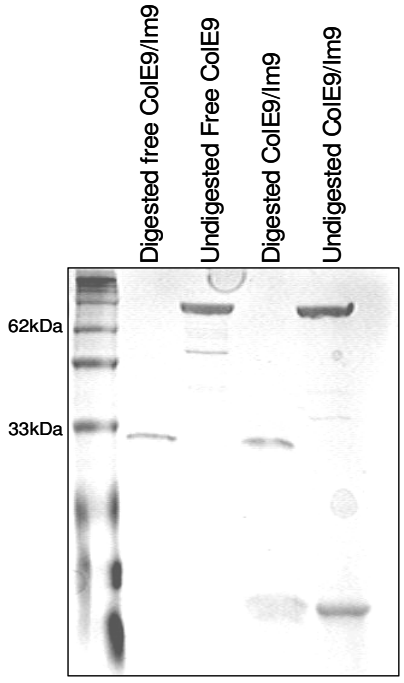


Figure 4.26 Silver-stained SDS-PAGE gel showing results of trypsin digest of colicin E9 vs colicin E9/Im9. In both digests a band at around 32 kDa can be seen. A second band can be seen in the colicin E9/Im9 digest at around 10 kDa corresponding to the immunity protein.

4.3. Discussion

4.3.1. Effect of Im9 on binding of colicin E9 to TolB

4.3.1.1. SPR

A preliminary SPR experiment, performed using a BIAcore J instrument, showed that free colicin E9 demonstrated enhanced binding to TolB, compared to the binding of colicin E9/Im9. The effect of the immunity protein was further demonstrated when the free colicin E9 was incubated with pure Im9 prior to injection across the TolB chip. Pre-incubation of the free colicin E9 with Im9 was shown to reduce the binding to almost exactly the same level as had been seen for the colicin E9 purified in complex with Im9. This shows that the increased binding seen for free colicin E9 is unlikely to be an effect of denaturation and refolding of the colicin. Further evidence that the free colicin E9 protein is intact is provided by the identical activity of the free colicin E9 and the colicin E9/Im9 complex seen in the spot test in Figure 3.7 of Chapter 3.

In order to quantitatively compare the affinities of colicin E9/Im9 and free colicin E9 for TolB, SPR was used to determine the kinetics for binding of free colicin E9 to TolB. The K_d value of 1.17 ± 0.025 μM was compared to the K_d value for binding of the colicin E9/Im9 complex to TolB, which had been determined as 13.8 ± 0.3 μM in Chapter 3. The results show that the affinity of colicin E9 for TolB is reduced more than 10-fold when the immunity is protein is present.

The good fit of the sensorgrams produced for binding of free colicin E9 to TolB to the Langmuir 1:1 binding model, suggests that the poor fit to this model seen in Chapter 3 for binding of free G36A colicin E9 to TolB, is an effect of the G36A mutation, rather than the lack of immunity protein. It is possible that the G36A mutation, increases the flexibility of the translocation domain and when Im9 is removed, the increased flexibility affects binding of the colicin to TolB. An apparent stabilising effect of Im9 was also seen for the G38A mutant, the activity of which was enhanced in the presence of Im9 (see Chapter 3).

4.3.1.2. AFM

Dynamic force spectroscopy was used to probe differences in the profiles of the dissociation landscapes for the colicin E9- TolB interaction and the colicin E9/Im9-TolB interaction.

Rupture forces required to separate the colicin E9/Im9-TolB complex and the colicin E9-TolB complex were recorded. In such experiments a distribution of forces is expected due to kinetics, but the distribution can be broadened due to experimental error, the most significant source of error likely to be determination of the spring constant of the cantilever. The spread in rupture forces is determined by the thermal force scale (f_β) and therefore the widths of the force distributions should match the slopes of linear regimes observed in the dynamic force spectrum (Evans and Williams 2002).

The modal or most probable rupture forces for the dissociation of the colicin E9-TolB complex were found to be augmented compared to the forces recorded for the dissociation of the colicin E9/Im9-TolB complex. Rupture forces were also recorded for both interactions at four different rates. Analysis of the distributions of forces recorded for the colicin E9-TolB and colicin E9/Im9-TolB complexes over the range of tip retraction rates produced a logarithmic relationship between rupture force and dissociation rate. This relationship is consistent with previous measurements for various receptor-ligand interactions, such as the streptavidin-biotin interaction (Yuan *et al* 2000).

The most probable rupture forces were plotted as a function of the logarithm of the tip retraction rate to produce dynamic force spectra for both interactions. A single linear regime was recorded for each interaction, which suggests that a single energy barrier to dissociation was probed. The linear regimes have comparable gradients, indicating that the same barrier to dissociation is being measured. The position of the energy barrier from the bound state has been calculated as approximately 1.5 Å for both interactions. The widths of the force distributions are also satisfyingly close to the gradients of the dynamic force spectra, hence further validating the obtained dynamic force spectra.

Although only a single gradient for each interaction is seen for this data, suggesting that only a single energy barrier is involved in the dissociation of colicin E9 from TolB, measurements of force over many orders of loading rates are required in order

to map the complete dissociation pathway (Evans, 1998). AFM is unable to achieve such a vast range of measurements, and therefore AFM can only probe barriers close to the bound state. Barriers further away from the bound state require lower loading rates which are inaccessible by AFM. In a landscape with more than one barrier, the more distant barriers are overcome first (at lower rates), and closer barriers become more dominant. The biomembrane force probe (BFP) could be used to achieve a wider spectrum of rates, and hence to build a more complete profile of the dissociation pathway.

If it is assumed that the same energy barrier is being measured for the colicin E9-TolB interaction and the colicin E9/Im9-TolB interaction, then the decreased rupture forces recorded for the colicin E9/Im9-TolB interaction are indicative of a decrease in the height of the energy barrier and hence a decrease in affinity of the interaction compared to the interaction between free colicin E9 and TolB.

Although the use of a polymer linker with an unknown spring constant prevents the calculation of k_{diss} values for the colicin E9-TolB interaction and colicin E9/Im9-TolB interaction, the comparable force scales for the two interactions, allows a comparison of the k_{diss} values by comparing the x-intercept values for the extrapolated linear regime. The regime for the colicin E9-TolB interaction has a lower x-intercept than the x-intercept of the regime for the colicin E9/Im9-TolB interaction, indicating that the colicin E9-TolB interaction has a lower k_{diss} and therefore a lower K_d than the colicin E9/Im9-TolB interaction.

The reduced k_{diss} value for the colicin E9-TolB interaction is consistent with the reduced K_d value for this interaction measured by SPR, confirming the effect of Im9 in decreasing affinity of colicin E9 for TolB and confirming that the effect of Im9 on TolB binding seen in SPR is not an artefact caused by, for example, non-specific binding to the sensor surface.

4.3.2. Effect of Im3 on binding of colicin E3 to TolB

The demonstration that the translocation domain of colicin E3 is involved in an interaction with Im3 in a thermodynamic study (Walker *et al* 2003), suggests that the immunity protein could affect binding of colicin E3 to TolB. This possibility was tested by obtaining kinetics for binding of colicin E3 and colicin E3/Im3 to TolB using SPR.

The K_d values determined for binding of colicin E3 (0.92 ± 0.02 μM) and colicin E3/Im3 (7.1 ± 0.1 μM) to TolB show that the affinity of colicin E3 for TolB is decreased by the presence of Im3.

A model for dissociation of Im3 from colicin E3 has been proposed in which translocation of at least part of the translocation domain into the periplasm allows binding of the translocation domain to TolB, displacing contacts between the translocation domain and Im3. This weakens the colicin E3-Im3 interaction, significantly increasing the dissociation rate of the complex (Walker *et al* 2003). The demonstration in this study that the presence of Im3 reduces the ability of colicin E3 to bind to TolB, supports the idea that the colicin E3/Im3 complex could bind to TolB, an interaction which promotes dissociation of Im3 from the translocation domain, which in turn increases the affinity of the colicin E3-TolB interaction.

The resolution of the colicin E3/Im3 structure does not allow detailed analysis of the molecular interactions between the translocation domain and Im3 (Soelaiman *et al* 2001). However, it has been noted there are relatively few electrostatic interactions and that the shape complementarity is lower than that for the RNase-Im3 interface (Walker *et al* 2003). It is unclear as to whether the immunity protein directly contacts the TolB box region due to the lack of electron density for residues 1-83, containing the TolB box. A structure of colicin E3, in the absence of Im3, is required to demonstrate the precise effect of Im3 on the translocation domain of the colicin, and to explain the effect of Im3 on binding of colicin E3 to TolB.

As there is no structure for the colicin E9/Im9 complex it is unclear whether the immunity protein interacts with the translocation domain of colicin E9, in a similar manner to the interaction of Im3 with the translocation domain of colicin E3. However, thermodynamic data suggest that if an interaction does occur between Im9 and the translocation domain, the residues must be making an energetically neutral contact, in contrast to the interaction of the translocation domain of colicin E3 with Im3 (Wallis *et al* 1995a, Walker *et al* 2003). It is interesting to note, however, that the residues in the translocation domain of colicin E3 that contact the immunity protein are conserved in DNase and rRNase colicins, despite the immunity proteins being structurally unrelated (Walker *et al* 2003), suggesting that residues in the translocation domain of colicin E9 may also contact the immunity protein. This is supported by the AFM and SPR data presented here, which show that the immunity

protein appears to affect the translocation domain of colicin E9, demonstrated by the effect of the immunity protein on binding of the colicin to TolB.

4.3.3. Investigating the reason for the effect of the immunity protein on binding of colicins to TolB

4.3.3.1. Effect of non-cognate immunity proteins

In order to test the specificity of the effect of the immunity protein on binding of colicin E9 to TolB, the effect of non-cognate immunity proteins on the binding of colicin E9 to TolB was investigated.

The immunity proteins, Im9, Im2, Im8 and Im7 bind to the DNase domain of colicin E9 with K_d values of 10^{-14} M, 10^{-8} M, 10^{-6} M and 10^{-4} M respectively (Wallis *et al* 1995b). Pre-incubation of colicin E9 with each of these immunity proteins before injection across TolB, immobilised to an SPR sensor chip, caused a relative reduction in binding that was related to the affinity of the immunity protein for the DNase domain of colicin E9. This is consistent with the binding of the immunity protein to the DNase domain of colicin E9 causing a change in the translocation domain. However, this experiment does not rule out the possibility that the reason for reduced binding of colicin E9 to TolB caused by the immunity protein is due to a direct effect of the immunity protein on the DNase domain.

4.3.3.2. Effect of Im9 on T & R domains of colicin E9

A possible explanation for the reduction in binding of colicin E9 to TolB caused by Im9 is that TolB interacts with colicin E9, not only via the translocation domain, but also via a second site, perhaps in the DNase domain. Binding of Im9 to the DNase domain would then obscure the second TolB binding site in this domain, reducing the affinity of the colicin for TolB. The presence of a binding site for TolB in the DNase domain of colicin E9 was tested by investigating the binding of the isolated T and R domains of colicin E9 to TolB, in the presence and absence of Im9.

The K_d values for binding of the T and R domain of colicin E9 are 1.64 μM, in the absence of Im9, and 2.96 μM in the presence of Im9. Therefore the affinity of the T and R domains of colicin E9 for TolB is very similar to the affinity of the full-length colicin E9 for TolB (K_d = 1.2μM). This strongly suggests that the only binding site for TolB is located in the translocation domain of colicin E9.

The effect of Im9 on the affinity of the T and R domains of colicin E9 for TolB is far less pronounced than the effect on the affinity of full-length colicin E9 for TolB. This suggests that it is the binding of Im9 to the DNase domain that is involved in reducing the binding of the colicin to TolB.

4.3.3.3. Effect of Im9 on the T_{61}-DNase colicin E9 construct

The binding of a T_{61}-DNase construct of colicin E9 to TolB, in the presence and absence of Im9 was used to investigate the hypothesis that binding of Im9 to the DNase domain causes a change in the helical hairpin of the receptor-binding domain which feeds a conformational change through to the translocation domain. If this feed-through theory were correct, the immunity protein would have no effect on the binding of this fusion protein to TolB because the receptor binding domain is not present to feed-through the required conformational change. However, SPR shows that the binding of the T_{61}-DNase colicin E9 construct is reduced in the presence of Im9.

This indicates that the receptor binding domain is not involved in the effect of the immunity protein on the translocation domain of colicin E9. It also suggests that if the reason for the reduction in binding to TolB caused by Im9 is due to a direct binding of Im9 to the translocation domain, Im9 must bind to the first 61 residues of the translocation domain. In the structure of colicin E3/Im3, the first 83 residues are absent but interactions between Im3 and residues downstream of the translocation domain can be seen to interact. Therefore, it appears unlikely that the only interaction between the translocation domain of colicin E9 and Im9, occurs within the first 61 residues of the translocation domain.

Although the affinity of the T and R domains of colicin E9 for TolB is similar to the affinity of full-length colicin E9 for TolB, the affinity of T_{61}-DNase colicin E9 fusion protein for TolB appears to be enhanced. This suggests that residues of the translocation domain or receptor-binding domain, downstream of residue 61, may restrict access of TolB to the TolB box of colicin E9, in a similar way to the masking of the TolA binding site by the rest of the colicin A protein, as suggested by Gokce *et al* (2000).

4.3.3.4. Binding of Im9 to the translocation domain of colicin E9

In an attempt to test whether the immunity protein binds directly to the translocation domain of colicin E9, the T and R domains of colicin E9 were covalently immobilised to an SPR sensor chip. Varying concentrations of Im9 were then injected across this chip but no response was recorded. The T and R domains immobilised on the chip were shown to be intact by the response recorded when TolB was injected across this chip. In agreement with the affinity data described above for the interaction of TolB with the T & R domains of colicin E9, Im9 was shown to have no effect on binding of TolB to the immobilised T and R domains of colicin E9.

However, this does not necessarily imply that the immunity protein does not interact with the translocation domain of colicin E9 *in vivo*. Firstly the interaction between the isolated translocation domain and Im9 could be of a lower affinity than can be measured by SPR. Alternatively, the binding of Im9 to the translocation domain, may require the initial high-affinity binding of Im9 to the DNase domain.

4.3.3.5. Trypsin digest

The possibility that removal of the immunity protein from colicin E9 caused a conformational change in the translocation domain was investigated by looking for an increase in trypsin cleavage of free colicin E9 compared to colicin E9/Im9. However, no extra cleavage products were identified after digestion of free colicin E9 compared to the cleavage products produced after digestion of colicin E9/Im9. This result is consistent with NMR experiments, which indicated that Im9 had no effect on the conformation of the translocation domain of colicin E9 (Collins *et al* 2002).

4.4. Summary

AFM and SPR experiments have shown that there is a clear reduction in affinity for the interaction of colicins E9 and E3 with TolB due to the presence of their respective immunity proteins, indicating that the immunity protein affects the translocation domains of these colicins. Although the crystal structure of colicin E3/Im3 clearly demonstrates that Im3 interacts with both the RNase domain and the translocation domain, thermodynamic experiments with colicin E9 had suggested that the only contacts the immunity protein had with the colicin were via the DNase domain.

A variety of methods have been used in an attempt to elucidate the reason for the effect of the immunity protein on binding of colicin E9 to TolB. No direct binding of the translocation domain of colicin E9 to Im9 could be detected. The lack of change in conformation in the translocation domain caused by Im9, shown by a trypsin digest of free colicin E9 and the colicin E9/Im9 complex, and by previous NMR data, suggests that the reason for the decreased affinity of colicin E9/Im9 for TolB compared to the affinity of colicin E9 for TolB, is not a conformational change.

A reduction in binding of the T_{61}-DNase domain to TolB, in the presence of Im9, suggests that the effect does not rely on binding of Im9 to residues of the translocation domain, further downstream than residue 61, whereas residues downstream of the colicin E3 translocation domain are shown in the crystal structure to interact with Im3.

It is therefore proposed that the presence of Im9 could hinder the access of TolB to the TolB binding site of colicin E9, without physically contacting the translocation domain. This would be consistent with the requirement, proposed due to results from thermodynamic experiments, for any contacts between the colicin and the immunity protein to be energetically neutral. The effect of Im3 on colicin E3 could be due to the same steric hindrance or could be due to conformational changes in the TolB box, caused by interactions of residues of the translocation domain further downstream with Im3.

However, crystal structures of colicin E9 in the presence and absence of Im9 and of an endonuclease colicin in complex with TolB, are required to fully understand the effect of the immunity protein on binding of colicin E9 and E3 to TolB.

CHAPTER 5 – Variations in binding to Tol proteins between pore-forming and endonuclease colicins

5.1. Introduction

5.1.1. Interaction between colicins and TolB

The interaction between colicin E3 and TolB has been observed *in vivo* and *in vitro* (Bouveret *et al* 1997) and the interaction between colicin E9 and TolB has been shown using the yeast-two hybrid technique (Carr *et al* 2000a). Both these interactions have been quantified in this thesis using surface plasmon resonance (see Chapters 3 and 4). An interaction between colicin A and TolB has also been demonstrated using *in vitro* cross-linking (Bouveret *et al* 1998).

The regions of colicins E3, E9 and A that interact with TolB have been identified. Chapter 3 shows that residues S34-E42 of colicin E9 are involved in the interaction with TolB and, as the sequence of colicin E3 in this region is identical to colicin E9, the equivalent residues of colicin E3 are likely to be involved in the interaction with TolB. Residues 11-20 in colicin A are predicted to be involved in interacting with TolB (Bouveret *et al* 1998). Residues 35-42 of colicins E3 and E9 are identical to residues 11-18 of colicin A, with the exception that residue 37 in colicin E3 and E9 is a serine, whereas the equivalent residue in colicin A is a threonine.

5.1.2. Interaction between colicins and TolA

TolA is a three-domain protein, consisting of an N-terminal domain I (residues 1-47), anchoring the protein to the cytoplasmic membrane, a central domain II, which tethers the anchoring domain to the functional domain III (Webster 1991).

Although an interaction has been detected between the N-terminal domain of colicins A and E1 with TolA by overlay Western blotting, gel-shift assays and SPR (Bénédetti *et al* 1991, Derouiche *et al* 1997), no interaction between TolA and the N-terminal domain of colicin E3 has been detected (Bouveret *et al* 1997, Bouveret *et al* 2002). The region of colicin A involved in interacting with TolA is between residues 52 and 97 (Bouveret *et al* 1998, Journet *et al* 2001). However, there is no sequence homology between the putative TolA binding region in colicin A and the region of

colicin N proposed to interact with TolA (residues 44-66, Raggett *et al* 1998, Gokce *et al* 2000). This suggests that these two colicins interact with TolA in different ways. SPR experiments indicated that the K_d for binding of the N-terminal domains of colicins A and E1 to TolA domain III were between 0.2 and 0.6 μM (Derouiche *et al* 1997). Similar values were obtained when domains II and III of TolA were used, and no interaction was seen between the colicins and domain II of TolA indicating that domain III of TolA is the only domain involved in binding to colicins (Derouiche *et al* 1997). Binding assays performed in varying ionic strengths indicated that the interaction between TolAIII and colicin A is not electrostatically driven (Deprez *et al* 2002).

The binding affinity of colicin N for TolA has also been measured, using isothermal titration calorimetry (ITC) and tryptophan fluorescence (Raggett *et al* 1998). The isolated N-terminal domain of colicin N was found to have a higher affinity (K_d = 1 μM) for TolA, compared to the full-length colicin (K_d = 18 μM). It has been suggested that this is due to an increase in accessibility of the TolA binding site in the isolated N-terminal domain, which could relate to the situation when the colicin has bound to its primary receptor (Raggett *et al* 1998). ITC was also used in an attempt to measure the affinity of the colicin A-TolA interaction, but this technique did not detect the expected binding (Gokce *et al* 2000).

5.1.3. Interaction between colicins and TolR

TolR is a 142-residue protein, consisting of three domains. Domain I (residues 1-43) anchors the protein to the inner membrane by a single transmembrane segment (Muller *et al* 1993). Domains II (44-116) and III (117-142) are located in the periplasm.

The production of a recombinant TolRII in the periplasm of *E.coli* cells induces tolerance of the bacteria to colicin A (Journet *et al* 1999). It has recently been shown by *in vitro* and *in vivo* cross-linking that this induction of tolerance is likely to be due to the fact that TolRII interacts with the translocation domain of colicin A (Journet *et al* 2001).

In vitro cross-linking has also demonstrated an interaction between the translocation domain of colicin E3 and TolRII (Journet *et al* 2001).

Deletion mutants have been used to localise the binding site for TolR to residues 7-14 of colicin A. This region of colicin A overlaps with the TolB box of colicin A

(residues 7-20), indicating that the interaction of colicin A with TolR and TolB may be closely related in space or time (Journet *et al* 2001).

However, this putative TolR box sequence is not well conserved in other colicins. This is similar to the situation for TolA, where no significant homology can be found for the regions of colicins interacting with TolA.

Attempts to detect the interaction between TolR and colicin A with the yeast-two hybrid technique and spectrofluorometric analysis have failed, indicating that this interaction could be weak and transient (Journet *et al* 2001). It has been suggested that the interaction of the colicin with TolB drives the interaction of the colicin with TolR, despite the lower affinity of the latter interaction and less conserved sequence homology (Journet *et al* 2001).

5.1.4. TolA-TolB interaction

An interaction between TolA and TolB has been shown by yeast-two hybrid screening and was confirmed by cross-linking with the purified proteins (Wallburger *et al* 2002). Mutagenesis experiments have identified domain III of TolA as the domain involved in interacting with TolB (Wallburger *et al* 2002, Dubuisson *et al* 2002). Although the N-terminal domain of TolB was shown to be sufficient for binding to TolA, the C-terminal domain was suggested to strengthen the interaction (Wallburger *et al* 2002). The identification of a suppressor mutant of *tolA* mutations in the *tolB* gene have provided further evidence that it is the N-terminus of TolB that interacts with TolA (Dubuisson *et al* 2002). The D120N mutation in TolB, located in the N-terminal domain, was able to restore colicin sensitivity to cells containing a *tolA* mutant, altered at residue F352 or Y353.

The physiological significance of this interaction has been shown by using cells expressing a Y354H V409E TolAIII mutant, which retains binding to the translocation domain of colicin A, but is unable to bind to TolB (Wallburger *et al* 2002). These cells exhibited a *tol* phenotype, being sensitive to SDS and tolerant to colicin A. The cells were also tolerant to colicin E3, but the sensitivity to colicin E1, which does not require TolB for translocation, was unaffected. Wallburger *et al* (2002) suggest that TolB links the outer and inner membranes of the cell via simultaneous interactions of its C-terminal domain with Pal and its N-terminal domain with TolA.

5.1.5. Ternary complexes

The demonstration of a ternary complex between colicin A, TolA and TolB has been used to suggest that colicin A can bind to TolA and TolB simultaneously (Bouveret *et al* 1998). As the TolA and TolB boxes are reasonably far apart in the colicin A translocation domain, this is likely to be the case. However, recent work showing that TolA binds to TolB (Wallburger *et al* 2002) means that although a ternary complex is formed between TolA, TolB and colicin A, the complex may be mediated by the interaction between the two Tol proteins, one of which is simultaneously interacting with colicin A, rather than the simultaneous interaction of colicin A with TolB and TolA.

Cross-linking has shown that colicin A can also interact with TolA and TolR simultaneously (Journet *et al* 2001).

5.1.6. Brownian ratcheting

It has been suggested that colicin translocation could occur by Brownian ratcheting. This hypothesis involves the movement of the translocation domain through the periplasm by diffusion, with interactions between the translocation domain and Tol proteins, preventing the colicin from moving backwards. Interactions between Tol proteins themselves may also drive the colicin through the periplasm (Journet *et al* 2001). No ATP is required for the ratcheting, consistent with the lack of ATP in the periplasm.

If Brownian ratcheting were the mechanism used for colicin translocation, the directionality of the translocation is likely to be driven by a series of interactions with Tol proteins with increasing affinity. Due to the association of TolB with the outer membrane via Pal, it has been suggested that TolB is the first TolB protein to interact with a colicin, on entering the periplasm. The colicin could then interact with TolA, via the TolB-TolA or Pal-TolA interactions. Finally the colicin would reach TolR, via the TolR-TolA interaction.

This mechanism has been demonstrated for translocation inside the endoplasmic reticulum of Prepro-α factor by BiP (Matlack *et al* 1999).

5.1.7. Pore formers vs endonucleases

Their differing mechanisms of cytotoxicity means that pore-forming colicins only need to cross the outer membrane and the periplasm to reach the inner membrane and

form pores in it, whereas endonuclease colicins need to cross the outer membrane, the periplasm and then the inner membrane to access the DNA or RNA in the cytoplasm. The different cellular targets therefore suggest the possibility that there may be differences between the two types of colicins in how they use the Tol system. This is supported by the observation that although colicin A interacts with TolA, no interaction of colicins E9 or E3 with TolA has been detected, indicating that these endonuclease colicins may not directly interact with TolA (Bouveret *et al* 2002). Wallburger *et al* (2002) also suggest that colicin A interacts with a different region of TolB than colicin E9.

The interaction of colicins with TolA in particular, appears to be variable between colicins. For example, two *tolA* mutations have been isolated which conferred tolerance to colicin A but not to colicins E1, E2, E3 and K, indicating that there may be more than one mechanism of interaction with TolA (Dubuisson *et al* 2002). In addition, the regions of colicins N and A predicted to interact with TolA, share no significant sequence homology (Raggett *et al* 1998, Journet *et al* 2001).

5.1.8. Aims of chapter

This chapter uses surface plasmon resonance as a quantitative technique to investigate differences between pore-forming colicins and endonuclease colicins in their interaction with Tol proteins. Much of the previous data on the interactions of colicins with Tol proteins has been from qualitative experimental techniques such as yeast-two hybrid screening and cross-linking studies. A dataset of affinity constants for several different colicins with different Tol proteins is likely to provide valuable information on the translocation mechanism(s) of group A colicins.

Previous chapters of this thesis have been concerned with the interaction between TolB and the endonuclease colicins E3 and E9. The interactions observed here will initially be compared to the interaction of the pore-forming colicin A.

The work is then extended to investigate the interactions of two other Tol proteins, TolA and TolR, with colicins A, E3 and E9. The capacity of SPR to determine affinity data for the interactions has been exploited to investigate the validity of the Brownian ratcheting model.

Finally gel filtration and SPR have been used to confirm the interaction between TolB and TolA, previously reported by Wallburger *et al* (2002), and kinetic parameters for this interaction have been obtained using SPR.

5.2. Results

5.2.1. Production of Tol proteins

Full-length TolB protein was prepared as described in earlier chapters.

The association of TolA and TolR proteins with the membrane presents complications for the purification of these proteins. Therefore, these proteins were purified in truncated form, without their membrane-associated domains.

Mutational analysis, cross-linking and yeast-two hybrid studies have shown that TolA interacts with colicins via domain III (Bénédetti *et al* 1991b, Derouiche *et al* 1997, Wallburger *et al* 2002). Previous SPR experiments investigating the interaction between TolA and colicins were performed with the isolated domain III of TolA and no interaction was detected between TolAII and colicin A (Derouiche *et al* 1997). Therefore, domain III of TolA was isolated and used in these studies.

In vitro and *in vivo* studies have indicated that it is domain II of TolR that is involved in the interaction with colicins (Journet *et al* 2001). Domain I is the only domain of TolR associated with the membrane and therefore domains II and III of TolR (residues 44-142) have been isolated and used in this study.

5.2.1.1. TolA domain III

TolA domain III (TolAIII) encoding DNA was prepared by digesting the plasmid pNP163 (Christopher Penfold), a pUC18 based plasmid containing TolA III DNA, with *Xho*I and *Nde*I restriction enzymes. The digestion products were run on an agarose gel and the TolAIII encoding DNA was purified from the gel. This DNA was then ligated into pET30c, in order to add a his-tag to the N-terminus of the encoded protein, and the ligated product was used to transform *E.coli* DH5α cells. The TolAIII DNA from the purified plasmid pSR12, obtained from the *E.coli* DH5α cells, was sequenced to ensure the cloning had been successful and the plasmid was then used to transform *E.coli* BL21 (DE3) cells. IPTG was used to express TolAIII in a large scale culture of these BL21 cells, with samples taken before and after induction and run on a 16% gel to ensure that expression had been successful (see Figure 5.1). Cells were harvested by centrifugation, lysed by sonication and the TolAIII protein was then purified using nickel affinity chromatography as described in Chapter 2. Following dialysis in 50 mM potassium phosphate buffer, pH 7.4, the purified

fractions were run on a 16 % SDS-PAGE gel to check the purity of the protein (see Figure 5.2).

Figure 5.1 16 % SDS-PAGE gel showing crude lysate samples taken from uninduced cells and induced cells containing pSR12. A band of approximately 12 kDa can be seen in the lane containing the induced sample, indicating that induction of TolA III protein has been successful.

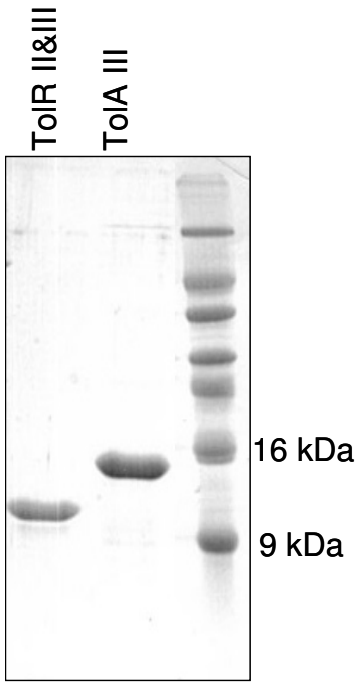


Figure 5.2 16 % SDS-PAGE gel showing purified TolR II&III (10.7 kDa) and TolAIII (12.1 kDa) proteins

5.2.1.2. TolR domains II & III

Plasmid ET1 (Elena-Stella Theophilou), a pET21a based plasmid containing domains II and III of TolR, was used to transform BL21 (DE3) cells. The expression of the TolR II&III protein was induced using IPTG in a large scale culture of these cells. The cells were then harvested by centrifugation, lysed by sonication and the TolR II&III protein was purified using nickel-affinity chromatography as detailed in Chapter 2. Following dialysis in 50 mM potassium phosphate buffer, pH 7.4, the purified fractions were run on a 16 % SDS-PAGE gel to ensure purity of the protein (see Figure 5.2).

5.2.2. Production of colicins

Full-length colicins E3 and E9 were purified as described in previous chapters. Cross-linking and SPR studies investigating the interaction of colicin A with Tol proteins mostly use the isolated N-terminal translocation domain of colicin A (Derouiche *et al* 1997, Bouveret *et al* 1998, Journet *et al* 2001).
Therefore, in order to be consistent with previous studies, the isolated N-terminal 172 residues of colicin A were also used in this study.
DNA encoding the translocation domain of colicin A was prepared by digestion of the pNP200 plasmid (Christopher Penfold), a pUC18-based plasmid containing DNA encoding the N-terminal domain of colicin A, with *Nco*I and *Xho*I restriction enzymes. The restriction digest was run on an agarose gel to separate the pUC18 vector from the insert and the colicin $A_{1\text{-}172}$ encoding digested DNA fragment was purified from the gel. The colicin $A_{1\text{-}172}$ DNA fragment was then ligated into pET21d, restricted with the same two restriction enzymes, in order to add a his-tag to the C-terminus of the protein, and the ligated product was used to transform *E.coli* DH5α cells. The colicin A encoding fragment of the plasmid purified from these cells was first sequenced to ensure the cloning had been successful and was then used to transform *E.coli* BL21 (DE3) cells. IPTG was used to induce expression of the colicin $A_{1\text{-}172}$ protein in a large scale culture of the *E.coli* BL21 pSR12 cells, with samples taken before and after induction and run on an SDS-PAGE gel to ensure expression had been successful (see Figure 5.3). The cells were then harvested by centrifugation, lysed and purified using nickel-affinity chromatography as detailed in

Chapter 2. Following dialysis in 50 mM potassium phosphate buffer, pH 7.4, the purified protein was run on a gel to ensure purity (see Figure 5.4).

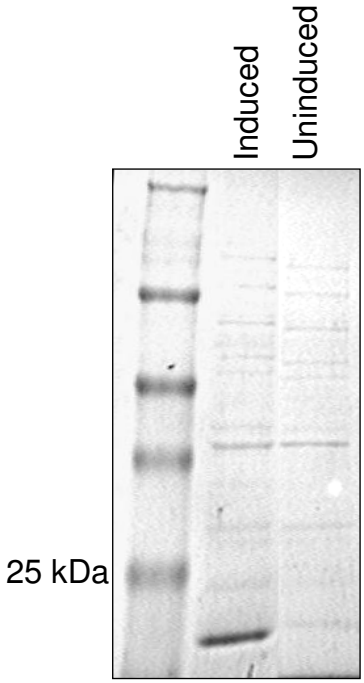


Figure 5.3 12% SDS-PAGE gel showing induction of colicin A_{1-172} protein. A band of approximately 18 kDa can be seen for the induced cells, indicating that induction of colicin A_{1-172} has been successful.

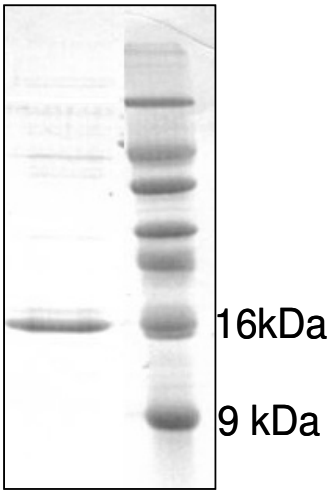


Figure 5.4 16 % SDS-PAGE gel showing purified colicin A_{1-172} (17.8 kDa) protein.

5.2.3. Interactions of colicins A, E3 and E9 with TolB

The previous two chapters of this thesis have demonstrated that an interaction can be detected between TolB and the endonuclease colicins E3 and E9. The K_d for the

interaction between colicin E9 and TolB is 13.8 μM in the presence of Im9 and 1.2 μM in the absence of Im9.

The K_d for the interaction between colicin E3 and TolB is 7.1 μM in the presence of Im3 and 0.9 μM in the absence of Im3. The same technique described in Chapter 3 has been used to determine the K_d for binding of colicin $A_{1\text{-}172}$ to TolB. Injections of 4 – 20 μM colicin $A_{1\text{-}172}$ were injected across the TolB chip and the sensorgrams produced were fitted to the Langmuir 1:1 binding model (see Figure 5.5). The kinetic parameters calculated for this fit are shown in Table 5.1. The linearity of the Scatchard plot (see Figure 5.6) and the low Chi squared value indicate that the data fit well to the model.

The K_d for binding of colicin $A_{1\text{-}172}$ to TolB was calculated as 10.5 ± 0.3 μM. Therefore the affinity of colicin $A_{1\text{-}172}$ is similar to the affinity of colicins E3 and E9 in the presence of the immunity proteins for TolB.

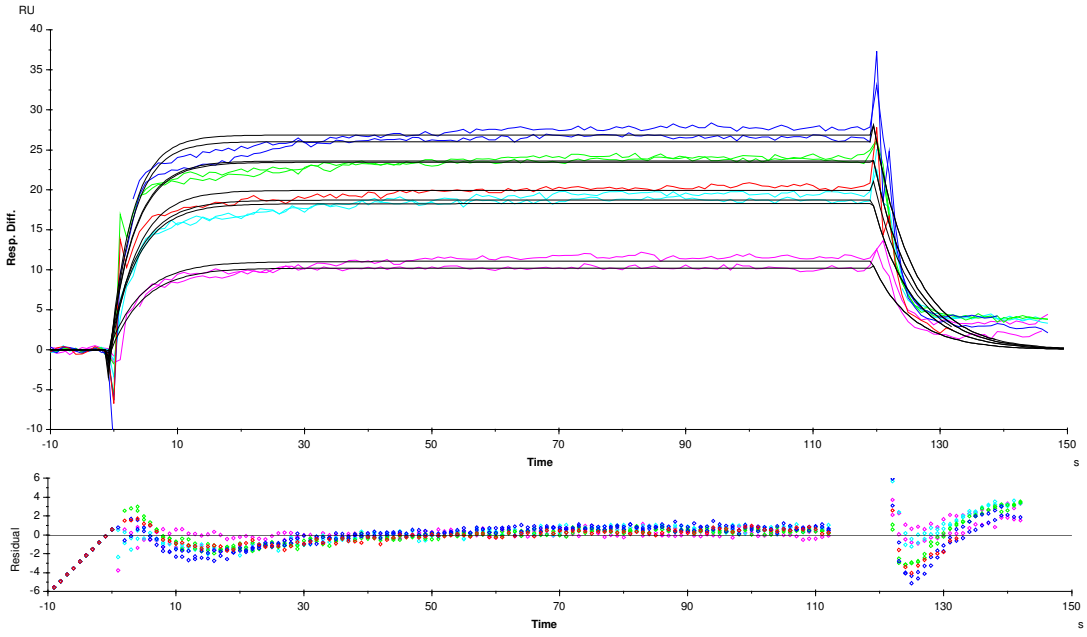


Figure 5.5 Corrected SPR sensorgrams and residual plots for titration of 4 μM (magenta), 8 μM (cyan), 10 μM (red), 16 μM (green) and 20 μM (blue) colicin $A_{1\text{-}172}$ against TolB at 25 ºC are shown. The fitted kinetic data for binding are superimposed onto each sensorgram in black. Residual plots highlight deviation of the experimental data from the theoretical fit.

Table 5.1 Kinetic data obtained from fitting of sensorgrams produced for binding of colicin A_{1-172} to TolB to the Langmuir 1:1 binding model. Errors for K_a and K_d were calculated by combining the standard errors on k_{ass} and k_{diss} in quadrature.

kass (1/Ms)	kdiss (1/s)	Ka (1/M)	Kd (μM)	Chi2
10100 ± 0.647	0.107 ± 0.00338	94900 ± 2998	10.5 ± 0.3	1.29

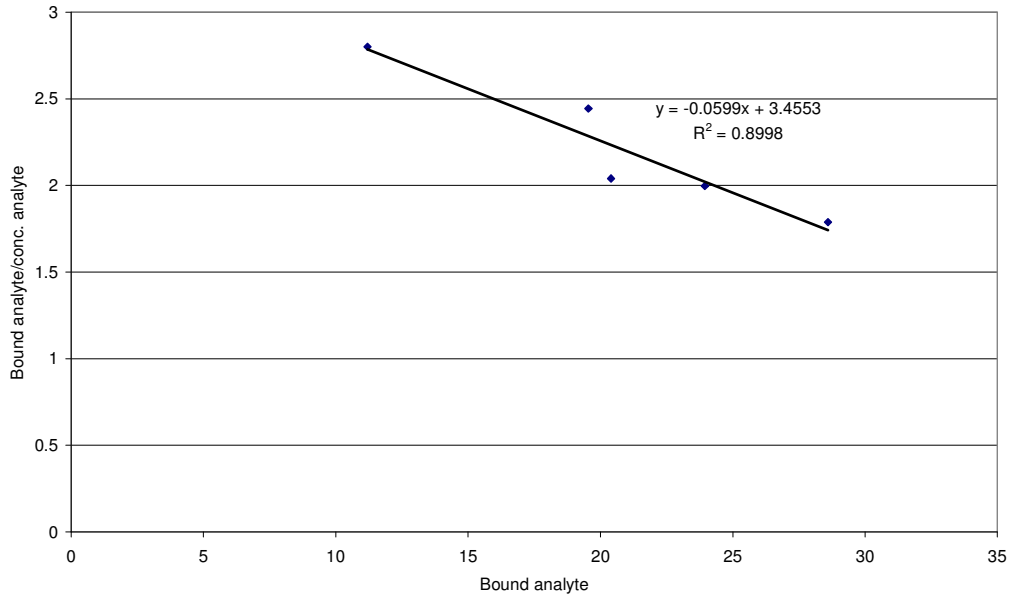


Figure 5.6 Scatchard plot for data for binding of colicin A_{1-172} to TolB. An R squared value of 0.9 is produced for a linear fit to the data indicating that the data fit well to the 1:1 Langmuir binding model. The estimated K_d for the interaction from this plot is 16.7 μM, a similar value to that produced for fitting of the data to the Langmuir binding model.

5.2.4. Interactions of colicins E3, E9 and A with TolA

Cross-linking studies have previously been used to show that colicin A interacts with TolA, but no interaction has been demonstrated between colicins E3 or E9 with TolA (Derouiche *et al* 1997, Bouveret *et al* 1997, Bouveret *et al* 2003, Christopher Penfold personal communication). In this study, SPR has been used to investigate the interaction between colicins A, E3 and E9 with TolA.

TolAIII was immobilised to a CM5 sensor chip by amine coupling as described in Chapter 2. The final response produced for immobilisation of TolAIII was 875 RU, producing an RUmax for colicin A_{1-172} of ~ 1280 and an RUmax for colicins E9 and E3 of ~ 4500. 2-10 μM colicin A_{1-172}, colicin E9 and colicin E3 were injected across the sensor chip. The sensorgrams in Figure 5.7 show that although a clear response was recorded on injection of 2 μM colicin A, no response was recorded at even the highest concentration (10 μM) of colicin E9 and E3. In order to check that the

immunity protein was not interfering with the interaction, injections of free colicins E3 and E9 were performed, but still no response was recorded.

Therefore, as expected from previous data (Derouiche *et al* 1997, Bouveret *et al* 1998), this study confirms that colicin A binds to TolAIII. The lack of binding of colicins E3 and E9 to TolAIII in the SPR experiments is consistent with the failure to detect an interaction between the T domain of colicin E9 and TolA III using the yeast-two hybrid technique (Christopher Penfold, personal communication) and the inability to cross-link TolA with the translocation domain of colicin E3 *in vitro* (Bouveret *et al* 1997).

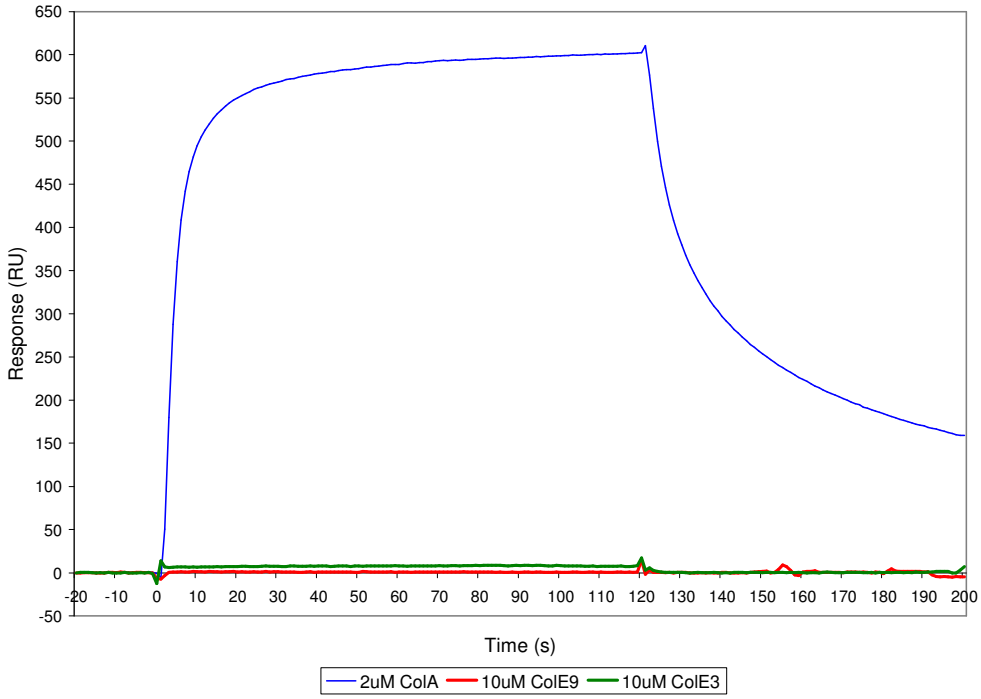


Figure 5.7 SPR sensorgrams produced on injection of 2 µM colicin A_{1-172}, 10 µM colicin E9 and 10 µM colicin E3 across TolA immobilised to a CM5 sensor chip.

In order to obtain kinetic data for the interaction between colicin A_{1-172} and TolAIII, a new SPR sensor chip was used to immobilise TolAIII at a lower concentration. The final response due to immobilisation of TolAIII was 61 RU, giving an RUmax for colicin A_{1-172} of 89 RU. 0.5 – 8 µM colicin A_{1-172} was injected across the TolAIII chip and the sensorgrams produced were fitted to the Langmuir 1:1 binding model (see Figure 5.8).

Two previous attempts at measuring kinetic parameters for the interaction between colicins indicated that TolAIII on a CM5 sensor chip has heterogeneous binding sites (Gokce *et al* 2000, Derouiche *et al* 1997). Therefore, the experiment was repeated in the opposite orientation with colicin $A_{1\text{-}172}$ immobilised on the chip. Injections of 0.25-3 µM TolAIII were performed across this chip. The sensorgrams produced, along with the fit to the Langmuir 1:1 binding model are shown in Figure 5.10. Kinetic parameters produced from fitting of the data to the model, for both experiments are shown in Table 5.2. The low Chi squared values and the high T-values indicate that the data fit the model well. Scatchard plots of the data also indicate a good fit (see Figure 5.9 and Figure 5.11).

When TolAIII was used as a ligand, the K_d is 0.5 ± 0.008 µM and when colicin $A_{1\text{-}172}$ was used as a ligand the K_d is 0.6 ± 0.002 µm. These values are very similar, giving good confidence that the K_d value is close to the true K_d value.

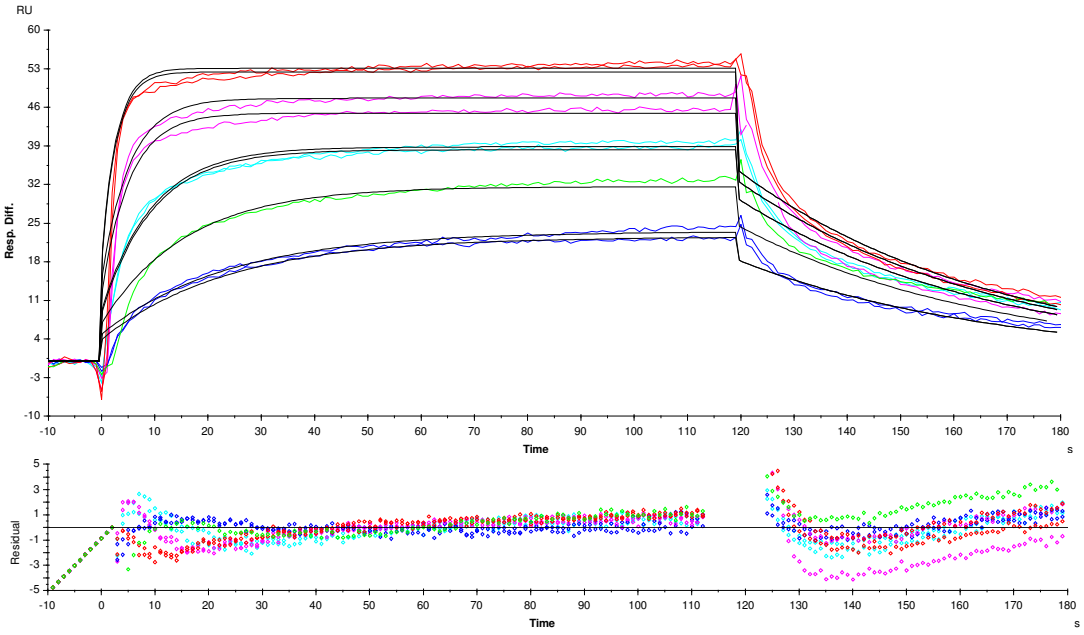


Figure 5.8 Corrected SPR sensorgrams and residual plots for titration of 0.5 µM (blue), 1 µM (green), 2 µM (cyan), 4 µM (magenta) and 8 µM (red) colicin $A_{1\text{-}172}$ against TolAIII at 25 ºC are shown. The fitted kinetic data for binding are superimposed onto each sensorgram in black. Residual plots highlight deviation of the experimental data from the theoretical fit.

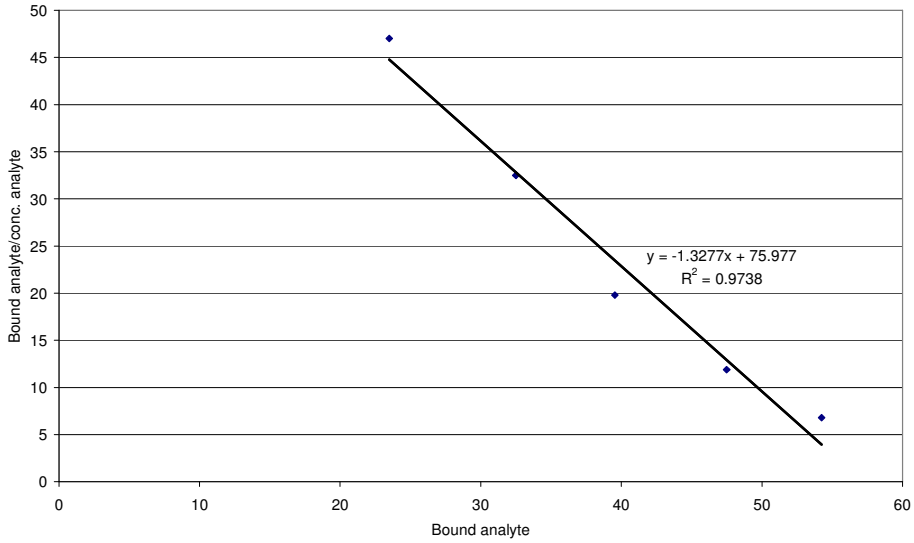


Figure 5.9 **Scatchard plot for data for binding of colicin $A_{1\text{-}172}$ to TolA. An R squared value of 0.97 is produced for a linear fit to the data indicating that the data fit well to the 1:1 Langmuir binding model. The estimated K_d for the interaction from this plot is 0.75 µM, a similar value to that produced for fitting of the data to the Langmuir 1:1 binding model.**

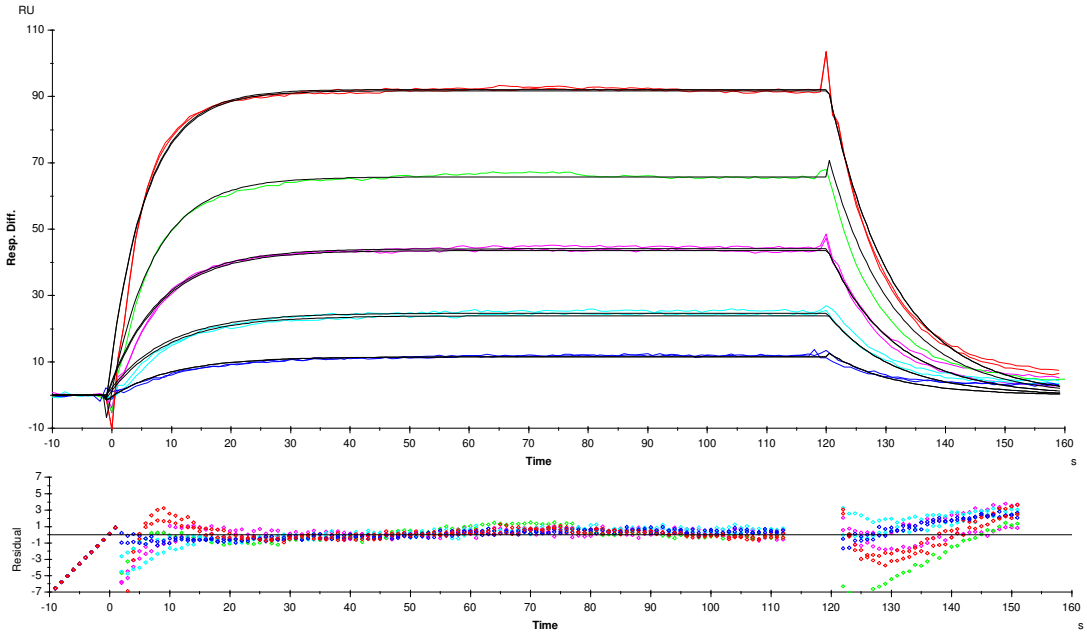


Figure 5.10 **Corrected SPR sensorgrams and residual plots for titration of 0.25 µM (blue), 0.5 µM (cyan), 1 µM (magenta), 2 µM (green) and 3 µM (red) TolAIII against colicin $A_{1\text{-}172}$ at 25 ºC are shown. The fitted kinetic data for binding are superimposed onto each sensorgram in black. Residual plots highlight deviation of the experimental data from the theoretical fit.**

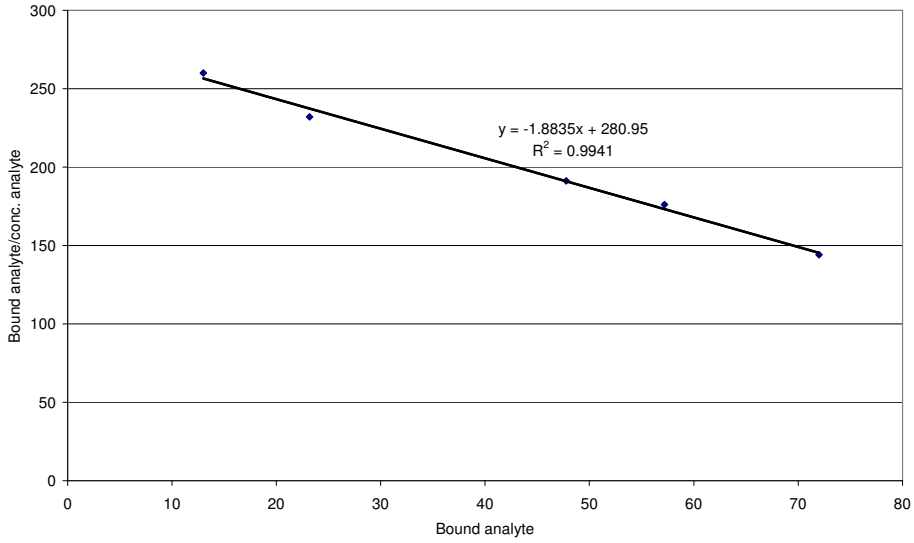


Figure 5.11 Scatchard plot for data for binding of TolA to colicin A_{1-172}. An R squared value of 0.99 is produced for a linear fit to the data indicating that the data fit well to the 1:1 Langmuir binding model. The estimated K_d for the interaction from this plot is 0.53 µM, a similar value to that produced for fitting of the data to the Langmuir 1:1 binding model.

Table 5.2 Kinetic data obtained from fitting of sensorgrams produced for binding of colicin A_{1-172} to TolA to the Langmuir 1:1 binding model. Errors for K_a and K_d were calculated by combining the standard errors on k_{ass} and k_{diss} in quadrature.

Ligand	kass (1/Ms)	kdiss (1/s)	Ka (1/M)	Kd (µM)	Chi2
TolAIII	42100 ± 611	0.0209 ± 0.000215	2020000 ± 35754	0.5 ± 0.008	1.44
Colicin A	135000 ± 3060	0.0814 ± 0.000751	1660000 ± 40620	0.603 ± 0.02	1.77

In order to confirm the lack of interaction between colicin E9 and TolA, a gel filtration experiment was performed to search for a colicin E9-TolA complex. Colicin E9 was first loaded onto a Superdex 75 gel filtration and the elution volume (Ve), shown by monitoring the absorbance at 280nm of the mobile phase, was recorded. This was repeated with TolAIII. Colicin E9 was then incubated for five minutes with equimolar amounts of TolAIII and the mixture was loaded onto the column. The Ve was recorded and compared to the Ve for TolAIII alone and colicin E9 alone. If a complex was formed between TolAIII and colicin E9, the complex would be eluted from the column earlier than the two proteins separately, *ie* the Ve would decrease, due to the increased size of the complex compared to the separate protein components (see Chapter 2).

However, Figure 5.12 shows that the absorbance peaks produced for the mixture are at the same position as the absorbance peaks produced for each protein separately,

demonstrating that the Ve values for the mixture correspond to the Ve values for TolAIII and colicin E9, eluting as separate proteins. This confirms the SPR work, indicating that there is no interaction between colicin E9 and TolAIII.

As a control, gel filtration was performed with TolAIII and colicin $A_{1\text{-}172}$. Figure 5.13 shows that when a TolAIII-colicin $A_{1\text{-}172}$ mixture is loaded onto the column, the absorbance peak produced shifts to the left, indicating that the mixture elutes earlier than the separate proteins *ie* the Ve is lower for the complex than the separate proteins. This shows that a complex is formed between colicin $A_{1\text{-}172}$ and TolAIII, consistent with previous data.

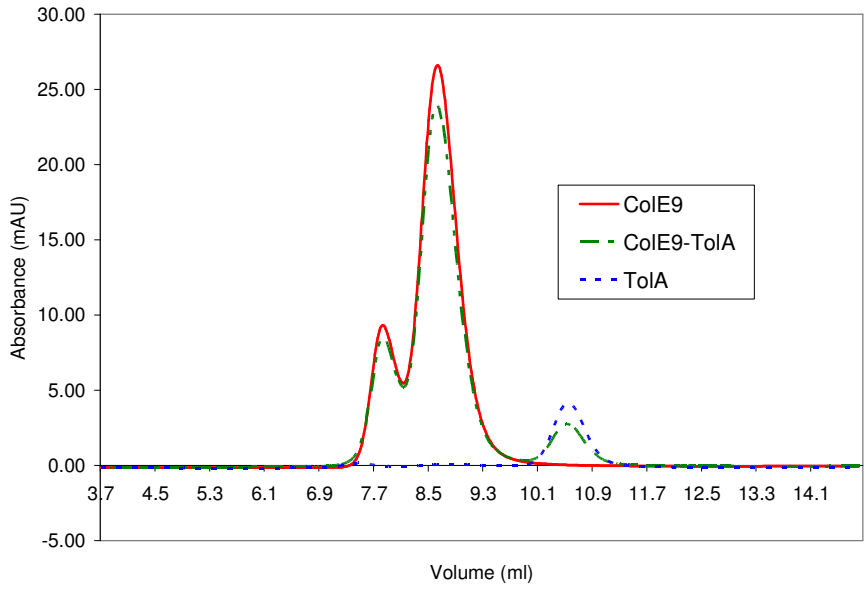


Figure 5.12 Gel filtration elution profiles of TolAIII alone, colicin E9 alone and TolAIII-colicin E9 together. The overlapping peaks indicate that there is no complex formed between TolAIII and colicin E9.

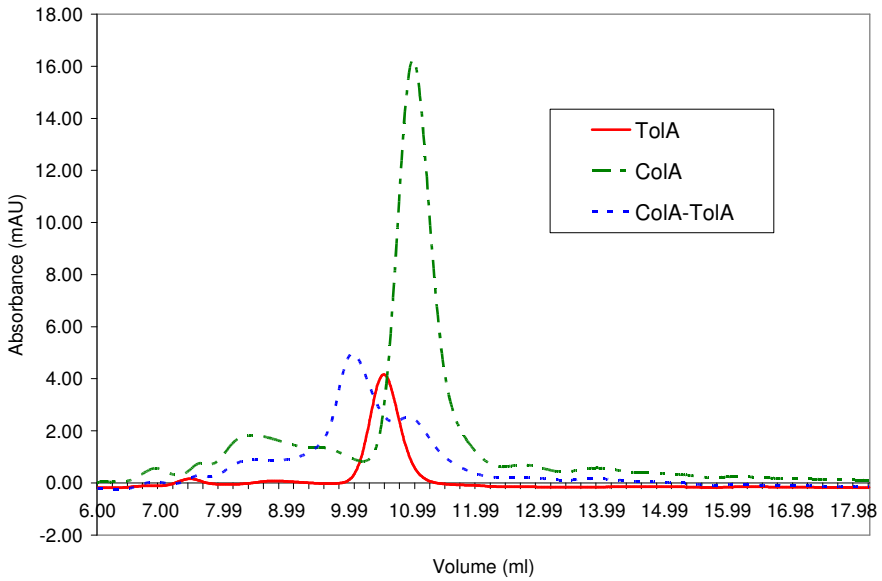


Figure 5.13 Gel filtration elution profiles of TolA alone, colicin A_{1-172} alone and TolA-colicin A_{1-172} together. The shift in peak position to the left when TolA and colicin A_{1-172} are run together, indicates an increase in size, demonstrating that TolA and colicin A form a complex via the translocation domain of colicin A.

5.2.5. Interactions of colicins E3, E9 and A with TolR

Cross-linking studies with purified proteins have indicated that colicins A and E3 interact with TolR (Journet *et al* 2001). The sequence homology between the translocation domains of colicins E9 and E3 suggests that colicin E9 also interacts with TolR. Therefore the binding of colicin E9 to TolR was investigated using SPR. TolR II&III was immobilised to a CM5 sensor chip via amine coupling. A final response due to immobilisation of TolR II&III of 657 was produced giving an RUmax for colicin E9/Im9 of 4421 RU. 2 µM colicin E9/Im9 was injected across the chip but no response was produced. Therefore increasing concentrations of colicin E9 up to 50 µM were injected, but still no response was recorded.

In order to check that the TolR II&III protein was intact on the chip, injections of varying concentrations of colicin A_{1-172} were performed. In this case a response was seen (see Figure 5.15). At 15 µM colicin A_{1-172}, the maximum response was 167 ± 2 RU, only 15 % of the RUmax for colicin A_{1-172} (1092 RU). This suggests that the interaction is of lower affinity than the previous interactions investigated in this thesis.

In order to check that Im9 was not interfering with the interaction between colicin E9 and TolR II&III, injections of free colicin E9 were performed but still no response

was produced (see Figure 5.14). Injections of colicin E3/Im3 also produced no response. This result is unexpected, as cross-linking studies had suggested that both colicin A and colicin E3 interacted with TolR II&III.

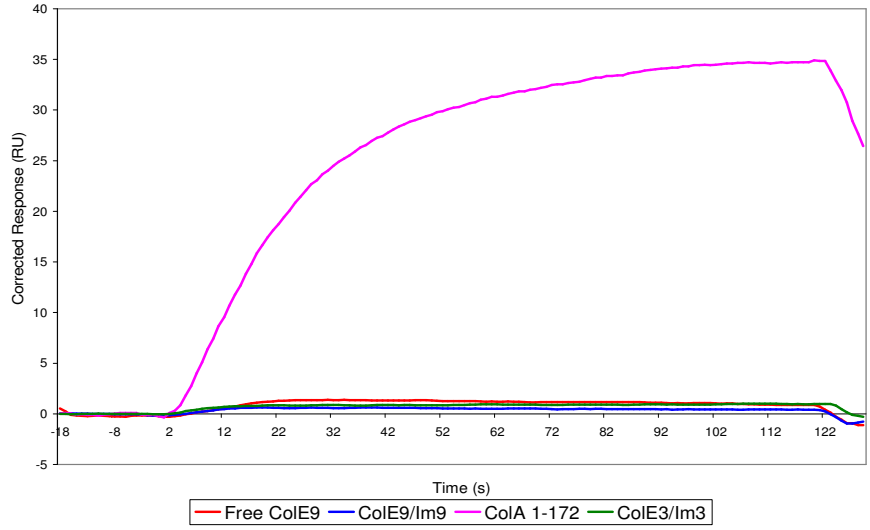


Figure 5.14 SPR sensorgrams produced on injection of 10µM free colicin E9, colicin E9/Im9, colicin E3/Im3 and 2µM colicin $A_{1\text{-}172}$ across TolR II&III immobilised on a CM5 sensor chip.

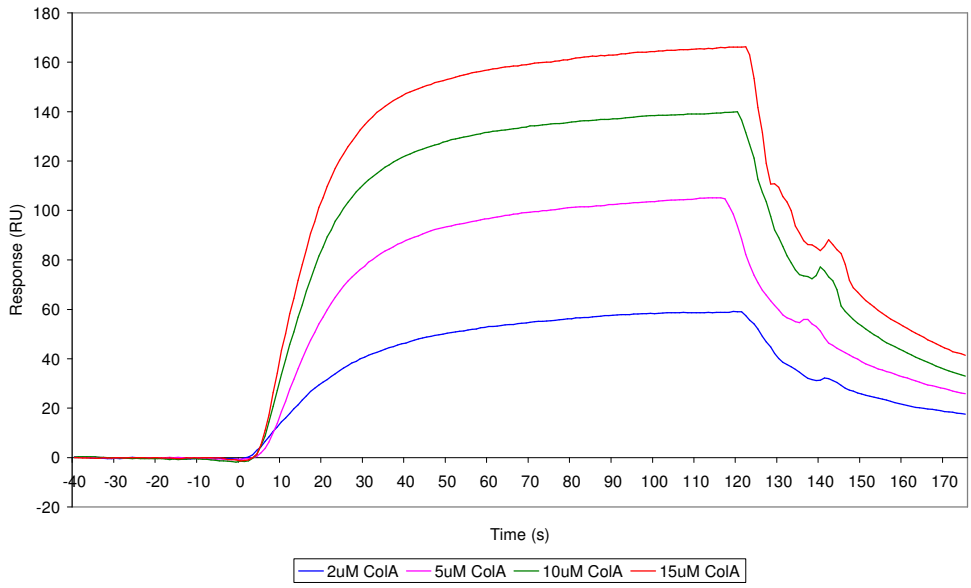


Figure 5.15 Sensorgrams produced for injection of 2, 5, 10 and 15 µM colicin $A_{1\text{-}172}$ across a TolR II&III sensor chip.

To ensure that immobilisation of TolR II&III was not affecting the interaction with colicin E9, the above experiment was performed in reverse, with free colicin E9 being immobilised on the sensor chip and TolRII&III being used as the analyte. Figure 5.16

shows that no response was produced on injection of 10 µM TolRII&III across the colicin E9 chip. TolB was injected across this chip, to check that the colicin E9 immobilised on the chip was intact. A response of 280 RU was produced, indicating that the colicin E9 translocation domain is intact and able to bind to TolB.

Gel filtration was also used to investigate the interaction between colicins and TolRII&III. Colicin E9 was first loaded onto a Superdex 75 gel filtration and the Ve was recorded. This was repeated with TolRII&III. Colicin E9 was then incubated for five minutes with equimolar amounts of TolRII&III and the mixture was loaded onto the column. The Ve was recorded and compared to the Ve for TolRII&III alone and colicin E9 alone. Figure 5.17 shows that the absorbance peaks produced for the mixture are at the same position as the absorbance peaks produced for each protein separately, demonstrating that the Ve values for the mixture correspond to the Ve values for TolRII&III and colicin E9, eluting as separate proteins. This supports the SPR work, indicating that colicin E9 does not interact with TolRII&III.

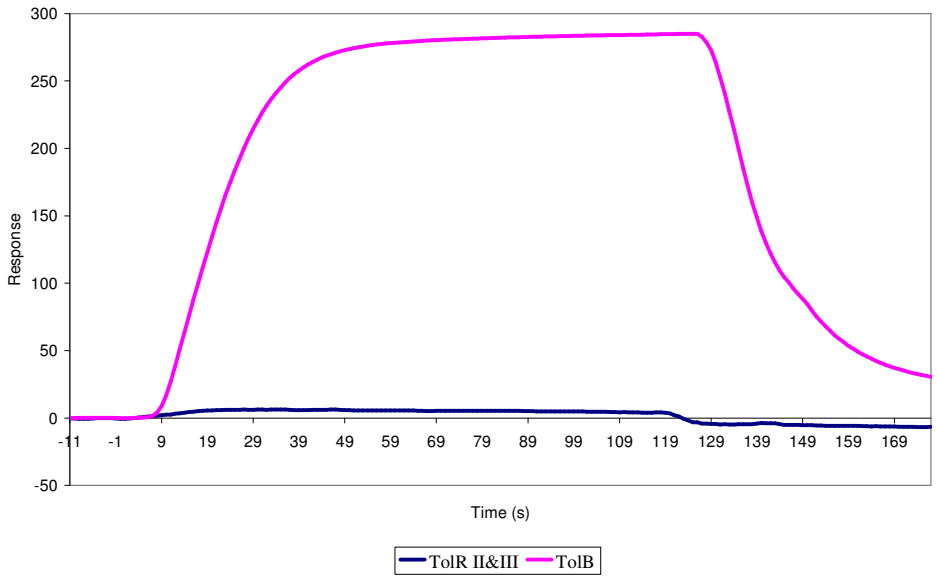


Figure 5.16 SPR sensorgrams produced on injection of 2 µM TolB and 10 µM TolR II&III across free colicin E9 immobilised on a CM5 sensor chip.

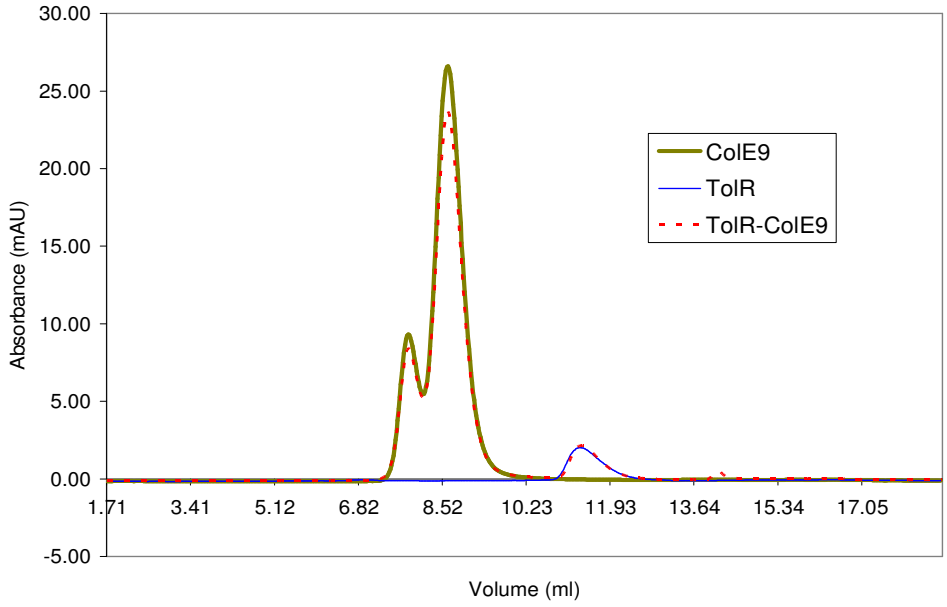


Figure 5.17 Gel filtration elution profiles of colicin E9, TolR II&III and a mixture of colicin E9/TolR II&III. The overlapping peaks for the mixture and separate proteins, indicate that colicin E9 and TolR II&III do not interact.

As a control, the same gel filtration experiment was performed with colicin $A_{1\text{-}172}$. However, Figure 5.18 shows that the peaks produced for the mixture overlap with the peaks produced for the individual proteins, suggesting that no complex between colicin $A_{1\text{-}172}$ and TolR II&III is being formed. However, this may be due to the apparent low affinity of the interaction suggested by the SPR data.

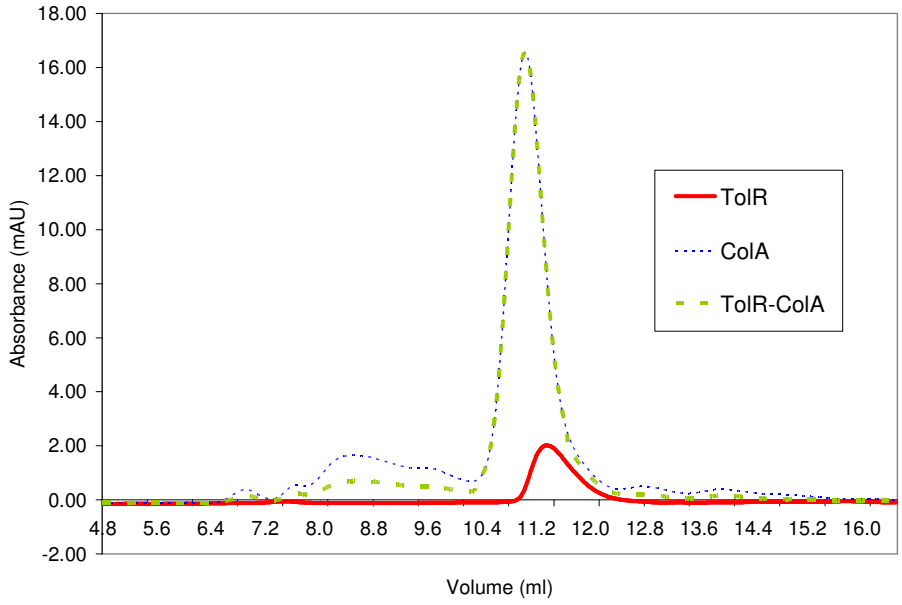


Figure 5.18 Gel filtration elution profiles of colicin A_{1-172}, TolR II&III and a mixture of colicin A_{1-172}/TolR II&III. The overlapping peaks for the mixture and separate proteins, suggest that no interaction is occurring between colicin A and TolR II&III.

The low responses produced in the preliminary SPR experiment for the binding of TolR II&III to colicin A_{1-172}, meant that no kinetic data could be determined for the interaction, as the concentrations of proteins required for the analysis are too high.

To investigate whether the binding of colicin E9 to TolAIII is dependent on binding of the colicin to TolR II&III or vice versa, colicin E9 was pre-incubated with 2 and 10 µM TolR II&III before being injected across a TolAIII chip. However, pre-incubation with TolR still produced no response. Pre-incubation of colicin E9 with 2 and 10 µM TolAIII before being injected across a TolR II&III chip, also produced no response.

5.2.6. Possible competition between TolR and TolB for binding to Colicin A

Journet *et al* (2001) suggest that the TolR box in colicin A overlaps the TolB box. Therefore, it is unlikely that TolR and TolB are able to bind to colicin A at the same time, in which case TolR could compete with TolB for binding to colicin A or vice versa. This hypothesis was tested using SPR.

Colicin A_{1-172} (2 or 4 μM) was incubated with varying concentrations of TolR II&III (2-16 μM) before being injected across a TolB chip. The responses produced were compared to the responses produced with injections of 2 and 4 μM colicin A_{1-172}, without TolR II&III, across the TolB chip. If colicin A_{1-172} and TolR II&III interact and are still able to bind to TolB, an increased response would be expected compared to injection of colicin A_{1-172} alone, as the TolB-colicin A_{1-172} complex has a greater mass than colicin A_{1-172} alone. If colicin A_{1-172} could not interact with TolB, whilst it was interacting with TolRII&III, a decrease in response would be expected compared to injection of colicin A_{1-172} alone. However, Figure 5.19 shows that there was no change in response when TolRII &III was present compared to when TolR II&III was absent. No response was produced on injection of TolR II&III across the TolB chip, indicating that TolR II&III does not interact with TolB. It is therefore possible that TolB is displacing TolR II&III for binding of colicin A_{1-172}. This seems plausible, as the affinity for binding of colicin A_{1-172} to TolB is much higher than the affinity for binding of colicin A_{1-172} to TolR II&III.

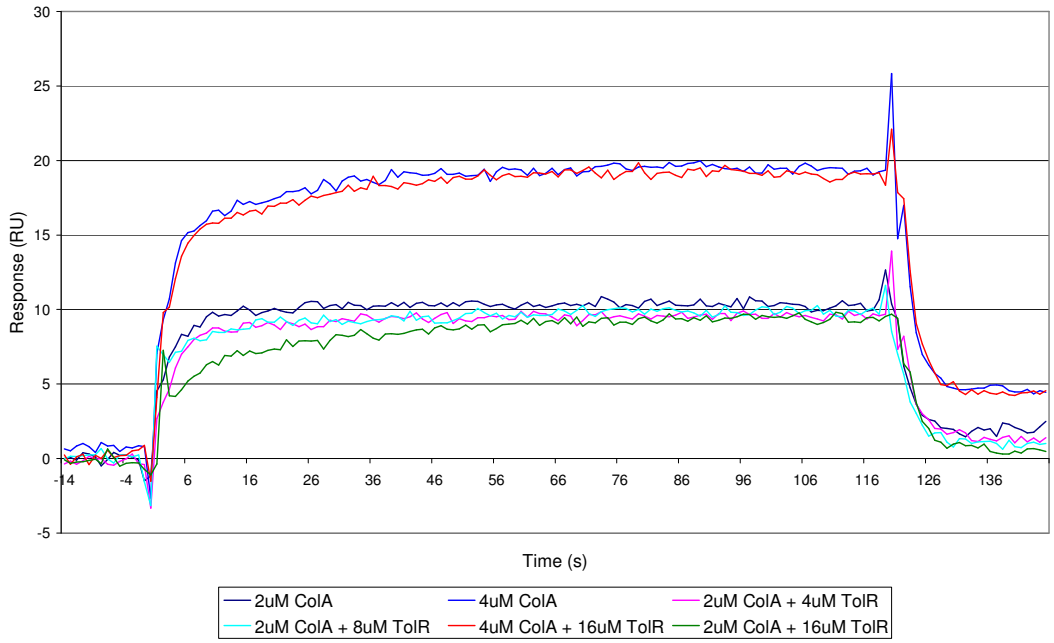


Figure 5.19 Sensorgrams produced for injection of 2 or 4 μM colicin A_{1-172} alone and pre-incubated with 4-16 μM TolR II&III.

5.2.7. Interaction between TolA and TolB

Wallburger *et al* (2002) showed, by yeast-two hybrid screening, that TolAIII interacts with the N-terminal domain of TolB. This interaction was confirmed by cross-linking

experiments on purified proteins. The biological significance of this interaction was shown by disruption of the interaction, which caused a disruption in membrane integrity and a tolerance to colicin A.

Given the significance of the interaction, this study set out to determine kinetic parameters for the interaction. Initially gel filtration was used to confirm the TolAIII-TolB interaction. Figure 5.20 shows that when a TolAIII-TolB mixture was loaded onto the column, the Ve was decreased compared to the Ve for TolAIII alone and TolB alone, confirming that TolAIII and TolB form a complex. This decrease in Ve could only be detected when concentrations of at least 15 μM of TolAIII and TolB were used.

SPR was then used to determine kinetic data for the interaction. Firstly TolB was amine coupled to a CM5 sensor chip as described previously. Concentrations of between 5 and 30 μM TolAIII were injected across the TolB chip and the sensorgrams produced were fitted to the Langmuir 1:1 binding model, shown in Figure 5.21. Table 5.3 shows the kinetic parameters calculated from the fit. The Chi squared value of <2 and the R squared value of 0.99 for a linear fit to the Scatchard plot (shown in Figure 5.22), indicate that the data fit the model well. A K_d for the interaction of 15.8 ± 1.1 μM was calculated.

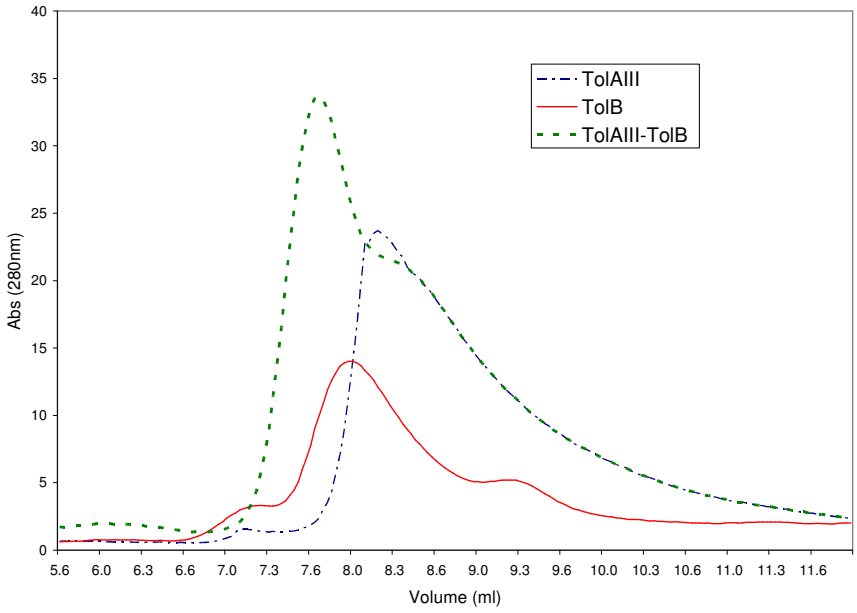


Figure 5.20 Gel filtration elution profiles for TolAIII, TolB and a TolAIII-TolB mixture. The shift in peak position to the left for the TolAIII-TolB mixture confirms that a complex is formed.

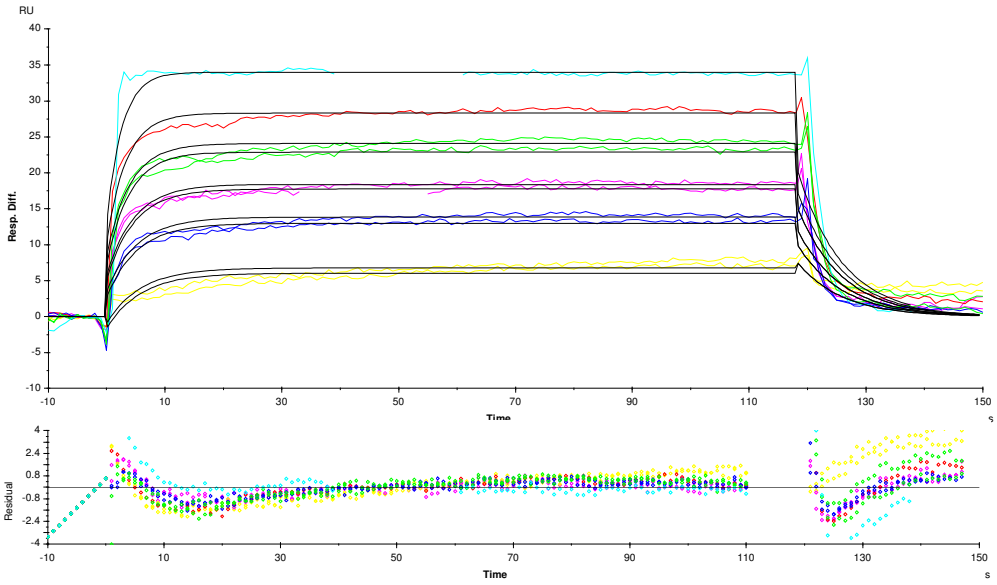


Figure 5.21 Corrected SPR sensorgrams and residual plots for titration of 5 µM (yellow), 7.5 µM (blue), 10 µM (magenta), 15 µM (green), 20µM (red) and 30 µM (cyan) TolAIII against TolB at 25 ºC are shown. The fitted kinetic data for binding are superimposed onto each sensorgram in black. Residual plots highlight deviation of the experimental data from the theoretical fit.

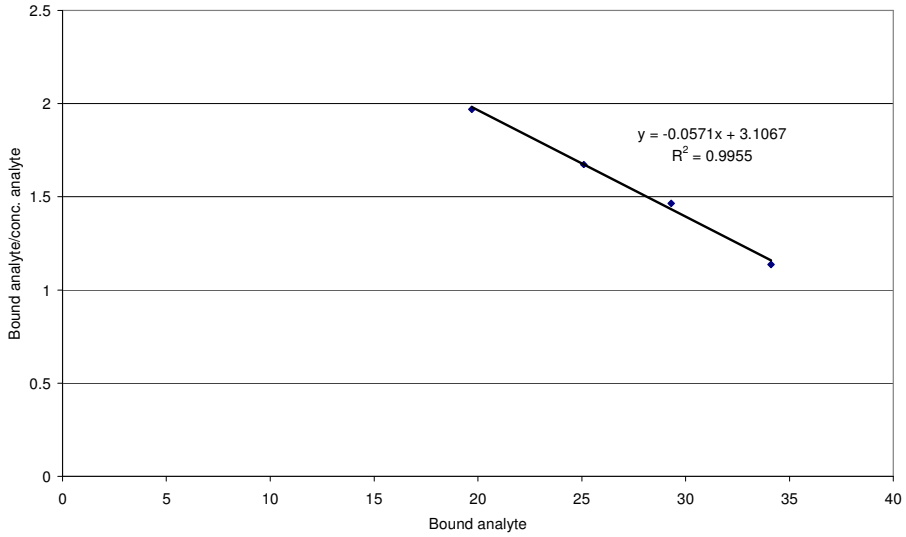


Figure 5.22 Scatchard plot for binding of TolAIII as the analyte to TolB on the sensor chip. An R squared value of 0.99 is produced for a linear fit to the data indicating that the data fit well to the 1:1 Langmuir binding model. The estimated K_d for the interaction from this plot is 17.5 µM.

The experiment was then repeated in reverse, using TolAIII as the ligand and TolB as the analyte. Concentrations of 2-20 µM TolB were injected across the TolAIII chip and the sensorgrams produced were fitted to the Langmuir 1:1 binding model (see Figure 5.23). The R squared value of 0.65 for a linear fit to the Scatchard plot of the

data, shown in Figure 5.24, suggests that the data do not fit particularly well to the model. However, the low Chi squared value and the high T-values for the calculated parameters (see Table 5.3) indicate that the fit is reasonable. A K_d of 15.3 ± 1.8 µM is calculated. This value is satisfyingly similar to the value produced when TolB is the ligand, giving good confidence that the calculated K_d is close to the true K_d value.

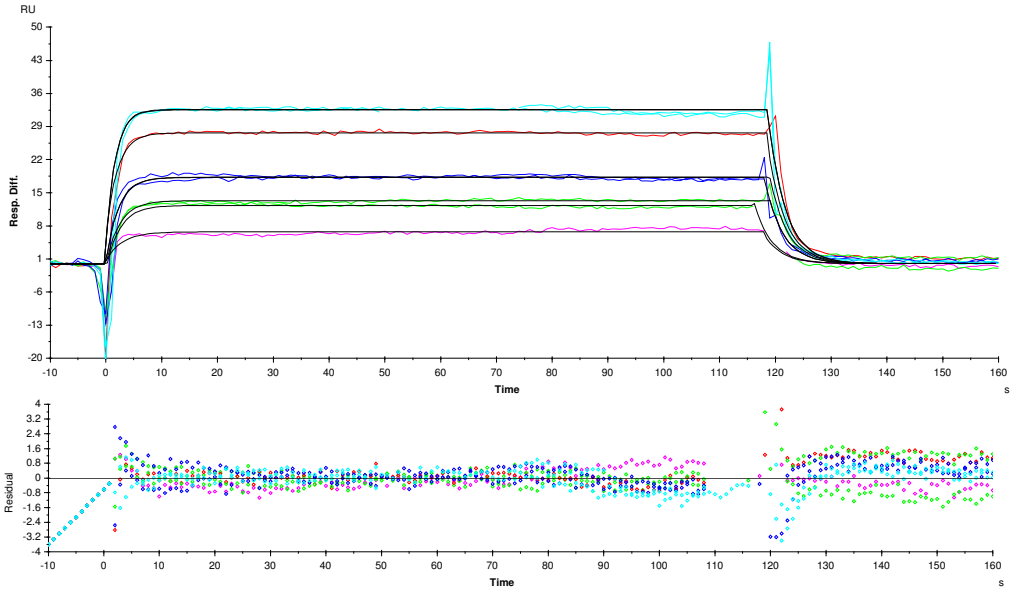


Figure 5.23 Corrected SPR sensorgrams and residual plots for titration of 2 µM (magenta), 5 µM (green), 10 µM (blue), 15 µM (red), and 20 µM (cyan) TolB against TolAIII at 25 ºC are shown. The fitted kinetic data for binding are superimposed onto each sensorgram in black. Residual plots highlight deviation of the experimental data from the theoretical fit.

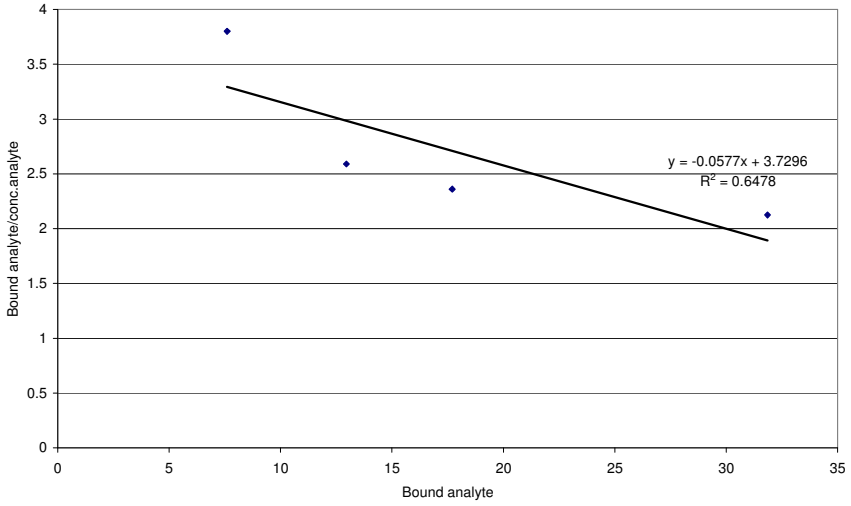


Figure 5.24 Scatchard plot for data for binding of TolB as the analyte to TolAIII on the sensor chip. An R squared value of 0.65 is produced for a linear fit to the data. This suggests that the data do not fit particularly well to the 1:1 Langmuir binding model. However, the Chi squared values and T-values for the data suggest that the fit is good and the interaction has been shown to fit the model with TolB on the surface. The estimated K_d for the interaction from this plot is 17.3 µM.

Table 5.3 Kinetic data obtained from fitting of sensorgrams produced for binding of TolB (analyte) to TolAIII (ligand) and for TolAIII (analyte) to TolB (ligand) to the Langmuir 1:1 binding model. Errors for K_a and K_d were calculated by combining the standard errors on k_{ass} and k_{diss} in quadrature.

Ligand	kass (1/Ms)	kdiss (1/s)	Ka (1/M)	Kd (μM)	Chi2
TolA	11000 ± 1210	0.169 ± 0.0078	65500 ± 7813	15.3 ± 1.8	1.25
TolB	8270 ± 515	0.131 ± 0.00422	63300 ± 4438	15.8 ± 1.1	1.54

5.2.8. TolAIII-TolB-Colicin E9 Ternary complex

The formation of a ternary complex between colicin A, TolA and TolB has been previously demonstrated by overlay Western blotting. To investigate whether a ternary complex is also formed between colicin E9, TolAIII and TolB, gel filtration was performed with the T_{61}-DNase colicin E9 fusion protein, TolAIII and TolB. TolAIII and TolB had been shown to interact together in gel filtration (see section 5.2.7). An interaction between T_{61}-DNase and TolB can also be shown by gel filtration (see Figure 5.25). No interaction between T_{61}-DNase and TolAIII was detected, consistent with SPR experiments.

When a combination of T_{61}-DNase, TolAIII, TolB was loaded onto the column, no shift in absorbance peak to the left was seen compared to the peaks from the TolAIII-TolB complex and the T_{61}DNase-TolB complex, indicating that no ternary complex is formed.

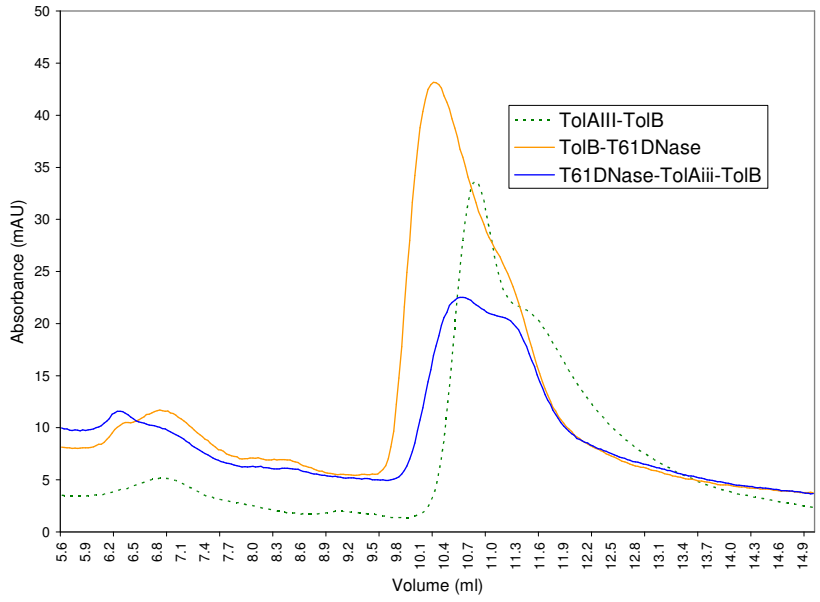


Figure 5.25 Gel filtration elution profiles of the TolAIII-TolB complex, the TolB-T_{61}DNase complex and a T_{61}DNase-TolB-TolAIII mixture. No shift in absorbance peak to the left is seen for the T_{61}DNase-TolB-TolAIII mixture, suggesting that no ternary complex is formed.

SPR was then used to investigate whether TolB is still able to bind to TolAIII, whilst bound to colicin E9. TolB was pre-incubated with varying concentrations of colicin E9, prior to being injected across a TolAIII sensor chip. The maximum responses produced were compared to the response produced when TolB alone was injected across the TolAIII chip to investigate whether the TolB remained bound to colicin E9 when it bound to TolAIII.

If TolB remained bound to colicin E9, an increased response would be expected compared to the response produced by TolB alone, due to the increased mass of the TolB-colicin E9 complex, compared to TolB. However, Figure 5.26 shows that as the concentration of colicin E9 increased, the response produced due to binding of TolB to TolAIII decreased. This suggests that colicin E9 is inhibiting the binding of TolB to TolAIII.

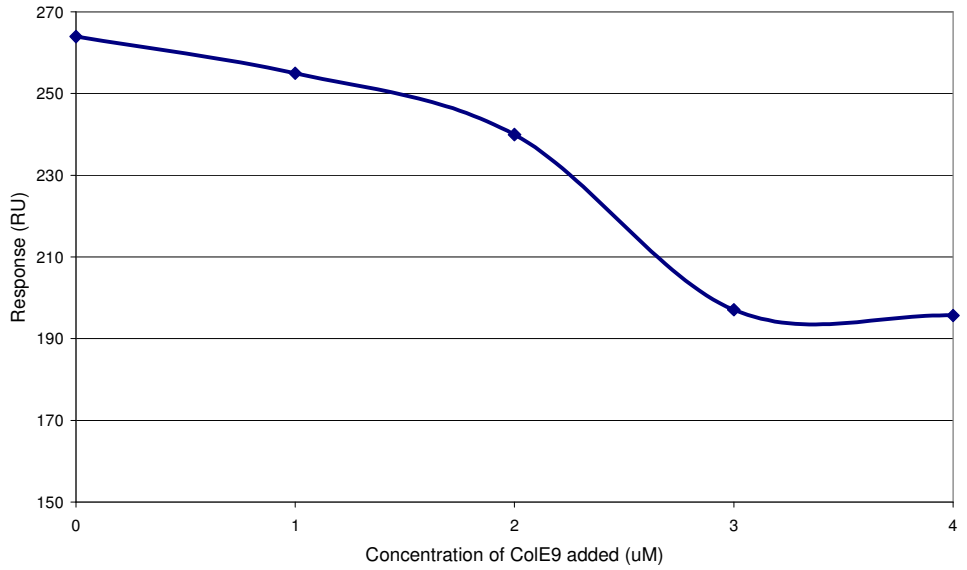


Figure 5.26 Maximum responses produced when 2 µM TolB was injected alone across a TolAIII chip, and when TolB was pre-incubated with colicin E9 at 1, 2, 3 and 4 µM concentrations before being injected across the TolAIII chip.

5.3. Discussion

5.3.1. Interactions between colicins and TolB

The K_d for binding of colicin A_{1-172} to TolB was found to be 10.5 ± 0.3 μM. ITC has previously been used to determine a K_d for the interaction of full-length colicin A with TolB of 10.4 ± 2.6 μM. Therefore the SPR data produced in this study appear to correlate very well with the ITC data.

Table 5.4 shows the K_d values for the binding of colicins A, E3 and E9 to TolB. The values from fitting the data to the Langmuir 1:1 model are similar to the values predicted from equilibrium analysis *ie* a Scatchard plot, giving good confidence in the parameters calculated.

Comparison of the K_d values for binding of the three colicins to TolB shows that the affinity of colicin A for TolB is comparable to the affinity of colicins E3 and E9 in the presence of their immunity proteins. The immunity protein for colicin A is situated in the inner membrane and therefore the colicin will not be in contact with the immunity protein, when it interacts with TolB. However, it is as yet unknown when the immunity protein is lost from endonuclease colicins, on entering a cell, and therefore it is unclear as to whether colicins E3 and E9 are in contact with their immunity proteins when they interact with TolB. A mechanism has been proposed for the loss of Im3 from colicin E3, in which the binding of the translocation domain to TolB, reduces the affinity of Im3 for colicin E3, facilitating dissociation of Im3 from the colicin (Walker *et al* 2003). This suggests the possibility that initially the immunity protein is in contact with the colicin when it interacts with TolB and then the immunity protein is lost, increasing the affinity of the colicin for TolB. As the immunity protein is not present in colicin A, it may therefore be expected that the affinity of colicin A for TolB is comparable to the affinity of free colicin E9 or E3 for TolB. It would therefore appear that an affinity of a colicin for TolB in the region of 10 μM is sufficient for translocation and that the increase in affinity of free colicin E9 or E3 for TolB may be part of a mechanism for release of the immunity protein.

The only difference between the TolB box of colicin A and the TolB box of colicins E9 and E3 is that position 37 in colicin E9 is a serine, whereas the equivalent residue in colicin A is a threonine. Chapter 3 describes a reduction on affinity for TolB due to mutation of S37 to T in colicin E9. The K_d for binding of wild-type colicin E9/Im9 to

TolB is 13.8 μM, whereas the K_d for binding of S37T colicin E9/Im9 to TolB is 24 μM. Therefore, the reduction in affinity of free colicin A for TolB compared to the affinity of free colicin E9 or colicin E3 for TolB could be, at least in part, due to the presence of a threonine residue in colicin A, in the place of the serine residue found in colicins E3 and E9.

Table 5.4 Predicted K_d values for binding of colicins A, E9 and E3 to TolB, obtained from fitting of sensorgrams to the Langmuir 1:1 binding model and from equilibrium analysis *ie* a Scatchard plot.

Colicin binding to TolB	Predicted K_d from Langmuir fitting (uM)	Predicted K_d from Scatchard plot (uM)
ColA	10.5	16.7
ColE9	1.2	1.6
ColE3	0.9	0.4
ColE9/Im9	13.8	12.2
ColE3/Im3	7.1	7.5

5.3.2. Interactions between colicins and TolA

Consistent with previous cross-linking studies, gel shift assays and SPR (Derouiche *et al* 1997, Bouveret *et al* 1998), this study has shown by gel filtration and SPR that the translocation domain of colicin A interacts with TolAIII.

However, no interaction of colicins E3 and E9 with TolAIII could be detected by SPR or gel filtration. A potential reason for this is that full-length colicins E9 and E3 were used, whereas only the translocation domain of colicin A was used in the experiment. Gokce *et al* (2000) were unable to detect an interaction between the full-length colicin A and TolA using ITC and suggested that this may be due to the TolA binding site of colicin A being blocked by other regions of the protein in the full-length colicin. However, a previous attempt to detect an interaction between the translocation domain of colicin E9 and TolAIII using the yeast-two hybrid technique failed (Christopher Penfold, personal communication) and it was not possible to cross-link TolA with the translocation domain of colicin E3 *in vitro* (Bouveret *et al* 1997). Therefore the failure to detect an interaction between colicins E3 or E9 and TolA using SPR and gel filtration, in combination with evidence from previous experiments, suggests that colicins E3 and E9 do not directly interact with TolAIII. These endonuclease colicins are, however, dependent on TolA for their translocation,

shown by their inactivity against cells containing lesions in the *tolA* gene (Davies and Reeves 1975a).

Therefore whilst TolA is directly involved in the translocation of Group A pore-forming colicins, TolA appears to be only indirectly involved in the translocation of the endonuclease colicins, perhaps by interacting with another Tol protein, such as TolB (see later).

5.3.2.1. Kinetic analysis of the TolA-colicin A interaction

Despite previous reports of a poor fit to a simple binding model, when TolAIII is immobilised on a CM5 sensor chip, (Gokce *et al* 2000, Derouiche *et al* 1997), in this SPR study the sensorgrams produced for the binding of colicin A_{1-172} to TolAIII immobilised to a CM5 sensor chip produced a good fit to the Langmuir 1:1 binding model, as demonstrated by the low Chi squared value (1.4) and the high R squared value (0.97) for a linear fit to the Scatchard plot of the data.

Derouiche *et al* (1997) immobilised TolAIII to a CM5 sensor chip by amine coupling. Concentrations of the translocation domain of colicin A from 0.2 to 2 μM were injected across the chip but the sensorgrams produced did not fit to the simple Langmuir 1:1 binding model. The authors used a more complex model, accounting for heterogeneity of immobilised ligand sites. This model produced K_d values for binding of the translocation domain of colicin A to TolAIII of 0.2 μM and 0.6 μM.

Gokce *et al* (2000) used SPR to demonstrate an interaction between TolA domains II and III and a GST-fusion protein, consisting of GST fused to residues 40-90 of colicin N (GSTN40). When GST was immobilised on a sensor chip via an anti-GST antibody, the sensorgrams produced on injection of varying concentrations of TolA II/III fitted well to the simple Langmuir 1:1 binding model producing a K_d of 0.94 μM, a k_{diss} of 0.085 s^{-1} and a k_{ass} of 9 x 10^4 M s^{-1}. However, when TolA was immobilised to the surface via amine coupling, the sensorgrams produced from injection of varying concentrations of GSTN40 across the surface, did not fit to the Langmuir 1:1 binding model. Again, the authors fitted the data to a "two-site heterogeneous parallel-reactions model", and suggested that GSTN40 bound independently to two ligand sites. The authors suggest that immobilisation of TolAIII on the CM5 chip produces heterogeneous binding sites.

In the first case, a large amount of TolAIII is immobilised on the sensor chip, producing responses in excess of 700 RU. With such a high concentration of TolAIII

on the chip, it is possible that mass transport effects could affect the kinetics of the interaction due to the rate at which the translocation domain of colicin A binds to the TolAIII on the surface exceeding the rate at which the colicin is delivered to the surface (see Chapter 2). In the current work, the RUmax for the interaction is kept below 100 RU, in order to reduce mass transport effects, which may account for the differences in the kinetic parameters.

In the second case, although the level of TolAIII immobilisation is lower, much higher concentrations of the GSTN40 are required than the concentrations of colicin $A_{1\text{-}172}$ used in the current study (12.5 – 100 μM, compared to 0.5 – 8 μM). This indicates that the GST could be affecting the binding of TolA to the translocation domain of colicin N. The GST itself may also interact with the TolAIII, which may explain the heterogeneity of the interaction observed. The calculated K_d values are of the order of 1 μM, and injections of GSTN40 at concentrations of 10-100 μM are very high compared to this value.

Despite the lack of agreement as to which model the interaction fits, this study produces a K_d value concurring with the previously calculated values (Gokce *et al* 2000, Derouiche *et al* 1997). The Kd for the interaction was calculated to be 0.5 μM in this study. Derouiche *et al* (1997) produced values of 0.2 μM and 0.6 μM whereas Gokce *et al* (2000) produced values of 0.85 μM and 0.19 μM when TolAIII was immobilised and 0.94 μM when GSTN40 was immobilised. The K_d for the interaction was confirmed by using colicin $A_{1\text{-}172}$ as the ligand and TolAIII as the analyte. In this case, the K_d was found to be 0.6 μM, again a value very similar to all the previously determined K_d values for this interaction. It would therefore appear that TolAIII is a suitable ligand, provided the surface concentration of TolAIII and the analyte concentrations used are low.

The K_d for the interaction of colicin $A_{1\text{-}172}$ with TolB (10.5 μM) is greater than the K_d for the interaction of colicin $A_{1\text{-}172}$ with TolAIII, indicating that the affinity of colicin A is greater for TolA than TolB.

5.3.3. Interactions between colicins and TolR

In vivo and *in vitro* cross-linking studies have shown that colicin A interacts with TolRII (Journet *et al* 2001). *In vitro* cross-linking also detected an interaction between colicin E3 and TolRII (Journet *et al* 2001) and from the sequence homology

between the translocation domains of colicins E3 and E9 it was assumed that colicin E9 also interacts with TolR (Bouveret *et al* 2002).

This SPR study detected an interaction between colicin A and TolR II&III, as expected, but was unable to detect an interaction between colicins E3 or E9 and TolR II&III. Gel filtration experiments did not detect an interaction between TolR II&III and colicin A or colicin E9.

The only previous data suggesting that colicins A and E3 interact with TolR is from cross-linking studies. These studies use millimolar concentrations of protein, rather than the micromolar concentrations used in the SPR and gel filtration experiments. Journet *et al* (2001) state that techniques other than formaldehyde cross-linking, including yeast-two hybrid screening and spectrofluorometric analysis have been unable to detect the TolR-colicin A interaction. The interaction between colicin A and TolR, detected using SPR, was much weaker than the interactions detected between colicin A and TolB or TolA with higher concentrations required for a reasonable response, and this prevented the completion of kinetic analysis. These observations suggest that the TolR-colicin A interaction could be of millimolar affinity rather than the micromolar affinity seen for the interaction of colicin A with TolB and TolA.

The detection of a colicin E3-TolRII interaction using *in vitro* cross-linking (Journet *et al* 2001) and the failure to detect the interaction using SPR and gel filtration raises the possibility that colicin E3 does interact with TolRII&III but with an even lower affinity than the interaction between colicin A and TolRII&III. SPR and gel filtration analysis was performed with full-length colicin E3, whereas only the translocation domain of colicin A was used. It is therefore possible that the region involved in binding to TolR in colicin E3 is less accessible in the full-length colicin, compared to the isolated translocation domain. In colicin A, the region of the colicin that is predicted to interact with TolR overlaps the region predicted to interact with TolB. If this was also the case for colicins E3 or E9, then it is unlikely that the TolR binding site is blocked, as the full-length colicins E3 and E9 are able to bind to TolB. Therefore, if a part of the full-length colicin E3 does block the TolR binding site, the TolR binding site must be in a different region to the TolB binding site. Removal of the immunity protein did not affect the detection of an interaction between colicin E9 or colicin E3 and TolRII&III, but did affect the affinity of the interaction between colicin E9 or colicin E3 and TolB. This supports the idea that, in contrast to the TolR

binding site of colicin A, the TolR binding site for the endonuclease colicins is located at a distance from the TolB binding site.

5.3.4. Competition between TolB and TolR for binding to colicin A

The suggestion that the TolR box in colicin A overlaps the TolB box (Journet *et al* 2001) led to the hypothesis that TolR may compete with TolB for binding to colicin A. This was tested by incubating colicin A_{1-172} with varying concentrations of TolRII&III before injecting the colicin across a TolB chip. As a control, TolRII&III was injected across the TolB chip. There is currently no published information on an interaction between TolR and TolB, and no interaction between TolRII&III and TolB was detected in this SPR study.

No change in response was seen, when colicin A was pre-incubated with TolRII&III, suggesting that TolB can compete with TolRII&III for binding to colicin A. This is plausible due to the low affinity between colicin A and TolRII&III. However, the low affinity of TolRII&III for colicin A, and hence the rapid dissociation rate constant, may mean that when TolB comes into contact with colicin A, TolRII&III has already dissociated from the colicin.

5.3.5. TolA-TolB interaction

A strong, physiologically relevant, interaction between TolA and TolB was reported by Wallburger *et al* (2002). SPR has therefore been used to determine kinetic parameters for this interaction.

The interaction between the purified proteins was initially confirmed using gel filtration. Kinetic parameters were then determined for TolAIII binding to immobilised TolB and for TolB binding to immobilised TolAIII. The parameters determined for the experiment in which TolB was the ligand, were very similar to those determined in the experiment in which TolAIII was the ligand. The rate constants, k_{ass} and k_{diss}, vary slightly but the ratios of k_{ass} to k_{diss} are the same, so that the K_d for the interaction in both cases was calculated to be approximately 15 μM. Therefore, the affinity of TolB for TolA is lower than the affinity of TolB for colicins A, E3 or E9.

5.3.6. TolA-TolB-colicin ternary complexes

A ternary complex between colicin A, TolB and TolA has previously been shown by a Western blot overlay method (Bouveret *et al* 1998). Therefore, gel filtration was used in this study to investigate whether a ternary complex is also formed between colicin E9, TolB and TolA. However, no ternary complex could be detected.
This suggests that the colicin A-TolB-TolA complex is mediated by colicin A and the fact that colicin E9 does not bind to TolA means that a ternary complex is unable to form. Alternatively colicin E9 could affect TolB to disrupt the interaction of TolB with TolA. This second hypothesis was tested by investigating the effect of incubating TolB with varying concentrations of colicin E9, prior to injection across a TolAIII sensor chip. The presence of colicin E9 appeared to reduce the binding of TolB to TolAIII, supporting the possibility that colicin E9 inhibits the TolB-TolA interaction. Although the N-terminal domain of TolB was shown to be sufficient to allow interaction with TolA by the yeast-two hybrid technique, the C-terminal domain strengthened the interaction. Therefore, if colicin E9 binds to the C-terminal domain of TolB, it may inhibit this strengthening of the interaction, reducing the binding of TolB to TolA.

5.3.7. Differences between pore-formers and endonucleases

Several differences in the interactions between pore-forming and endonuclease colicins have been highlighted in this chapter.
Firstly, the reduced affinity of the colicin A translocation domain compared to the affinity of free colicin E9 and colicin E3, indicates that the interactions of pore-forming and endonuclease colicins with TolB may differ. This is consistent with the suggestion by Wallburger *et al* (2001) that colicin A may interact differently with TolB than colicin E9. This was based on the failure to detect an interaction between colicin A and the isolated N-terminal or C-terminal domains of TolB, in contrast to the detection of an interaction between the C-terminal domain of TolB and colicin E9 (Carr *et al* 2000a). The presence of a threonine residue in the TolB box of colicin A in place of a serine residue in the TolB box of colicin E9 may go some way to explain these differences. It would be interesting to investigate whether the affinity of the colicin A-TolB interaction could be increased by mutation of residue T13 of colicin A to serine.

Secondly, the SPR and gel filtration experiments have confirmed the lack of a direct interaction between TolA and colicins E9 and E3. This is in contrast to the direct interaction observed for colicin A and TolA. Interactions between TolA and the pore-forming colicins E1 and N have been reported (Derouiche *et al* 1997, Raggett *et al* 1998). Therefore, a difference in the use of TolA for translocation between endonuclease colicins and pore-forming colicins is emerging.

Although an interaction between colicin A and TolR has been confirmed in this study, no interaction between colicins E3 and E9 and TolR could be detected. The suggestion that the interaction between colicin A and TolR is transient is supported by the SPR and gel filtration results. It is possible that colicins E3 and E9 also interact with TolR but in an even more transient way, suggesting that colicins E3 and E9 require another factor, possibly another Tol protein to increase the affinity of their interaction with TolR. A ternary complex between TolR, TolA and colicin A has been reported (Journet *et al* 2001) but a similar complex is unlikely to form for colicins E3 and E9, as no interaction was detected between these colicins and TolA and any interaction between these colicins and TolR is likely to be transient.

The roles of YbgF, TolQ and Pal have not been considered in this study but are likely to be involved, either directly or indirectly, in translocation of endonuclease and/or pore-forming colicins.

5.3.8. Stoichiometry and concentrations of Tol proteins

With the possible exception of the TolR-colicin A interaction, all the interactions in this thesis have been of micromolar affinity. The calculated affinity constants should be qualified by an approximation of the concentrations of the interacting proteins in the periplasmic space. The inhibition or enhancement of interactions by other interacting proteins should also be considered.

The stoichiometry of the Tol system is not well defined, as the expression levels have not been simultaneously measured in a given strain. However, the number of copies of each Tol protein per cell has been estimated (see Table 5.5).

Assuming that the periplasmic space has a volume of 6.5×10^{-17} litres (CyberCell Database, Sundararaj *et al* 2004), the concentrations of Tol proteins can be calculated from the estimations of the number of molecules of each protein per cell.

Table 5.5 Concentration of proteins of the Tol system in a typical *E.coli* cell

Protein	Number of molecules per cell	Reference	Concentration
TolA	800	Levengood *et al* 1991	20 μM
TolR	2000 - 3000	Muller *et al* 1993	51 – 76 μM
TolQ	6000 - 9000	Guihard *et al* 1994	153 – 230 μM
TolB	10000 - 30000	Cascales *et al* 2000, Lloubes *et al* 2001	256 – 512 μM
Pal	10000 - 30000	Cascales *et al* 2000, Lloubes *et al* 2001	256 – 512 μM

Table 5.5 shows that the concentrations of all the proteins of the Tol system are estimated to be at least micromolar. From the definition of K_d for the interaction

$A + B \leftrightarrows AB$,

ie $$Kd = \frac{[A]\,[B]}{[AB]}$$

when the concentration of A equals the K_d, half the binding sites of B will be occupied, at equilibrium, in other words if the concentration of Tol proteins is above the K_d, more than half the colicin molecules in the cell will be bound to them. Therefore the micromolar concentrations of Tol proteins in the cell are consistent with the micromolar K_d values calculated for the interactions of these Tol proteins with colicins.

5.3.9. Mechanism of translocation

5.3.9.1. Colicin A

Analysis of the affinity of the interactions between colicin A and three Tol proteins, has provided further insights into the mechanism of colicin A translocation. The association of TolB with the outer membrane via Pal, suggests that on entering the periplasm, the translocation domain of colicin A could first interact with TolB. The association of TolA with TolB and Pal, indicates that colicin A could then interact with TolA. A ternary complex may form between TolA, TolB and colicin A, as shown by cross-linking studies (Bouveret *et al* 1998). If colicin A first interacts with TolB and then TolA, the affinity constants calculated for the colicin A-TolB and

colicin A-TolA interactions are consistent with the Brownian ratcheting model, as the affinity of colicin A for TolA is greater than the affinity of the colicin for TolB. However, if colicin A then interacts with TolR, the low affinity constant for the colicin A-TolR interaction is inconsistent with the Brownian ratcheting model, although the association of TolR with TolA and the formation of a colicin A-TolA-TolR ternary complex, may enhance the affinity of the colicin A-TolR interaction.

5.3.9.2. Colicins E9 and E3

In contrast to colicin A, the only interaction detected between colicin E3 and E9 with a Tol protein, was with TolB. Although a ternary complex between colicin A, TolA and TolB has been reported, no such ternary complex has been reported for an endonuclease colicin. In this study attempts to show an interaction between colicin E9, TolA and TolB using gel filtration did not detect a ternary complex, despite the detection of a TolA-TolB complex and the colicin E9-TolB complex. In addition, SPR showed that incubation of TolB with colicin E9 prior to injection across a TolA chip partially inhibits the TolB-TolA interaction. The affinity of the interaction between colicins E9 and E3 is greater than the interaction of TolB with TolA. These observations suggest the possibility that binding of colicins E9 and E3 to TolB could displace the TolB-TolA interaction. If the interaction between TolB and TolA is stabilised by the C-terminal domain of TolB, it is possible that binding of colicin E9 or E3 to this domain of TolB, could destabilise the interaction. The disruption of the TolB-TolA interaction may interfere with the Tol system stoichiometry, which has previously been proposed to cause a change in the cellular envelope, to trigger penetration of the cytotoxic domain of the colicin across the outer membrane. Further studies on the precise stoichiometry of the Tol system are required to determine the role of the stoichiometry in colicin translocation.

As colicins E3 and E9 only appear to directly bind to TolB, the mechanism of reaching the inner membrane, which they need to cross in order to enter the cytoplasm, remains unclear. TolB has been proposed to act as a shuttle between the inner and outer membranes, as binding of colicin E3 prevents TolB from interacting with Pal (Clavel *et al* 1998, Bouveret *et al* 1999). Therefore binding of colicins E9 and E3 to TolB could disrupt the TolB-Pal and TolB-TolA interactions, allowing TolB to shuttle the colicin from the outer to the inner membrane.

5.4. Summary

This chapter has provided further insight into the interactions between proteins of the Tol system and Group A colicins, and hence into the mechanisms of translocation for both endonuclease and pore-forming colicins.

SPR has been used to confirm the interactions of colicin A with TolR, TolB and TolA. The affinity of colicin A for TolB was shown to be decreased compared to the affinity of colicins E9 and E3 for TolB. This reduction in affinity may be attributed to a threonine residue in colicin A in place of a serine residue in colicin E9. The requirement for a higher affinity interaction between colicin E3 and TolB may reflect the role of the TolB-colicin E3 interaction in displacing the immunity protein from the colicin. SPR was used to show that the affinity of colicin A for TolA is higher than the affinity of colicin A for TolB. The transient nature of the interaction between colicin A and TolR was also confirmed by SPR.

The demonstration that colicin A interacts with TolB and TolA with increasing affinity, supports the Brownian ratcheting model for colicin translocation. A ternary complex between TolB, TolA and colicin A may increase the binding affinity further. However, if colicin A then interacts with TolR, the interaction must be stabilised by another factor in order to increase the affinity. A ternary complex between colicin A, TolA and TolR may provide this extra stabilisation.

Although colicins E3 and E9 were shown to interact with TolB with micromolar affinity, no interaction between these colicins and TolA was detected. In addition, in contrast to previous cross-linking evidence, no interaction could be detected between colicins E9 or E3 and TolR. This indicates that if colicins E9 and E3 do interact with TolR, the interaction is particularly transient and may require stabilisation by another Tol protein. In contrast to colicin A, no ternary complex could be detected between colicin E9, TolB and TolA, suggesting that there is a difference in the translocation mechanism between pore-forming and endonuclease colicins. It is proposed that binding of colicin E9 to the C-terminal domain of TolB could destabilise the TolA-TolB interaction and the TolB-Pal interaction, allowing the cytotoxic domain to penetrate the inner membrane and shuttling of the colicin or from the outer membrane to the inner membrane by TolB.

CHAPTER 6 – General Discussion

6.1. Introduction

Colicins are multi-domain proteins, which exhibit several fundamental biological properties and they have therefore been exploited in several ways to address fundamental biological questions. The mechanism by which colicins kill cells is a rare example of uptake of polypeptides into bacterial cells. Colicins initially bind to at least one outer membrane receptor. The translocation domain of the colicin then traverses the outer membrane and the periplasm, a process which allows translocation of the cytotoxic domain across the outer membrane and, for endonuclease colicins, the inner membrane. The mechanisms used for entry of colicins into cells, share similarities with the mechanisms used by the cell for uptake of essential metabolites. Therefore a detailed understanding of this process has potential for delivery of recombinant polypeptides into bacterial cells and for development of new antibiotics. Colicins are also emerging as an interesting example of a protein with an intrinsically disordered region *ie* the N-terminus of the translocation domain, which is functionally important and which adopts a folded structure upon binding to its biological target. In addition, the combination of high binding affinities between a colicin and its cognate immunity protein, together with the relatively low affinities between the colicin and non-cognate immunity proteins, makes it an attractive system for investigating molecular recognition events between proteins.

The work presented in this thesis has focussed on the process of translocation of Group A colicins across the periplasm, which requires interactions between the translocation domain of the colicin with the periplasmic and membrane-bound proteins of the Tol system. Although there is now a substantial amount of published information about the translocation process, a number of important details remain unresolved.

One of the best characterised interactions involved in colicin translocation is the interaction between endonuclease colicins, particularly colicin E9, and TolB. However, several issues regarding this interaction remained unresolved such as the region of TolB involved in the interaction with colicins, the possibility that an extended region of colicin E9 could be involved in the interaction with TolB and the

suggestion that binding of the translocation domain to colicin E9 could induce structure in this disordered region of the colicin. This thesis set out to further characterise the interaction of colicin E9 with TolB, in an attempt to resolve some of these issues.

There have now been a number of studies on the interactions of several colicins with various Tol proteins and it is tempting to apply information obtained for one colicin to another colicin. However, recent evidence suggests that different Group A colicins, may use the Tol system in different ways in order to access their site of cytotoxic action. There is currently a lack of quantitative analysis of interactions of Tol proteins with endonuclease and pore-forming colicins, measured under the same experimental conditions. Therefore this thesis has investigated differences in the interactions with Tol proteins between the Group A pore-forming colicin A and the endonuclease colicins E9 and E3.

6.2. TolB

Yeast-two hybrid experiments and mutagenesis experiments identified a pentapeptide sequence, DGSGW, from residues 35-39 of colicin E9 that interacts with TolB (Carr *et al* 2000a, Garinot-Schneider *et al* 1997). By sequence homology and mutagenesis experiments, the same sequence in colicin E3 had also been shown to interact with TolB (Bouveret *et al* 1997). An interaction between colicin A and TolB has also been demonstrated using *in vitro* cross-linking with residues 11-20 of colicin A predicted to be involved in the interaction (Bouveret *et al* 1998).

6.2.1. Roles of residues of the established TolB box

Chapter 3 of this thesis confirms that residues D35, S37 and W39 of colicin E9, and, by homology, of colicin E3, are crucial for the interaction with TolB. Revertant mutations of these three essential residues showed that at least one other residue could be tolerated in these positions without substantial loss of activity or loss of TolB binding. The chemical nature of the tolerated residues suggests that the roles of residues at positions 35, 37 and 39 differ. It is speculated that residue D35 is involved in hydrogen bonding with another residue in colicin E9 or a residue in TolB. Residue S37 may be involved in maintaining local structure of the TolB box and residue W39 may form stacking interactions with aromatic residues in TolB.

In addition to these three essential residues, this study has shown for the first time that G36 and G38 also play a role in binding of colicin E9 to TolB. It is suggested that these glycine residues may be important in maintaining flexibility of the TolB box and it would be interesting to investigate the role of other glycine residues in the glycine-rich translocation domain.

6.2.2. Extension of the TolB box

Consistent with previous evidence, including NMR data and mutagenesis studies (Collins *et al* 2002, Holland 2003), the region of colicin E9 involved in interacting with TolB has been shown in this study to be larger than the previously identified pentapeptide sequence. Mutation of residues S34, S40, S41, E42 and N43 abolish activity and significantly reduce binding of colicin E9 to TolB. Therefore the TolB box extends at least from residue S34 to N43. Further mutagenesis is required to investigate whether the TolB box extends further upstream of residue S34 and further downstream of residue N43, although residue N44 appears not to be involved in the interaction with TolB and may therefore form the C-terminal boundary of the TolB box. Residues 34 – 42 are conserved in all endonuclease colicins, suggesting that the TolB box defined for colicin E9 is also applicable to other endonuclease colicins.

6.2.3. Differences between pore-forming and endonuclease colicins in interacting with TolB

Residues 35-42 of colicin E9 are also conserved in colicin A, with the exception of a threonine residue in colicin A, in place of a serine residue at position 37 in colicin E9. Mutation of residue S37 to a threonine residue in colicin E9, has been shown in this study to significantly reduce binding of colicin E9 to TolB. The affinity of colicin A for TolB is lower than the affinity of endonuclease colicins for TolB, suggesting that the presence of the threonine residue in colicin A could be at least partially responsible for the reduction in affinity of colicin A for TolB, compared to the affinity of colicin E9 for TolB. Colicin K also contains a threonine residue at this position of the TolB box and it would be interesting to compare the affinity of colicin K for TolB to the affinities of colicins A, E3 and E9 for TolB.

However, the presence of an alanine residue at the fifth position of the TolB box of colicin K, instead of a tryptophan residue in colicin E9, indicates that this colicin may interact differently with TolB than colicin E9, as mutation of the tryptophan residue to

alanine in colicin E9 has been shown previously, by yeast-two hybrid studies, and in this study, by SPR, to abolish binding of colicin E9 to TolB. Residues S34, S40, S41 and E42 of colicin E9, which are proposed to also be involved in the interaction with TolB, are conserved in other colicins known to interact with TolB, such as colicins E2, E3, E6, E7, colicin A and colicin U but are not conserved in colicin K, providing further evidence that the interaction between colicin K and TolB is different to the interaction between other TolB-dependent colicins with TolB.

Colicins A and K are pore-forming colicins, indicating that the proposed difference in the interactions of colicin K with TolB and colicin A with TolB are not due to a difference in cytotoxic target.

The increased affinity of endonuclease colicins for TolB compared to the affinity of pore-forming colicins for TolB may reflect a crucial role of TolB in allowing the cytotoxic domain of endonuclease colicins to cross the cytoplasmic membrane. This increased affinity for TolB would not be required for pore-forming colicins, as they could firstly bind to TolB and then to TolA, releasing TolB.

6.3. TolA

This study has confirmed the interaction between colicin A and domain III of TolA, and determined a K_d for the interaction of 0.5 μM, a value very similar to the values determined in previous studies. The data presented here also supports evidence from yeast-two hybrid and cross-linking experiments that colicins E9 and E3 do not directly interact with TolA. This suggests a possible difference between pore-forming colicins and endonuclease colicins in their use of the Tol system.

6.4. TolR

The interaction between colicin A and TolR has also been confirmed in this study. However, the interaction was the weakest interaction measured for colicin A with a Tol protein.

Despite a previous cross-linking study that suggested colicin E3 interacted with TolR, no interaction of colicin E3 with TolR could be detected by gel filtration or SPR (Journet *et al* 2001). These results suggest that the interaction of colicins with TolR is transient. High concentrations, *ie* millimolar concentrations, used in *in vitro* cross-linking studies, of colicin E3 or TolR may be required to detect an interaction between colicin E3, and by homology colicin E9, with TolR. This suggests that *in*

vivo, TolR may be recruited at a specific site, to increase the apparent local concentration of TolR. This may be achieved by binding of colicin A to TolA, and binding of TolA to TolR, consistent with the detection of a TolA-TolR-colicin A ternary complex (Journet *et al* 2001).

6.5. Mechanisms of translocation

6.5.1. Colicins E9 and E3

The only interaction between colicin E9 and the Tol system that has been detected in this study and previous studies is the interaction with TolB. However, despite the lack of a direct interaction between colicins E9 or E3 with TolA or TolR, these Tol proteins are still required for translocation of these colicins. This suggests that binding of colicin E9 to TolB triggers a set of events, involving interactions between Tol proteins that allows translocation of colicin E9 across the periplasm. It has been suggested that intermittent contact of TolB with Pal could allow TolB to act as a shuttle between the outer and inner membranes. The region of TolB, involved in binding Pal has been localised to the β-propeller domain (Ray *et al* 2000, Abergel *et al* 1999). The same domain of TolB is proposed to be involved in interacting with colicin E9 (Carr *et al* 2000a), and the binding of colicin E3 to TolB has been shown to prevent the interaction of TolB with Pal (Bouveret *et al* 1997) suggesting the possibility that binding of colicin E9 to TolB, disrupts the TolB-Pal interaction, allowing the colicin E9-TolB complex to move across the periplasm towards the inner membrane.

This study has also confirmed the interaction between TolA and TolB and has provided kinetic parameters for the interaction. The K_d for the interaction was determined as 15 μM, indicating that the affinity of TolB for TolA is lower than the affinity of TolB for colicins.

Although a ternary complex for colicin A, TolB and TolA has been reported, no such complex has been reported for colicin E9, and attempts to detect a colicin E9-TolB-TolA complex in this study, using gel filtration, failed. The binding of colicin E9 to TolB, was also shown to inhibit the TolB-TolA interaction. These observations have lead to the proposal that binding of colicin E9 or E3 to TolB could displace the TolB-TolA interaction, altering the stoichiometry of the Tol/Pal complex and possibly

disrupting the outer membrane integrity, to allow penetration of the cytotoxic domain of the colicin.

6.5.1.1. Immunity proteins for endonuclease colicins

An unexpected reduction in binding of colicins E9 and E3 to TolB, caused by the immunity protein, has been demonstrated by SPR and AFM.

The immunity protein of endonuclease colicins must be lost during entrance of the colicin to a susceptible cell, to allow the C-terminal domain to exert its cytotoxic action. However, the affinity of the immunity protein for the colicin is one of the highest ever measured and there is little information available as to how this high affinity interaction may be overcome to allow loss of the immunity protein.

The immunity protein of colicin E3 has been shown to interact with the cytotoxic domain and the translocation domain by thermodynamic and structural analysis (Walker *et al* 2003, Soelaiman *et al* 2001). With the contacts that Im3 makes with the translocation domain of colicin E3, an effect of Im3 on this domain to reduce binding of the colicin to TolB is conceivable. It has been suggested that the binding of the translocation domain of colicin E3 to TolB, could allow dissociation of Im3 from the translocation domain, reducing the affinity of Im3 for the colicin and promoting dissociation of the immunity protein (Walker *et al* 2003). The increase in affinity of colicin E3 for TolB, when the immunity protein is removed, is consistent with this hypothesis.

However, it has been suggested that the immunity protein of colicin E9 only interacts with the cytotoxic domain of the colicin, and, if contacts did occur between the immunity protein and the translocation domain, they must be energetically neutral (Walker *et al* 2003). Therefore the effect of the immunity protein on the translocation domain of colicin E9, reducing binding of the colicin to TolB, is more puzzling. It is suggested, that rather than directly contacting the translocation domain of colicin, Im9 may sterically hinder the translocation domain, partially inhibiting the binding of colicin E9 to TolB.

Structures of colicin E9 in complex with Im9 and colicin E3 and E9 in the absence of their respective immunity proteins and an *in vivo* assay to confirm the stage at which the immunity protein is lost, would allow a more detailed understanding of the effect of immunity proteins on the translocation domains of endonuclease colicins and on their binding to Tol proteins.

6.5.2. Colicin A

It has been proposed that colicin A translocation could occur via Brownian ratcheting. This mechanism requires each interaction between the colicin and a Tol protein, to be of a higher affinity than the previous interaction, to prevent the colicin from moving backwards. This study has shown that the affinity of the interaction between colicin A and TolB is lower than the affinity of the interaction between colicin A and TolA, consistent with the ratcheting model, assuming that colicin A first interacts with TolB, associated with the outer membrane, and then TolA. This assumption was based on the localisation of TolB to the outer membrane via its interaction with Pal. However, it has recently been shown that TolA also interacts with Pal (Cascales *et al* 2000), indicating that the C-terminus of TolA is also linked to the outer membrane. The binding of colicin A to TolAIII has been shown to cause an increase in flexibility of this domain, possibly disrupting the TolA-Pal interaction, affecting the integrity of the outer membrane and allowing the cytotoxic domain to traverse the outer membrane. The demonstration in this study that the colicin A-TolR interaction is of low affinity, is, ostensibly, inconsistent with the ratcheting model. However, the suggestion that colicins interact with one protein at a time is likely to be an over-simplification of the *in vivo* situation. The identification of a TolA-TolB-colicin A ternary complex and a TolA-TolR-colicin A ternary complex, suggests that the colicin recruits a Tol protein and then interactions between different Tol proteins occur, facilitating another interaction of the colicin with a different Tol protein and, in effect, pulling the colicin across the periplasm.

6.6. Comparison of translocation mechanisms

A summary of the proposed mechanisms for translocation of endonuclease and pore-forming colicins is shown in Figure 6.1 and Figure 6.2.

The two major requirements for colicin translocation are translocation of the cytotoxic domain across the outer membrane and movement of the colicin across the periplasm. The proposed systems that endonuclease and pore-forming colicins use in order to fulfil these requirements are given below.

1. Translocation of the cytotoxic domain across the outer membrane

For colicin A, it is proposed that this occurs by the interaction of the colicin with TolA, which disrupts the TolA-Pal interaction, disrupting the integrity of the outer

membrane. For colicin E9/E3, it is proposed that binding of the colicin to TolB disrupts the TolB-TolA and the TolB-Pal interactions, again causing a disruption of the outer membrane.

2. Transport of the translocation and cytotoxic domains across the periplasm.

For colicin E9 and E3 it is proposed that TolB, initially tethered to the outer membrane via its interaction with Pal, shuttles the colicin from the outer membrane to the inner membrane due to disruption of the Pal-TolB interaction on binding of the colicin to TolB. For colicin A, it is proposed that colicin A binds to TolB and then forms a complex with TolB and TolA. The association of TolA with TolR via domain III of TolR, then allows an interaction between colicin A and domain II of TolR, effectively pulling the colicin towards the inner membrane.

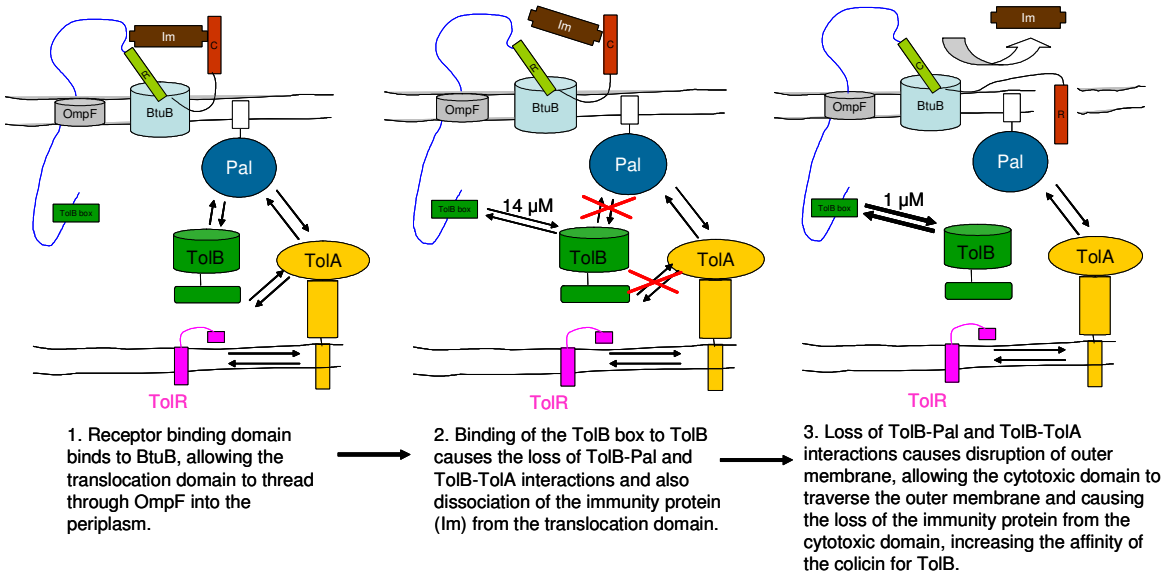


Figure 6.1 **Suggested mechanism for translocation of endonuclease colicins.**

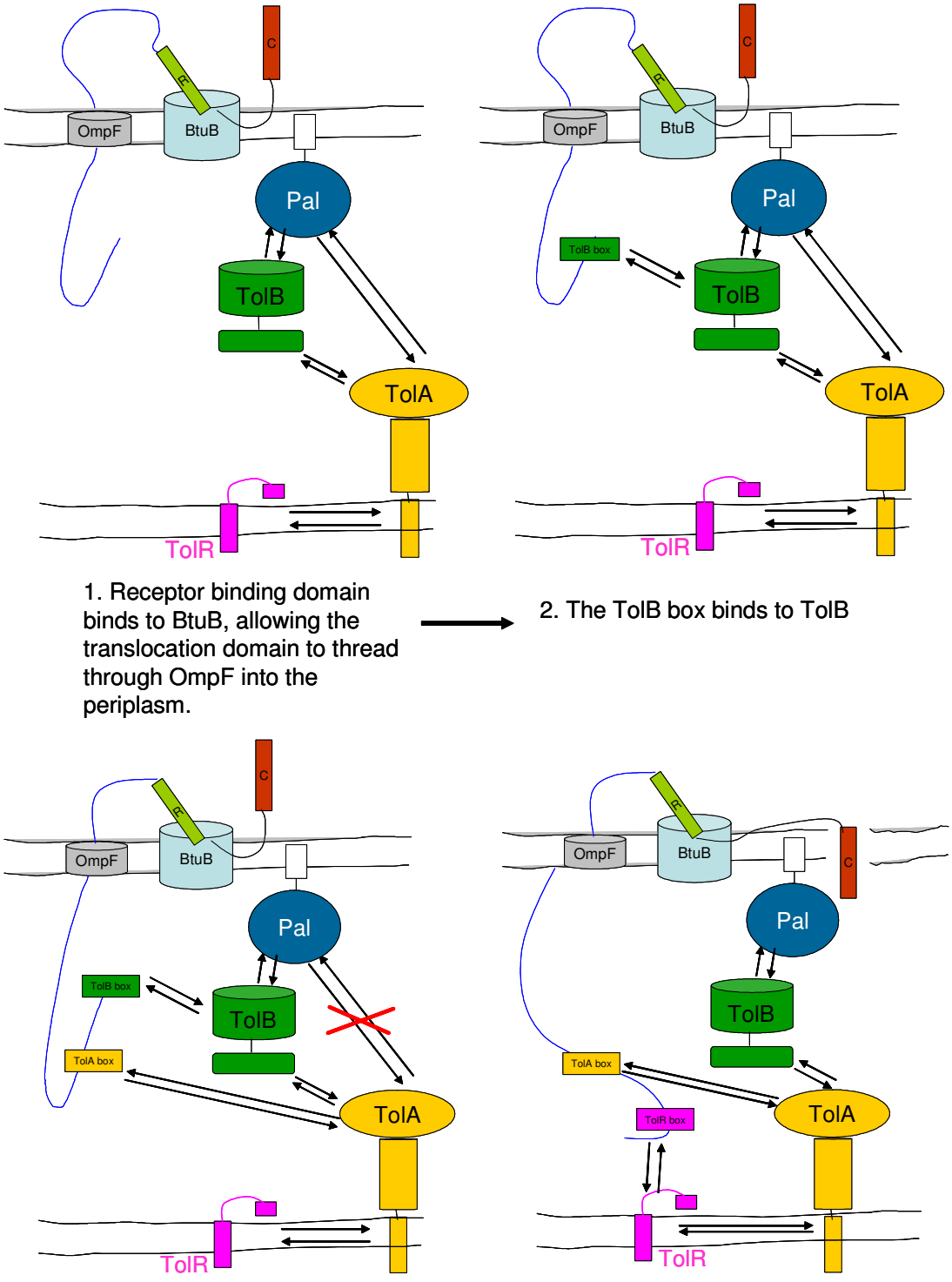


Figure 6.2 **Proposed mechanism for translocation of pore-forming colicins.**

6.7. Future directions

The models proposed above are still incomplete and further work is required to modify and validate the models.

Although it is useful to obtain affinity constants for the interactions between colicins and Tol proteins, it is, in general, likely to be too simplistic to consider individual interactions between a single Tol protein and the colicin. Rather, a situation in which interactions between a colicin and a Tol protein promote varying and transient interactions between different protein partners. Information on the stoichiometry of the Tol system in the presence and absence of colicins may prove invaluable in understanding how colicins use the Tol system for translocation. Further information on the cellular role of the Tol/Pal system may also provide clues as to why colicins have parasitized the system for purposes of translocation and hence clarify the role of the Tol proteins in colicin translocation.

The proposal that at least part of the translocation domain is unfolded to allow it to enter the periplasm via OmpF (Kurisu *et al* 2003) together with the possibility that partial folding occurs on binding of the translocation domain to TolB and TolA (Collins *et al* 2002, Anderluh *et al* 2003, 2004), suggests that the colicin may be in different states of folding, when it interacts with each Tol protein. This may help direct the colicin through the periplasm. NMR studies on the conformation of colicins in complex with varying Tol proteins may provide further information on the role that different conformations of the colicin play in the translocation process. *In vivo* localisation of TolB in the presence and absence of colicin, using confocal microscopy for example, may help to verify whether TolB can act as a shuttle, to transport endonuclease colicins from the outer to the inner membrane.

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