THE TONB AND TOLA TRANSMEMBRANE DOMAINS: CONTRIBUTIONS OF NON-ESSENTIAL SIDE-CHAiNS TO ENERGY TRANSFER SPECIFICITY

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A Dissertation

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ABSTRACT

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The gram-negative bacterium *Escherichia coli* has two systems that transduce energy from the cytoplasmic membrane to the outer membrane, the TonB system (TonB, ExbB, ExbD) and the TolA system (TolA, TolQ, TolR). The energy-harvesting complexes of the two systems are ExbB/ExbD and TolQ/TolR, with the energy-transducers being TonB and TolA, respectively. Sequence homologies between the transmembrane domains of ExbB/TolQ and ExbD/TolR proteins are high. TonB and TolA share only a conserved Ser-X(3)-His-X(6)-Leu-X(3)-Ser (SHLS) motif in their transmembrane domain, with the first Ser and the His residues of this motif being essential for efficient energization by the energy-harvesting complexes in *E. coli*. In cells lacking the energy-harvesting complex from one system, the energy-transducer is able to be energized (less efficiently) by the energy-harvesting complex from the opposite system, a phenomenon called crosstalk. Protein capture and bacteria two-hybrid assays were unable to identify interactions between proteins in the TolA system; therefore, evaluation of crosstalk interactions between the TolA and TonB systems required another approach. Crosstalk is inefficient, the result of a system specificity possibly defined by side group differences between the transmembrane domain residues of TonB and TolA. The relative contributions of such side groups were examined by systematically removing the differences between the energy-transducers via multiple alanyl substitutions, creating a generic transmembrane domain retaining only the essential SH residues and their relative spatial relationships. TonB derivatives containing up to 14 alanyl substitutions still properly partitioned to the cytoplasmic membrane, retained the ability to interact with ExbB, to be energized, and to participate in crosstalk.
Replacement of residues 12-20 caused a decrease in TonB protein function, but that decrease was not the result of instability of those derivatives. In contrast, changes to residues 21-27 did affect the stability of TonB derivatives. These data indicated that the amino terminal residues in the transmembrane domain of the energy-transducers play an important, as yet unidentified role for proper energization of the energy-transducer by either energy-harvesting complex. Further, system specificity between the transducers and their respective energy-harvesting complexes did not appear to involve the residues of the TonB/TolA transmembrane domain.
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Bacteria are the most abundant organisms on the planet. The majority of bacteria are gram-negative. Members of the Family Enterobacteriaceae (from the word enteric, meaning bowel related) are relatively small gram-negative rods. Enteric bacteria are able to grow in a variety of habitats such as soil, water (both fresh and marine), as well as being part of the flora found in the intestines of humans and other animals (Neidhart, 1996). While these organisms are commonly found as commensals of intestinal tracts, some species are pathogenic. A historical division in the family is based on the ability to utilize lactose; those that cannot are often associated with pathogenic processes, while those that can are often less pathogenic. This latter group came to be referred to as “coliform” bacteria, as typified by Escherichia coli (Janda & Abbott, 2006).

Much of the knowledge about gram-negative bacteria comes from research on E. coli. The distinct characteristic of gram-negative bacteria is the presence of a double membrane surrounding the bacterial cell, often referred to as the cell wall. The cell wall of a typical gram-negative bacterium is comprised of two concentric membranes. The inner, cytoplasmic membrane (CM) serves as a permeability barrier, while the outer membrane (OM) acts primarily as a diffusion barrier. Between these two membranes is an aqueous compartment, the periplasmic space, which includes a thin layer of peptidoglycan (Figure 1).

The OM is an asymmetric bilayer containing two types of lipids. The outer leaflet of the OM is rich in lipopolysaccharide [LPS], while the inner layer is composed of phospholipids, as
Figure 1: Cartoon schematic of a gram-negative envelope. Illustrated is the outer membrane, periplasmic space, and cytoplasmic membrane and their major components.
well a set of unique proteins (Nikaido, 1996). The LPS in the outer leaflet of the OM forms an
anionic surface that hinders the passage of hydrophobic molecules into the cell, while the lipid
component prevents diffusion of hydrophilic solutes. The OM prevents lipophilic molecules,
such as bile salts, from being able to penetrate the cell, allowing these bacteria to flourish in the
intestinal tracts (Nikaido & Vaara, 1985). The reason many gram-negative are more resistant to
antibiotics, is because they are excluded from entering the cell by the OM. Indiscriminately, the
OM also inhibits the diffusion of nutrients into the cell. The protein composition of the outer
membrane of *Escherichia coli* is well characterized, and many are involved in the uptake of these
nutrients that are hindered by the OM. There are two basic groups of proteins, lipoproteins and
beta-barrels. In *E. coli*, about 90 species of lipoproteins have been experimentally confirmed to
exist in the OM (Junker *et al.*, 2003; Brokx *et al.*, 2004; Miyadai *et al.*, 2004). The major
lipoprotein in the OM is the Braun lipoprotein (LPP), with about 400,000 copies of LPP
covalently attached to the peptidoglycan layer anchoring the OM to the cell (Reviewed in
references Braun, 1975; Wu & Tokunaga, 1986; Nikaido, 1994). There are several classes of
beta-barrel proteins in the OM; including porins that allow passive diffusion of solutes, and beta-
barrel receptors involved in active transport of larger molecules. Porins are a family of trimeric
proteins that form aqueous pores of a fixed diameter through the lipid bilayer. Hydrophilic
molecules up to about 600 Daltons are able to passively diffuse through the OM of *E. coli* via
porins (Nikaido & Rosenberg, 1981; Nikaido & Rosenberg, 1983), while larger nutrient
molecules and/or harmful hydrophobic compounds (such as bile salts in the intestinal tract) from
the environment are excluded from entering the cell. Since larger nutrients, such as vitamin B$_{12}$
and iron-bearing siderophores, can not readily access the periplasm by passive diffusion through
these porin protein channels, these molecules require active transport via specific receptors to
In addition to the traditional porin proteins mentioned above, *E. coli* has a unique porin-like protein, OmpA, that is expressed in a relative high copy number of 100,000 per cell (Nikaido, 1996). OmpA acts as a porin with low permeability that allows slow penetration of small solutes, and can be found in two conformations. The majority of the protein is found folded with two domains (Ried *et al.*, 1994), and has be shown to be important in binding the OM to the peptidoglycan (Koebnik, 1995; de Cock *et al.*, 1999). A minor portion of the protein folds to form an open β-barrel that can form a traditional trimeric protein channel (Nikaido, 2001).

While the majority of the proteins in the OM discussed here have been those involved in bringing molecules into the cell, there are various systems involved in exporting molecules out of the cell. Once such protein, TolC, is located in the OM and acts to export a variety of proteins (Wandersman & Delepelaire, 1990). The resolved crystal structure shows TolC exists as a trimer with a β-barrel structure in the OM and a large periplasmic tunnel (Koronakis *et al.*, 2000). TolC has been shown to be involved in the efflux of several molecules, including the *E. coli* siderophore enterobactin (Bleuel *et al.*, 2005).

Between the OM and the CM is the periplasmic space. This aqueous compartment has its own unique set of proteins as well as a thin layer of peptidoglycan. While referred to as a “space” and often depicted in cartoon schematics as being void, it actually contains numerous proteins and other molecules that allow a variety of vital functions to occur in this compartment. Several categories of periplasmic proteins include: (1) proteins that work with ABC transporters or chemotaxis receptors for the sensing and uptake of ions and solutes such as sugars and amino acids; (2) catabolic enzymes responsible for degrading large, complex molecules into smaller
ones for easier transport across the CM; (3) those enzymatic proteins involved in cell protection; and (4) those proteins involved in promoting envelope protein or polymer biogenesis for the OM (Oliver, 1996). The periplasm plays an important role in maintaining turgor pressure in the cell by the use of membrane-derived oligosaccharides (MDO) that help the periplasm maintain its volume during changes in osmotic pressure (Kennedy, 1996). This is extremely important for the enteric bacteria as they go from the high osmolarity found in the intestinal tracts, to relatively low osmolarity of fresh water (Csonka & Epstein, 1996). MDO are large hydrophilic oligosaccharides (containing ~6 to 12 glucose units) that are too large for porins and too charged to permeate the OM. Thus, they are retained in the periplasm and help maintain the volume of periplasm space under a wide range of osmotic conditions (Oliver, 1996). Another important component in the periplasmic space is the thin layer of peptidoglycan. The peptidoglycan is a polymer of disaccharides that are cross-linked by short peptides and its structure and arrangement in E. coli is representative of all Enterobacteriaceae, as well as many other gram-negative bacteria (Weidel & Pelzer, 1964) and provides the cell rigidity and shape. Interactions between the peptidoglycan layer and the LPP molecules anchor the OM to the peptidoglycan, thereby causing the OM to be adhered to the cell.

The cytoplasmic membrane (CM) is a typical phospholipid bilayer (Kadner, 1996). The cytoplasmic membrane is rich in a diversity of proteins that generate, harvest, and utilize ion electrochemical gradients to support essential cellular processes. The lipid bilayer forms a hydrophobic barrier that prevents the uncontrolled movement of polar molecules, thereby allowing the accumulation and retention of metabolites and proteins on one side of the CM (Kadner, 1996). The composition of the CM allows for the creation of a transmembrane electrochemical gradient of protons referred to as proton motive force (PMF). This PMF plays a
key role in many of the biosynthetic and transport processes in *E. coli* (Kadner, 1996).

The structure of the gram-negative envelope complicates the cell’s access to nutrients. To further understand the effect of the OM on nutrient uptake, the Nikaido laboratory performed experiments comparing the diffusion rates of very hydrophobic test solutes (i.e. steroids) between cells with and without an OM of LPS. These experiments showed the diffusion rates of the test solutes to be about two orders of magnitude slower in cells with an OM containing LPS than the diffusion rate across a typical phospholipid bilayer membrane (Plesiat & Nikaido, 1992; Plesiat *et al.*, 1997). Thus, while the LPS portion of the OM is an effective diffusion barrier to a variety of substances, this barrier seems to be the rate limiting factor in the diffusion of required molecules into the cell. The main route of entry for small molecules into the cell is through porins; however, these porin channels prevent the OM from generating its own energy that is required to bring in large nutrients via active transport, as well as the energy required in maintaining the integrity of the OM barrier. In order for the cell to acquire larger nutrients, such as vitamin B12 and iron-bearing siderophores, the OM must somehow access energy derived by the CM to carry out the active transport of these nutrients. Two systems have been shown to support such energy-requiring events as this at the OM, the TonB system and the TolA system (Figures 2 & 3, respectively). The proteins of the TonB system support the active transport of iron-siderophore complexes, other iron-complexes, and vitamin B$_{12}$ across the OM, while the TolA system plays an unidentified role in OM maintenance (Reviewed in references Lazzaroni *et al.*, 2002; Postle & Kadner, 2003).

*Iron Uptake and the TonB System*

Iron is an important nutrient. Iron plays a crucial role in many metabolic processes involved in microbial replication such as DNA synthesis (pyrimidine biosynthesis), the
Figure 2: **Known components of the TonB system.** (A.) A cartoon representation of the proteins in the TonB system, including the receptor for the *E. coli* produced siderophore enterochelin, FepA (Buchanan *et al.*, 1998), as well as an iron-siderophore complex. (B.) Maps of the *exb* and *tonB* operons, in which the proteins of the system are transcribed from two promoters from separate locations on the chromosome. The *exbB* and *exbD* genes are located at ~65 minutes on the chromosome, while the *tonB* gene is expressed at ~28 minutes on the chromosome (B. Bachman, *E. coli* Genetic Stock Center).
Figure 3: Known components of the TolA system. (A.) A cartoon representation of the proteins in the TolA system, including the apparent non-essential proteins in the operon, YbgC and YbgF, as well as an outer membrane porin, OmpF (Cowan et al., 1992). (B.) A map of the tol-pal operon in which all the proteins of the system are transcribed from two promoters from the operons located next to one another on the E. coli chromosome. (Operon modified from Lazdunski, et al., 1998, Vianney et al., 1996). Genes of the TolA system are expressed from two distinct promoters (represented here as P and PB) clustered at ~17 minutes on the chromosome (B. Bachman, E. coli Genetic Stock Center).
tricarboxylic acid cycle, as well as being an essential cofactor of many enzymes with redox activity such as iron-sulfur (Fe-S) proteins and the heme groups of cytochromes. The majority of bacteria require micromolar levels ($10^{-5}$ to $10^{-7}$ M) of iron for optimum growth (Guerinot, 1994). There are low levels of bioavailable iron (approximately $10^{-9}$ M) in terrestrial and aqueous environments (Ratledge and Dover, 1999) as most of the iron occurs as ferric iron [Fe(III)] and under aerobic conditions Fe(III) is unavailable (Neilands, 1981). Pathogenic bacteria are dependent on their host for nutrients, and must obtain iron in order to survive. Host have been shown to have a variety of defense mechanisms to withhold iron from infecting bacteria (Payne, 1993; Weinberg, 1993) with only $10^{-8}$ M amounts of iron available in biological fluids (Griffiths, 1978; Wienk et al., 1999). Within a host, the iron is usually bound to specific proteins such as transferrin (TF), lactoferrin (LF), and ferritin, or in complex with heme (Kaplan, 2002; Henze et al., 2004). Yet even with low iron bioavailability, bacteria are still able to multiply within a host, indicating that bacteria have strategies for acquiring that bound iron (Schaible & Kaufman, 2004). In order to cause the release of iron from the cells, *E. coli* and *Vibrio cholerae* secrete hemolysins and cytolysins causing the heme groups to be accessible for uptake by the bacteria (Andrews et al., 2003). Many *Escherichia* (Andrews et al., 2003), *Salmonella*, *Klebsiella*, and *Shigella* species (Grass, 2006) produce siderophores to acquire any available iron. Siderophores are high-affinity iron chelators (Neilands, 1981) of low molecular weight that are synthesized by various bacterial and fungal species, with approximately five hundred different siderophores have being chemically characterized (Drechsel and Winkleman, 1997). There are three distinct families of siderophores that include catecholates, hydroxamates, and $\alpha$-hydroxamate (or mixed ligands) that all bind Fe(III) (Carrano et al., 2001; Winklemann, 2002). In another iron-acquiring strategy, *Neisseria* species and *Haemophilus influenzae* express receptors on their cell
surfaces that can interact directly with the mammalian iron-binding proteins such as TF and LF (Cornelissen & Sparling, 1994; Andrews et al., 2003) capturing the host’s iron-binding proteins. Whether the bacteria cells are taking up iron as an iron-siderophore complex or via receptors for host iron proteins such as TF and LF, getting these iron complexes released into the periplasm from the various OM receptors requires energy. The various strategies for uptaking iron at the OM are dependent on the TonB protein (Postle, 1999, Ratledge and Dover, 2000, Andrews et al., 2003).

The organism used in this dissertation research is *Escherichia coli*. The most common strategy for acquiring iron from the environment used by *E. coli* is using high affinity siderophore-mediated iron uptake. Today we recognize that the capture and retrieval of iron-siderophore complexes in *E. coli* is mediated by a set of outer membrane receptors that bind iron-siderophore complexes with nanomolar affinity, allowing cells to efficiently recover iron (Postle, 1999). While this high affinity affords efficient iron recovery, it also creates a potential problem, as the release of the iron-siderophore complex into the periplasm requires energy. This energy is transduced from the cytoplasm by the TonB protein, which works in concert with two CM proteins, ExbB and ExbD. While the role of this system is for the uptake of iron siderophores and vitamin B$_{12}$, it is parasitized by group B colicins (Table 1) and a variety of bacteriophage (Reviewed in Postle & Kadner, 2003; Postle & Larsen, 2004).

The *tonB* gene was discovered during the fluctuation studies of Luria and Delbruck (1943) as one of two *Escherichia coli* genes that conferred resistance to bacteriophage T1. One (*tonA*) encoded the receptor for phage attachment, while the function of the other gene (*tonB*) was to remain unclear for many years. Strains carrying mutations in *tonB* were subsequently
Table 1: Representative members of group B colicins

<table>
<thead>
<tr>
<th>Colicin</th>
<th>Receptor</th>
<th>Translocation</th>
<th>Cytotoxic activity</th>
<th>Molecular Weight (kDa)</th>
<th>Original producer</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>FepA</td>
<td>TonB, ExbB, D</td>
<td>Pore formation</td>
<td>54.732</td>
<td>E. coli</td>
</tr>
<tr>
<td>D</td>
<td>FepA</td>
<td>TonB, ExbB, D</td>
<td>Inhibits RNA Synthesis</td>
<td>74.688</td>
<td>E. coli</td>
</tr>
<tr>
<td>G</td>
<td>Fiu</td>
<td>TonB, ExbB, D</td>
<td>ND (Membrane lysis?)</td>
<td>5.5</td>
<td>E. coli</td>
</tr>
<tr>
<td>H</td>
<td>Fiu</td>
<td>TonB, ExbB, D</td>
<td>ND (Membrane lysis?)</td>
<td>100</td>
<td>E. coli</td>
</tr>
<tr>
<td>Ia</td>
<td>Cir</td>
<td>TonB, ExbB, D</td>
<td>Pore formation</td>
<td>69.406</td>
<td>E. coli</td>
</tr>
<tr>
<td>Ib</td>
<td>Cir</td>
<td>TonB, ExbB, D</td>
<td>Pore formation</td>
<td>69.963</td>
<td>Shigella sonneii</td>
</tr>
<tr>
<td>M</td>
<td>FhuA</td>
<td>TonB, ExbB, D</td>
<td>Inhibits Murein synthesis</td>
<td>29.453</td>
<td>E. coli</td>
</tr>
<tr>
<td>Q</td>
<td>Cir</td>
<td>TonB, ExbB, D</td>
<td>ND</td>
<td>ND</td>
<td>E. coli</td>
</tr>
<tr>
<td>S1</td>
<td>Cir</td>
<td>TonB, ExbB, D</td>
<td>Pore formation</td>
<td>ND</td>
<td>S. boydii</td>
</tr>
<tr>
<td>V</td>
<td>Cir</td>
<td>CvaA, CvaB, CvaA*</td>
<td>Pore formation</td>
<td>9</td>
<td>E. coli</td>
</tr>
</tbody>
</table>

ND = Not Determined
Table adapted from review by Cursino et. al, 2002.
found resistant to the lambdoid phage φ80 (Matsushrio, 1963), and tolerant to a subclass (Group B) of colicins, toxic proteins produced by certain E. coli strains (Davies and Reeves, 1975a). Most importantly, it was shown that these strains were unable to support either high affinity siderophore-dependent iron transport (Frost & Rosenberg, 1975; Hantke & Braun, 1975; Williams, 1979) or vitamin B₁₂ transport (Bassford et al., 1976). Further studies found these TonB-dependent processes to require an intact electrochemical gradient at the CM (Hancock & Braun, 1976; Reynolds et al., 1980; Bradbeer, 1993). The main role for TonB is siderophore-dependent iron transport and the transport of vitamin B₁₂ by utilizing energy from the CM (Reviewed in Postle & Kadner, 2003).

The TonB system tranduces potential energy, generated as protonmotive force, across the periplasm to provide the necessary energy for active transport by the OM receptors, such as FepA and FhuA (Reynolds et al., 1980; Bradbeer, 1993; Postle, 1993; Braun, 1995). In E. coli, at least nine different OM receptors mediate TonB–dependent transport of specific ligands, (Table 2), allowing E. coli to use a variety of siderophores in addition to the one it produces (enterochelin). These OM receptors all contain a five residue phylogenetically conserved “TonB box” sequence (consensus: D/ETXXV) essential for productive interaction with TonB and with many of the OM receptors having TonB boxes near the extreme amino-terminus (Heller et al., 1988; Gudmundsdottir et al., 1989; Bell et al., 1990). The solved crystal structures of three OM receptors (FepA, FhuA, and BtuB) reveal these transporters have a 22-stranded anti-parallel β-barrel structure and a globular amino-terminal “cork” domain (residues ~1-150) that obstructs the periplasmic face of the barrel (Buchanan et al., 1998; Locher et al., 1998; Postle, 1999; Postle & Kadner, 2003). A charged-TonB protein provides the energy to the OM receptor required to drive a postulated conformational shift of this cork domain, releasing the ligand into
Table 2: TonB-dependent receptors of *E. coli* and their corresponding ligands

<table>
<thead>
<tr>
<th>Name</th>
<th>Mol wt</th>
<th>Solute Transported – Siderephore utilized</th>
<th>Approx $K_d$ of ligand</th>
<th>Utilization by phage and colicins</th>
</tr>
</thead>
<tbody>
<tr>
<td>BtuB</td>
<td>66,400</td>
<td>Vitamin B12</td>
<td>3 nM</td>
<td></td>
</tr>
<tr>
<td>Cir</td>
<td>69,839</td>
<td>Catechols</td>
<td></td>
<td>Colicins Ia, Ib, Q, S1, V</td>
</tr>
<tr>
<td>IutA</td>
<td>74,500</td>
<td>$\text{Fe}^{3+}$ - aerobactin</td>
<td>0.4 $\mu$M</td>
<td>DF13</td>
</tr>
<tr>
<td>FhuE</td>
<td>76,000</td>
<td>$\text{Fe}^{5+}$ - coprogen</td>
<td>0.3 $\mu$M</td>
<td></td>
</tr>
<tr>
<td>FhuA</td>
<td>78,992</td>
<td>Ferrichrome</td>
<td>~0.6 nM$^b$</td>
<td>T1, T5, φ80, Colicin M</td>
</tr>
<tr>
<td>FecA</td>
<td>81,718</td>
<td>Ferric dicitrate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FepA</td>
<td>79,908</td>
<td>$\text{Fe}^{3+}$ - enterobactin</td>
<td>~0.2 nM$^a$</td>
<td>Colicins B, D,</td>
</tr>
<tr>
<td>Fiu</td>
<td>83,000</td>
<td>Catechols</td>
<td></td>
<td>Colicins G, H</td>
</tr>
<tr>
<td>ChuA$^c$</td>
<td>80,624</td>
<td>Heme</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Listed are the nine known outer membrane receptors of *E. coli* that contain a TonB-box, including the approximate binding constant of solute/ligand, as well as Group B colicins and bacteriophage that utilize that receptor for access into the cell. The table is modified from Nikaido, 1996, with updated $K_d$ values for ligands from $^a$Hoegy *et al.*, 2005; $^b$Annamalai *et al.*, 2004; and additional information from $^c$Torres & Payne, 1997.
the periplasm (Buchanan et al., 1998; Locher, 1998).

The TonB protein of *E. coli* is a 239 amino acid protein (~26 kDa), with the majority of the protein predicted to occur in the periplasmic space, and having only a single transmembrane domain (Hannavy et al., 1990; Roof et al., 1991). This topology allows TonB to interact with components of both the outer and the cytoplasmic membranes. TonB consists of three distinct domains, the amino terminal domain (aa 1-32), the central domain (aa 33-100), and the carboxyl terminal domain (aa 100-239). The amino terminal domain serves as a signal anchor (Postle & Skare, 1988; Skare et al., 1989) with a predicted 21 residue (aa 12-32) hydrophobic transmembrane domain that contains a conserved Ser16, His20, Leu27, Ser31 motif (SHLS; Koebnik, 1993) essential for efficient energization of TonB (Figure 4) (Larsen & Postle, 2001). The central domain has a proline-rich region, containing a series of Glu-Pro and Lys-Pro repeats. This region is important in allowing TonB to span the periplasm and make contact with the OM, but is not essential for activity in most laboratory settings (Larsen et al., 1993). The carboxyl terminus of TonB is required for its association with the outer membrane. A deletion of the carboxyl terminus causes TonB to only be found in association with the cytoplasmic membrane (Letain & Postle, 1997).

ExbB and ExbD are CM-localized products of the *exb* operon; however, the gene for the TonB protein is located in a different operon (Figure 2). In *E. coli*, ExbB is a 244 amino acid protein (26 kDa), with three predicted transmembrane domains, the bulk of the protein occupying the cytoplasm, and the unusual topology of displaying its amino-terminus in the periplasm (Kampfenkel & Braun, 1993a; Karlsson et al., 1993a). The *E. coli* ExbD is a 141 amino acid protein (~17 kDa) that, like TonB, is predicted to mostly occupy the periplasm, having only a single transmembrane domain as its signal anchor (Kampfenkel & Braun, 1992).
Figure 4: A space-filled model of the TonB transmembrane domain. The residues of the conserved SHLS motive are shown in red, with these conserved residues residing on the same face of the protein. (Larsen et al., 1994)
In vivo formaldehyde crosslinking studies show ExbB and ExbD form homodimers and homotrimers, and that TonB physically interacts with both ExbB and ExbD (Higgs et al., 1998). ExbB is thought to serve as a chaperone for the insertion of TonB into the CM (Karlsson et al., 1993a); and it appears that ExbB and ExbD contribute to the stability of the TonB protein (Fischer et al., 1989; Skare et al., 1993; Ahmer et al., 1995) and that TonB is extremely unstable in the absence of ExbB and ExbD.

In TonB, there are two residues (Ser16 and His20) in the amino-terminal transmembrane domain that facilitate ExbB/D-mediated conversion of uncharged TonB to its energized form (Larsen & Postle, 2001). Deletion or substitution of either conserved residue (Ser16 and His20), causes a loss of physical interaction with ExbB as detected by in vivo crosslinking. Interaction between TonB and ExbB is also lost if there is an alteration of the spatial relationship between Ser16 and His20. This phenotype can be suppressed by mutations that map to the first transmembrane domain of ExbB. Three such suppressor alleles have been isolated in ExbB where a hydrophobic amino acid is replaced by an acidic residue (Ala39Glu, Val35Glu, and Val36Asp), restoring in vivo cross-linking and at least partial function to TonB TMD mutants (Larsen et al., 1994, Larsen et al., 1999). The transduction of energy by TonB from the CM to the OM occurs by a set of conformational changes in TonB that are responsive to PMF and are dependent on ExbB interacting with the signal anchor of TonB, thereby providing for transduction of potential energy to energy-requiring OM targets (Larsen et al., 1999). The current working model incorporating the contributions of ExbB and ExbD to the TonB-dependent energy transduction cycle is summarized in Figure 5 (Postle & Kadner, 2003). This schematic model involves the TonB protein starting in an uncharged conformation and interacting with the ExbB/D complex in the CM. In the CM, the energy-harvesting complex of
Figure 5: Working model for energy transduction between membranes in gram-negative bacteria. Uncharged TonB (1) is converted to charged TonB (2) by proton passage through the ExbB/D complex. Charged TonB can shuttle to the outer membrane and dock with non-transporter proteins, Lpp and OmpA (3). Ligand binding to the TonB-gated transporter FepA results in a conformational change in the FepA that induces productive interaction with the charged TonB, and release of the conformationally stored potential energy from TonB (4). Discharged TonB is recovered at the cytoplasmic membrane as uncharged TonB (1) by ExbB/D. (Postle & Kadner, 2003).
ExbB/ExbD uses the PMF generated at the CM to energize the TonB protein. TonB is able to associate with the OM even in the absence of PMF (Letain & Postle, 1997), appropriate ligand, and many of the OM receptors (Higgs et al., 2002); however, only a charged TonB protein can transduce energy to the ligand-bound OM receptors (Moeck et al., 1996 & 1997; Larsen et al., 1999; Moeck and Letellier, 2001; Higgs et al., 2002). A charged TonB protein interacts with the OM, and it appears that the initial docking at the OM occurs via interaction with Lpp and OmpA (Higgs et al., 2002). While the carboxyl-terminus is interacting with the OM, the amino-terminus is still associated with the CM via the ExbB/ExbD complex. Once contact is made with the OM, the amino-terminus of the charged TonB is released from the CM and the energy-harvesting complex. TonB would then be available to interact with a ligand-bound receptor, transducing that energy to the receptor to bring the ligand into the periplasm; thereby allowing the iron-siderophore complex to gain entry into the periplasmic space through the receptor. The now discharged TonB protein leaves the outer membrane, regaining its uncharged conformation where it can again interact with the ExbB/ExbD energy-harvesting complex in the CM.

**Energy Requirements of Outer Membrane Maintenance and the TolA System**

Colicins parasitize *E. coli* systems for transport across the OM and cell entry. Early colicin uptake studies in *E. coli* found several mutants that were tolerant to various colicins, with those mutants tolerant to Group A colicins (Davis & Reeves, 1975b) and to certain single-stranded DNA phages (M13, fd, and f1) termed *fii* mutants (Reviewed in Webster, 1991). These mutants had pleomorphic phenotypes, with the cells highly sensitive to a variety of detergents, drugs, and dyes. This appeared to reflect a loss of outer membrane integrity, as evidenced by blebbing of the outer membrane (Bernadac *et al.*, 1998), and leakage of periplasmic contents into the medium (Lazzaroni *et al.*, 1989). These observations suggested that maintenance of the
structural integrity of the cellular envelope of *E. coli* involves the products of the predicted *tol-pal* operon (Bernadac *et al.*, 1998; Lazdunski, *et al.*, 1998). The TolA system consists of seven known proteins, organized into two complexes: a CM complex and an OM complex in which all the genes for the proteins are located in the same operons (Figure 3) (Lazdunski, *et al.*, 1998, Vianney *et al.*, 1996). The OM complex involves associations with the OM proteins Lpp and OmpA, which also can interact with TonB, but also includes two proteins with no counterparts in the TonB system, TolB and Pal. Conversely, the CM complex is very similar to that of the TonB system, involving three proteins that interact through their transmembrane domains, TolA, TolQ and TolR (Derouiche *et al.*, 1995; Koebnik, 1993; Germon, *et al.*, 1998; Germon, *et al.*, 2001; Journet, *et al.*, 2001), with topologies (Bourdineaud *et al.*, 1989; Benedetti *et al.*, 1991; Levengood *et al.*, 1991; Kampfenkel & Braun, 1993b; Levengood-Freyermuth *et al.*, 1993; Muller *et al.*, 1993; Vianney *et al.*, 1994) similar to those of their TonB system counterparts (Figure 6).

The OM complex is composed of TolB and Pal, proteins that also interact with Lpp, OmpA, and the peptidoglycan layer (Bouveret *et al.*, 1995; Koebnik, 1995; Clavel *et al.*, 1998). The TolB protein of *E. coli* is a 408 amino acid periplasmic protein (Isnard *et al.*, 1994) while Pal is a 173 amino acid OM peptidoglycan-associated protein (Lazzaroni & Portalier, 1992) that is anchored by an amino-terminally located lipid moiety and through a carboxyl-terminal region that interacts strongly with the peptidoglycan layer (Lazdunski, *et al.*, 1998; Bouveret *et al.*, 1999). The carboxyl-terminal region of Pal also interacts with TolB (Clavel *et al.*, 1998). The TolA carboxyl-terminal domain has been shown to associate with Pal (Cascales *et al.*, 2000) and TolB (Dubuisson *et al.*, 2002; Walburger *et al.*, 2002) and appears to be involved in interactions between CM and OM complexes.
Figure 6: Predicted topology of the energy-transducers and the energy-harvesting complexes. (A) The energy-transducers (TonB and TolA) have a single TMD with the bulk of the protein believed to be in the periplasm and (B) the energy-harvesting complexes (ExbB/ExbD and TolQ/TolR). ExbB and TolQ have three TMDs with the unique topology of the amino-terminal being in the periplasm, while both ExbD and TolR have a single TMD with the majority of the protein located in the periplasm (Original diagram courtesy of Dr. Kathleen Postle, diagram above has been modified slightly).
The CM complex is comprised of TolA, TolQ, and TolR proteins. TolA of *E. coli* is a 421 amino acid protein (~43kDa) having three domains: an amino-terminal domain (aa ~1-42), a central, periplasmic domain (~43-272), and a carboxyl-terminal domain (~273-421), with each domain separated by a stretch of Gly residues (Levengood *et al*., 1991). While the predicted topologies of TolA and TonB are similar (Figure 4) (Roof *et al*., 1991; Levengood *et al*., 1991; Levengood-Freyermuth *et al*., 1993), only the amino-terminal transmembrane domains of these two proteins show actual sequence homology. TolA is anchored to the cytoplasmic membrane by the amino-terminal domain that contains a hydrophobic segment of 21 amino acids (from Ala14 to Phe34), with the highly conserved SHLS motif (identical to that described above for TonB) (Figure 4). The large, central domain is predicted to assume an alpha-helical conformation of sufficient length to span the periplasm, allowing TolA to access the OM (Levengood, 1991). The carboxyl-terminal domain is thought to interact with OM proteins and has been shown to be involved in colicin transport (Levengood-Freyermuth *et al*., 1993) and to directly interact *in vitro* with colicins A and E1 (Benedetti *et al*., 1991) and shown to interact and co-crystallize with an f1 phage tail protein (Click & Webster, 1997; Riechmann & Hollinger, 1997).

*In vivo* studies have found that the amino-terminal domain of TolA can be crosslinked to TolQ and TolR (Derouiche *et al*., 1995). Mutagenesis of the highly conserved transmembrane domain SHLS motif in the amino-terminal identified only Ser18 and His22 of the SHLS motif and Phe26 as playing an important role in TolA function. Suppressor mutations in TolQ, Gly26Asp and Ala30Val were found for the TolA mutants at Ser18 and His22, respectively (Germon *et al*., 1998). These suppressor mutations in the TolA system are similar to the suppressor mutations found in ExbB of the TonB system (Larsen *et al*., 1994 & 1999).
TolQ is a 230 amino acid protein (~26 kDa) that, like ExbB, has a predicted topology with a periplasmic amino-terminal followed by three transmembrane domains (Kampfenkel & Braun, 1993b). TolR is a 142 amino acid protein (~16kDa) comprised of a short cytoplasmic amino-terminal with the majority of the protein predicted to occur in the periplasm (Kampfenkel & Braun, 1993b). Suppressor mutations experiments suggest that the transmembrane domain of TolR interacts with the third TMD of TolQ (Lazzaroni et al., 1995).

Until recently, most of the data about the TolA system came from studies that focused on the transport of Group A colicins (Table 3) into the cell, with TolA involvement in energy transduction predicted on the basis of similarities to the TonB system (Braun & Herrmann, 1993; Larsen et al., 1999). A PMF-dependent conformational change has recently been demonstrated for TolA (Germon et al., 2001) and requires the presence of TolQ and TolR, similar the previously described requirement of TonB for EXB and ExbD (Larsen et al., 1999). Studies have shown that TolA interacts with Pal and it appears this interaction requires both the PMF and the TolQ and TolR proteins (Cascales et al., 2000).

There are two additional genes, ybgC and ybgF, in the Tol-Pal operon. YbgC is a 134 amino acid protein found in the cytoplasm functioning as an Acyl-CoA thioester hydrolase (Zhuang et al., 2002), while YbgF is a 221 amino acid periplasmic protein with an unknown function (Walburger et al., 2002). Deletion of these genes has no apparent affect on the function of the TolA system under the conditions examined to date.

Crosstalk between the TonB and TolA Systems

As described above, there are similarities between the TonB and TolA system. Both systems have an energy-harvesting complex located in the CM (ExbB/ExbD and TolQ/TolR) that harvest the CM’s energy and a specific energy transducer (TonB and TolA, respectively)
Table 3: Representative members of group A colicins

<table>
<thead>
<tr>
<th>Colicin</th>
<th>Receptor/Assisting Protein</th>
<th>Translocation</th>
<th>Cytotoxic activity</th>
<th>Molecular Weight (kDa)</th>
<th>Original producer strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>BtuB/OmpF</td>
<td>TolQRAB</td>
<td>Pore formation</td>
<td>62.989</td>
<td>Citrobacter freundii</td>
</tr>
<tr>
<td>E1</td>
<td>BtuB/TolC</td>
<td>TolQRA</td>
<td>Pore formation</td>
<td>52.279</td>
<td>E. coli</td>
</tr>
<tr>
<td>E2</td>
<td>BtuB/OmpF</td>
<td>TolQRAB</td>
<td>DNA endonuclease</td>
<td>61.561</td>
<td>S. soneii</td>
</tr>
<tr>
<td>E3</td>
<td>BtuB/OmpF</td>
<td>TolQRAB</td>
<td>rRNA endonuclease, RNase hydrolyzing 16S rRNA</td>
<td>57.960</td>
<td>E. coli</td>
</tr>
<tr>
<td>E4</td>
<td>BtuB/OmpF</td>
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<td>rRNA endonuclease, RNase hydrolyzing 16S rRNA</td>
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<td>E. coli</td>
</tr>
<tr>
<td>E5</td>
<td>BtuB/OmpF</td>
<td>TolQRAB</td>
<td>RNase hydrolyzing the anticodon loop of tRNA, translocation block</td>
<td>ND</td>
<td>E. coli</td>
</tr>
<tr>
<td>E6</td>
<td>BtuB/OmpF</td>
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</tr>
<tr>
<td>K</td>
<td>Tsx/OmpF, OmpA</td>
<td>TolQRAB</td>
<td>Pore formation</td>
<td>59.611</td>
<td>S. soneii</td>
</tr>
<tr>
<td>L*</td>
<td>ND/OmpA</td>
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ND = Not Determined

*Colicin L and Bacteriocin 28b are very similar, if not identical (Guasch et al., 1995)

Table adapted from review by Cursino et al., 2002 with additional information and data incorporated from Lazzaroni et al., 2002
that then delivers that energy to targets at the OM. The energy-harvesting complexes from these two systems bear a great degree of similarity. This similarity is sufficient to allow these complexes to engage in crosstalk, meaning in the absence of a system’s energy-harvesting complex, the energy-transducer can be energized by the energy-harvesting complex from the opposite system. The initial observation of cross-talk came from the TonB system, where the absence of ExbB and ExbD did not entirely prevent activity (Braun, 1989), and that the residual activity of TonB in the absence of ExbB and ExbD was dependent on the presence of TolQ and TolR. Later studies demonstrated that not only could TolQ and TolR partially fill the role of ExbB and ExbD, but also the reverse was true in that ExbB and ExbD could partially fill the role of TolQ and TolR and provide for partial support of TolA activity (Braun & Hermann, 1993).

Most studies of this cross-talk phenomenon have focused on colicin sensitivity. Colicins parasitize OM receptors that are dependent on the energy transducer, either TonB- or TolA-dependent (Table 1 & Table 3) for CM-energy. If an energy-transducer is unable to transduce that CM-energy to the OM, the uptake of colicins by the cell does not occur; and the cell is protected from their lethal effects. The absence of ExbBD reduces the sensitivity of the cell to TonB-dependent colicins, while the absence of TolQR reduces the cell’s sensitivity to TolA-dependent colicins. While the sensitivity at higher dilution to these colicins is reduced in the absence of the system’s energy-harvesting complex, these cells are still not fully resistant to the colicin. It is only when the remaining energy-harvesting complex is also absent, that the cells become fully resistant to both TonB- and TolA-dependent colicins. This residual colicin activity represents crosstalk and suggests the two systems must share some basic mechanisms for harvesting and transducing energy.

The proteins in the energy-harvesting complexes of the TonB and TolA system not only
have a common topology but they also share a large amount of sequence homology in their transmembrane domains (Figure 7), with almost 70% sequence identity in the second transmembrane domain of ExbB/TolQ and in the TMD of ExbD and TolR. The high homology of these transmembrane domains may be in part due to their common role of harvesting energy, based on the fact that the TMDs of the energy-harvesting complexes ExbB/ExbD and TolQ/TolR share homology with the energy-harvesting portions of the transmembrane domains of MotA and MotB (Cascales et al., 2001). MotA and MotB are proteins that have been shown to be involved in the harvesting of energy from the proton gradient as part of the flagellar motor (Blair & Berg, 1990; Blair & Berg, 1991; Stolz & Berg, 1991; Zhou et al., 1998; Braun et al., 1999; Van Way et al., 2000). The TolA and TonB protein have very different sequences in their periplasmic regions, most probably because they serve different OM targets. The only area in which TolA and TonB proteins share sequence homology is in their amino-terminal transmembrane domains, containing the conserved SHLS motif (Figure 4 & Figure 8). Point mutation studies have shown the Ser16 and His20 residues are essential for efficient function, and that deletion or substitution of either residue or alteration of the spatial relationship between the two results in a loss of TonB activity (Larsen et al., 1994; Larsen et al., 1999; Larsen & Postle, 2001). Similarly, mutations of TolA at Ser18 and His 22 result in a loss of function of TolA activity (Germon et al., 1998). This shared SH motif may account for both the ability of energy-transducer proteins to interact with the energy-harvesting complexes (ExbBD and TolQR) at the CM as well as their ability to become charged.

While cross-talk does occur, it does so with less efficiency. Studies in the Higgins laboratory demonstrated that a TolA/TonB chimera protein of Salmonella typhimurium (amo-
A.

TMD 1

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<tr>
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TMD2

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<td>TolR</td>
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Figure 7: Sequence alignment of the transmembrane domains of the energy-harvesting complex proteins. The sequence alignment of the transmembrane domains of the (A) ExbB and TolQ proteins, and (B) the ExbD and TolR proteins from *E. coli*. Homologous amino acid residues are highlighted. Sequences aligned with the predicted transmembrane domain of the ExbB and ExbD protein, indicated by the black line at the top of the sequences. Sequences obtained from UniProtKB/Swiss-Prot entries and predicted transmembrane domains of proteins correspond to those from Zhai et al., 2003.
Species

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<td>Y. pestis</td>
<td>WSLIFS*GLHGSVV*AALVYSVE</td>
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<td>LGLLI*SLIAHGI*VGFIL*W*NW*N*E</td>
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<td>P. putida</td>
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<tr>
<td>P. aeruginosa #1</td>
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TolA

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Figure 8: Sequence alignment of the transmembrane domain of TonB from various species. The sequences are aligned relative to residues 11 to 33 of E. coli TonB, with the predicted transmembrane domain of the protein in E. coli being residues 12 to 32 (which are indicated by the black line at the top of the sequences). The TonB analog (TolA) of two species is shown at bottom. Conserved amino acids in the SHLS motif are highlighted. The SHLS motif in TonB is conserved in closely related gram-negative species, while the SHLS motif is less conserved in others. V. cholerae and P. aeruginosa have two TonB proteins, with the second TonB containing none of the SHLS motif (Figure adapted from Postle & Larsen, 2004, with additional sequences from UnitProtKB/Swiss-Prot entries).
terminal replacement of the first 32 residues TonB with the first 34 residues of TolA = TolA\textsubscript{1-34}
TonB\textsubscript{33-239} had the ability to interact with the various energy-harvesting complexes using a \(\phi80\)
adsorption assay. This data suggests the derivative was able to be energized better by the TolQR complex than by the ExbBD complex. While the data was limited, the results do provide a possible role for the first 32 residues of TonB (as well as the first 34 residues of TolA). These other “non-essential” residues may actually be involved in determining the specificity of interaction of the energy-transducer with the energy-harvesting complex (Karlsson \textit{et al}, 1993b).

\textit{Research Aims}

The inefficiency of crosstalk between the TonB and TolA systems suggest there may be specificity for preferred interaction of the energy-transducing protein with the energy-harvesting complex within the system versus interactions with proteins of the opposite system. The transmembrane domains of the energy-transducers, TonB and TolA, contain conserved Ser and His residues that are involved in energization of the proteins. While a need for the conserved Ser and His residues is apparent, a role for the remaining residues in the amino-terminus of the energy-transducers has yet to be clearly established. Based on the limited data from the Higgins laboratory (Karlsson \textit{et al}, 1993b), one possible role for these remaining “non-essential” residues is for determining the specificity of interaction between the energy-transducers and the energy-harvesting complexes; therefore, a thorough examination of the non-essential transmembrane residues of TonB and TolA could reveal sequences that alter the specificity of each system, thus providing insight into the functional portions of the proteins in both systems. The research conducted for this dissertation was divided into three specific aims:

1. Develop assays to detect physical interactions between proteins of the TolA and TonB systems engaged in energy transduction.
(2) Identify the minimal TMD components required for energization efficiency of TonB.

(3) Develop various amino-terminal domain chimeric proteins of TonB and TolA for identifying domains responsible for the specificity by the energy-harvesting complexes.

My dissertation research was divided into the above mentioned aims and an introduction, a complete methodology, results, as well as a conclusion for aims one and two are thoroughly described in the following chapters, while the progress made on aim three is presented in the form of an appendix.
CHAPTER II
DEVELOPMENT OF ASSAYS TO DETECT PHYSICAL INTERACTIONS OF PROTEINS INVOLVED IN CROSSTALK BETWEEN THE TONB AND TOLA SYSTEMS

INTRODUCTION

In gram-negative bacteria, such as *Escherichia coli*, there are two systems involved in the transfer of energy from the cytoplasmic membrane to the outer membrane, the TonB and TolA systems. The proteins of these two systems include an energy-transducer (TonB/TolA), as well as a cytoplasmic membrane energy-harvesting complex (ExbBD/TolQR). The physical interaction of the proteins within each system has been demonstrated (Derouiche *et al.*, 1995; Higgs *et al.*, 1998; Journet *et al.*, 1999). In the absence of the specific energy-harvesting complex for a system, cross-talk between the two systems has been shown to occur in which the energy-transducers (TonB and TolA) are energized by energy-harvesting complex from the opposite system (Braun, 1989; Braun & Hermann, 1993). This cross-talk indicates the proteins from these two systems must interact in some manner; however direct interaction between proteins in opposite systems has not been demonstrated. The purpose of the research in this aim was to develop various assays that could identify the physical interactions of the proteins in the TonB and TolA systems that occur during crosstalk. Before attempting to identify protein/protein interactions during crosstalk, it is important to clearly identify the protein/protein interactions that occur within each system.

*In vivo* formaldehyde chemical cross-linking is one method that has been used to identify protein/protein interactions in both the TonB (Skare *et al.*, 1993; Higgs *et al.*, 1998, Higgs *et al.*, 2002) and the TolA system (Journet *et al.*, 1999). Formaldehyde is a “zero-length” cross-linker,
forming a single methyl bridge between proteins that have reactive residues (Trp, His, Arg, Csy, Lys, Tyr) (Means & Feeney, 1971) within 2 Å of one another. With in vivo formaldehyde chemical crosslinking, if proteins are interacting with one another in such a manner that the specific reactive residues from both proteins are within 2 Å to one another, these can produce cross-linked complexes that are able to be visualized using Immunoblot analysis. This method has been used to examine interactions of the proteins in the TonB system (Skare et al., 1993) and has revealed complexes representing ExbB and ExbD homomultimers, as well as TonB/ExbB and TonB/ExbD heterodimers (Higgs et al., 1998, Higgs et al., 2002). This technique has also been used to identify a TolR homodimer, an apparent TolR dimer/TolQ monomer, as well as TolA/TolQ and TolA/TolR heterodimers (Journet et al., 1999). While this methodology worked well for detecting the interactions of proteins within a system, the low efficiency of crosstalk makes this an unfavorable method for looking at crosstalk because of the difficulty in detecting the low number of protein complexes involved in crosstalk.

In addition to the low efficiency of crosstalk that may affect the use of in vivo chemical crosslinking, there are other reasons that crosslinking may not be the best means to identify protein/protein interactions. In order for in vivo chemical crosslinking to identify protein/protein interactions, the part of the proteins that are within proximity of one another must have available reactive residues from both proteins must be close enough to one another to form a methyl-bridge. Another potential problem is the site at which that crosslink occurs, even if proteins are able to form an in vivo chemical crosslink. If the crosslink obscures or interferes with the epitope the antibody would bind, the protein/protein complex would not be able to be detected by Immunoblot analysis. It is important to note that due to the restrictions of in vivo chemical crosslinking, the absence of crosslinked complexes is not direct evidence for the absence of
protein/protein interactions. For example, even though several complexes in the TonB system have been identified using in vivo chemical crosslinking, this method has been unable to identify an ExbB/ExbD heterodimer complex, nor identify TonB in complex with TolQ/TolR. For those reasons, in vivo formaldehyde chemical crosslinking is not an assay that will be utilized in this research for the detection of protein/protein complexes that occur during crosstalk.

A protein capture assay was utilized by the Braun laboratory (Braun et al., 1996) to determine that ExbB interacts with both ExbD and TonB. This protein capture assay involved a nickel-affinity chromatography column and a recombinant ExbB protein that contain a Histidine-rich (His$_6$) region. The assay worked by the recombinant ExbB (His$_6$) protein being first bound to the nickel in the column, and then the solubilized proteins of interest (ExbD and TonB) were applied to the column. Subsequent washes removed any solubilized proteins that did not interact with the ExbB (His$_6$) protein bound to the column. Elution of the fractions would remove the ExbB (His$_6$) protein as well as any and all proteins that interacted with the ExbB (His$_6$) protein. Due to the fact this method was successfully used for determining protein/protein interactions in the TonB system, a slightly modified method of this capture assay was used in this research in order to gain more information about the protein/protein interaction of the proteins of the TolA system. Once interactions of the proteins of the TolA system are identified, the assay was to be used to identify interactions of the proteins involved in crosstalk.

The general principle of the protein capture assay used in this research aim is that a “bait” protein containing a His$_6$ region is first applied to a Ni-charged column/matrix and the His$_6$ region allows the protein to bind to the Ni. After washing any unbound His$_6$ protein from the column/matrix, a second “target” protein is applied that lacks a His$_6$ region, so it can not bind to the column on its own. If the proteins have the ability to interact, the bait protein will be able to
capture the target protein and the two proteins will be eluted off the column/matrix.

Another common molecular technique used to identify protein-protein interactions is two-hybrid systems, whether yeast or bacteria. The BacterioMatch 2-Hybrid system is a bacterial system that is based on transcriptional activation (Stratagene). It was used in this research as an additional method to examine in vivo protein/protein interactions within the TolA system. The BacterioMatch 2-Hybrid system functions so that the gene of a “bait” protein of interest is fused to the gene for the full-length bacterial lambda repressor protein. The gene corresponding to a “target” protein is fused to the coding region of the N-terminal domain of the α-subunit of RNA polymerase. The constructs are assayed in reporter strains that have been engineered such that when the two fusion-proteins interact, they recruit and stabilize the binding of RNA polymerase close to the promoter, activating transcription of reporter genes (Figure 9) (Stratagene). The pBT and pTRG plasmids both carry antibiotic resistance genes (pBT plasmid carries the cat gene, while the pTRG plasmid carries the tetA gene) allowing for their selection. The genes for the “fusion protein” in each plasmid are under the regulation of the lac-UV5 promoter. This allows the fusion products to be transcribed and translated when the gene for the fusion-protein is de-repressed by the addition of IPTG (Figure 13 and 14). The test strains are constructed to contain two reporter genes under a modified lac promoter with a single λ operator at position −62. This keeps the reporter genes from being induced simply by the addition of IPTG and explains the need for λ.cI for transcription (Stratagene). The reporter genes for the XL1-VCR test strain are HIS3 (a component of the histidine biosynthetic pathway) and aadA (confers resistance to streptomycin). In the presence of bait and target proteins that interact, the RNA polymerase is recruited and allows for transcription of these genes. Therefore, testing for the ability of XL1-VCR cells containing bait and target proteins to grow on selective
**Figure 9: Diagram of the mechanism of the BacterioMatch two-hybrid system.** Interaction of the “bait” and “target” fusion proteins provide the proper orientation of the RNA Polymerase subunit resulting in the transcription (and ultimately translation) of the downstream reporter genes used for determination of protein-protein interaction. The system used involved using two reporter strains (A) BacterioMatch® II Validation Reporter Competent Cells, referred to as XL1-VCR and used for growth on the dual selective media and (B) XL1-Blue Strain, referred to as XL1-AX, and used for the beta-galactosidase experiments (Diagrams from Stratagene).
Figure 10: The pBT vector plasmid. (A.) Diagram of pBT plasmid, demonstrating the position the gene for the bait protein would be inserted to form the fusion protein with λcI. (B.) Diagram of the pBT plasmid indicating the position of the Multiple Cloning Site Region (MCS) (C.) The MCS for pBT with the various restriction sites denoted. The pBT plasmid contains a chloramphenicol resistance gene as a selection marker (Diagrams from Stratagene).
Figure 11: The pTRG vector plasmid. (A.) Diagram of the pTRG plasmid, demonstrating the position the gene for the “target” protein would be inserted to form the fusion protein with the alpha-subunit of RNA polymerase. (B.) Diagram of the pTRG plasmid indicating the position of the Multiple Cloning Site Region (MCS) (C.) The MCS for pTRG with the various restriction sites denoted. The pTRG plasmid contains a tetracycline resistance gene as a selection marker (Diagrams from Stratagene).
screening medium (5 mM 3-AT, with tetracycline and chloramphenicol for plasmid selection) and dual selective screening medium (5 mM 3-AT, 12.5 μg ml⁻¹ streptomycin, with tetracycline and chloramphenicol for plasmid selection) allows for the determination interaction of the bait and target proteins (Stratagene). The XL1-AX test strain contains amp⁺ and lacZ as the reporter genes, allowing interactions of the bait and target proteins to be identified utilizing an assay for β-galactosidase activity (Stratagene). Reporter strains carrying plasmids bearing fusions known to interact (pBT-LGF2 encodes the dimerization domain of the Gal4 protein fused to λcI repressor while pTRG-Gal11P encodes a Gal11 derivative fused to α-subunit of RNA polymerase) serve as a positive control. Parent control vector plasmids lacking any fusion partners serve as negative controls (Stratagene).

The pBT and pTRG plasmids encoding the TolQ- and TolR-fusion proteins were constructed and transformed into reporter strains, and then tested either for growth on antibiotic selective media or for the levels of β-galactosidase, and compared to the positive control of pBT-LGF2 and pTRG-Gal11P. The intent was to quickly identify interactions between the various proteins in the TolA system, then use the assay to determine the proteins interactions that occur during crosstalk between the two systems.
MATERIAL AND METHODS

Media

Bacterial strains and plasmids were maintained on Luria-Bertani (LB) medium (Sambrook et al., 1989), containing ampicillin at 100 μg ml\(^{-1}\), tetracycline at either 20 μg ml\(^{-1}\) or 12.5 μg ml\(^{-1}\), kanamycin at 30 μg ml\(^{-1}\), and chloramphenicol at either 34 μg ml\(^{-1}\) or 25 μg ml\(^{-1}\) where indicated. Media were also supplemented with isopropyl-β-D-thiogalactopyranoside (IPTG) as indicated. Additional media were utilized for the BacterioMatch Two-Hybrid assays, made according to the Stratagene Instruction Manual (Stratagene). These included: M9+ His-dropout Broth (0.4% w/v glucose, 200 μM adenine HCl, 1X His-dropout supplement amino acids (from BD Biosciences Clontech), 1 mM MgSO\(_4\), 1 mM Thiamine HCl, 100 μM CaCl\(_2\), 50 μM IPTG in 1X M9 minimal salts); Nonselective medium (M9+ His-dropout Broth, 1.5% w/v agar, chloramphenicol 25 μg ml\(^{-1}\) tetracycline 12.5 μg ml\(^{-1}\)); Selective Screening Medium (nonselective media with 5 mM 3-amino-1,2,4 triazole [3-AT]); and Dual Selective Screening Medium (selective screening media with 12.5 μg ml\(^{-1}\) streptomycin). For cloning reactions, SOC medium (2% w/v tryptone, 0.5% w/v yeast extract, 0.05% w/v NaCl, 20 mM glucose, 10 mM MgCl\(_2\), 10 mM MgSO\(_4\)) was used as indicated.

Bacterial strains and plasmids

The principal bacterial strains and plasmids used in this research are summarized in Table 4 and Table 5. Growth of bacterial strains was monitored and measured as \(A_{550}\) on a Spectronic 20 spectrophotometer with a pathlength of 1.5 cm. As part of the pET expression system from Novagen (Figure 12) the host strain (BL21-λDE3) for the Bacteria 2-Hybrid System encodes the T7 RNA polymerase (λDE3 lysogen) for the expression of target genes in a pET vector (that are
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</tr>
<tr>
<td>pBT-LGF2</td>
<td>Encodes dimerization domain of Gal4 protein fused to λ.cI repressor</td>
<td>Cam</td>
</tr>
<tr>
<td>pTRG-Gal11P</td>
<td>Encodes Gal11 derivative fused to α-subunit of RNA polymerase</td>
<td>Tet</td>
</tr>
<tr>
<td>pRA0028</td>
<td>Encodes TolQ protein fused to λ.cI repressor</td>
<td>Cam</td>
</tr>
<tr>
<td>pRA0029</td>
<td>Encodes TolQ protein fused to α-subunit of RNA polymerase</td>
<td>Tet</td>
</tr>
<tr>
<td>pRA0030</td>
<td>Encodes TolR protein fused to λ.cI repressor</td>
<td>Cam</td>
</tr>
<tr>
<td>pACYC184</td>
<td>Vector (Control plasmid)</td>
<td>Cam, Tet</td>
</tr>
<tr>
<td>pTPS202</td>
<td>8.7-kb BglII fragment carrying the tolQ, tolR, tolA, and tolB genes cloned in pJII12, Ap'</td>
<td>Amp</td>
</tr>
</tbody>
</table>
Figure 12: The pET Expression System. Cartoon illustration of the control elements found in the pET system including the T7 RNA gene, the T7 promoter in the pET vectors, and the plasmid pLysS that provides stringent control of the of the genes contained on the pET vector under the T7 promoter. The pET24a plasmid contains a 25 bp lac operator sequence immediately downstream from the T7 promoter. The binding of the lac repressor (lacI) helps to effectively reduce transcription from the T7 RNA polymerase. Both the T7 RNA polymerase gene and the T7 promoter for the gene of interest are under lac repression, rendering the system inducible by IPTG (Figure from Novagen).
under the control of a T7 promoter). The gene for T7 RNA polymerase is under the control of the lacUV5 in the λDE3 lysogens; therefore, in the presence of IPTG, the T7 polymerase gene is no longer repressed by the lacI repressor and transcription the T7 polymerase gene can occur. In addition to the T7 polymerase gene, the expression of the gene of interest cloned into the pET24a plasmid is under a T7 promoter also repressed by the lacI repressor. Addition of IPTG to BL21-λ.D3 cells containing a gene of interest cloned into the pET24a plasmid allows transcription of the T7 polymerase gene as well as making the promoter for the gene of interest accessible to the T7 polymerase. For the capture assay, recombinant tolQ and tolR genes were constructed as described below, to create modified TolQ and TolR proteins that contained both an 11-amino acid N-terminal T7 epitope and a C-terminal His-tag (containing 6 histidyl residues), as well as creating modified TolQ and TolR proteins that contained only the 11-amino acid N-terminal T7 epitope. The T7 epitope allows the proteins to be detected with a monoclonal α−T7 antibody (Novagen). Plasmids for the BacterioMatch 2-Hybrid system were constructed so that the tolQ and tolR genes were fused to the genes encoding λcI (λ repressor) or the α-subunit of RNA polymerase. Once the genes were cloned into the appropriate plasmids, the plasmids were transformed into reporter strains to test for protein/protein interactions as described below.

Construction of the plasmids for protein capture assays

For protein capture assay experiments, the pET System from Novagen was utilized for the cloning and expression of recombinant TolQ and TolR proteins in E. coli strain BL21-λ.D3 (Moffatt & Studier, 1986; Rosenberg et al., 1987, Studier et al., 1990). The modified TolQ and TolR proteins that contained both an 11-amino acid N-terminal T7 epitope and a C-terminal His-tag (referred to as T7-TolQ-His6 and T7-TolR-His6 proteins, respectively) were constructed by R. A. Larsen by cloning into the pE24a to form plasmids pRA0005 and pRA0006. The
modified TolQ and TolR proteins containing only the 11-amino acid N-terminal T7 epitope (referred to as T7-TolQ and T7-TolR, respectively) were generated by cloning the genes from pRA0005 and pRA0006 into pET14b plasmids.

To generate the T7-TolQ-His6 protein, the \textit{tolQ} gene was amplified by the polymerase chain reaction (PCR) from the pTPS202 plasmid using primers oRA0100R and oRA0101R (Table 6) to generate a 708 bp product. Multiple PCR reactions were pooled and purified using the Qiaquick PCR purification kit (Qiagen); the purified fragment was digested with \textit{EcoRI} and \textit{HindIII} at 37\textdegree C for 120 minutes and transferred to 65\textdegree C for 20 minutes to heat inactivate the restriction enzymes. The digestion mixture was purified using the Qiaquick PCR purification kit (Qiagen). The digested fragment was then ligated into pET24a (Figure 13) that had also been digested with \textit{EcoRI} and \textit{HindIII} (the same as the \textit{tolQ} DNA fragment), dephosphorylated by incubation with shrimp alkaline phosphatase (SAP) for 15 minutes at 37\textdegree C, followed by inactivation of SAP by incubation at 65\textdegree C for 15 minutes. Ligation reactions containing the digested/dephosphorylated pE24a, digested \textit{tolQ} fragment, T4 ligase, and T4 ligase buffer (total volume of 10 \mu l) were incubated at room temperature (~25\textdegree C) for one hour, then placed at ~20\textdegree C overnight. Ligation reactions were thawed and transformed by adding 2 \mu l of ligation reactions to 40 \mu l of chemically competent DH5-\alpha cells (from InVitrogen) and incubating for 30 minutes, followed by heat shock at 42\textdegree C for 90 seconds, then 4\textdegree C for 2 minutes, followed by the addition of 900 \mu l of SOC medium and the mixture incubated at 37\textdegree C for 1 hour. After the 1 hour, 100 \mu l of the transformation was plated on LB agar plates containing kanamycin at 30 \mu g ml\textsuperscript{-1} and incubated overnight at 37\textdegree C. Fourteen of the colonies obtained by the transformation were screened by growing the colonies overnight in LB containing kanamycin at 30 \mu g ml\textsuperscript{-1}, taking
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Gene &amp; direction</th>
<th>Restriction site created</th>
</tr>
</thead>
<tbody>
<tr>
<td>oRA0100R</td>
<td>5’ cccgaattcggtgacatgaatatc 3’</td>
<td>tolQ forward</td>
<td>EcoRI</td>
</tr>
<tr>
<td>oRA0101R</td>
<td>5’ gcgaaggcccttttggtgtctc 3’</td>
<td>tolQ reverse</td>
<td>HindIII</td>
</tr>
<tr>
<td>oRA0102R</td>
<td>5’ cggcgtcggccagagcgcgtgg 3’</td>
<td>tolR forward</td>
<td>BamHI</td>
</tr>
<tr>
<td>oRA0103R</td>
<td>5’ cccagcttgactagggtcgc 3’</td>
<td>tolR reverse</td>
<td>HindIII</td>
</tr>
<tr>
<td>oRA0119R</td>
<td>5’ gcatcgaattcgactgaatatcc 3’</td>
<td>tolQ forward</td>
<td>EcoRI</td>
</tr>
<tr>
<td>oRA0123R</td>
<td>5’ caaggacttcactgaatatcc 3’</td>
<td>tolQ forward</td>
<td>EcoRI</td>
</tr>
<tr>
<td>oRA0131R</td>
<td>5’ cccgaattctacctgacatgaatatc 3’</td>
<td>tolQ forward</td>
<td>EcoRI</td>
</tr>
<tr>
<td>oRA0121R</td>
<td>5’ gcatcgaattcgccagagcgcgtgacg 3’</td>
<td>tolR forward</td>
<td>EcoRI</td>
</tr>
<tr>
<td>oRA0122R</td>
<td>5’ caggactccgtaggtcgtcattaaacc 3’</td>
<td>tolR reverse</td>
<td>BamHI</td>
</tr>
</tbody>
</table>

Sequence of the primers used in PCR reactions. The primers were designed to include restriction enzyme sites (restriction sites are underlined and listed in far right column) in order to clone the genes of interest into the various plasmids. The italicized nucleotides represent any additional nucleotides designed as part of the primer that are not part of the original gene. Relative positions (in codons) are indicated below each primer.
Figure 13: The pET24a plasmid vector. (A.) Diagram of pET24a plasmid, the sequence of the plasmid is numbered with the pBR322 convention; therefore the T7 expression region is reversed on this plasmid map. (B) The cloning/expression region in the pET24a plasmid contains an N-terminal T7•Tag sequence as well as an optional C-terminal His•Tag sequence. An important feature of the plasmid is that the cloning/expression region of the coding strand is transcribed by T7 RNA polymerase. Other unique sites for this plasmid are shown on the circle map and include: T7 promoter 311-327; T7 transcription start 310; T7•Tag coding sequence 207-239; Multiple cloning sites (BamHI - Xho I) 158-203; His•Tag coding sequence 140-157; T7 terminator 26-72; lacI coding sequence 714-1793; pBR322 origin 3227; Kan coding sequence 3936-4748; f1 origin 4844-5299 (Diagram from Novagen).
1.5 ml of that culture and isolating plasmid DNA by the alkaline lysis method (Sambrook et al., 1989), with recovery of the plasmids in 40 μl of ddH₂O. Using 10 μl of those plasmid preps, digestions with EcoRI and HindIII were performed to determine which plasmids contained the tolQ insert (11 out of 14 contained the proper ~700 bp fragment). To confirm the presence of the tolQ gene in these plasmids, further restriction mapping of two isolates was performed with BglI and AvaI. Both produced bands corresponding to those expected with a tolQ insert into pET24a, one was chosen and named pRA0005.

The T7-TolQ construct was made by digesting pRA0005 with BglII and XhoI, releasing a fragment that includes the tolQ gene along with the T7 promoter, the lac operator, and the T7 tag. The XhoI restriction site in pRA005 is upstream to the His-tag region; therefore, the modified protein produced from the gene could be detected in the same manner as the T7-TolQ-His6 protein, but would lack the His-tag. Purified pRA0005 and pET14b (Figure 14) were digested with BglII and XhoI at 37°C for 100 minutes. Reactions were extracted using 45 μl of phenol, chloroform, isoamyl alcohol in a 25:24:1 ratio, mixing and then centrifuging for 1 minute. The aqueous phase was recovered, with 10 μl of 3M sodium acetate and 200 μl 100% ethanol then added and mixed. After sitting for 5 minutes at room temperature, the mixture was centrifuged and the supernatant removed. Pellets were rinsed in 70% ethanol, air dried, then suspended in 40 μl H₂O for the pRA0005 digestion, and 45 μl H₂O for the pET14b digest. Digested pRA0005 plasmid was resolved on a 1% w/v agarose gel, the DNA band from corresponding to 873 bp was excised from the gel and purified using the QIAquick gel extraction kit (Qiagen) with recovery
Figure 14: The pET14b plasmid vector. (A.) Diagram of pET14b plasmid, the sequence of the plasmid is numbered with the pBR322 convention; therefore the T7 expression region is reversed on this plasmid map. (B.) The cloning/expression region in pET-14b containing an N-terminal His•Tag sequence, after which is a thrombin site as well as three cloning sites. An important feature of the plasmid is that the cloning/expression region of the coding strand is transcribed by T7 RNA polymerase. Other unique sites for this plasmid are shown on the circle map and include: T7 promoter 646-662; T7 transcription start 645; His•Tag coding sequence 554-571; Multiple cloning sites (Nde I - BamH I) 510-526; T7 terminator 404-450; pBR322 origin 2845;bla coding sequence 3606-4463 (Diagram from Novagen).
of the DNA fragment in ~50 μl in 10 mM Tris-HCl, pH 8.5. Digested pET14b was dephosphorylated and ligation reactions with the purified, digested pRA0005 fragment set up as described above, with the exception that these ligation reactions were incubated at room temperature for 18 hours. After ligation, 2 μl of the reactions were transformed into chemically competent DH5-α cells as above, except the 100 μl of cells were plated on LB plates containing ampicillin at 100 μg ml⁻¹, and grown overnight at 37°C. Alkaline lysis (Sambrook et al., 1989) of twelve colonies was performed, and restricted with BglII and XhoI. One plasmid having the proper size fragment was chosen and named pRA0011.

The TolR modified protein (T7-TolR-His6) was built by R.A. Larsen in a similar fashion as the T7-TolQ-His6 protein. The tolR gene was amplified by PCR from the pTPS202 plasmid using primers oRA0102R and oRA0103R to generate a 441 bp oligonucleotide. PCR products were pooled and purified as stated above but in this case recovered in 80 μl of 10 mM Tris-Cl, pH 8.5. The purified fragment (8 μl) was digested with BamHI and HindIII at 37°C for 120 minutes, phenol/chloroform extracted, and the digested fragment (429 bp) purified using the Qiagen column (Qiagen) recovering it in 40 μl of 10 mM Tris-Cl, pH 8.5. The pET24a plasmid (2 μl) was digested in the same manner as the tolR fragment, then dephosphorylated with SAP as described above. Ligation reactions with the BamHI/HindIII digested tolR fragment and pET24a were performed as described, and transformed into the chemically competent DH5-α cells as above. Alkaline lysis (Sambrook et al., 1989) of twelve colonies was performed, and restriction with BamHI and HindIII revealed 12 out 12 colonies contained plasmids having the proper size fragment, with one chosen as pRA0006.

Construction of the plasmid containing the gene for the modified T7-TolR protein
occurred by cloning the fragment from a \textit{Bgl} II to \textit{Xho}I of pRA0006 into the \textit{Bgl}II/\textit{Xho}I sites of pET14b in the same manner as pRA0011. Digestion of pRA0006 with \textit{Bgl}II and \textit{Xho}I at 37°C for 100 minutes resulted in a 587 bp DNA fragment that was gel purified using a Qiagen Gel Extraction Kit (Qiagen). The digested, dephosphorylated pET14b plasmid prep made from the construction of pRA0011 was used for these ligation reactions with the \textit{Bgl}II/\textit{Xho}I digested, purified pRA0006 DNA fragment. The ligation reactions were incubated for 18 hours at room temperature after which 2 \( \mu l \) was used to transform into chemically competent DH5-\( \alpha \) cells as described, and plated on LB plates containing ampicillin at 100 \( \mu g \) ml\(^{-1}\). Alkaline lysis (Sambrook \textit{et al.}, 1989) of twelve colonies was performed, and used for digestion with \textit{Bgl}II and \textit{Xho}I. One plasmid having the proper size fragment was chosen as pRA0012.

After verifying the plasmids pRA0005, pRA0006, pRA0011, and pRA0012 contained the proper size fragments, the plasmids were purified and transformed into BL21-\( \lambda \)DE3 using TSS transformation (Chung \textit{et al.}, 1989) so that the genes of interest on the plasmids could be expressed and the protein extracts used with the capture assay. Plasmids pRA0005 and pRA0006 were maintained on LB with kanamycin at 30 \( \mu g \) ml\(^{-1}\), while plasmids pRA0011 and pRA0012 were maintained on LB with ampicillin at 100 \( \mu g \) ml\(^{-1}\).

\textit{Construction of plasmids for BacterioMatch 2-Hybrid System}

Primers were designed to incorporate restriction enzyme sites (Table 6) that would allow the \textit{tolQ} and \textit{tolR} genes to be cloned into the multiple cloning site (MCS) of either pBT (Figure 10) or pTRG (Figure 11) for use in the BacterioMatch 2-hybrid system. Construction of pBT-TolQ, pTRG-TolQ, and pBT-TolR was performed by R.A. Larsen. The \textit{tolQ} gene was PCR amplified from genomic DNA of the \textit{E. coli} K12 strain MG1655 with primers oRA0119R and
oRA0131R for cloning into pBT plasmid. Additional PCR amplification using the genomic DNA of MG1655 was performed with primers oRA0123R and oRA01131R for cloning the tolQ gene into pTRG plasmid. The tolR gene was PCR amplified from genomic DNA of MG1655 with primer oRA0121R and oRA0122R for cloning into pBT plasmid. PCR amplified products were digested with EcoRI for the tolQ amplimers, and with both EcoRI and BamHI for the tolR amplimer. Both the pBT and pTRG vector plasmids were digested with EcoRI, pBT was also digested with both EcoRI and BamHI. Digestions were performed at 37°C for 90 minutes and purified through a Qiagen Purification kit (Qiagen), recovering in 9 μl of 10 mM Tris-HCl, pH 8.5. Digested, purified vectors were dephosphorylated with SAP as previously described and used in ligation reactions with the digested, purified amplimers performed at 14°C overnight. Ligation reactions were precipitated with sodium acetate and ethanol, washed, dried, and suspended in 20 μl of ddH$_2$O. Aliquots of 2 μl were transformed into 50 μl of XL1-PC cells using electroporation, recovered in 900 μl of SOC medium for 1 hour at 30°C, and 100 μl plated on LB containing chloramphenicol at 34 μg ml$^{-1}$ for pBT ligations and on LB containing tetracycline 20 μg ml$^{-1}$ for pTRG and grown overnight at 30°C. Resultant colonies were screened by restricting plasmids recovered by alkaline lysis (Sambrook et al., 1989) with EcoRI to verify insertion of tolQ gene. A second digestion was performed on those containing an insert using the restriction endonuclease RsaI to determine the orientation of the inserts. Colonies from the pBT-TolR transformation were screened by digesting plasmids from alkaline lysis (Sambrook et al., 1989) with EcoRI and BamHI to verify insertion of tolR gene.

*Expression of the genes from the plasmids used in the protein capture assays*

The pRA0005 and pRA0006 plasmids were also transformed (Chung et al., 1989) into
BL21-λDE3 that carried an additional pLysS plasmid which encoded a T7 lysozyme gene. Overnight cultures of pRA0005 and pRA0006 in BL21-λDE3 and BL21-λDE3 with pLysS strains were diluted 1:200 into 40 ml of fresh LB containing kanamycin at 30 μg ml⁻¹ and then grown to an $A_{550} = 0.4$. Cells were then either induced with 1 mM IPTG or not (uninduced) and allowed to grow for an additional 90 minutes. At 90 minutes post-induction, 750 μl samples were removed and precipitated by adding 750 μl of 20% w/v cold (4°C) trichloroacetic acid (TCA) and incubating at 4°C for 15 minutes, then centrifuged for 5 minutes at ~13,000 x g. Pellets were washed in 1 ml of 100 mM Tris-HCl, pH 8.0, centrifuged for 5 minutes at ~13,000 x g, and the supernatant removed. Pellets were resuspended in 25 μl 1 M Tris-HCl, pH 8.0 and 25 μl of 2X Laemmli sample buffer (LSB) (Laemmli, 1970), then incubated at 97°C for 5 minutes. Samples were resolved on 12.5% sodium dodecyl sulfate-polyacrylamide (SDS-PA) gels, transferred to polyvinylidiene fluoride (PDVF) membranes and immunodecorated with α-T7 antiserum performed as described below.

Overnight cultures of pRA0011 and pRA0012 were diluted into 5 ml fresh LB containing ampicillin at 100 μg ml⁻¹ and allowed to grow at 37°C until the cultures reached an $A_{550} = 0.4$. The cultures were then either induced with 1 mM IPTG or not (uninduced) and allowed to grow for an additional 2 hours at 37°C. At 90 minutes post-induction, 750 μl samples were removed and TCA precipitated as described above, 10 μl samples were resolved on a 12.5% SDS-PA gel, transferred to PDVF membranes and immunoblots with α-T7 performed.

Protein extracts for use with the protein capture assays were made by growing BL21-λDE3 with the plasmids in 40 ml of fresh LB medium with the appropriate antibiotics. Cultures were allowed to grow at 37°C to $A_{550} = 0.2$, then induced with 1 mM IPTG and incubated at
37°C for 2 hours. The entire culture was then centrifuged at ~17,400 x g for 10 minutes at 4°C and the supernatant removed. Protein extracts were obtained from the pellets using the BugBuster Protein Extraction System (Novagene). The last step in the extraction was centrifuging the culture at ~31,000 x g for 20 minutes. The pellets were placed at 20°C and the supernatant was removed, stored until used in the protein extracts in the capture assays.

Protein capture assay using a His-binding matrix in an Eppendorf tube

The pilot capture assay was performed using a His-binding resin matrix in an Eppendorf tube. The His-binding resin matrix was generated in a sterile Eppendorf tube by taking 200 μl of the bead slurry solution His-bind Resin (from Novagen) and centrifuging at ~70 x g for 30 seconds, then removing the supernatant. To the His-bind resin (now ~100 μl), 1 ml of wash buffer (500mM NaCl, 20 mM Tris-HCl pH 8.0, 2 mM imidazole) was added, centrifuged at ~70 x g for 30 seconds, and the supernatant removed. To charge the resin, 200 μl of 50 mM NiSO₄ was added, centrifuged at ~70 x g for 30 seconds and the supernatant removed, processed repeated to ensure the resin was charged to 10 μmol Ni. To the charged resin, 200 μl of bait protein extract was added and mixed using a rotational mixer at room temperature for 20 minutes. Samples were then centrifuged and supernatant saved. Samples were washed three times with 300 μl of wash buffer, and the supernatants saved and pooled with the supernatant following addition of the bait extract. To the resin matrix, 50 μl of target protein extract was added and mixed using a rotational mixer at room temperature for 20 minutes. Sample were centrifuged and supernatant saved. Pellets were washed three times with 300 ml of wash buffer, and the supernatants saved and pooled with the supernatant following addition of the target extract. Proteins were eluted from the charged resin matrix by adding 500 μl of Elution Buffer (500mM NaCl, 20 mM Tris-HCl pH 8.0, 200 mM imidazole) centrifuging, and removing the
supernatant (considered the elution fraction) twice and these elution fractions were pooled. All samples were precipitated with TCA and 20 µl samples resolved on 12.5% SDS-PA gels, transferred to PDVF membranes and immunoblotted and probed with α-T7 antibodies as described below.

Additional modifications were made to the protein capture assay in an Eppendorf tube as described above. Various changes to the wash buffer and the elution buffer were made as noted in the figure legends. In some instances, the bait and target protein extracts were pre-cleared prior to being added to the matrix. Pre-clearing the protein extract included taking 500 µl of the protein extract obtained from the BugBuster Protein Extraction (Novagene) and centrifuging it for 5 minutes at ~13,000 x g. The supernatant was removed, saved, and added to the matrix according to the protocol. In some instances, the bait and target protein extracts were pre-incubated prior to adding them to the matrix. This involved taking 100 µl of the pre-cleared bait protein extract and 100 µl of the pre-cleared target protein extract and incubating them together at 37°C for 1 hour, and then adding the entire mixture to the matrix.

Protein capture assay using a His-binding matrix in a column

To allow proteins a better chance of interacting, the column protein capture assay (Braun et al., 1996) was modified slightly and utilized in this research. The column used was a Pasteur pipette with a small amount of glass wool in the bottom. To produce a His-binding bead bed, 0.5 ml of His-binding bead slurry was placed in a microcentrifuge tube, and spun at ~1700 x g for 1 minute and the supernatant removed. Beads were washed with 1 ml of ddH₂O, centrifuged at ~1700 x g for 1 minute and the supernatant removed. To charge the beads, 1 ml of 50 mM NiSO₄ solution was added, centrifuged at ~1700 x g for 1 minute and the supernatant removed. Beads were washed twice with 1 ml of ddH₂O, centrifuged at ~1700 x g for 1 minute and the
supernatant removed. Beads were then washed with 0.750 ml of adsorption buffer (0.5 mM NaCl, 20 mM Tris-HCl pH 8.0, 5 mM imidazole) and entire mix poured onto the column. The column was washed with ~5 ml of adsorption buffer until the beads had settled into a bed in the column. Once the column had settled, 0.5 ml of T7-TolQ-His6 protein extract (from cells containing pRA0005) was applied to column, washed with 5 ml of adsorption buffer and 1 ml fractions collected as they came off the column. Next, 0.5 ml of T7-TolQ protein extract (from cells containing pRA0011) was applied to column, then the column washed and 1 ml fractions collected as stated above. The column was washed with 5 ml wash buffer (0.5 mM NaCl, 20 mM Tris-HCl pH 8.0, 40 mM imidazole) and 1 ml fractions collected. Proteins were eluted from the column by washing with 5 ml of elution buffer (0.5 mM NaCl, 20 mM Tris-HCl pH 8.0, 1 M imidazole) with 1 ml fractions collected. Fraction samples were processed by mixing samples with 0.5 ml volumes of 2X LSB and incubating at 97°C for 5 minutes. Samples of 150 μl were resolved on 12.5% SDS-PA gels, then transferred to PDVF membranes, immunoblotted and probed with α-T7 antibody as described below. Additional column assays were run in the same manner, using different bait and target extracts.

**Immunoblot analysis**

The proteins used for this capture assay were engineered to contain an 11-amino acid amino-terminal epitope tag derived from the T7 capsid protein (T7 tag) that can be detected using specific antibodies (T7 antibody, Novagen). Samples were processed as above for the various protein capture assays, and resolved by loading on either 11% or 12.5% SDS polyacrylamide gels. The proteins were electro-transferred onto polyvinylindene fluoride (PVDF) membranes overnight at 4°C at 100 mA in transfer buffer (25 mM Tris, 192 mM glycine, 15% v/v methanol, 0.1% SDS). The membranes were placed in blocking buffer (5 %
evaporated milk, 0.05% Tween 20, Phosphate Buffered Saline [PBS = 10 mM sodium phosphate, 145 mM NaCl; pH ~7.4]) for 30 minutes. The α-T7 monoclonal antibody was used at a concentration of 1:10,000 in blocking buffer and incubated with the membrane for 1 hour at room temperature. The membranes were washed three times with ~50 ml of blocking buffer for ~7 minutes each. The secondary antibody was a goat α-mouse IgG + IgM alkaline phosphatase conjugate used at a concentration of 1:12,500 in blocking buffer, and incubated with the membrane at room temperature for 1 hour. The membranes were rinsed with ~25 ml blocking buffer, and then washed four times with final rinse buffer (0.05% v/v Tween 20, PBS), using ~50 ml for 5 minutes for each wash. Following the final rinse, membranes were incubated with enhanced chemifluorescence (ECF) substrate at 24 μl per cm\(^2\) at room temperature for 5 minutes. The membrane was then placed protein-side down on the screen of the STORM 860 system. The image was obtained using the fluorescence acquisition mode, with settings of Blue (450), Normal Sensitivity 600, and pixel size of 100 microns. Following immunoblot analysis, the membrane was stained with 0.1% w/v Coomassie blue in 50% methanol, 7% glacial acetic acid for 5 – 10 minutes, and then destained with 50% methanol, 7% glacial acetic acid with a knotted Kimwipe to adsorb dye for 30 – 60 minutes, then allowed to air dry.

**BacterioMatch Two-Hybrid**

Once the genes for tolQ and tolR were cloned into the pBT and pTRG plasmids, the plasmids were purified using alkaline lysis (Sambrook et al., 1989). Using these constructed plasmids in conjunction with the positive control plasmids (pBT-LGF2 and pTRG-Gal11P) and the empty pBT and pTRG vectors, various pBT and pTRG plasmid pairings were made to identify protein/protein interactions between the Tol fusion proteins (pBT-TolQ, pBT-TolR, and pTRG-TolQ). Recombinant transformants in pBT were originally maintained on LB containing
chloramphenicol at 34 μg ml⁻¹, while recombinant transformants in pTRG were originally maintained on LB containing tetracycline 20 μg ml⁻¹ and grown at 30°C to minimize potential toxicity. Eventually, antibiotic concentrations were changed and recombinant transformants in pBT were then maintained on LB containing chloramphenicol at 25 μg ml⁻¹ while recombinant transformants in pTRG were maintained on LB containing tetracycline 12.5 μg ml⁻¹. The change in concentrations is noted in the figure legends.

The reporter genes for the XL1-VCR test strain are \textit{HIS3} (a component of the histidine biosynthetic pathway) and \textit{aadA} (confers resistance to streptomycin). In the presence of bait and target proteins that interact, RNA polymerase is recruited, and the reporters transcribed. Therefore, testing for the ability of XL1-VCR cells containing bait and target proteins to grow on selective screening medium (5 mM 3-AT, with tetracycline and chloramphenicol for plasmid selection) and dual selective screening medium (5 mM 3-AT, 12.5 μg ml⁻¹ streptomycin, with tetracycline and chloramphenicol for plasmid selection) allowed for the determination interaction of the bait and target proteins.

As a pilot 2-hybrid growth assay, various pBT and pTRG plasmid pairings were co-transformed into XL1-VCR using TSS transformation (Chung \textit{et al}., 1989) and plated onto nonselective medium containing both tetracycline at 20 μg ml⁻¹ and chloramphenicol at 34 μg ml⁻¹ and allowed to grow at 37°C overnight. Individual isolates growing on the nonselective medium were replated on nonselective medium, selective screening medium, and dual selective screening medium and allowed to grow at 37°C overnight. Growth was recorded and the plates scanned (Figure 20).

Other 2-hybrid growth assays using the lower antibiotic concentrations (tetracycline at
12.5 μg ml\(^{-1}\) and chloramphenicol at 25 μg ml\(^{-1}\)) were performed as described above. Various pBT- and pTRG- plasmids co-transformed into the XL1-VCR strain by TSS transformation (Chung et al., 1989) were plated onto nonselective medium containing both tetracycline at 12.5 μg ml\(^{-1}\) and chloramphenicol at 25 μg ml\(^{-1}\) and allowed to grow at 37°C overnight. Multiple isolates were plated on nonselective medium, selective screening medium, and dual selective screening medium and allowed to grow at 37°C overnight. Growth on the plates was scored as the number of isolates out of eight that were able to grow on the media. Determination of protein/protein interactions was made by comparing the growth of the TolQ/TolQ and TolQ/TolR pairings to the positive control containing pBT-LGF2 and pTRG-Gal11P, and the various negative controls.

**β-galactosidase assays used in BacterioMatch 2-Hybrid system**

XL1-AX strains encoding various combinations of recombinant pBT fusion proteins and recombinant pTRG fusion proteins were analyzed using the colorimetric β-galactosidase assay (Miller, 1972). Overnight cultures of XL1-AX containing either pBT-LGF2 and pTRG-Gal11P or pBT and pTRG-Gal11P were diluted 1:200 into fresh Nonselective medium without IPTG and grown at 30°C to an A\(_{550}\) = 0.3. Cultures were then induced with either 0 or 50 μM of IPTG and incubated at 30°C for 1 hour, and the samples placed on ice for 20 minutes. The OD\(_{550}\) reading was taken to determine the cell density just before assay using a Beckman Coulter DU 520 general purpose UV/Vis Spectrometer. For the assay, 0.1 ml of culture added to 0.9 ml Z buffer (Miller, 1972) and 1 drop of toluene added and tubes vortexed for 10 seconds. Samples were placed in 37°C water bath for 40 minutes, then transferred to a 28°C water bath for 5 minutes. Reactions were started by adding 0.2 ml of o-nitrophenyl-β-D-galactoside (ONPG). Pilot studies to determine optimal incubation times included various lengths of incubation (20, 60, 120, 1440
minutes), with reactions stopped by adding 0.5 ml of 1 M Na$_2$CO$_3$ solution and optical density recorded at both 420 and 550 Å. Subsequent assays were incubated for 20 minutes, using 0, 10, 50, or 100 μM IPTG. Assays were performed in triplicate and the mean of three experiments graphed, with error bars representing one standard deviation. Units of β-galactosidase were determined by using the following equation (modified slightly from Miller, 1972):

\[
\text{Miller Units} = 1000 \times \frac{\text{OD}_{420} - (1.75 \times \text{OD}_{550} \text{[from reaction mixture]})}{t \times v \times \text{OD}_{550} \text{ (cell density before starting assay)}}
\]

\(t = \) time of assay reaction in minutes
\(v = \) volume of culture used in assay, in ml.
RESULTS

Testing expression of plasmid constructs to be used in capture assays

The pET system (Figure 12) works based on the regulation of the T7 RNA polymerase by lacUV5 in the λDE3 lysogens. Genes on pET plasmids are readily transcribed by inducing with IPTG, allowing for transcription of the T7 RNA polymerase gene, and expression of the test gene, as the T7 promoter is repressed by lacI. Tighter regulation can be obtained by inclusion of a pLysS plasmid (encoding a T7 lysozyme) that inhibits T7 RNA polymerase (Inouye et al., 1973, Huang et al., 1999, Zhang & Studier, 1997), reducing expression of the gene of interest to levels less likely to disrupt cell function (Novagen).

The genes for the TolQ and TolR proteins were first cloned into the pET24a plasmid to form two new plasmids named pRA0005 and pRA006. After determining the plasmids contained the proper size fragment (data not shown), the plasmids (pRA0005 contains the gene for T7-TolQ-His6 and pRA0006 contains the gene for T7-TolR-His6) were transformed into BL21-λDE3 either with or without pLysS and the cells either induced or not induced with 1 mM IPTG (Figure 15). In both cells with (Figure 15 - A) or without (Figure 15 - B) pLysS, plasmids were inducible by the addition of IPTG (lanes 2 and 4) compared to those that were not (lanes 1 and 3). The T7-TolQ-His6 protein was detectable at ~29.7 kDa, while the T7-TolR-His6 as detectable at ~27 kDa. Specific proteins were not detected from the pET24a parent vector either with or without IPTG (lanes 5 and 6), as expected. In the absence of pLysS (Figure 15/B), there did seem to be a slight increase in the amount of T7-TolR-His6 protein when induced with IPTG (Figure 15/B – lane 4) relative to the comparable pLysS-bearing strain (Figure 15/A – lane 4), while no real difference was seen in the amount of T7-TolQ-His6 protein produced following induction (Figure 15/ lane 4 in A and B). These results demonstrate that the pRA0005 and
Figure 15: Testing the ability of the pRA0005 and pRA0006 plasmids to induce the proper size proteins compared to vector plasmid pET24a. BL21-λDE3 bearing either the pRA0005 or pRA0006 plasmid were grown to OD = 0.4 and then either induced with 1 mM IPTG (Lanes 2, 4, and 6) or not (lanes 1, 3, 5). The experiment was done either (A) in the presence, or (B) in the absence of pLysS. At 90 minutes post-induction, 750 μl samples were removed and TCA precipitated, resolved on a 12.5% SDS-PA gel, transferred to PDVF membrane and probed with α-T7 antibody. Lanes 1 & 2 – pRA0005 (T7-TolQ-His6); lanes 3 & 4 – pRA0006 (T7-TolR-His6); and lanes 5 & 6 – pET24a. Size standards in kilodaltons are indicated at the left of the figure.
pRA0006 are inducible. Further, the presence of pLysS did not appear to make a significant difference on expression.

Following verification of pRA0005 and pRA0006, the genes were cloned into pET14b to produce recombinant TolQ and TolR proteins that only contained the T7-tag. It is important to note that while this did remove the 6 residue His-tag from the protein, the overall length of the protein increased by 14 residues reflecting the distance to the closest in-frame stop codon (Figures 10 and 11, respectively). The new plasmids (pRA0011 and pRA0012) were transformed into BL21-λDE3 to test for proper expression of the genes and production of the T7-TolQ and T7-TolR proteins (Figure 16). The plasmids pRA0011 (containing T7-TolQ) and pRA0012 (containing T7-TolR) both showed an increase in the amount of protein produced following induction with 0.5 mM IPTG (Figure 16 – lanes 3 and 5, respectively). As expected, these proteins are larger than those in Figure 13, the T7-TolQ protein detectable at ~32 kDa and the T7-TolR protein detectable at ~ 29 kDa, due to the additional 14 residues relative to proteins with the His-tag. These plasmids were transformed into a strain that lacking pLysS, with a small of amount of protein evident from each plasmid even when not induced by IPTG (Figure 16 – lanes 2 and 4). As expected, no protein was detected from the pET14b vector whether induced or not (Figure 16 - lanes 6 and 7). To verify that what was being detected were the T7-tagged proteins, commercial T7 capsid protein standards were included (lane 1).

A detectable size difference between the T7-TolQ-His6 and the T7-TolQ proteins

While there was a noticeable size difference between the T7-TolQ-His6 protein in Figure 15 and the T7-TolQ protein in Figure 16, it was important to verify that the size difference between would be distinguishable when run together on the same gel. Various amounts (10, 5, and 2 μl) of protein extracts prepared for Figure 15 and Figure 16 were resolved on a 12.5%
Figure 16: Testing the ability of the pRA0011 and pRA0012 plasmids to induce the proper size proteins compared to vector plasmid pET14b. BL21-λDE3 bearing either the pRA0011 or pRA0012 plasmid were grown to OD = 0.4 and then either induced with 0.5 mM IPTG (lanes 3, 5, 7, 9, 11) or not (lanes 2, 4, 6, 8, 10). At 90 minutes post-induction, 750 μl samples were removed and TCA precipitated 10 μl samples were run on a 12.5% SDS-PAGE gel, transferred to PDVF membrane and probed with α-T7 antibody. Lane 1 – 2 μl of T7-tagged control protein (to ensure detection by antibody); lanes 2 & 3 – pRA0011 (T7-TolQ); lanes 4 & 5 – pRA0012 (T7-TolR); and lanes 6 & 7 – pET14b. Size standards in kilodaltons are indicated at the left of the figure.
SDS-PAGE gel and transferred to a PDVF membrane for immunoblot analysis (Figure 17). As seen in lanes 1-4, the two proteins could be resolved with sample loads of 10 and 5 μl (Figure 17).

*Protein capture assay performed using a His-binding resin matrix in an Eppendorf tube*

Protein extracts were obtained from BL21-λDE3 cells bearing the indicated plasmid using the BugBuster Protein Extraction System from Novagen, including from the control vectors pET24a and pET14b. Preliminary protein capture assays were performed in an Eppendorf tube using a His-binding resin matrix. Various protein extract combinations were examined in order to determine the best parameters to use for the capture assay. Experiments with the protein extract from BL21-λDE3 cells bearing pRA0006 (T7-TolR-His6) was used as bait with the protein extract from cells bearing pET14b as target, a band corresponding to T7-TolR-His6 was visible in the elution fraction (data not shown). Thus, the T7-TolQ-His6 protein was binding and remaining bound to the matrix through the various washes, and was able to be eluted as expected. However, when the control protein extract from BL21-λDE3 cells bearing pET24a was used as bait and the protein extract from cells bearing pRA0011 (T7-TolQ) as target, there was a noticeable amount of T7-TolQ found in the elution fraction (data not shown). Apparently, the T7-TolQ protein (which contains no His-tag) has an affinity for non-specific binding to the His-binding matrix.

To optimize binding and protein/protein interactions, a capture assay was performed (Figure 18) in which supernatants from centrifugation following loading of bait (and ultimately target) were kept separate from washes and changes were made to the wash (20 mM Tris-HCl pH 8.0, 10 mM imidazole) and elution (500 mM NaCl, 20 mM Tris-HCl pH 8.0, 400 mM imidazole) buffers. Additionally, prior to adding the bait and target protein extracts to the His-
Figure 17: Determining if there is a detectable size difference between the T7-TolQ-His6 protein and the T7-TolQ protein. The TCA precipitated samples from BL21-λDE3 bearing either the pRA0005 (lanes 1, 3, and 5) or pRA0011 (lanes 2, 4, and 6) plasmid prepped for figure 15 and Figure 16 were resolved on a large-format (14 cm long) 12.5% SDS-PA gel, transferred to PDVF membrane, and probed with α-T7 antibody. Various amounts TCA prepared samples (10, 5, and 2 μl - displayed at the top) of T7-TolQ-His6 (from pRA0005) and T7-TolQ (from pRA0011) were loaded in order to determine the sensitivity of detection of the antibody for low amounts of protein. Size standards in kilodaltons are indicated at the left of the figure.
Figure 18: Capture assay using a resin matrix in an Eppendorf tube. (A) Bait protein extract from pE24a and target protein extract from pRA0011 (T7-TolQ); (B) Bait protein extract from pRA0006 (T7-TolR-His6) and target protein extract from pET14b; and (C) Bait protein extract from pRA0006 (T7-TolR-His6) and target protein extract from pRA0011 (T7-TolQ). Capture assay was performed as stated in Material and Methods. Briefly, the bait and target protein extracts were pre-cleared by centrifugation for 5 minutes at 14,000 rpm prior to adding to the resin mixture and the supernatant removed and used as the bait and target protein in the assay. 200 μl of bait protein extract was added to 100 μl of His-binding resin charged to 10 μmol Ni. Following addition of the bait and target protein, sample centrifuged and the supernatant saved separate from the washes. Samples were washed 3X with wash buffer (20 mM Tris-HCl pH 8.0, 10 mM imidazole), after adding the bait and target, washes collected and pooled. Proteins were eluted off the resin matrix by 2X adding 500 μl of Elution Buffer (500 mM NaCl, 20 mM Tris-HCl pH 8.0, 400 mM imidazole), elution fractions were collected and pooled. 2X LSB was added to the samples and they were incubated at 97°C for 5 minutes. 25 μl samples were resolved on a 12.5% SDS-PAGE gel, transferred to PDVF membrane and an immunoblot with α-T7 performed. In addition to the capture assay samples, 2 μl of T7-tagged control protein (lane 2) was also included to verify detection by antibody. Lanes: 3 – pre-cleared bait protein, 4 – centrifugation sample following addition of bait protein, 5 – pre-cleared target protein, 6 – supernatant from centrifugation following addition of target protein, and 7 – pooled elutions. Size standards in kilodaltons are indicated at the left of the figure.
binding matrix, extracts were pre-cleared by centrifugation. When extracts from BL21-λDE3 cells bearing pET24a were mixed with extracts from cells bearing pRA0011 (T7-TolQ) as a target (Figure 18/A), T7-TolQ was seen only in the sample load (lane 5) and the supernatant following addition of the extract (lane 6). Unlike the preliminary experiments, in this capture assay T7-TolQ was not visible in the elution fractions (lane 7). As expected, no protein was seen in the sample load or centrifugation sample for the extract from the bait pET24a (lanes 3 and 4). When the protein extract from BL21-λDE3 cells bearing pRA0006 (T7-TolR-His6) was used as bait with extract from cells bearing pET14b as a target (Figure 18/B), the T7-TolR-His6 protein was detectable in the sample load (lane 3), the centrifugation sample (lane 4) and in small amounts in the elution fraction (lane 7). Lanes 5 and 6 showed no detectable protein, as expected from the extract from the cells bearing pET14b. In the capture assay designed to examine interaction between TolQ and TolR (Figure 18/C), the extract from BL21-λDE3 cells bearing pRA0006 (T7-TolR-His6) was used as bait to capture T7-TolQ from the extract of cells bearing pRA0011. The T7-TolR-His6 protein was detected in the sample load (lane 3), the centrifugation sample (lane 4), and in the elution fraction (lane 7); however, the amount of T7-TolR-His6 seen in this capture assay appeared to be lower than the amount seen in the preliminary capture assay. The T7-TolQ was detected in both the sample load (lane 5) and the centrifugation sample (lane 6); however, in this capture assay the protein was not detectable in the elution fraction (lane 7).

Modifications were again made to the wash buffer (20 mM Tris-HCl pH 8.0, 5 mM imidazole) to obtain refine the capture assay using the His-binding method in an Eppendorf tube. Four protein capture assays were performed using various combinations of protein extracts as bait and target proteins and the pooled elution fractions analyzed by immunoblot (Figure 19). In
this capture assay, equal amounts of the bait and target protein extracts were pre-incubated together for one hour at 37°C and this entire mixture was then added to the His-binding matrix. Samples of the protein extractions used in the capture assays were also ran for comparison (Figure 19 – lanes 2, 3, and 8.), including the extracts from the empty plasmid vectors (lanes 1 and 9).

Evaluation of the elution fractions from the various combinations of Tol proteins showed that a band corresponding to the T7-TolQ protein was seen in the presence of T7-TolQ-His6 (Figure 19 – lane 6) as well as in the presence T7-TolR-His6 (Figure 19 – lane 7) and when it was combined with the extract from pET24a vector plasmid (Figure 19 – lane 5). Due to the fact that the T7-TolQ protein was recovered in elutions when paired with the extract from pET24a vector plasmid (Figure 19 – lane 5), shows that the T7-TolQ protein has an affinity for non-specific binding to the matrix, as seen in the preliminary capture assays and; therefore, it can not be clearly determined if are detectable interactions between TolQ/TolQ and TolQ/TolR from these results.

Protein capture assay using performed using a His-binding column

Even with several modifications, the protein capture using a His-binding matrix in an Eppendorf tube was unable to give clear results regarding TolQ/TolQ and TolQ/TolR protein interactions. T address some of these problems, a protein capture assay using a Ni-charged column was developed as described in the material and methods. Fractions were collected as they came off the column and analyzed by immunoblot. Since the results from Figure 18 did not exclude a possible TolQ/TolQ protein interaction, this was further tested using an extract from BL21-λDE3 cells bearing pRA0005 (T7-TolQ-His6) as bait in an attempt to capture the T7-TolQ
**Figure 19: The elutions from a capture assay using a resin matrix in an Eppendorf tube.**

Protein extractions were pre-cleared as in the previous capture assay, then a mixture of 100 μl each of the pre-cleared bait and the pre-cleared target protein extractions was incubated together at 37°C for 1 hour. This mixture was then added to the His-binding resin and mixed via rotation at room temperature for 20 minutes. Following incubation, the mixtures were centrifuged, washed (3X) with wash buffer (20 mM Tris-HCl pH 8.0, 5 mM imidazole), and the proteins eluted from the matrix by washing twice with 200 μl of elution buffer (500 mM NaCl, 20 mM Tris-HCl pH 8.0, 400 mM imidazole) for each elution. Elution fractions were pooled and 200 μl of 2X LSB added, samples incubated at 97°C for 5 minutes and 150 μl of those samples loaded and resolved on 12.5% SDS-PAGE gel, transferred to PDVF membrane, and an immunoblot using α-T7 performed. For comparison, just the pre-cleared protein extracts were run (lanes 1, 2, 3, 8 & 9) to allow distinction between the various TolQ and TolR proteins in the capture assay elution fractions (lanes 4, 5, 6, & 7). The contents of the lanes are identified in the table below the image of the immunoblot. Size standards in kilodaltons are indicated at the left of the figure.

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protein from extracts of cells bearing pRA0011. In preliminary experiments (data not shown), there were detectable T7-TolQ and T7-TolQ-His6 proteins in the elution fractions. To verify the TolQ/TolQ interaction result, a control capture assay was performed using the extract from BL21-λDE3 cells bearing pET24a as the bait and the extract from cells bearing pRA0011 (T7-TolQ) as the target to evaluate the presence of T7-TolQ in the elution fraction. The fractions of all washes and elutions were collected and evaluated by immunoblot (Figure 20). The T7-TolQ was found in all of the adsorption washes (lanes 6-10), as well as all the wash fractions (lanes 11-14). A band corresponding to T7-TolQ was seen in the first elution fraction (lane 15), indicating this protein was able to remain in the Ni-charged column. Therefore, the TolQ/TolQ protein interaction seen in the preliminary column protein capture assay (data not shown) did not provide clear evidence, again reflecting the ability of the T7-TolQ protein to adhere to the matrix.

BacterioMatch 2-Hybrid does not indicate Tol protein interactions

Recombinant bait and target fusion proteins for the BacterioMatch 2-Hybrid system were made by cloning the \( tolQ \) and \( tolR \) genes into the multiple cloning site regions of the pBT and pTRG vector plasmids (Figures 10 and 11, respectively). The “bait” proteins that were produced from the pBT plasmids are fusions of the experimental protein and the \( \lambda cl \) repressor (Figures 9 and 10); while the “target” proteins produced from the pTRG plasmid were fusions of the experimental protein and the \( \alpha \)-subunit of RNA polymerase (Figures 9 and 11).

In the pilot 2-Hybrid growth assay, various pBT and pTRG plasmids were co-transformed into the XL1-VCR test strain and plated onto nonselective medium. Individual isolates from that transformation were streaked onto nonselective medium, selective screening
**Figure 20: Protein capture assay using a column with extracts from pET24a and T7-TolQ protein.** To determine if the T7-TolQ protein was remaining in the His-binding resin even with the absence of a His-tag, a capture assay was performed as stated in materials in methods. Briefly, the column used was a Pasteur Pipette with a small amount of glass wool in the bottom. A 0.5 ml His-binding bead bed was prepared, poured into column, washed with adsorption buffer and allowed to settle. Once the column had settled, 0.5 ml of protein extract (from pET24a) was applied to column as “bait”, washed with adsorption buffer and 1 ml fractions collected as they came off the column (lanes 3-6). Next, 0.5 ml of T7-TolQ protein extract (from pRA0011) was applied to column as “target” and washed with adsorption buffer and 1 ml fractions collected (lanes 7-10). Column was then washed with 5 ml of wash buffer and 1 ml fractions collected (lanes 13-16). Elution of proteins from the column occurred by adding 5 ml of elution buffer and collecting 1 ml fractions as they came off the column (lanes 17-20). Fraction samples were prepared by taking 100 μl samples from the fractions collected and adding 50 μl of 2X LSB. To 200 μl of the protein extracts, 100 μl of 2X LSB was added. All samples were then placed at 97°C for 5 minutes. 150 μl samples were resolved on a 12.5% SDS-PA gel, then transferred to PVDF membrane and an immunoblot with α-T7 performed. Lanes: 1 and 10 are 75 μl each of T7-TolQ-His6 and T7-TolQ protein extract, lanes 2-5 are fractions from adsorption wash following addition of “bait” protein extract, lanes 6-9 are fractions from adsorption wash following addition of T7-TolQ protein extract, 11-14 are fractions from wash, and lanes 15-18 are the elution fractions. Size standards in kilodaltons are indicated at the left of the figure.
medium, and dual selective screening medium and the growth on the plates observed (Figure 21). Plates were scanned and the results showed that only XL1-VCR cells containing the positive control bait and target proteins (pBT-LGF2 + pTRG-Gal11P) grew on both types of media, indicative of a strong protein/protein interaction. XL1-VCR cells containing an empty pBT vector paired with pTRG, pTRG-Gal11P, or pTRG-TolQ did not show growth on either screening medium, as expected for these control pairings. The same was true when pTRG was paired with pBT-LGF2 or pBT-TolR as controls. When the TolR-fusion was expressed as a bait protein and the TolQ-fusion was expressed as a target protein in XL1-VCR, no growth occurred on either the selective or the dual selective screening media (Figure 21). This would indicate no protein/protein interaction between TolQ and TolR; however, being the pilot experiment many factors may have affected this result.

In experiments involving growth of cells in liquid medium, problems in growth occurred for cells carrying both plasmids (including the positive control plasmids, pBT-LGF2 + pTRG-Gal11P) with some cultures requiring nearly 48 hours to reach saturation (data not shown). Realizing that Stratagene recommended antibiotic concentrations lower than those used in the pilot study for maintaining the 2-hybrid plasmids, drug level were adjusted accordingly. Additional liquid growth experiments with tetracycline at 12.5 μg ml⁻¹ and chloramphenicol at 25 μg ml⁻¹ resulted in better growth of XL1-VCR cells bearing both pBT and pTRG plasmids (data not shown). An interesting note in those experiments is that the combination of pBT-TolR and pTRG-TolQ grew at a rate less than cells with combination of pBT + pTRG. As expected, XL1-VCR cells carrying the positive control plasmids, pBT-LGF2 + pTRG-Gal11P, had the greatest rate of growth (data not shown). Therefore, the results from the pilot 2-hybrid growth assay may have been affected by the fact that all media utilized for that experiment
Figure 21: Pilot bacteria two-hybrid experiment using the XL1-VCR reporter strain.
Various pBT and pTRG plasmid pairings were co-transformed into XL1-VCR onto Nonselective medium containing tetracycline at 20 μg ml\(^{-1}\) and chloramphenicol at 34 μg ml\(^{-1}\). Individual isolates from those growing on the Nonselective Medium plates were selected and plated on (A) Nonselective Medium, (B) Selective Screening Medium (5 mM 3-AT, tetracycline at 20 μg ml\(^{-1}\), and chloramphenicol at 34 μg/ml\(^{-1}\)), and (C) Dual Selective Screening Medium (5 mM 3-AT, 12.5 μg ml\(^{-1}\) streptomycin, tetracycline at 20 μg ml\(^{-1}\), and chloramphenicol at 34 μg/ml\(^{-1}\)). The plates were grown overnight at 37°C. Scanned images of the plates are shown above, with the plasmid pairings label above the image. Growth on the selective screening (B) or dual selective screening (C) medium indicating interaction of the bait and target proteins.
contained tetracycline at 20 μg ml⁻¹ and chloramphenicol at 34 μg ml⁻¹ for the selection of cells carrying the pBT and pTRG plasmids.

All media adjusted to lower antibiotic concentrations, and another 2-hybrid growth assay performed by plating eight individual isolates from the transformation onto the nonselective, selective screening, and dual selective screening media. This experiment showed a large amount of growth from the various control plasmid pairings on the selective and dual selective screening media (data not shown). For example, in XL1-VCR cells carrying pBT-LGF2 + pTRG on the dual selective medium all eight of the isolates plated were able to form substantial growth (data not shown). As a result of the large amount of growth on the selective screening and the dual selective screening media of control plasmid pairings, new dual selective media was made and the amount of streptomycin increased from 12.5 μg ml⁻¹ to 25 μg ml⁻¹ and another 2-hybrid growth assay performed.

The 2-hybrid growth assay was performed as stated above, with eight individual isolates from the co-transformation of the plasmid plated on nonselective, selective screening, and dual selective screening (streptomycin at 25 μg ml⁻¹) media and the growth recorded (Table 7). Three sets of pBT-TolQ + pTRG-TolQ were performed to fully evaluate the possibility of a TolQ/TolQ interaction. As expected, for XL1-VCR cells containing the positive control plasmids, pBT-LGF2 + pTRG-Gal11P, eight out the eight isolates plated were able to grow on each of the media (Table 7). All plasmid pairings were able to grow on the selective screening medium, with the pBT-TolQ + pTRG-TolQ combination being as effective as the control plasmids having growth from all eight isolates. On the selective screening media, there was growth of XL1-VCR cells carrying the negative control pairings, pBT + pTRG-Gal11P and pBT + pTRG, having growth from six and four out of eight isolates, respectively. Only the positive control plasmids, pBT-
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<td>pBT-TolQ + pTRG-TolQ</td>
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<td>8/8</td>
<td>0/8</td>
</tr>
</tbody>
</table>

Bacteria 2-Hybrid experiment using the XL1-VCR reporter strain. Various pBT and pTRG plasmid pairings were co-transformed into XL1-VCR onto Nonselective medium containing tetracycline at 12.5 μg ml⁻¹ and chloramphenicol at 25 μg ml⁻¹. Multiple individual isolates from those on the nonselective plates following transformation were selected and then plated on nonselective, selective screening medium (5 mM 3-AT, tetracycline at 12.5 μg ml⁻¹ and chloramphenicol at 25 μg ml⁻¹) and dual selective screening medium (5 mM 3-AT, 25 μg ml⁻¹ streptomycin, tetracycline at 12.5 μg ml⁻¹ and chloramphenicol at 25 μg ml⁻¹) and incubated overnight at 37°C, then another 16 hours at room temperature in the dark, and then growth evaluated. Numbers in the table represent the number of isolates out of the 8 plated that were able to grow on that particular medium. Plasmid pairings included 3 sets of pBT-TolQ + pTRG-TolQ in order to fully evaluate in triplicate protein-protein interactions amongst Tol proteins.

* Dual Selective Screening plates contained streptomycin at 25 μg ml⁻¹.
LGF2 + pTRG-Gal11P were able to grow on the dual selective medium. All other plasmids pairing had no isolates that were able to grow the dual selective medium. Thus, these results did not substantiate a TolQ/TolQ interaction.

To further test the ability of this 2-hybrid system to identify protein/protein interactions amongst the proteins in the TolA system, TolQ and TolR protein pairings were made in the XL1-AX test strain where increases in β-galactosidase levels from the negative controls would indicate protein/protein interactions. Experiments, either in the presence or absence of IPTG at 50 mM, were performed in XL1-AX cells carrying either the positive control plasmids, pBT-LGF2 + pTRG-Gal11P, or the negative control plasmids, pBT + pTRG-Gal11P, and the β-galactosidase assay allowed to run for various lengths of time (20, 60, 120, and 1440 minutes) before stopping the reactions. The results of this assay indicated a reaction time of 20 minutes was sufficient to obtain maximum levels of β-galactosidase (data not shown).

To determine if the amount of IPTG affected the level of β-galactosidase activity, a β-galactosidase assay was performed. XL1-AX cells containing various plasmid pairings were induced with 10 μM, 50 μM, and 100 μM IPTG for one hour prior to the assay, with the β-galactosidase assay reactions stopped after 20 minutes (Figure 22). The cells containing the positive control plasmids, pBT-LGF2 + pTRG-Gal11P, showed an increase in β-galactosidase levels from the negative control pBT + pTRG-Gal11P. Due to the high standard deviation of the positive control with 0 mM IPTG (Figure 22 – solid bar), the levels in these cells compared to the others was not determined to be significant. There was no evident difference in the β-galactosidase levels for cells with the negative control plasmid pairing between the various levels of IPTG induction (Figure 22 – open bars). For cells carrying the pBT-TolQ + pTRG-TolQ,
Figure 22: Beta-galactosidase assays of XL1-AX cells carrying various pBT and pTRG plasmid pairings were induced with various levels of IPTG. Plasmid combinations of solid bar (pBT-LGF2 + pTRG-Gal11P); open bar (pBT + pTRG-Gal11P); checkered bar (pBT-TolQ + pTRG-TolQ); and dashed bar (pBT-TolR + pTRG-TolQ) were co-transformed into XL1-AX to perform beta-galactosidase assays as part of the BacterioMatch 2-Hybrid system. Cells were grown overnight and cultures diluted 1:200 into fresh Nonselective medium (tetracycline at 12.5 μg ml\(^{-1}\) and chloramphenicol at 25 μg ml\(^{-1}\)) without IPTG and grown at 30°C to an A\(_{550}\) = 0.3, sets were then induced with various levels of IPTG (0, 10, 50, 100 μM). All reactions were stopped after 20 minutes, and OD readings taken. Miller units were determined using the formula by Miller (1972), and the average Miller units from a triplicate set of experiments were graphed with standard deviation values. Assays performed with the assistance of Lindsey Milliken.
there was no difference between the β-galactosidase levels following induction by 0 or 10 mM IPTG; however, there was increase in the β-galactosidase levels following induction with 50 and 100 mM IPTG (Figure 22 – checkered bars). These results show a dose-dependent increase in β-galactosidase levels with a TolQ and TolQ pairing; however, the overall levels produced by this pairing were not significantly different than the negative control (Figure 22 – checkered bars compared to open bars). The β-galactosidase levels for XL1-AX cells carrying the pBT-TolR and pTRG-TolQ were indistinguishable from that of the negative control for all levels of IPTG induction (Figure 22 – dashed bars compared to open bars). Thus, these results indicated no detectable interactions between TolQ and TolR.
DISCUSSION

In this research aim, protein capture assays and the bacteria two-hybrid system were used to investigate the interactions between the proteins of the TolA system. The research performed for this aim of my dissertation had two goals. The first goal was to determine interactions between proteins in the TolA systems, in particular interactions between the TolQ and TolR proteins. The second goal of this aim was once interactions between proteins in the TolA system and been identified to use these assays to determine interactions between the proteins of the TolA and TonB systems during crosstalk.

For the protein capture assays, the genes for \(\text{tolQ}\) and \(\text{tolR}\) were placed into the pET24a and pET14b plasmids of the pET system from Novagen. Since there are not currently antibodies to the TolQ and TolR proteins, the pET system allowed the genes to be constructed in a manner so the proteins would contain an 11-amino acid T7-tag for detection by Immunoblot analysis using a \(\alpha\)-T7 mAb. The genes were also constructed so that a subset of TolQ and TolR proteins contained a His6-tag to allow the proteins to bind to a nickel-charge bead matrix, as well as the T7-tag for detection. The genes were properly constructed because when BL21-\(\lambda\)DE3 cells containing the pRA0005, pRA0006, pRA0011, and pRA0012 plasmids were induced with IPTG, T7-tagged proteins of predicted size were detectable (Figure 15 and 2H). The manner in which the genes were constructed resulted in the T7-TolQ being a larger protein that the T7-TolQ-His6 protein (Figure 17), the same was true for the T7-TolR and T7-TolR-His6 proteins (data not shown).

The results for the protein capture assays using an Eppendorf tube indicated that neither the T7-TolR-His6 nor T7-TolQ-His6 proteins were able to capture T7-TolQ (Figure 18). In experiments in which the T7-TolQ protein was found in the elutions with T7-TolQ –His6 this
was not indicative of protein/protein interaction because the T7-TolQ protein was also found in the elution fractions of control experiments (Figures 19 & 20). Modifications that caused the lack of detectable T7-TolQ protein in the elutions for control experiments, eliminated any T7-TolQ and T7-TolQ-His6 interactions (Figure 18 –A). Together, these data indicate that the T7-TolQ protein has an adhesive property that allowed it to remain in matrix under various conditions and buffers. This property of the T7-TolQ became more apparent when this T7-TolQ protein was used in other experiments in the laboratory by other members attempting to make antibodies to the TolQ (personal communications with R. A. Larsen and K. K. Brinkman). The adhesive property of the T7-TolQ protein most likely reflects the low solubility of the protein in the buffers used for purification for chromatography.

Braun was able to use the protein capture assay using a nickel-affinity column to identify interactions of ExbB with both ExbD and TonB (Braun et al., 1996). In contrast, the similar protein capture methods used in this research were unable to clearly identify interactions of TolQ with TolR or with itself in forming dimer complexes. A previous crosslinking study revealed the interaction involving a TolR dimer/TolQ monomer (Journet et al., 1999); however, there was no evidence of TolR/TolQ interaction was seen in this research. The TolR dimerization noted by Journet et al., (1999) was seen in the absence of amino acids 1 – 43, indicating that is actually residues 44–117 that are involved in TolR dimerization. Journet et al., (1999) also noted that interaction of TolR with TolQ or TolA required residues 1 – 43, indicating the transmembrane domain of TolR is important for interaction between proteins. One explanation for the differences seen in this research from that of Journet et al. (1999) is that the interaction between TolQ/TolR sought in this assay might occur in membrane-resident portion of the proteins and thereby the soluble versions of the proteins used in this assay might not be able to interact, as
regions may be conformationally sequestered. This notion is supported by the suppressor mutations studies by Lazzaroni et al. (1995) that showed that TolQ and TolR interact via their transmembrane domains. Another potential reason for the difference is that the protein complexes may form at certain concentrations or in a particular order, normal requirements not replicated in this assay, which used over-expressed proteins and mixed lysates. The ultimate goal of the protein capture assay was to detect physical interaction between the proteins of the TonB and TolA system; however, because the assay is limited by several factors in the detection of protein interactions amongst proteins within a system it could not be used for proper evaluation of protein interactions involved in crosstalk.

The initial pilot BacterioMatch 2-Hybrid growth assay did not indicate TolQ/TolR protein/protein interactions (Figure 20) when compared to the positive control. One problem with this initial pilot study was that the antibiotic concentrations (tetracycline at 20 μg ml⁻¹ and chloramphenicol at 34 μg ml⁻¹) used to maintain the plasmids were higher than those recommended (tetracycline at 12.5 μg ml⁻¹ and chloramphenicol at 25 μg ml⁻¹) in the Stratagene manual (Stratagene). The higher antibiotic concentrations could have affected the ability of the cells to grow if the protein/protein interactions were not as strong as those of the positive controls of the pBT-LGF2 + pTRG-Gal11P (Figure 20). Since this was a pilot study, only an individual isolate was streaked on the various selective media; therefore, the results could be the result of that single isolate and may not be true representations of the protein/protein interactions occurring in those pBT and pTRG pairings.

Additional BacterioMatch 2-Hybrid growth assays were performed using the Stratagene recommended antibiotic concentrations (tetracycline at 12.5 μg ml⁻¹ and chloramphenicol at 25 μg ml⁻¹). These assays indicated there may be some TolQ/TolQ interaction, but due to the high
amount of interaction of the negative controls the ability to clearly detect those interactions may not be obtainable in this assay (Table 7). Similarly, the beta-galactosidase assays that were performed using the BacterioMatch 2-Hybrid test strain XL1-AX implied a slight IPTG dose-dependent increase for TolQ-TolQ pairing (Figure 22 – checked bars) but the beta-galactosidase levels were not significantly different than that of the negative control pBT + pTRG-Gall11P pairing (Figure 22 – open bars). This again implies a possible TolQ-TolQ interaction, but due to the large amount of interaction noted in the negative controls, a TolQ-TolQ interaction was not clear.

Together, neither the results from the protein capture assay nor from the BacterioMatch 2-Hybrid system identified any TolQ/TolQ or TolQ/TolR protein interactions. The data obtained in this research is in direct contrast to that of the crosslinking data of Journet et al., (1999) that indicated a TolR dimer/TolQ monomer. This difference in results is most likely due to the problems that occurred with the assays performed here affecting the results. The effectiveness of the BacterioMatch 2-Hybrid system was limited by several factors, including its dependence on the fusion products being able to interact when associated with DNA. A potential problem is that the fusion proteins used in this research are interacting in their nature state and inserting themselves in the membrane (as all proteins being evaluated here have transmembrane domains). This would make the protein unavailable for transcription and detection in this system indicating a lack of protein/protein interactions. It is important to note that carrying TolQ and TolR derivatives in a strain that already encodes a wild-type TolQ and TolR seemed to cause various growth issues for the bacterial strains. These problems ranged from lack of growth of the cells even in nonselective medium, to the inability to form full bacterial lawns in top agar experiments. One possible interpretation of this observation is that TolQ and/or TolR-fusion
proteins are interacting with wild-type TolQ and/or TolR, poisoning the system – thus indirectly suggesting interactions between proteins but interfering with the interpretation of the results. This problem may not have been an issue if a yeast 2-hybrid method was used. Walburger et al. (2002) used a yeast 2-hybrid screen and was able to clearly show TolB dimerizes and that the periplasmic domain of TolA is able to interact with YbgF and TolB. It is possible that utilizing the yeast 2-hybrid system for further evaluation of the Tol proteins may have alleviated the problems of performing the BacterioMatch 2-hybrid and the possible poisoning of the cells with excess TolQ and TolR proteins.

Due to their limitations and restrictions, the protein capture assay and the Bacteria 2-Hybrid systems are unlikely to provide for quantitative evaluation of interactions between proteins in the TolA and TonB systems. The fact that the proteins in the TolA system are membrane proteins further complicates the use of other standard protein/protein interact assays. Further, because the constructs used in this research are all plasmid-encoded it is unlikely that appropriate stoichiometries would be achieved and maintained, complicating interpretation; therefore, other methods need to be utilized to clearly evaluate the interactions that occur during crosstalk.
CHAPTER III
DETERMINING THE EFFECT OF ALANYL SUBSTITUTIONS IN THE
TRANSMEMBRANE DOMAIN OF TONB ON THE EFFICIENCY OF ENERGIZATION

INTRODUCTION

The cytoplasmic membrane (CM) of Escherichia coli is rich in proteins that generate, harvest, and utilize ion electrochemical gradients to power essential cellular processes. The outer membrane (OM) cannot itself generate energy due to its porosity, yet harbors various energy-requiring functions that must somehow access CM-derived energy. Two systems have been shown to support events that occur at the OM, the TonB system and the TolA system. The proteins of the TonB system energize the active transport of iron-siderophore complexes (Frost & Rosenberg, 1975; Hantke & Braun, 1975; Williams, 1979) and vitamin B$_{12}$ (Bassford et al., 1976) across the OM, while the TolA system plays an unidentified role in OM maintenance (Webster, 1991; Bernadac et al., 1998; Lazzaroni et al., 1999).

The TonB system (TonB, ExbB, ExbD) and the TolA system (TolA, TolQ, TolR) are both comprised of a cytoplasmic membrane energy harvesting complex and an energy-transducer. These two systems contain proteins that share the same basic topology, with the transducers of energy in the systems being TonB and TolA, respectively. The energy-harvesting complex for the TonB system is comprised of the ExbB and ExbD proteins, with their paralogs TolQ and TolR comprising the energy-harvesting complex for the TolA system (Braun, 1989; Braun & Hermann, 1993). The TolQ and ExbB proteins have similar membrane topologies (Kampfenkel and Braun, 1993; Karlsson et al., 1993b; Vianney et al., 1994), each with three transmembrane domains and the unique feature of the amino-terminus located in the periplasmic
space. A high degree similarity is shared between the three transmembrane domains, having 38%, 68%, and 79% sequence homology among the first, second, and third TMD, respectively. The TolR and ExbD proteins also have similar topologies, each with a single transmembrane domain with a cytoplasmic amino-terminus and the bulk of the protein located in the periplasmic space. There is 70% sequence identity in the TMD of the ExbD and TolR proteins. The TMDs of the energy-harvesting complexes ExbB/ExbD and TolQ/TolR also share features with the energy-harvesting portions of the transmembrane domains of MotA and MotB (Cascales et al., 2001). The high homology of the transmembrane domains of the energy-harvesting complexes may be in part due to their common role of harvesting energy.

The topology of the TolA and TonB proteins is also similar, with each having a single TMD and the bulk of the protein residing in the periplasmic space (Hannavy et al., 1990; Roof et al., 1991, Levengood et al., 1991). Unlike the energy-harvesting complexes, the only sequence homology TonB and TolA share is in their amino-terminal transmembrane domains, which contains the conserved S-X(3)-H-X(6)-LX(3)-S (SHLS) motif (Koebnik, 1993), which includes Ser16 and His20 in TonB and Ser18 and His22 in TolA. TolA and TonB also have very different sequences in their periplasmic regions, most probably because these proteins serve different OM targets. The deletion of individual amino acids between positions 9-25 of the TonB TMD revealed that only Ser16, His20, and the relative spacing between these residues was essential to efficient function (Larsen et al., 1994; Larsen et al., 1999; Larsen et al., 2001). Likewise, mutagenesis of the transmembrane domain of TolA identified that only Ser18 and His22 of the SHLS motif, as well as Phe26, as playing an important role in TolA function (Germon et al., 1998). The activity of TonB was eliminated when residues 1-32 of TonB were either absent (Karlsson et al., 1993b), replaced with a transmembrane domain from the TetA
protein, or removed post-translationally by the inclusion of an engineered peptidase cleavage site (Jaskula et al., 1994).

Normally, the TonB protein of *E. coli* is exported from the cytoplasm without proteolytic cleavage of its amino terminus (Postle & Skare, 1988). Mutational studies in the amino terminus of a hybrid TrpC-TonB protein identified the TMD of TonB as a signal sequence for the export of the protein (Postle & Skare, 1988; Skare et al., 1989). Research on the individual residues of the transmembrane domains of TonB and TolA has provided only slight insight into the role of the remaining residues within the TMD than those found to be important for efficient function.

A Gly26Asp mutation of TonB, the TetA₁-2₈TonB₃₄-2₃₉ mutant or the deletion of the amino terminus (residues 1-32) all together prevents the export of TonB from the cytoplasm (Jaskula et al., 1994; Karlsson et al., 1993b), indicating the role of amino terminus as an export signal.

Interactions between TonB/ExbB and TolA/TolQ have been identified using *in vivo* crosslinking (Derouiche et al., 1995; Higgs et al., 1998; Journet et al., 1999). Such interactions between TonB and ExbB were undetectable with *in vivo* crosslinking in the TetA₁-2₈TonB₃₄-2₃₉ protein (Jaskula et al., 1994), further suggesting the importance of the residues of the transmembrane domain. The shared Ser-His-Leu-Ser motif of TonB and TolA may account for the ability of these two proteins to interact with the energy-harvesting complexes (ExbBD and TolQR) at the CM; as TonB and TolA are able to interact with the energy-harvesting complexes from the others system. Thus, in the absence of ExbB/ExbD, TolQ/TolR can be used to energize TonB, likewise, in the absence of TolQ/TolR, ExbB/ExbD can energize TolA; however, energization by such “crosstalk” is less efficient (Braun & Hermann, 1993). Suppressor mutations in ExbB have been found for the Ser16 and His20 mutations of TonB that restore at least partial TonB function and *in vivo* crosslinking between TonB and ExbB (Larsen et al.,...
Likewise, suppressor mutations in TolQ have been found that restore functions in Ser18 and His22 mutants of TolA, as well as in vivo crosslinking between TolA and TolQ (Germon et al., 1998).

The sequence differences found between the transmembrane domains of TonB and TolA may contribute to the specificity of interaction between the energy transducers and their respective energy-harvesting complexes. The low efficiency of cross-talk is most likely due to system specificity that is defined by the side groups of non-essential amino acids. One method to identify structural features important to the efficient energization of TonB and TolA is to create a generic transmembrane domain where the normal residues of the transmembrane domain of TonB, except the Ser16 and His20, were replaced with a relatively low impact residue, such as Alanine. Initial studies involved substitutions of TMD residues 17-19 by three alanyl residues (Larsen & Postle, 2001), with no apparent loss of energy transfer efficiency. That study focused only on interactions with the ExbBD complexes, while energization by TolQR complexes was not examined. This research examined the ability of a set of TonB derivatives with successively more alanyl substituted transmembrane domains to receive energy from either ExbBD or TolQR complexes. Various functional assays were used to determine the activity level of these TonB derivatives/energy transfer complex pairing.

The research in this chapter was facilitated by collaboration with G.E. Deckert and K. Postle (at WSU and PSU). The ultimate goal of the collaboration was to identify the minimal TMD components required for energization efficiency of TonB by changing large portions of the transmembrane domain to alanines. This approach would lead to the construction of a TonB derivative with an all alanyl transmembrane domain except for the important Ser_{16} and His_{20} residues. In the process of building that construct, many other derivatives were produced and
their function, stability, and their protein/protein interactions examined to determine the effects of the various alanyl substitutions on the ability of each TonB derivative to transduce energy. As part of a collaborative team, testing of the various derivatives was divided into two subsets. The majority of the research for the first subset of derivatives (pKP441, pKP453, pKP558, pKP559, and pRA0022) was performed by me in the laboratory of R.A. Larsen, while the testing of the second subset of derivatives (pKP559, pKP647, pKP674, and pKP712) was performed by me and others in the Larsen and Postle laboratories. The data generated from the second subset of derivatives is in the process of being submitted as a manuscript (In preparation – Larsen et al., 2006). This chapter focuses on those data generated from experiments with the first subset of derivatives.
MATERIALS AND METHODS

Media

Bacterial strains and plasmids were maintained on Luria-Bertani (LB) medium (Sambrook et al., 1989), containing ampicillin at 100 μg ml⁻¹, tetracycline at 20 μg ml⁻¹, kanamycin at 30 μg ml⁻¹, and chloramphenicol at 34 μg ml⁻¹ where required and indicated. Media were also supplemented with L-arabinose as indicated to induce the plasmids. Cells for specific assays were grown on supplemented M9 minimal medium (M9 salts [42 mM sodium phosphate, 22 mM potassium phosphate, 9 mM sodium chloride, 19 mM ammonium chloride] supplemented with 0.4% w/v glycerol, 0.8% w/v casamino acids, 40 μg ml⁻¹ tryptophan, 0.4 μg ml⁻¹ thiamine, 10 mM MgSO₄, 0.5 mM CaCl₂, and 1.85 μM iron; provided as FeCl₃·6H₂O). During cloning reactions, SOC medium (2% w/v tryptone, 0.5% w/v yeast extract, 0.05% w/v NaCl, 20 mM glucose, 10 mM MgCl₂, 10 mM MgSO₄) was used as indicated for the recovery of transformants.

Bacteria strains and plasmids

All bacteria are derivatives of the Escherichia coli K-12 strain W3110 (Hill & Harnish, 1981) and the principal bacterial strains used in this research are described in Table 8. The pKP315 plasmid was constructed by R.A. Larsen by inserting the tonB gene from the transcriptional start to past the rho-independent termination site (basepairs 302-1148) into the multiple cloning site of the pBAD18 plasmid. The cloning was performed so that tonB is controlled by the arabinose promoter, with the ribosome binding site of the tonB transcript provided (Larsen et al., 1999). All other plasmids are derivatives from pKP325 (Larsen et al., 1999), a construct in which the tonB (including its transcriptional start and rho-independent terminator) and the araC genes flanking a bidirectional araBAD promoter from pKP315 was
<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α</td>
<td>F− φ80dlacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17(γ−, mK+) phoA supE44 λ− thi-1 gyrA96 relA1</td>
<td>InVitrogen</td>
</tr>
<tr>
<td>W3110</td>
<td>F−, IN(rrnD−, rrnE)1</td>
<td>M. Berlyn, <em>E. coli</em> Genetic Stock Center</td>
</tr>
<tr>
<td>KP1344</td>
<td>W3110 ΔtonB::blaM</td>
<td>Larsen <em>et al.</em>, 1999</td>
</tr>
<tr>
<td>KP1406</td>
<td>W3110 ΔtonB::blaM, aroB::Tn10</td>
<td>Larsen <em>et al.</em>, 2003a</td>
</tr>
<tr>
<td>KP1423</td>
<td>W3110 ΔtonB::blaM, tolQ&lt;sub&gt;AM37&lt;/sub&gt; (nadA::Tn10, ~80% linked to tolQ)</td>
<td>R. Larsen, WSU</td>
</tr>
<tr>
<td>RA1003</td>
<td>W3110 ΔexbBD::kan (deletion of bases 585-1745 replaced with kan&lt;sup&gt;+&lt;/sup&gt; gene from pACYC184)</td>
<td>R. Larsen, BGSU</td>
</tr>
<tr>
<td>RA1006</td>
<td>W3110 ΔtonB::blaM, ΔexbBD::kan via P1 transduction from RA1003 into KP1344</td>
<td>Present Study</td>
</tr>
<tr>
<td>RA1007</td>
<td>W3110 ΔtonB::blaM, tolQ&lt;sub&gt;AM37&lt;/sub&gt; (nadA::Tn10, ~80% linked to tolQ), ΔexbBD::kan via P1 transduction from RA1003 into KP1423</td>
<td>Present Study</td>
</tr>
<tr>
<td>RA1010</td>
<td>W3110 ΔtonB::blaM, aroB::Tn10, ΔexbBD::kan via P1 transduction from RA1003 into KP1406</td>
<td>Present Study</td>
</tr>
<tr>
<td>RA1020</td>
<td>W3110 ΔexbBD::kan, aroB::Tn10 via P1 transduction from RA1406 into RA1003</td>
<td>Present Study</td>
</tr>
<tr>
<td>RA1023</td>
<td>W3110 aroB::Tn10 via P1 transduction from RA1406 into W3110</td>
<td>Present Study</td>
</tr>
</tbody>
</table>
subcloned into pACYC184, disrupting the tetracycline resistance gene (\textit{tetC}). Plasmids encoding TonB derivatives with transmembrane domains bearing multiple alanyl substitutions were generated by R. A. Larsen and G. A. Deckert using a three-primer polymerase chain reaction (PCR)-based site-directed mutagenesis approach as described below. Several of the plasmids used in this study were assembled by sequential rounds of mutagenesis, with Ala codons added in blocks of 4 to 7 codons. All plasmids encoding TonB derivatives were confirmed by DNA sequencing. The plasmids used in this research are summarized in Table 9 with the basic design of the plasmid depicted in Figure 23, and the alanyl substitutions shown in Figure 24.

\textit{Construction of TonB derivatives using 3-primer PCR-based site-directed mutagenesis}

Plasmids encoding TonB derivatives with various amounts of alanyl substitutions were generated using a three-primer PCR-based site-directed mutagenesis approach adapted from Michael (1994) by Larsen & Postle (2001). The majority of the alanyl-substituted TonB derivatives were generated by R. A. Larsen and G. E. Deckert; however, using this approach a construct encoding a TonB derivative with the amino acids 12 – 15 substituted with alanyl residues (pRA0022) was generated as part of this dissertation research. The basic strategy was the same for all the derivatives generated, the method described here is for the construction of the TonB\textsubscript{Ala12-15}-encoding plasmid (Figure 25). To generate the \textit{tonB}\textsubscript{Ala12-15} allele, the \textit{tonB} gene was amplified by a modified polymerase chain reaction from a pKP315 template using the mutagenic 5’ phosphorylated primer oRA0148psm (Table 10) as well as with the standard primers oKP176 and oKP177 (Table 10; Larsen \textit{et al.}, 2001) to generate a 963 bp product. The oRA0148psm primer has designed to contain nucleotide substitutions that encode four alanyl residues, with homologous regions to \textit{tonB} flanking the mutagenic 12 nucleotides on both sides.
Table 9. Plasmids used in Chapter III

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Characteristics of Plasmid</th>
<th>Antibiotic Marker</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBAD18</td>
<td>AraBAD promoter, AraC</td>
<td>Amp</td>
<td>Guzman et al., 1995</td>
</tr>
<tr>
<td>pBAD24</td>
<td>AraBAD promoter, AraC</td>
<td>Amp</td>
<td>Guzman et al., 1995</td>
</tr>
<tr>
<td>pKP315</td>
<td>pBAD regulated wild type TonB</td>
<td>Amp</td>
<td>Larsen et al., 1999</td>
</tr>
<tr>
<td>pKP325</td>
<td>Encodes wild type TonB, AraC regulated by pBAD promoter</td>
<td>Cam</td>
<td>Larsen et al., 2001</td>
</tr>
<tr>
<td>pKP368</td>
<td>Encodes wild type TonB (unique NcoI site in TMD), AraC regulated by pBAD promoter</td>
<td>Cam</td>
<td>Larsen et al., 2001</td>
</tr>
<tr>
<td>pKP441</td>
<td>Encodes TonB Ala(_{17,19}) derivative of pKP368</td>
<td>Cam</td>
<td>Larsen et al., 2001</td>
</tr>
<tr>
<td>pKP453</td>
<td>Encodes TonB Ala(_{17,19,21-27}) derivative of pKP441</td>
<td>Cam</td>
<td>R. Larsen</td>
</tr>
<tr>
<td>pKP558</td>
<td>Encodes TonB Ala(_{12,15,17,19}) derivative of pKP441</td>
<td>Cam</td>
<td>K. Postle, WSU</td>
</tr>
<tr>
<td>pKP559</td>
<td>Encodes TonB Ala(_{12,15,17,19,21-27}) derivative of pKP558</td>
<td>Cam</td>
<td>K. Postle, WSU</td>
</tr>
<tr>
<td>pKP647</td>
<td>Encodes TonB Ala(_{12,15,17,19,21-31}) derivative of pKP559</td>
<td>Cam</td>
<td>K. Postle, WSU</td>
</tr>
<tr>
<td>pKP674</td>
<td>Encodes TonB Ala(_{12,15,17,19,28-31}) derivative of pKP558</td>
<td>Cam</td>
<td>K. Postle, WSU</td>
</tr>
<tr>
<td>pKP712</td>
<td>Encodes TonB Ala(_{17,19,21-31}) derivative of pKP453</td>
<td>Cam</td>
<td>K. Postle, WSU</td>
</tr>
<tr>
<td>pRA0022</td>
<td>Encodes TonB Ala(_{12,15}) derivative of pKP368</td>
<td>Cam</td>
<td>Present Study</td>
</tr>
<tr>
<td>pKP477</td>
<td>pKP325 ΔTonB, religated with BamH1 sites, so still has AraC promoter with no TonB</td>
<td>Cam</td>
<td>K. Postle, WSU</td>
</tr>
<tr>
<td>pACYC184</td>
<td>Vector (Control plasmid)</td>
<td>Cam, Tet</td>
<td>Chang &amp; Cohen, 1978</td>
</tr>
</tbody>
</table>
Figure 23: The basic structure of the pKP325 plasmid and derivatives. The various TonB derivatives were constructed using the three-primer site-directed mutagenic PCR and ultimately cloned into pKP325 using the BamHI sites (Plasmid pKP325 information from Larsen et al., 2001).
Wild type TonB = WPTLLSVCHGAVVAGLLYTSVH
pKP325/pKP368 = S H L S
pKP441 = S---H L S
pKP453 = S---H----- S
pKP558 = ----S---H L S
pRA022 = ----S H L S

pKP712 = S---H--------
pKP674 = ----S---H L----
pKP559 = ----S---H----- S
pKP647 = ----S---H--------

Figure 24: Sequence of the transmembrane domains of the various TonB derivatives. The sequences of the wild type TonB is residues 11 to 33, with the predicted transmembrane domain of the protein being residues 12 to 32 (indicated by the black line at the top of the sequence) with the conserved SHLS motif of the TonB protein depicted in the single letter amino acid abbreviations. The dashes indicate each amino acid that was replaced by an alanyl residue.
A.  
oRA0148psm $^{\text{Phos}(5')}\text{cgc cgc ttc ccc tgg gca gct gca gcg tgc gtc tgc att c}^3$:  
\hspace{1cm} \overset{\text{Arg Arg Phe Pro Trp Ala Ala Ala Ala Ser Val Cys Ile}}{\text{7 8 9 10 11 12 13 14 15 16 17 18 19}} 

TonB $^{(5')}\text{cgc cgc ttc ccc tgg ccg acg tta ctt tcg gtc tgc att c}^3$:  
\hspace{1cm} \overset{\text{Arg Arg Phe Pro Trp Pro Thr Leu Leu Ser Val Cys Ile}}{\text{7 8 9 10 11 12 13 14 15 16 17 18 19}} 

\hspace{1cm}

B. 

\hspace{1cm}

**Figure 25: Three primer site-directed mutagenesis.** (A) Primer oRA0148psm and the corresponding TonB sequence. The primer designed to create the TonB derivative with amino acids 12-15 substituted for alanines (oRA0148psm) is shown at the top, while the TonB wild type sequence is shown along the bottom. It is important to note the primer was designed to have a 5' - phosphate to allow for ligation during the PCR-reaction by the Taq DNA ligase. (B) Cartoon illustration of the steps in site-directed mutagenesis with the various primers, as well as the Taq DNA ligase and Deep Vent polymerase all indicated. Due to the large regions of homology flanking the substituted residues, the mutagenic primer (shown in black with the residue substitutions signified in red) is able to anneal to the tonB gene. The gap left between the normal primer and mutagenic primer, is able to be ligated during the PCR reaction by the thermostable Taq DNA ligase. Ultimately, after a few rounds of PCR, the residues are substituted and are merely amplified from simply the two flanking, standard primers oKP176 and oKP177. Amplified products will contain BamHl sites flanking the tonB gene that are then used for cloning the tonB allele into pKP325.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>oRA0148psm</td>
<td>(5') PO₄-cgccgctcccctgg<strong>gcaagctgacg</strong>tctgggtctgcattc3' 7 AlaAlaAlaAla 18</td>
</tr>
<tr>
<td>oKP176</td>
<td>5' tctgagaaccggtgccc3' 877 893</td>
</tr>
<tr>
<td>oKP177</td>
<td>5' atcagaccgcttctgcg3' 1433 1416</td>
</tr>
</tbody>
</table>

Sequence of the primers used in PCR reactions. The mutant primer was designed to include *pvu*II restriction enzyme site (underlined). The bold nucleotides represent the nucleotides designed for the incorporation of the alanines into the *tonB* gene. Below the primer sequence are numbers that correspond to the numeric amino acid sequence of the TonB protein (oRA0148psm) or the numbers that correspond to the residue sequence of the pBAD24 plasmid (oKP176 and oKP177).
For this approach, an excess of the standard flanking primers (50 pmol of primers oKP176 and oKP177) were used relative to the mutagenic primer (5 pmol of the oRA0148psm) with 200 pmoles of each dNTP, 10 units thermostable Taq DNA ligase and 2 units of Deep Vent DNA polymerase, in a 1X Taq DNA thermoligase buffer (20 mM Tris-HCl [pH 7.6 @ 25°C], 25 mM potassium acetate, 10 mM magnesium acetate, 10 mM dithiothreitol, 1 mM Nicotinamide Adenine Dinucleotide (NADH), and 0.1% Triton X-100). Reaction volumes of 50 μl were subjected to 35 cycles of melting (94°C for 30 seconds), annealing (52°C for 30 seconds), and extension (67°C for 240 seconds). Reactions were evaluated by electrophoresis in 1% agarose gels, with those containing the predicted 963 bp product pooled and purified using the Qiaquick PCR purification kit (Qiagen). The purified 963 bp amplimer was digested with BamHI at 37°C for 135 minutes, then resolved on a 1% agarose gel and the band migrating at ~900 bp excised and recovered using the Qiaquick gel extraction kit (Qiagen). This ~900 bp digested fragment was then inserted into pKP325 (Figure 23) that had been previously digested with BamHI at 37°C for 195 minutes and dephosphorylated by incubating with shrimp alkaline phosphatase (SAP) for 15 minutes at 37°C (with subsequent inactivation of the SAP by incubating at 65°C for 15 minutes). Standard ligation reactions (total volume of 20 μl) containing various combinations of the digested/dephosphorylated pKP325, digested tonBAla12-15 fragment, and 20 units of T4 ligase, in T4 ligase buffer (50 mM Tris-HCl [pH 7.5], 10 mM MgCl₂, 10 mM dithiothreitol, 1 mM Adenosine 5’-triphosphate (ATP), 25 μg/ml bovine serum albumin) were incubated at room temperature (~25°C) for 18 hours. Ligation reactions were ethanol precipitated and then suspended in 20 μl of ddH₂O. Ligation products were recovered by transformation with 2 μl of the ligation reactions added to 50 μl of electro-competent DH5-α cells in 0.1 cm electroporation cuvettes and pulsed in an MicroPulser™ Electroporation Apparatus (from BIORAD) using the
“Ec1” setting. Cells were recovered in 900 μl SOC medium and transferred to sterile 12 ml culture tubes. Following an one hour incubation at 37°C with aeration, transformants were selected by plating 100 μl of recovered cells on LB agar plates supplemented with chloramphenicol at 34 μg ml⁻¹, with incubation overnight at 37°C. Twelve colonies obtained in this manner were then screened by growing the colonies overnight in LB containing chloramphenicol at 34 μg ml⁻¹, taking 1.5 ml of that culture and isolating plasmid DNA by the alkaline lysis method (Sambrook et al., 1989), with recovery of the plasmids in 50 μl of ddH₂O. Using 10 μl of those plasmid preps, digestions with BamHI were performed to determine which plasmids contained the 900 bp tonB Alα12-15 insert. Eleven isolates had the insert. To confirm that the insert contained the mutant tonB Alα12-15 gene, further restriction mapping was performed by two individual digestions, one using PvuII and the other using BstEII. Two plasmids produced bands corresponding to those predicted for a correct tonB Alα12-15 construct. The identity of these two plasmids was confirmed by sequence determination (by contract with the University of Iowa DNA Facility). One plasmid encoding the Alα12-15 mutation was chosen and named pRA0022.

**Expression levels of TonB derivatives**

Cells bearing plasmids encoding individual TonB derivatives and isogenic cell strains that encode wild type TonB from the native chromosomal promoter bearing pKP447 or pACYC184 were grown with aeration at 37°C in supplemented M9 minimal media containing 34 μg ml⁻¹ chloramphenicol and various levels of L-arabinose. When cells reached a density of ~1.6 x 10⁸ colony-forming units ml⁻¹ (corresponds to an A₅₅₀ of 0.4 as determined with a Spectronic 20 spectrometer with a path length of 1.5 cm), 0.5 ml samples were harvested for immunoblot analysis as described below. Visual comparisons of various the ECL results were used to determine the L-arabinose concentrations that produced full-length TonB derivatives at levels
equivalent to the levels achieved for chromosomally-encoded wild type TonB under native regulation. The L-arabinose concentrations that produced TonB levels similar to that of the chromosomally encoded TonB were thus identified for use in subsequent experiments ranging for a final concentrations of 0.001% to 0.01%). As steady state half life experiments were to be performed on both subsets of derivatives, expression levels for KP1406 cells bearing all of the various TonB derivatives were determined prior to steady state half life experiments.

**Colicin Spot Titer Assay**

The colicin spot titer assays were performed as previously described by Larsen *et al.*, (1999) as adapted from Gudmundsdottir *et al.*, (1988), with the following modifications. Overnight cultures were diluted 1:100 into fresh medium supplemented with 34 μg ml⁻¹ chloramphenicol and L-arabinose as indicated for each derivative and then allowed to grow for 3 hours. Then a 200 μl aliquot of cell culture was added to 3 ml of molten T-top agar containing 34 μg ml⁻¹ chloramphenicol and L-arabinose where indicated and poured onto an LB plate 34 μg ml⁻¹ containing chloramphenicol. Serial 5-fold dilutions of colicin B were made in M9 salts, and 5 μl volumes of these dilutions were applied to the surface of the top agar and incubated at 37°C overnight. Plates were evaluated for clearing in bacteria lawn and scored using the highest dilution in which sensitivity to the colicin was evident. All assays were performed in triplicate.

**φ80 phage adsorption assay**

The φ80 phage adsorption assays were performed as previously described by Larsen *et al.*, (1993), with the following modifications. Overnight KP1344 cultures bearing the TonB derivative plasmids were diluted 1:100 into 10 ml of supplemented M9 Minimal medium containing Fe to 1.85 μM, chloramphenicol 34 μg ml⁻¹ (as described above), and L-arabinose as indicated for each individual plasmid. Cultures were grown in side-arm flasks with aeration at
37°C to an A<sub>550</sub> of 0.4, at which time 1 ml samples were taken, centrifuged and the supernatant removed. Pellets were suspended in 100 μl of LB containing 5 mM CaCl<sub>2</sub> and incubated at 37°C for 5 minutes, and then 4 μl φ80 phage were added. At 0, 5, 10, and 20 minutes, 20 μl samples were harvested to 980 μl of LB containing 5% v/v CHCl<sub>3</sub> and kept on ice until all time points were collected. The residual phage in each sample were titered on W3110. Briefly, samples were diluted by placing 25 μl of the stopped phage assay mixture into 2.0 ml of LB and mixed. To 100 μl of the saturated <i>E. coli</i> K12 strain W3110, 25 μl of the phage dilution was added and allowed to incubate for 10 minutes at 37°C. Three ml of molten T-top agar were then added to the phage/W3110 mixture and poured onto a T-plate. Plates were incubated at 37°C overnight, and the number of phage plaques present on the bacteria lawns were then determined. The experiment was performed in triplicate and the number of plaques at each given time point were divided by the number of plaques at time 0 and then multiplied by 100 to calculate the percent of phage remaining.

*TonB pulse synthesis half-life assay*

The TonB pulse synthesis half-life assays were performed as previously described by Larsen <i>et al.</i> (1999). Briefly, overnight cultures of KP1344 containing either pKP368 or pKP559 were diluted 1:100 into 10 ml of supplemented M9 Minimal medium containing Fe to 1.85 μM and chloramphenicol at 34 μg ml<sup>-1</sup> (but no L-arabinose) in side-arm flasks and grown with aeration at 37°C to an A<sub>550</sub> of 0.4. Samples (0.5 ml) were taken as uninduced controls and precipitated with TCA as described below. The pulse synthesis of TonB was generated by inducing messenger synthesis from the plasmid with 10 mM L-arabinose at 37°C with shaking for 3 minutes. Messenger synthesis was then halted by addition of L-fucose to 12mM and D-glucose-6-phosphate to 7 mM to repress the araBAD promoter, and incubated at 37°C with
shaking for 2 minutes. After halting synthesis from the *tonB* gene, a 0.5 ml sample was immediately taken (0 minute) and TCA precipitated. Additional 0.5 ml samples were taken at 15, 30, 60 and 90 minutes post-pulse and TCA precipitated as described below. Because many of the TonB derivatives required varying amounts of L-arabinose, a pilot pulse synthesis experiment for each of the TonB derivatives was performed in KP1406. An Immunoblot analysis using α-TonB mAb (as described below) was performed using only the 0 minute samples of the various derivatives to determine if the pulse would be sufficient to produce equivalent amounts of TonB.

*TonB steady state half-life assay*

Overnight cultures were diluted 1:100 into 10 ml of supplemented M9 Minimal medium containing 1.85 μM Fe, chloramphenicol at 34 μg ml⁻¹, and various L-arabinose levels dependent upon the plasmid (Table 3E), and grown to A₅₅₀ of 0.4 at which time a 0.5 ml sample was taken (time = 0). Messenger synthesis from plasmid-borne *tonB* was halted by addition of L-fucose to 12mM and D-glucose-6-phosphate to 7 mM to repress the *araBAD* promoter (Larsen et al., 1999), while protein synthesis in the cell was halted by the addition of spectinomycin to 500 μg ml⁻¹ (modified from Claret & Hughes, 2000). Additional 0.5 ml samples were harvested at 15, 30, 60, and 120 minutes following additions. Samples were TCA precipitated and evaluated by immunoblot analysis as described below.

*Iron Uptake Assay*

Iron transport assays were performed as adapted by Larsen and Postle (2001) from Koster and Braun (1990), with the following modifications. Overnight cultures were diluted 1:100 in 20 ml of supplemented M9 Minimal medium containing 1.85 μM Fe, chloramphenicol at 34 μg ml⁻¹, and L-arabinose levels as indicated and grown to an A₅₅₀ of 0.5 at which time 10 ml of cells
were harvested and centrifuged for 10 minutes. Supernatants were removed and the cells suspended to $2 \times 10^8$ ml$^{-1}$ in 10 ml of M9 salts containing 0.1 mM nitrilotriacetate and 4% w/v d-glucose. Cell suspensions were equilibrated for 5 minutes at 37°C. A 0.5 ml sample was removed just prior to starting assay and precipitated with TCA as described below for immunoblot analysis. Transport was started by adding 10 μl of 130 pmol of $[^{55}\text{Fe}]$-ferrichrome (produced by incubating 67 μl of 1 mM iron-free ferrichrome from *Ustilago sphaerogena* (from Sigma) with 10 μl of 1 mM $[^{55}\text{Fe}]$ in 10 mM HCl for 15 minutes at 37°C), and incubated at 37°C with triplicate 0.5 ml samples removed at 2, 6, 10, and 14 minutes. Samples were collected on Whatman GF/C filters in a vacuum manifold and washed three times with 3 ml of 100 mM LiCl. Filters were air-dried, then placed in plastic scintillation bottles with 2.5 ml Cytoscint and incubated overnight at room temperature. The incorporation of $[^{55}\text{Fe}]$ was determined as counts per minute (CPM) by using a Beckman LS 3801 Liquid Scintillation counter read on a channel with a lower limit of 0 keV and an upper limit of 1000 keV. Experiments were performed in triplicate.

**In vivo chemical crosslinking**

*In vivo* chemical crosslinking was performed as previously described by Skare *et al.* (1993), with the following modifications. Overnight cultures were diluted 1:100 in 5 ml of M9 Minimal medium supplemented with chloramphenicol 34 µg ml$^{-1}$ and arabinose (as indicated), grown to $A_{550}$ of 0.4, and 1.0 ml samples harvested and centrifuged for 5 minutes at room temperature. Supernatants were removed and cells resuspended in 1 ml of 100 mM NaPO$_4$ (pH 6.8), then 62.5 μl of 16% paraformaldehyde was added and the mixture incubated for 15 minutes at room temperature. Cells were then centrifuged for 5 minutes at room temperature, the supernatant removed, and cells resuspended in 20 μl of 1 M Tris-Cl pH 8.0 and 20 μl Laemmli
sample buffer (LSB) (Laemmli, 1970) then incubated at 60°C for 5 minutes. Samples (20 μl) were resolved on degassed 11% SDS-PA gels and evaluated by immunoblot analysis as described below.

**TCA Precipitation**

Samples for SDS-PAGE analysis were routinely precipitated by adding cells to an equal amount of 4°C 20% w/v TCA. Mixtures were incubated at 4°C for 15 minutes, then centrifuged for 5 minutes at ~13,000 x g, and the supernatants removed. Pellets were washed with 1 ml of 100 mM Tris-HCl, pH 8.0, and suspended in 25 μl 1 M Tris-HCl, pH 8.0 and 25 μl of 2X Laemmli sample buffer (LSB) (Laemmli, 1970) and the samples incubated at 97°C for 5 minutes. The pellets produced during these TCA precipitations were often very soft and occasionally lost during aspiration. To reduce the possibility of sample loss during later experiments, where indicated, pellets were washed with 1 ml of 80% acetone (Claret & Hughes, 2000) instead of 1 ml of 100 mM Tris-HCl. In these later experiments, the remainder of the TCA precipitation procedure remained the same, only the wash was changed.

**SDS-PAGE and Immunoblot analysis**

Immunoblot analyses were performed as described by Towbin *et al.*, (1979) using TonB-specific monoclonal antibodies and enhanced chemiluminescence (ECL). Samples were prepared as stated above for the various assays, and were subjected to electrophoresis on 11% SDS-PA gels. Resolved proteins were electrotransferred to Immunobilon-P polyvinylindene fluoride (PVDF) membranes overnight at room temperature at 100 mA in transfer buffer (25 mM Tris, 192 mM glycine, 15% v/v methanol, 0.1% SDS). The membranes were placed in blocking buffer (5 % w/v milk, 0.05% v/v Tween 20 in Phosphate Buffered Saline [PBS = 10 mM sodium phosphate, 145 mM NaCl; pH ~7.4]) for 30 minutes. The monoclonal antibody 4F1, (specific
for TonB residues 120-128; Larsen et al., 1996) was used at a concentration of 1:1,000 in blocking buffer (unless otherwise indicated) and incubated with the membranes for 1 hour at room temperature. Membranes were washed three times with ~50 ml of blocking buffer for 7 minutes. The secondary antibody was a goat α-mouse IgG (H + L) human horseradish peroxidase conjugate used at a concentration of 1:2,500 (unless otherwise indicated) in blocking buffer, incubating with the membrane at room temperature for 1 hour. Membranes were rinsed with ~25 ml blocking buffer, then washed four times with final rinse buffer (0.05% v/v Tween 20 in PBS), using ~50 ml for 5 minutes for each wash. Equal amounts of the ECL reagents were mixed and poured so to directly cover the membrane. The membrane was rocked gently for about 1 minute. The membrane was carefully placed between two pieces of clean, plastic transparencies to avoid any air bubbles. Signals were visualized by exposing membranes to either HyBlot CL autoradiography film or Kodak BioMax XAR film for the stated times. Following immunoblot analysis, membranes were placed in a glass dish and stained with 0.1% w/v Coomassie blue in 50% methanol, 7% glacial acetic acid for 5 – 10 minutes, then destained with 50% methanol, 7% glacial acetic acid with a knotted Kimwipe to adsorb dye for 30 – 60 minutes. Membranes were removed from the destaining solution, placed on piece of Whatman paper, and allowed to air dry.
RESULTS

*Sensitivity of TonB derivatives to colicin B when utilizing ExbB/ExbD*

Mutations in tonB have been shown to make *E. coli* strains tolerant to Group B colicins (Davies and Reeves, 1975a). The dependence of a functional TonB protein for the entry of colicin B into the cell allows a means to assay the affect of various mutations on the functionality of the TonB protein. Cells with a functional TonB are sensitive to the killing affects caused by colicin, even at with diluted amounts of colicins. These types of colicin-dependent killing assays show a moderate degree of sensitivity to TonB activity. For example, a nine-fold reduction in the level of TonB resulted in approximately a five-fold decrease in sensitivity to the group B colicins (Larsen *et al.*, 2003a). TonB derivatives containing alanyl substitutions in the TMD were tested for their sensitivity to colicin B compared to that of wild type chromosomal TonB (Figure 26) in KP1344 (ΔtonB) cells. In cultures that were not induced with L-arabinose, those derivatives with alanyl substitutions of residues 12-15 (pKP558 and pKP559) were approximately one dilution less sensitive to the colicin (5\(^4\), 5\(^4\), 5\(^3\), 5\(^3\), 5\(^3\), respectively) than the wild type (5\(^5\), 5\(^5\), 5\(^5\)) or other alanyl substitutions (Figure 26, A). Derivatives with alanyl substitutions of residues 17-19 (pKP441) or 17-19, 21-27 (pKP453) showed a sensitivity to colicin B (5\(^5\), 5\(^5\), 5\(^4\) and 5\(^5\), 5\(^5\), 5\(^5\), respectively) similar to the plasmid encoded wild type TonB (pKP368). The induction of the pKP558 and pKP559 plasmids with L-arabinose (0.01% w/v) increased the sensitivity of cells to the colicin (Figure 26, graph B) relative to uninduced cells (Figure 26, graph A). In contrast, induction of pKP368, pKP441, and pKP453 plasmids with L-arabinose resulted in a decrease in activity. This decrease in TonB activity is consistent with the previously documented occurrence of a dominant negative gene dosage effect for TonB (Kadner & McElhaney, 1978; Mann *et al.*, 1986; Larsen *et al.*, 2003a). Studies have shown
Figure 26: Colicin spot titer of TonB derivatives using colicin B and their ability to use ExbB/ExbD energy-harvesting complex. Plasmids containing the various TonB derivatives were transformed into KP1344 (ΔTonB) and colicin spot titers performed either when the cells in the T-Top agar where (A) uninduced or (B) induced with 0.01% L-arabinose. W3110 is the TonB chromosomal control (carrying pACYC184), while pACYC184 represents the negative control and is the empty vector in KP1344. Bars represent the average of the highest 5-fold dilution in which cells were still sensitive to the colicin of a triplicate experimental set. A dilution of (-1) represents cells that were totally resistant (R) to colicin B.
overexpressed TonB is actually unstable and the resulting degradation products potentially inhibit efficient energy transfer (Larsen et al., 2003a). In the presence of L-arabinose, the overexpression of the TonB derivatives from the pKP558 and pKP559 increased the sensitivity of the cells to the colicin, probably due to an increase in the amount of TonB present; however, this increase still did not equal the sensitivity of uninduced wild type cells.

_Sensitivity of TonB derivatives to colicin B when utilizing TolQ/TolR in cross-talk_

In the absence of ExbB/ExbD, TonB has been shown to utilize TolQ/TolR via crosstalk (Braun, 1989). The TonB derivatives were transformed into RA1006 (ΔtonB, ΔexbBD) and their sensitivity to colicin B examined to determine the effect of the alanyl substitutions on the ability of TonB to participate in crosstalk with TolQ/TolR (Figure 27). As seen with the energy-harvesting complex ExbB/ExbD (Figure 26), the TonB derivatives of pKP441 and pKP453 had the same sensitivity as the plasmid encoded wild type TonB (pKP368), while the derivatives from pKP558 and pKP559 were not as sensitive (Figure 27). The overall sensitivity of those cells containing only the energy-harvesting complex TolQ/TolR (Figure 27) to the colicin was decreased relative to the strains containing ExbB/ExbD (Figure 27) when supported by TolQ/TolR. This decrease in sensitivity of the derivatives between being in KP1344 and RA1006 strains represented the inefficiency of crosstalk. Derivatives with alanyl substitutions in residues 12-15 (pKP558 and pKP559) were equally less sensitive relative to wild type, whether energized by ExbB/ExbD or TolQ/TolR (Figure 26 and Figure 27). Thus, the relative decrease in sensitivity of these derivatives with alanyl 12-15 substitutions was independent of the energy-harvesting complex with which they interact.
Figure 27. Colicin spot titer of TonB derivatives using colicin B and their ability to use the TolQ/TolR energy-harvesting complex via crosstalk. Plasmids containing the various TonB derivatives were transformed into RA1006 (ΔTonB, ΔExbBD) and colicin spot titers performed either when the cells in the T-Top agar were induced with 0.01% L-arabinose. RA1006 containing pACYC184 represents the negative control as it is an empty vector. This allowed all cells to be grown in the presence of chloramphenicol at 34 μg ml⁻¹. Bars represent the average of the highest 5-fold dilution in which cells were still sensitive to the colicin of a triplicate experimental set. A dilution of (−1) represents cells that were totally resistant (R) to colicin B.
Sensitivity of TonB derivatives to φ80 bacteriophage

Certain bacteriophage, such as φ80, use the TonB system to gain access to the cell (Matsushrio, 1963). This parasitism of the TonB system provides another means to evaluate the activity of the various TonB derivatives compared to wild type TonB. Irreversible phage adsorption is essentially a transport assay, and studies have shown this assay is able to distinguish between chromosomal, 1/8-, and 1/200-fold levels of TonB (Larsen et al., 2003a). Irreversible phage adsorption as described by Larsen et al. (1993) was performed on the same derivatives that were tested using the colicin spot titer assay. Unlike colicin spot titer assays, irreversible phage adsorption is a liquid-based assay, allowing TonB function to be correlated with the amount of TonB present. Cells with a functional TonB protein will be able to adsorb phage efficiently, thereby reducing the amount of residual. The assay is thus scored as the percent of phage remaining, with the amount of phage for 0 minute time point considered 100% and percent values determined based on that number. For the various TonB derivatives, there was significant variance among the triplicate samples, with large standard errors (Figure 28 – A). Because of the large standard error, there is no statistical significance between the various TonB derivatives and the wild type TonB at any time point (Figure 28); however, the trend of the mean averages (Figure 28 – B) is similar to those seen in the colicin spot titer assays. As with the colicin spot titer results, the percent of phage adsorbed by the Ala₁₇₋₁₉ derivative (pKP441) was nearly identical to that of the wild type TonB from pKP368, both going from 100% to ~45% over the 12 minute assay (Figure 28 - B). The remaining derivatives (Ala₁₇₋₁₉, Ala₂₁₋₂₇, Ala₁₂₋₁₅, Ala₁₂₋₁₅, Ala₂₁₋₂₇ − from pKP4453, pKP558, and pKP559, respectively) showed less ability to adsorb phage than the wild type TonB at 8 and 12 minutes (~65% at 12 minutes); however, little difference was seen between the any of the derivatives (all being ~80%) at the 4 minute time...
Figure 28: **Irreversible phage adsorption assay.** KP1344 cells (ΔTonB) transformed one of the various test plasmids were subjected to irreversible phage assay as stated in Material and Methods. Phage measurements were taken, and the percent of phage remaining calculated. Graphs are the average of three experiments (A) with standard error bars and (B) without.
point (Figure 28 – B). All derivatives were able to irreversibly adsorb phage compared to the pACYC184-bearing negative control, (Figure 28 – B) and there was a significant difference between the negative control and all of the TonB derivatives, including wild type at the 12 minute time point (Figure 28 – A).

*Stability of wild type and Ala_{12-15,17-19,21-27} TonB using pulse synthesis*

Since cells containing pKP559 (Ala_{12-15,17-19,21-27}) were the least sensitive to colicin B in the spot titer assays compared to those containing pKP368 (WT), a pulse synthesis assay of those two TonB derivatives was performed to determine if that difference was caused by a change in the stability of protein. Following pulse synthesis of TonB, the wild type TonB (pKP368) had a half life of ~ 90 minutes (Figure 29 – A). The Ala_{12-15,17-19,21-27} TonB derivative also had a half life of approximately ~ 90 minutes (29 – B). The difference in sensitivity from the colicin assays (Figures 26 & 27) did not appear to be the result of instability of the Ala_{12-15,17-19,21-27} TonB derivative. The only other derivative that showed a decrease in sensitivity colicin B compared to wild type TonB (pKP368) was the Ala_{12-15,17-19} TonB derivative (pKP558). Based on the decrease in sensitivity of the TonB derivatives with alanyl substitutions for residues 12-15 in the colicin spot titer assays (Figure 26 & 27) and the stability of the Ala_{12-15,17-19,21-27} TonB derivative, an additional derivative containing only alanyl substitutions in residues 12-15 (pRA0022) was made using three-primer mutagenic PCR (Figure 25).

*Sensitivity of Ala_{12-15} TonB derivative to colicin B*

Once the new Ala_{12-15} TonB derivative had been constructed, a colicin spot titer was performed to determine if the Ala_{12-15} substitution was the reason the Ala_{12-15,17-19} (pKP558) and Ala_{12-15,17-19,21-27} (pKP559) had a decreased sensitivity to colicin B compared to wild type (pKP368). KP1406 cells (∆tonB, aroB') carrying the wild type derivative (pKP368) showed the
Figure 29: TonB stability assay using pulse synthesis. Pulse synthesis half live performed (as described in Material and Methods) on pKP1406 cells harboring either (A) pKP368 or (B) pKP559. Samples of 500 μl were collected prior to pulse (uninduced) as well as at 0, 15, 30, 60, 90 minutes following the pulse. Exposure times for each immunoblot were chosen so that the 0 minutes time points would be relatively equivalent and those times were (A) 1 minute and (B) 5 minutes. Following immunoblot analysis, membranes were stained with Coomassie Blue to ensure protein loads were equivalent (data not shown). Size standards in kilodaltons are indicated at the right of the figure.
same sensitivity (sensitive at a colicin B dilution of $5^7$) whether induce with L-arabinose (Figure 30 – A, diamond grid) or not (Figure 30 – A, vertical stripes). The Ala_{12-15} TonB derivative (pRA0022) (Figure 30 – A, horizontal stripes) had the same sensitivity (a dilution of $5^7$) as the plasmid encoded wild type TonB, pKP368. Both derivatives were slightly lower than the chromosomal TonB control RA1023 cells ($aroB^+$) that had a sensitivity out to a dilution of $5^8$ (Figure 30 – A, solid bar) and the cells containing no TonB (pKP477) were totally resistant to killing by the colicin (Figure 30 – A, checker board). These results indicate that an Ala_{12-15} substitution alone did not affect the sensitivity of the cells to the TonB-dependent colicin B in the presence of the ExbBD energy-harvesting complex.

Since there was no difference in sensitivity with the wild type derivative (pKP368) in the presence or absence of L-arabinose induction (Figure 30 – A), remaining spot titers were performed without absence of L-arabinose. RA1010 cells ($\Delta tonB, \Delta exbBD, aroB^+$) carrying the plasmid encoded wild type TonB (pKP368) were sensitive only to dilutions of $5^4, 5^4, 5^5$ (Figure 30 – B, diamond grid). The Ala_{12-15} TonB derivative (pRA0022) (Figure 30 – B, horizontal stripes) had the nearly the same sensitivity (a dilution of $5^4$) as the wild type derivative (pKP368). Both derivatives were lower than the chromosomal TonB control RA1020 ($\Delta exbBD, aroB^+$) cells that had a sensitivity out to a dilution of $5^7$ (Figure 30 – B, solid bar) and the RA1010 cells containing no TonB (pKP477) were totally resistant to killing by the colicin (Figure 30 – B, checker board). These results indicated that an Ala_{12-15} substitution alone does not affect the sensitivity of cells to colicin B when the TonB is involved in crosstalk utilizing the TolQR energy-harvesting complex.

**Alanyl substitution of residues 12-15 of TonB alters efficiency of iron transport**

Since TonB is involved in high affinity iron-siderophore transport (Frost & Rosenberg,
Figure 30: The effect of TonB Ala{sub}12{sub}15 substitution on colicin B uptake. A colicin spot titer using various dilutions of colicin B was performed as described in Materials and Methods on (A) KP1406 (ΔTonB, AroB{sup}−) cells containing either the pKP368 (WT TonB) uninduced in the top agar (Vertical lines), pKP368 induced in the top agar (Diamond grid), pRA0022 (TonB{sub}Ala12-15) (Horizontal lines), or pKP477 (Vector) (Checkerboard) along with the chromosomal wild type RA1023 (AroB{sup}−) cells (solid bar), as well as in (B) KP1010 (ΔTonB, ΔExbBD, AroB{sup}−) cells containing pKP368 (Vertical lines), pRA0022 (Horizontal lines), or pKP477 (Checkerboard) along with the chromosomal wild type RA1020 (ΔExbBD, AroB{sup}−) cells (solid bar). The bars represent the highest dilution (5{sup}[N]) at which the cells still showed sensitivity to the colicin, with cells that were totally resistant represented as −1. The average of three experiments is graphed.
1975; Hantke & Braun, 1975; Williams, 1979), a more direct measure of TonB activity would be an iron transport assay. The bacterial strain KP1406 (ΔtonB, aroB) was used for the iron transport assay because the aroB genotype insures that the cells cannot make their own enterochelin (the sole siderophore produced by K12 E. coli). Upon the addition of [55Fe]-ferrichrome, these cells can take up the radioactive iron-siderophore complex in the presence of a functional TonB protein, thus providing a direct measure of TonB activity. All the TonB derivatives maintained the ability to transport iron with rates much higher than those seen during crosstalk conditions of RA1020 (ΔexbBD, aroB) cells (Figure 31). Only the Ala12-15 TonB derivative (pRA0022) transported iron at a significantly lower rate than wild type TonB (30%) (Figure 31 & Table 11). The other derivatives transported less iron overall than the wild type (Figure 31 & Table 11); however, the actual transport rates for those derivatives (from pKP453, pKP441, & pKP558) were similar to that of wild type TonB (pKP368), transporting iron at least 70% that of wild type TonB levels (Table 11). Based on these data, it appears that substituting residues 12-15 of TonB with alanyl residues did have an effect on the ability of TonB to effectively transport iron compared to wild type TonB.

Various TonB derivatives still maintain TonB-protein interactions

The differences in iron transport of the TonB derivatives could be the result of either protein instability or interference with protein/protein interactions. Since the TonB protein has been shown to shuttle (Letain & Postle, 1997; Larsen et al., 2003b), a possibility for the difference in transport rates may be a result of the substituted residues being involved in the interaction of TonB with other proteins during energy transduction. Alanyl residues at those positions may be affecting the various protein/protein interactions that occur with TonB during shuttling and the transduction of energy to the OM. In vivo chemical crosslinking has revealed
Figure 31: The transport of $^{55}$Fe-ferrichrome by the various TonB derivatives. Plasmids carrying the various TonB derivatives were transformed into KP1406 (ΔTonB, aroB−) and then their ability to uptake the radioactive iron-siderophore complex ($^{55}$Fe-ferrichrome) measured using liquid scintillation as described in the Material and Methods. Graphed is the average of three experiments for the linear portion of the graph used for the linear regression analysis of the slopes (Table 11) which is 2 minute through 10 minute time point samples.
Table 11: Transport rates for iron of the various TonB derivatives

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Intercept</th>
<th>CPM/minute (Slope)</th>
<th>R-Square</th>
<th>Iron Transport Rate&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKP368</td>
<td>1274.58</td>
<td>761.15</td>
<td>0.9958</td>
<td>100%</td>
</tr>
<tr>
<td>pKP453</td>
<td>1582.8</td>
<td>648.97</td>
<td>0.9937</td>
<td>85%</td>
</tr>
<tr>
<td>pKP441</td>
<td>1174.4</td>
<td>568.38</td>
<td>0.9857</td>
<td>75%</td>
</tr>
<tr>
<td>pKP558</td>
<td>561.61</td>
<td>533.41</td>
<td>0.9804</td>
<td>70%</td>
</tr>
<tr>
<td>pRA0022</td>
<td>1046.12</td>
<td>230.88</td>
<td>0.9351</td>
<td>30%</td>
</tr>
</tbody>
</table>

<sup>a</sup>Iron transport rates were determined as percent transport compared to wild type using the slope calculations for each TonB derivative compared to the wild type TonB from the plasmid pKP368. The CPM measurements from the 2, 6, and 10 minute time points were used to calculate the slope from the transport experiment and was calculated using linear regression analysis on SAS (Statistics via SAS performed with the assistance of Bradley A. Pickens).
complexes representing TonB/ExbB and TonB/ExbD heterodimers, as well as interactions with proteins in the OM, TonB/FepA, TonB/Lpp, and TonB/OmpA (Skare et al., 1993; Higgs et al., 1998, Higgs et al., 2002). In vivo formaldehyde crosslinking was used in this research to determine if these alanyl substitutions have affected the physical interactions of these TonB derivatives with other proteins wild type TonB normally interacts during energy transduction (Figure 32 & 33).

When RA1023 cells are not treated with paraformaldehyde, only the TonB monomer is evident as a band at 36 kDa (Figure 33 – lane 1). With paraformaldehyde treatment, the TonB chromosomal control, RA1023 (aroB), forms the characteristic complexes with Lpp (43 kDa), ExbB (59 kDa), OmpA complex (77 kDa) and FepA (195 kDa) (Figure 32 – lane 1, Figure 33 – lane 2), with some complexes more visible at longer exposures (data not shown). To ensure the TonB derivatives were able interact with the energy-harvesting complex ExbB/ExbD, a control RA1020 (ΔexbBD, aroB) was treated with paraformaldehyde treatment. Treated RA1020 clearly shows an absence of the 59 kDa TonB-ExbB complex (Figure 32 – lane 2, Figure 33- lane 3). This control confirms the complex migrating to 59 kDa is TonB-ExbB at this position.

Treated KP1406 cells containing the wild type TonB from the pKP368 plasmid (Figure 32 – lane 3, Figure 33 – lane 4) forms complexes identical to those of the chromosomal control, RA1023 (Figure 32 – lane 1, Figure 33- lane 2). The crosslinking profiles of the remaining TonB derivatives tested (pKP441, pKP453, pKP558, and pKP559) were similar to those of the wild type TonB from the pKP368 plasmid and the chromosomal control RA1023 (Figure 32 – lanes 4 – 7) forms the characteristic complexes with Lpp (43 kDa), ExbB (59 kDa), OmpA complex (77 kDa) and FepA (195 kDa). In addition to those TonB derivatives, the Ala12-15 derivative (pRA0022) in KP1406 (Figure 33 – lane 5) produced crosslinked complexes that were
Figure 32: Formaldehyde in vivo crosslinking of TonB derivatives. Various TonB derivatives were transformed into KP1406 (ΔTonB, AroB⁻) cells and chemically crosslinked as stated in the Materials and Methods. The Immunoblot analysis using α-TonB (4F1) shows wild type TonB (36 kDa) as well as other TonB-protein complexes. Known TonB-protein complexes from previous research (Skare et al., 1993; Higgs et al., 1998; Higgs et al., 2002) are noted. Lanes: (1) RA1023 (AroB⁻) w/ pKP477; (2) RA1020 (ΔExbBD, AroB⁻); (3) KP1406 w/ pKP368; (4) KP1406 w/ pKP441; (5) KP1406 w/ pKP453; (6) KP1406 w/ pKP558; and (7) Kp1406 w/ pKP559.
The Immunoblot analysis using α-TonB (4F1) shows wild type TonB (36 kDa) as well as other TonB-protein complexes. Known TonB-protein complexes from previous research (Skare et al., 1993; Higgs et al., 1998; Higgs et al., 2002) are noted. Lanes (1) RA1023 cells (aroB<sup>−</sup>) w/ pKP447, not crosslinked; (2) RA1023 cells w/ pKP447, crosslinked; (3) RA1020 (ΔexbBD, aroB<sup>−</sup>) w/ pKP477, crosslinked; (4) KP1406 (ΔtonB, aroB<sup>−</sup>) cells w/ pRA0022, crosslinked; and (5) KP1406 (ΔtonB, aroB<sup>−</sup>) cells w/ pKP368, crosslinked.
nearly identical to the plasmid encoded wild type. For some of the derivatives, the characteristic TonB complexes were more evident at longer exposures (data not shown). For the TonB derivatives containing the Ala21-27 substitution, pKP453 and pKP559, the crosslinking profiles contained a more pronounced amount of TonB degradation product detected (Figure 32 – lane 5 & lane 7) as the TonB chromosomal control (Figure 33 – lane 2) or the wild type plasmid TonB (Figure 33 – lane 5). Taken together, the crosslinked TonB-complexes for the derivatives from pKP441, pKP453, pKP558, pKP559, and pRA0022 are all nearly identical to those complexes of the crosslinked wild type chromosomal control (Figure 32 & 33).

An attempt to evaluate the stability of the TonB derivatives using pulse synthesis

The pulse synthesis method had already been used in this research to evaluate the stability of the derivatives produced from pKP368 and pKP559 (Figure 29). Based on that data, pulse synthesis half life studies were to be performed on the majority of the TonB derivatives constructed (including additional constructs [pKP647, pKP674, & pKP712] built by G.E. Deckert in the laboratory of K. Postle) to determine the stability of these TonB derivatives. Pulse synthesis of all the constructs was performed, but only the 0 minute time sample was analyzed using α-TonB immunoblot analysis. This was to determine if all of the derivatives had been induced to similar levels and corresponding to chromosomal amounts of TonB (Figure 34). The plasmids pKP441 and pKP453 produced chromosomal levels when pulsed (Figure 34 – Lanes 1,3,4); while the plasmids pKP368, pKP558, and pKP712 produced TonB levels slightly less than that of chromosomal (Figure 34 – Lanes 1,2,5,7). The amount of TonB produced by pKP559 when pulsed was much lower than the chromosomal control; however, the amount of protein produced was still clearly visible on the immunoblot (Figure 34 – Lanes 1, 6). The
Figure 34: Comparison of zero minute samples from the various TonB derivatives following pulse synthesis. KP1406 cells carrying one of the various test plasmids were pulsed by 1 mM L-Arabinose (as described in the Material and Methods) to compare the amount of TonB produced following a pulse induction of the TonB gene. The zero minute samples were TCA precipitated and resolved on a 12.5% SDS-PA gel, transferred to a PVDF membrane and an Immunoblot analysis performed using α-TonB (4F1). Lanes: (1) RA1023 w/ pKP477; (2) KP1406 w/ pKP368 (WT TonB); (3) KP1406 w/ pKP441 (Ala<sub>17-19</sub>); (4) KP1406 w/ pKP453 (Ala<sub>17-19</sub>, 21-27); (5) KP1406 w/ pKP558 (Ala<sub>12-15</sub>, 17-19); (6) KP1406 w/ pKP559 (Ala<sub>12-15</sub>, 17-19, 21-27); (7) KP1406 w/ pKP647 (Ala<sub>12-15</sub>, 17-19, 21-31); (8) KP1406 w/ pKP674 (Ala<sub>12-15</sub>, 17-19, 28-31); and (9) KP1406 w/ pKP712 (Ala<sub>17-19</sub>, 21-31). Size standards in kilodaltons are indicated at the right of the figure.
amount of protein produced from the pulse of cells containing either pKP647 or pKP674 was barely detectable on the immunoblot (Figure 34 – Lanes 7, 8). The expression:turnover ratio of these derivatives reduces the amount of proteins, and thus creates the inability to pulse many of the multiple alanyl derivatives to even detectable levels. Due to the problems obtaining a 0 time minute pulse, it was determined the pulse synthesis assay would not be an effective method for determining the stability of these TonB derivatives.

*Steady state half life assay*

Since several derivatives were not able to be pulsed to chromosomal levels, another method to determine the half life and stability of these TonB derivatives was needed. One possible approach would be steady state half-life experiments, in which protein synthesis is halted in growing cells and the degradation of the proteins evaluated over time. Many of the steady state half life experiments use large amounts of chloramphenicol to inhibit protein synthesis; however, this approach could not be used in this research because the test plasmids for this research conferred chloramphenicol resistance. Some steady state half studies used spectinomycin to inhibit protein synthesis in cells (Claret and Hughes, 2000). Pilot steady state experiments that used only spectinomycin were not sufficient to halt protein synthesis and cell growth (data not shown). The method developed for the evaluation of the steady state half life of the TonB derivatives in this research used a combination of spectinomycin, D-glucose-6P and fucose. Spectinomycin was used to halt protein synthesis within the cell, while D-glucose-6P and fucose (used in the pulse synthesis assay) were used to halt messenger synthesis from the *tonB* gene on the plasmid. Before the steady state half experiments could be performed, it was important to determine the L-arabinose levels that would produce TonB levels equivalent to chromosomal TonB levels. The nine TonB derivatives were grown in supplemented M9
minimal medium (as described above) containing various amounts of \( L \)-arabinose and subjected to \( \alpha \)-TonB immunoblot analysis to determine the level of arabinose that produced a signal at \( \sim 36 \) kD of equivalent intensity to that of chromosomal wild type TonB (from RA1023) (data not shown). Various immunoblots were performed and the \( L \)-arabinose amount that produced levels closest to that of chromosomal TonB levels for each derivative is recorded in Table 12. Using these adjusted arabinose levels that induced the various TonB derivatives to chromosomal levels; steady state half life experiments were performed on all nine TonB derivatives to determine their stability.

*The effect of alanyl substitutions on the stability of the various TonB derivatives*

To allow for a direct comparison between the iron transport levels and the stability of the TonB derivatives, steady state assays were performed with KP1406 cells bearing the TonB derivative plasmids. Individual steady state time point samples were resolved on an 11% SDS-PA gel and an \( \alpha \)-TonB mAb immunoblot analysis performed. Exposures times from the ECL results were chosen so the bands of wild type chromosomal TonB controls were equivalent. In a visual determination, the wild type TonB had an apparent chemical half life greater than one hour but less than 120 minutes (Figure 35, part A). The Ala_{12-15,17-19} derivative from pKP558 had a chemical half life equal to that of the wild type TonB (Figure 35 – D). The derivatives from pKP441 and pKP453 seemed to be extremely stable, appearing to have chemical half lives of over 120 minutes (Figure 35 – C & E), while the TonB derivative from pRA0022 seemed to have a chemical half life that is slightly less than wild type TonB (Figure 35, part B). In the TonB derivatives, there does appear to be a \( \sim 28 \) kDa degradation product that was not seen in the chromosomal wild type TonB (Figure 35), even at longer exposures (date not shown). For each of the TonB derivatives (except pKP453), the amount of degradation products at all time points
Table 12: The L-arabinose levels used to induce various TonB derivatives to levels similar to that of chromosomal TonB

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>TonB Derivative (Alanine Substitutions)</th>
<th>L-arabinose level&lt;sup&gt;a&lt;/sup&gt; (Final concentration)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKP368</td>
<td>Wild type TonB from plasmid</td>
<td>0.001%</td>
</tr>
<tr>
<td>pKP441</td>
<td>Ala&lt;sub&gt;17-19&lt;/sub&gt;</td>
<td>0.001%</td>
</tr>
<tr>
<td>pKP453</td>
<td>Ala&lt;sub&gt;17-19&lt;/sub&gt;, Ala&lt;sub&gt;21-27&lt;/sub&gt;</td>
<td>0.001%</td>
</tr>
<tr>
<td>pKP558</td>
<td>Ala&lt;sub&gt;12-15&lt;/sub&gt;, Ala&lt;sub&gt;17-19&lt;/sub&gt;</td>
<td>0.0025%</td>
</tr>
<tr>
<td>pKP559</td>
<td>Ala&lt;sub&gt;12-15&lt;/sub&gt;, Ala&lt;sub&gt;17-19&lt;/sub&gt;, Ala&lt;sub&gt;21-27&lt;/sub&gt;</td>
<td>0.0025%</td>
</tr>
<tr>
<td>pRA0022</td>
<td>Ala&lt;sub&gt;12-15&lt;/sub&gt;</td>
<td>0.0025%</td>
</tr>
<tr>
<td>pKP647</td>
<td>Ala&lt;sub&gt;12-15&lt;/sub&gt;, Ala&lt;sub&gt;17-19&lt;/sub&gt;, Ala&lt;sub&gt;21-31&lt;/sub&gt;</td>
<td>0.01%</td>
</tr>
<tr>
<td>pKP674</td>
<td>Ala&lt;sub&gt;12-15&lt;/sub&gt;, Ala&lt;sub&gt;17-19&lt;/sub&gt;, Ala&lt;sub&gt;28-31&lt;/sub&gt;</td>
<td>0.005%</td>
</tr>
<tr>
<td>pKP712</td>
<td>Ala&lt;sub&gt;17-19&lt;/sub&gt;, Ala&lt;sub&gt;21-31&lt;/sub&gt;</td>
<td>0.005%</td>
</tr>
</tbody>
</table>

<sup>a</sup>This table is an accumulation of various immunoblots using α-TonB (4F1). The final concentration level of L-arabinose required for each TonB derivative was determined by comparing the amount of TonB expressed from the induced plasmids to that of the chromosomal wild type TonB at A<sub>550</sub> = 0.4. The arabinose levels shown in this table were used for the steady state half life data experiments (Figures 36 & 37). Levels are reported as a weight/volume percentage.
Figure 35: Determination of protein stability of the first set of TonB derivatives using steady state half life experiments. The 0 minute sample was taken just prior to halting protein synthesis from the plasmid and within the cell. The exposures time were selected from immunoblots of the various TonB derivatives A. pKP368, (B) pRA022, (C) pKP441, (D) pKP558, and (E) pKP453 in order to have similar levels of chromosomal wild type TonB (CH) for comparison. Exposures times were 5 seconds for A and D, and 15 seconds for all other derivatives. Size standards in kilodaltons are indicated at the right of the figure.
seem to be equal. This is different than the degradation found from pKP453, as there seems to be a slight increase in the degradation over time, implying this product is slightly more stable than the product seen in the other derivatives.

As part of this dissertation research, half life data for the second subset of TonB derivatives were determined (Figure 36). The Ala_{17-19,21-31}, Ala_{12-15,17-19,28-31}, and Ala_{12-15,17-19,21-27} TonB derivatives from pKP712, pKP674, and pKP559 plasmids, respectively, each had an apparent chemical half life slightly less than that of the wild type derivative (Figure 36 – A,B,C,D). The most pronounced difference in the chemical stability was that of the Ala_{12-15,17-19,21-31} TonB derivative, where the apparent chemical half life was approximately 30 minutes (Figure 36 – E). The amount of degradation products for this subset of largely alanyl substituted TonB derivatives was much more apparent than with the derivatives from the first subset (Figure 35), with more visible intermediates. As was seen with the degradation products of the TonB derivatives from pKP453 (Figure 35 – E), the degradation products seen here tend to increase over time, indicating these degradation products are more stable than the full-length TonB derivative.

_Evaluating crosstalk ability of TonB derivatives using iron transport_

Both TonB and TolA contain the conserved Ser, His motif, known to be essential for efficient function of the proteins. If the residues replaced with alanines in these various derivatives are important in determining the specificity of interaction of the energy-transducers with the corresponding energy-harvesting complex, then changing those residues to alanines could reduce the specificity; thus, allowing the TonB derivatives to function equally with the TolQ/TolR complex as with the ExbB/ExbD complex. On the other hand, the removal of the residues that are involved in specificity of interactions between the TonB derivatives and the
Figure 36: Determination of protein stability of the second set of TonB derivatives using steady state half life experiments. The 0 minute sample was taken just prior to halting protein synthesis from the plasmid and within the cell. The exposures time were selected from immunoblots of the various TonB derivatives A. pKP368, (B) pKP712, (C ) pKP674, (D) pKP559, and (E) pKP647 in order to have similar levels of chromosomal wild type TonB (CH) for comparison. Exposures times were 5 seconds for A, B, and D; 10 seconds for E; and 30 seconds for D. Size standards in kilodaltons are indicated at the right of the figure. Figure is part of manuscript in preparation by Larsen et al., 2006
energy transducers could diminish those interactions altogether. A pilot iron transport assay was
performed comparing the chromosomal wild type strain, RA1020 (ΔexbBD, aroB'), to RA1010
(ΔtonB, ΔexbBD, aroB') cells carrying either the plasmid encoded wild type TonB, pKP368, or
the most alanyl substituted derivative of set one, pKP559 (Ala12,15,21-27) (Figure 37). The amount
of iron transported by chromosomal wild type TonB during crosstalk with TolQ/TolR was
significantly less than the amount of iron the TonB derivatives were able to transport in 2
minutes using ExbB/ExbD as the energy-harvesting complex (Figure 31), demonstrating the low
efficiency of crosstalk. After 10 minutes, RA1020 cells transported iron to less than 700 CPM,
having an overall iron transport rate of 40.375 CPM/minutes (Figure 37, Table 13). There was a
significant difference in transport between the wild type TonB of pKP368 and the chromosomal
wild type, with pKP368 transporting iron at only 41.5% that of chromosomal. The transport rate
of the pKP559 TonB derivative was nearly undetectable, transporting iron at only 1.2% of
chromosomal (Figure 37, Table 13a). When comparing the transport rates from the plasmids, the
TonB derivative pKP559 transport rate was only 3% of that of the plasmid-encoded wild type
TonB (Table 13b). Based on the pilot assay, testing of the remaining derivatives could be used
to evaluate crosstalk of the various alanyl TonB derivatives. But based on research by Karlsson
et al. (1993b) another, more direct, approach of making chimeric TolA/TonB proteins would
utilized.
Figure 37: The transport of $^{55}$Fe-ferrichrome during crosstalk using only the TolQ/TolR energy-harvesting complex. Chromosomal wild type RA1020 ($\Delta exbBD$, $aroB^{-}$), or RA1010 ($\Delta tonB$, $\Delta exbBD$, $aroB^{-}$) cells carrying either the wild type TonB (pKP368) or the alanyl 12-15, 21-27 TonB derivative (pKP559) were evaluated for their ability to uptake the radioactive iron-siderophore complex ($^{55}$Fe-ferrichrome), which was measured using liquid scintillation as described in the Material and Methods. Graphed is the average of three experiments for the 2 minute through 10 minute time point samples.
### Table 13a: Transport rates for iron during crosstalk compared to chromosomal TonB

<table>
<thead>
<tr>
<th>Plasmid/Strain</th>
<th>CPM/Minute (Slope)</th>
<th>Iron Transport Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKP477 in RA1020</td>
<td>40.375</td>
<td>100%</td>
</tr>
<tr>
<td>pKP368 in RA1010</td>
<td>16.75</td>
<td>41.5%</td>
</tr>
<tr>
<td>pKP559 in RA1010</td>
<td>0.5</td>
<td>1.2%</td>
</tr>
</tbody>
</table>

### Table 13b: Transport rates for iron for crosstalk compared to plasmid induced wild type TonB

<table>
<thead>
<tr>
<th>Plasmid/Strain</th>
<th>CPM/Minute (Slope)</th>
<th>Iron Transport Rate$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKP368 in RA1010</td>
<td>16.75</td>
<td>100%</td>
</tr>
<tr>
<td>pKP559 in RA1010</td>
<td>0.5</td>
<td>3.0%</td>
</tr>
</tbody>
</table>
DISCUSSION

The Ser16 and His20 residues are important for TonB activity (Larsen et al., 1994; Larsen et al., 1999; Larsen & Postle, 2001) as are the Ser18 and His22 residues for TolA activity (Germon et al., 1998). The purpose of the research performed for this dissertation aim was to identify the minimal TMD components required for energization efficiency of TonB. Using colicin spot titers, irreversible phage adsorption assays, iron transport assays, in vivo formaldehyde crosslinking, and steady state half life experiments, the effect of substituting portions of the transmembrane domain of TonB with alanyl residues on energy transduction and protein/protein interactions was evaluated for various TonB derivatives. The dissertation research performed for this aim had two goals. The first goal was to determine if any of the various TonB alanyl derivatives affected the function of the protein when interacting with the energy-harvesting complex ExbBD. The second goal of this aim was to determine if the various alanyl substitutions would be more efficient at utilizing TolQR via crosstalk.

The TonB TMD derivatives bearing up to 14 alanyl substitutions retained the basic characteristic functions of TonB. This is evident in the fact that all the TonB derivatives conferred sensitivity to colicin B, both in the presence and absence of ExbBD (Figure 26, 27, 31). The two TonB derivatives containing TonB Ala12-15,17-19 substitutions (pKP558 and pKP559) were the least sensitive to colicin B, even though the TonB Ala12-15 and TonB Ala17-19 derivatives (pRA0022 and pKP441) both showed the same sensitivity as the wild type derivative (Figure 26, 27, 31). These colicin data indicate that residues 12-19 may play an important role in the uptake of colicin B, and the process is similar whether TonB is utilizing ExbBD or TolQR.

All the TonB derivatives tested in this research were able to transport iron at levels much higher than the amount observed by the chromosomal TonB in the ΔexbBD strain (Figure 26).
The TonB derivative containing the Ala$_{12-15,17-19}$ substitution was one of the least sensitive to the colicin B (Figure 26, 27); however, the iron transport rate for that derivative was only slightly less than wild type TonB (Figure 31 & Table 11). The TonB derivative that showed the most diminished activity relative to identically-expressed wild type TonB was the Ala$_{12-15}$ TonB derivative (Figure 31 & Table 11). These data imply that the remaining residues in the TMD (especially 12-15) played an undetermined role in TonB activity of iron transport.

The TonB derivatives tested in this research indicated that, for the most part, the TonB TMD derivatives engaged in the protein interactions normally detected by in vivo chemical crosslinking, forming complexes characteristic of wild type TonB (Skare et al., 1993; Higgs et al., 1998; Higgs et al., 2002). In steady-state half-life experiment and in vivo chemical crosslinking experiments, the amount of a ~ 28 kDa N-terminal degradation product relative to full-length monomeric TonB was increased for derivatives that included substitutions at residues 21-27. While the derivatives bearing the alanyl 21-27 mutation caused an increase in their degradation, those TonB derivatives still maintained relatively efficient TonB function. The increase in a degradation product by TonB derivatives with the Ala$_{21-27}$ substitution could be due to a change in the cycling of the TonB, decreasing the amount of time TonB interacts with the ExbB/ExbD complex. The ExbB and ExbD proteins have been shown to contribute to the stability of TonB (Fischer et al., 1989; Skare et al., 1993; Ahmer et al., 1995). If the residues 21-27 interfere with the proper cycling, the TonB protein could be getting “stuck” at the outer membrane during the shuttling cycle (Letain & Postle, 1997; Larsen et al., 2003b); thereby, making those derivatives more susceptible to degradation.

The apparent chemical steady state half life of most of substituted TonB derivatives was either equal or only slightly only less than that of wild type TonB (Figure 35 & Figure 36). With
the derivatives being stable, the differences in activity that were noted in this research for the
colicin spot titers as well as the iron transport data did not correspond to differences in the
relative stability of the TonB derivatives (Figure 26, 27, 31, 32 compared to Figure 35 & 37).
These findings suggest that TonB residues 12-20 are important in colicin B uptake while TonB
residues 12-15 make an undefined contribution to the TonB activity of transporting iron.
Together, these data indicate that it is the more amino portion of the TMD of TonB that is
important in efficient TonB function.

The OM processes, such as high affinity transport of iron siderophore and vitamin B_{12}, and
infection by T1, φ80, and colicins infection, are driven by the proteins TonB, ExbB and ExbD
that work to harvest and deliver cytoplasmic energy to these process (reviewed in Postle 1993,
processes are blocked by protonphores (Hancock and Braun, 1976; Reynolds et al., 1980;
Bradbeer et al., 1993) indicating the source for this energy is protonmotive force (PMF). This
energization of TonB requires the presence of ExbB/ExbD (Larsen et al., 1999) that occurs as a
heteromultimer (Braun, 1996; Higgs et al., 1998). The delivery of energy to the OM appears to
involve the physical shuttling of TonB to the OM (Letain & Postle, 1997; Larsen et al., 1999;
Higgs et al., 2002; Larsen et al., 2003b), in which TonB obtains an energized conformation
through interaction with ExbB/ExbD at the cytoplasmic membrane (Figure 5). The amino
terminus of TonB has bee shown to be essential for both TonB activity (Karlsson et al., 1993b;
Jaskula et al., 1994) and formation of TonB-ExbB complexes by in vivo chemical crosslinking
(Jaskula et al., 1994). The overexpression of a ΔSer16, ΔVal17, or ΔHis20 mutant has been
shown to produce a small amount of TonB activity, indicating that at least some TonB can achieve an active conformation regardless of the presence of those residues (Larsen et al., 2001).

The more alanyl substitutions in the TMD of TonB, the closer the TMD becomes to a generic TMD that would be representative of multiple alanyl substitutions in the TMD of TolA. Therefore, the data from this research can also be used in the evaluation of the TMD of TolA. Protein complexes for TolA/TolQ and TolQ/TolR have been shown using in vivo crosslinking (Derouiche et al., 1995, Journet et al., 1999) with the TMD of TolR involved in the TolA and TolQ interactions Journet et al., 1999). The mutagenesis of TolA revealed that Ser18, His22, and Phe26 are important for function of TolA (Germon et al., 1998). Using a helical wheel projection of the alpha helix of the transmembrane domain of TolA, Germon et al. (1998) predicted that the Ser18, His22, and Phe26 would most likely be clustered in a less hydrophobic space that would be more optimal for a protein-protein interaction with TolQ. Suppressor mutations in TolQ at Ala30Val and Ile29Ser restored function of the TolA Ser18Leu mutant, while the TolQ suppressor mutations at Gly26Asp restored function of the TolA His22Arg (Germon et al., 1998). The colicin data from this research indicate that residues 12-20 may play an important role in the uptake of colicin B, and the process is similar whether TonB is utilizing ExbBD or TolQR (Figure 26, 27, 31). Together, this data further indicates that TolA and TolQ interact with the energy-harvesting complex through their transmembrane domains.

The conserved Ser16, His20, L27, Ser31 (SHLS) motif of TonB is predicted to comprise one face of the proposed transmembrane alpha helix, as are the Ser18, His22, L29, Ser33 residues of TolA (Koebnik, 1993). It has been thought that since the conserved SHLS motif of TonB is also found in TolA that the ability of ExbB/ExbD, and subsequently TolQ/TolR during crosstalk, to interact with the TonB must occur in the TonB transmembrane. This idea was supported by data
that a TonB-ΔSer16 or TonB-ΔHis20 mutation, or the spatial relationship between the two residues, rendered TonB inactive and unable to obtain the PMF-dependent conformation (Larsen & Postle, 2001). This inactivity of the above TonB mutants was able to be restored by suppressor mutations in the predicted first transmembrane domain of ExbB (Larsen et al., 1994; Larsen et al., 1999). The ExbB suppressor mutations V36D, V35E, and A39E were all able to restore at least partial activity to the TonB mutants, ΔSer16, ΔHis20, or ΔVal (Larsen et al., 1994; Larsen et al., 1999). The use of in vivo crosslinking has revealed that TonB interacts with ExbB through transmembrane domains (Skare et al., 1993; Larsen et al., 1994; Higgs et al., 1998), and that there is interaction between TonB and ExbD (Higgs et al., 1998).

While the data from the various alanyl residues provided some insight that certain residues may contribute to TonB function and protein interaction, a more direct approach to evaluate the residues involved in the specificity of an energy-transducer with the corresponding energy-harvesting complex of that system is needed. Research in Salmonella typhimurium by Karlsson et al., (1993) involved the substitution of residues 1-32 of TonB with residues 1-34 of TolA, which produced a chimeric protein that was able to utilize TolQ/TolR better than ExbB/ExbD in the transport of φ80 (Karlsson et al., 1993b). That research further demonstrates the importance of the amino terminus of the energy-transducers for the interactions with the energy-harvesting complexes. Yet to date, the bulk of the research regarding the interactions of the energy transducer has largely focused on the predicted transmembrane domain of TonB (residues 12-32) and TolA (residues 14-34), or included the entire amino-terminus (residues 1-32 and 1-134, respectively). In the evaluation of energy-transducer/energy-harvesting complex interaction, the role of the extreme amino-terminal (residues 1-11 in TonB and residues 1-13 in TolA) has not been directly evaluated. Based on the experiment by Karlsson et al., (1993b) the identification
of contribution of “non-essential” residues in the amino-terminus of the energy-transducers could better be evaluated by the creation of chimeric TonB and TolA proteins. The construction of the various chimeric proteins (TolA\textsubscript{1-34}/TonB\textsubscript{33-239}; TolA\textsubscript{1-13}/TonB\textsubscript{12-239}; and TonB\textsubscript{1-11}/TolA\textsubscript{14-34}/TonB\textsubscript{33-239}) will also allow for the direct evaluation certain residues, including the role of the very amino terminal portion of TonB (residues 1-11) and TolA (residues 1-13) as well as the residues of the predicted transmembrane domain. The building of these TonB/TolA chimeric proteins is described in detail in Appendix A and their importance in understanding protein/protein interactions of the protein interactions of the TonB and TolA systems is discussed in chapter four.
CHAPTER IV
SUMMARY AND GENERAL CONCLUSIONS

In the gram-negative bacterium *Escherichia coli*, there are two known systems involved in the transfer of energy from the cytoplasmic membrane (CM) to the outer membrane (OM), the TonB and TolA systems. The TonB system (TonB, ExbB, ExbD) and the TolA system (TolA, TolQ, TolR) are both consist of a cytoplasmic membrane energy harvesting complex and an energy-transducer. The energy-harvesting complex of the TonB system is comprised of the ExbB and ExbD proteins, with the energy-harvesting complex of the TolA system is comprised of the TolQ and TolR proteins; and the transducers of energy in the systems are TonB and TolA, respectively. This dissertation encompasses projects that were designed to examine the physical interactions of the proteins in the TolA and TonB systems, with particular interest in the less studied TolA system. A portion of this research involved the development of assays to examine the physical interactions of the proteins of the TolA system (Chapter II). The majority of the research involved a collaborative effort with the laboratory of Dr. Kathleen Postle in examining the roles of the “non-essential” residues in the transmembrane domain (TMD) of TonB using multiple alanyl substitutions to identify the minimal TMD components required for efficient energization of TonB (Chapter III). Based on the results from the multiple alanyl substitutions, the final portion of this research involved the construction of chimeric TolA/TonB proteins to directly examine the role of the residues in the amino-terminal and TMD regions of TonB and TolA as specific targets for recognition by the energy-harvesting complexes (Appendix A).

In Chapter II, protein capture assays and the bacteria two-hybrid system were used to investigate the interactions between the proteins of the TolA system, in particular interactions
between the TolQ and TolR proteins. The protein capture assays (Figures 18, 19, 20) were unable to clearly demonstrate interaction between TolQ/TolQ or between TolQ/TolR, even though a similar method had been used by Braun et al. (1996) to demonstrate that ExbB interacts with both ExbD and TonB. The results from the bacteria two-hybrid system (Figures 21, 23; Table 7) also did not clearly demonstrate interactions between the TolQ and TolR proteins. While an interaction involving a TolR dimer/TolQ monomer has previously been shown by in vivo crosslinking experiments by Journet et al., (1995), similar results were not demonstrated in this research. The Journet data demonstrated that residues 1 – 43 of TolA are involved in interaction with TolR, TolQ and TolA dimerization; indicating the transmembrane domain (TMD) of TolA is important interaction between proteins. Together these data indicate the importance of the transmembrane domain, and the lack of a membrane in this assay may have inhibited such interactions.

Many problems were encountered in the physical assays used in this research while using the TolQ and TolR proteins. The results from this research were in direct contrast to the crosslinking data of Journet et al., (1999) that indicated a TolR dimer/TolQ monomer and mutational experiments that indicated both TolQ/TolQ and TolQ/TolR protein interactions (Lazzaroni et al., 1995). While Braun was able to use the protein capture assay to identify interactions of ExbB with both ExbD and TonB (Braun et al., 1996), the protein capture in this research failed to show any interactions between the TolQ and TolR proteins. Unlike the yeast two-hybrid system used by Walburger et al. (2002) that was able to show protein interactions (TolA/TolB, TolA/YbgF, TolB/Pal, and TolR/TolR), the bacteria two-hybrid system used in this research was unable to clearly indicate any protein/protein interactions. While the data from Chapter II did not indicate any clear protein/protein interactions between TolQ and TolR.
proteins, the research provides indirect information about the TolQ and TolR proteins. There is indirect data from this research that supports the transmembrane requirement of TolR demonstrated by Journet et al. (1995). If the interactions of the TolA, TolQ proteins with the TolR proteins occur in the transmembrane, then the presence of a membrane may be a requirement for the appropriate protein conformation and interaction of the residues in those transmembrane domains. If such interactions require a membrane, then both the protein capture assays and the bacteria two-hybrid assays would be unable to appropriately detect protein/protein interactions. Previous experiments have revealed that tol and pal mutants are hypersensitive to drugs and detergents (Lazzaroni et al., 1999) as well as having an increase in the release of outer membrane vesicles (Bernadac et al., 1998), indicating TolA system involvement in maintaining the integrity of the outer membrane. During bacteria two-hybrid experiments the expression of TolQ and TolR from plasmids in strains already containing wild type TolQ and TolR proteins severely inhibited the ability of those cells to grow and form lawns in top agar experiments. This indirectly implied the interaction of Tol proteins, as it was unlikely that appropriate stoichiometries would be achieved and maintained, complicating interpretation. Due to their limitations and restrictions, the protein capture assay and the Bacteria 2-Hybrid systems are unlikely to provide for quantitative evaluation of interactions between proteins in the TolA and TonB systems.

The uptake of iron is very important for bacteria to survive. For E. coli, iron uptake is mainly accomplished by the retrieval of iron-siderophore complexes. This is mediated by a set of outer membrane receptors that bind iron-siderophore complexes with nanomolar affinity, allowing cells to efficiently recover iron (Postle, 1999). The uptake of these iron-siderophore complexes requires TonB-transduced energy to release these complexes from their various OM
receptors into the periplasm (Hancock & Braun, 1976; Reynolds et al., 1980; Bradbeer, 1993; Postle, 1999, Ratledge and Dover, 2000, Andrews et al., 2003). TonB is energized at the CM by the ExbB/ExbD energy-harvesting complex (Letain and Postle, 1997; Larsen & Postle, 2001) and requires the PMF from the CM (Hancock & Braun, 1976; Reynolds et al., 1980; Bradbeer, 1993). There have been many models of “energized” TonB and how that results in the transfer of energy to the OM targets from the CM. These have included: TonB as a lever that cranks/turns open the OM receptors that have bound ligand (Chang et al., 2001); a diffusible mobile messenger (Reynolds et al., 1980); TonB acting as a protein permease (Wookey, 1982) and a TonB shuttling model (Letain and Postle, 1997). All of the above models require TonB to remain associated with the CM with the exception of the shuttling model. In addition to the sucrose density gradient fractionation data (Letain and Postle, 1997), data from proteinase K resistant experiments (Larsen et al., 1999) and experiments with Oregon Green(R) 488 maleimide (Larsen et al., 2003b) reveal the extreme amino-terminal of an energized TonB conformation does become accessible in the periplasm, thus supporting the physical shuttling of TonB to the OM through interaction with ExbB/ExbD at the cytoplasmic membrane (Chapter I – Figure 5).

The proteins of the TonB and TolA systems share the same basic topology (Chapter I – Figure 4) (Braun, 1989; Hannavy et. al., 1990; Roof et al., 1991, Levengood et al., 1991; Braun & Hermann, 1993, Kampfenkel and Braun, 1993; Karlsson et al., 1993b; Vianney et al., 1994). Besides having similar topologies, the proteins in the energy-harvesting complexes share a high degree of sequence identity in their transmembrane domains. TolQ and ExbB have 38%, 68%, and 79% sequence homology among the first, second, and third TMD, respectively, while the ExbD and TolR proteins share 70% sequence identity in their TMD (Chapter I – Figure 8).
Unlike the energy-harvesting complexes, the only sequence identity TonB and TolA share is in their amino-terminal transmembrane domains, which contains the conserved S-X(3)-H-X(6)-LX(3)-S (SHLS) motif (Koebnik, 1993), which includes Ser16 and His20 in TonB and Ser18 and His22 in TolA (Chapter I – Figures 6 & 7).

The interactions between TonB/ExbB and TolA/TolQ identified using in vivo crosslinking (Derouiche et al., 1995; Higgs et al., 1998; Journet et al., 1999) were undetectable by in vivo crosslinking in the TetA1-28TonB34-239 protein (Jaskula et al., 1994), suggesting the importance of the residues of the transmembrane domain. Research has determined that the Ser-XXX-His residues in the transmembrane domains of TonB and TolA are essential to efficient function and mutations to these residues causes a substantial loss of function and loss of in vivo crosslinking (Larsen et al., 1994; Germon et al., 1998; Larsen et al., 1999; Larsen et al., 2001). Suppressor mutations in the first transmembrane domains of ExbB and TolQ have been identified that restore function for the Ser16 and His20 mutations of TonB and the Ser18 and His22 mutants of TolA, respectively, as well as to restore the ability to form complexes with in vivo crosslinking between TonB/ExbB and TolA/TolQ (Larsen et al., 1994; Germon et al., 1998). This indicates that while the Ser-XXX-His residues are important for efficient function, they are not an absolute requirement for TonB and TolA function.

In the absence of a system’s energy-harvesting complex, the energy-transducer of that system can be energized by the energy-harvesting complex from the opposite system. Thus, in the absence of ExbB/ExbD in a cell, TolQ/TolR can energize TonB; likewise, in the absence of TolQ/TolR, ExbB/ExbD can energize TolA; however, energization by such “crosstalk” is less efficient (Braun, 1989; Braun & Hermann, 1993). The shared Ser-XXX-His motif of TonB and TolA may account for the ability of these two proteins to interact with the energy-harvesting
complexes (ExbBD and TolQR) at the CM; as TonB and TolA are able to interact with the energy-harvesting complexes from the others system. Not fully understood are the minimal requirements for energization of the energy-transducers and what determines the specificity of interaction between a system’s energy-transducer and energy-harvesting complex that makes crosstalk so inefficient.

Previous research has focused on the Ser-XXX-His residues in the transmembrane domains of TonB and TolA (Larsen _et al._, 1994; Germon _et al._, 1998; Larsen _et al._, 1999; Larsen _et al._, 2001). Thus, the role of most of the remaining residues in the transmembrane domain and in the extreme amino-terminus remains unknown. A large portion of the research for this dissertation focused on the transmembrane domain of the energy-transducer TonB (Chapter III). In Chapter III, multiple alanyl substitutions in the transmembrane domain of the energy-transducer TonB revealed that these various TonB derivatives were still functional. Thus, the various TonB derivatives were still sensitive to colicin B, still able to adsorb phage, and still maintained the ability to transport iron (Figures 26-28, 30, & 31). The results of colicin experiments (Figure 26, 27, 30) indicated that residues 12-20 together might be important in the uptake of colicin B, and the process was similar whether TonB was energized by ExbBD or TolQR. The TonB derivatives with multiple alanyl substitutions were able to transport iron at near wild type levels (Figure 31 and Larsen _et al._, in preparation). In contrast, the TonB derivatives with just Ala_{12-15} substitutions or that had Ala_{17-19,21-31} substitutions (wt 12-15 residues) had significantly lower levels of iron transport relative to wild type TonB (Figure 31). The stability of most of the TonB alanyl derivatives was equal to or only slightly less than that of wild type TonB based on steady state half-life experiments (Figure 35 & 36). Only the all alanyl TonB derivative had a half life significantly different than that of wild type, yet the derivative
still had a half life of ~30 minutes (Figure 36). However, the instability of this derivative did not seem to affect function, as it maintained almost wild type levels of iron transport retained sensitivity to most group B colicins (Larsen et al., in preparation). The steady state half-life experiments did showed an increase in an ~28 kDa degradation product for those derivatives bearing alanyl 21-27 substitutions, implying residues 21-27 of TonB might be involved in the proper shuttling of TonB (Figure 35). The current shuttling model involves recycling of the TonB protein once it has delivered energy to its OM target (Chapter I – Figure 5). If the residues 21-27 are involved in the TonB being re-inserted into the cytoplasmic membrane from the periplasm for the delivery of more energy, mutations in this region of the protein might inhibit the protein from reinserting into the CM and ExbB/ExbD complex. Since previous data indicated that ExbB and ExbD contribute to the stability of the TonB protein (Fischer et al., 1989; Skare et. al., 1993; Ahmer et al., 1995), if TonB was unable to cycle back into the ExbB/ExbD complex it would explain the increase in degradation products of TonB derivatives with 21-27 alanyl substitutions. The result is that these so-thought “non-essential” residues of the TMD appear to make important contributions to efficient TonB shuttling and function. Changes in the transmembrane domain up to Ala_{12-15,17-19,21-27} of TonB did not alter the ability of the derivatives to participate in those protein interactions normally detected by in vivo chemical crosslinking (Figure 32, 33), forming complexes characteristic of wild type TonB (Skare et al., 1993; Higgs et al., 1998; Higgs et al., 2002). The in vivo crosslinking profile for the all alanyl TonB derivative was unable to identify a TonB/ExbB complex; however, other studies revealed this TonB derivative protein was more stable in the presence of ExbB/ExbD complex (Larsen et al., in preparation), suggesting a TonB/ExbB interaction that is consistent with the findings that ExbB and ExbD proteins contribute to the stability of TonB (Fischer et al., 1989; Skare et. al.,
1993; Ahmer et al., 1995). Taken together, possible roles for various groups of residues can be incorporated into our current understanding of the how TonB transduces energy (Figure 38).

The data from this research indicated that:

- TonB residues 12-20 make an undefined contribution to the TonB activity of transporting iron and colicin B uptake.
- TonB residues 21-27 affect the stability of the protein and may play a role in the efficient cycling of TonB.
- The TMD does not dictate crosstalk specificity.

Previous mutagenic research had indicated that the individual residues of the TonB transmembrane domain were not essential for function (Larsen et al., 1994; Larsen et al., 1999; Larsen et al., 2001); however, the Ser16-XXX-His20 is important for efficient TonB function. In this research, TonB proteins with multiple alanyl substitutions in the TMD were still able to transport iron at levels ranging from 30 – 75% that of wild type TonB, indicating that various groups of residues are still not essential for function. Even though the Ser-XXX-His residues are important for efficient function, they are not an absolute requirement for TonB and TolA function. What is the absolute minimum requirement for proper transduction of energy by TonB? The requirements appear may be only a few residues, and perhaps it is the residues of the very amino terminal of the TonB protein. This phenomenon of only a few residues of a protein being absolutely essential for the function of that protein has been previously demonstrated, one such example is in the lac permease of *E. coli*. Cys-scanning and site-directed mutagenesis indicates that only four residues (Glu269, Arg302, His322, and Glu325) are irreplaceable in respect to the coupling of lactose and H⁺ translocation (Dunten et al., 1993; Sahin-Toth &
Figure 38: Possible roles for residues of the transmembrane domain of TonB in energy transduction. Colicin uptake, iron transport, and steady-state stability assays indicate possible roles for the various “non-essential” residues in the transmembrane of TonB. (A.) Alanyl substitutions in the TMD that are to the amino-terminal of the His20 residue have an affect on TonB function while (B.) alanyl substitutions in the TMD that are to the carboxy-terminal of the His20 residue effect TonB stability. The decrease in stability of the TonB derivatives having alanyl substitutions in residues 21-27, may indicate the involvement of those residues in the “recycling” of TonB. (Figure modified from Postle & Kadner, 2003).
Kaback, 1993; Sahin-Toth et al., 1994a; Sahin-Toth et al., 1994b; Sahin-Toth et al., 1994c; Frillingos et al., 1994; Jung et al., 1995; Weitzman & Kaback, 1995; Frillingos & Kaback, 1996; Frillingos et al., 1997a; Frillingos et al., 1997b; Frillingos et al., 1997c; Kaback, 1997), and only two (Glu126 and Asp237) are important for the ability of lac permease to catalyze lactose influx down a concentration gradient (Frillingos et al., 1997a). The large amount of alanyl substitutions in the TMD of this research indicates that the absolute minimum requirement of residues for function in TonB may be very few.

While only six residues in the lac permease of *E. coli* are irreplaceable, about 40 residues appear to be involved in other interactions (like with the substrate) or with conformationally active helical faces of the various TMD of the protein (Kaback et al., 1997). The same may be true with the various residues of the TonB protein. It is known that Ser16 and His20 (and their relative spatial relationships) are important for efficient energization (Larsen et al., 1994; Larsen et al., 1999; Larsen et al., 2001). The data from this research indicates that the remaining “non-essential” residues in the TMD may play other “supporting” roles. While not essential for function, it appears residues 12-20 make an unidentified contribution to the TonB activity of transporting iron and colicin B uptake. Since the actual mechanism of the delivery of energy from TonB to the various OM receptors is still unknown, one possibility is that these residues could be involved in the “charged confirmation” and delivery of TonB energy to the OM receptors. These residues may not be directly involved in energization, but they may play a role in producing or stabilizing a conformation that is required for the proper transduction of energy from TonB to the OM receptors. In such a case, residues 12-20 would not be involved directly in energy transduction; however, they may be important in allowing TonB to obtain a “charged conformation” or possibly the conformation of TonB that allows it to deliver the CM energy to
the OM receptor as it goes from its “charged conformation” to its “discharged conformation” (Figure 38 – A). Further research will need to be performed to clearly determine the role of residues 12-20 in TonB function and if these residues are truly involved in the allowing TonB to obtain the proper conformation to deliver energy to the various OM receptors. These residues may not be directly involved in energization, but they may play a role in producing or stabilizing a conformation that is required for the proper transduction of energy from TonB to the OM receptors. Further research will need to be performed to clearly determine the role of residues 12-20 in TonB function and if these residues are truly involved in the allowing TonB to obtain the proper conformation to deliver energy to the various OM receptors. The other “non-essential” TonB residues 21-27 affect the stability of the protein, but seem to have little effect on the activity of the protein. Due to the instability of TonB derivatives with alanyl substitutions in residues 21-27, data from this research indicates that residues 21-27 may play a role in the efficient cycling of TonB (Figure 38 – B). In such a model, the TonB derivatives would be able to successfully deliver energy, but would not be able to “recycle” and re-enter the ExbB/ExbD energy-harvesting complex. This would make these TonB derivatives a “one shot” delivery system, and explain why they required such higher levels of L- arabinose levels (Table 12) to induce these various TonB derivatives to levels similar to that of chromosomal TonB. Other portions of the TonB protein appear to be involved in yet other interactions, not necessarily involved in energy transduction. Recent resolved crystal structures of the C-terminal portion of TonB associated with two of the OM receptors, FhuA and BtuB (Pawelek et al., 2006; Shultis et al., 2006). These data demonstrate interactions of the TonB protein with an OM receptor; however, only the carboxy-terminal portion of the TonB protein was used in the crystallizations, and therefore can not represent the interaction of energized TonB with the OM receptors.
The residues of the transmembrane domain of TonB most likely play some role in the interaction with the proteins of the energy-harvesting complexes, as a TetA<sub>1-28</sub> – TonB<sub>34-239</sub> protein was unable to form in vivo crosslinked complexes with ExbB (Jaskula <i>et al.</i>, 1994). It is important to note is that there are various reactions between residues within a protein having multiple transmembrane domains, as well as various reactions between residues in the transmembrane domains of various proteins within a membrane protein complex. One of the best studied systems regarding how proteins insert themselves into the membrane and the various membrane domains involved in that process is the alkaline phosphatase fusions to the MalF protein of the maltose transport system (Boyd <i>et al.</i>, 1987; Boyd & Beckwith, 1990; Traxler <i>et al.</i>, 1992). Those experiments determined there were several features that affect membrane topology including: the basic residues within a TMD (Boyd & Beckwith, 1990); the presence of positively charged cytoplasmic domains, with the TMD acting as export signal (Traxler <i>et al.</i>, 1992); and the cell’s protein translocation machinery (Prinz <i>et al.</i>, 1998). MalF-alkaline phosphotase fusions experiments suggested when hydrophobic segments of a protein act as a export signaling sequences, these usually result in rapid export of the following portion of the protein (Traxler <i>et al.</i>, 1992). It is important to note that crosslinking profiles for TonB derivatives pKP441, pKP453, pKP558, pKP559, and pRA0022 are all nearly identical to those complexes of the crosslinked wild type chromosomal control (Figures 32 and 33).

In interpreting data, it is important to remember that all of the proteins in the TonB system are membrane proteins and that they all must be inserted into the membrane. The manner in which this occurs not only affects that protein, but also affects all the other proteins in that protein complex with which it interacts. While in vivo crosslinking data indicated that all derivatives (with the exception of the all alanyl derivative – Larsen <i>et al.</i>, in preparation) were
able to form complexes with ExbB, TMD substitutions could have affected the rate at which the TonB protein was assembled into the CM. The rate at which the TonB protein was assembled into the CM would not necessarily affect \textit{in vivo} crosslinking; however, it could affect the ability and rate at which TonB is able to interact (and be energized) by the ExbB/ExbD protein complex.

The multiple alanyl substitution research did not address the role of the very amino terminal residues of the energy-transducers (residues 1-11 in TonB and residues 1-13 in TolA), nor did it fully address the role those residues involved in crosstalk. The fact that residues 12-20 have been show to affect the function of TonB indicates the possible importance of the more amino-terminus residues in interaction of TonB with ExbB. The interactions that occur between TonB and ExbB could reside in the very amino terminal residues, 1-20. One possible model for TonB/ExbB interaction is if the ExbB proteins form some sort of “pocket” or “container” into which a TonB protein in the appropriate confirmation “fits” nicely. When the amino terminal residues of TonB (residues 1-11) are able to interact with the ExbB protein (perhaps it’s the cytoplasmic loop), the energy-transducer protein may be able to be more easily energized. If the ExbB proteins do form some sort of “pocket” or “container” into which the TonB protein is inserted, then mutations that change the confirmation of the TMD domain of TonB would cause a loss of TonB interaction of those amino-terminal residues with the ExbB protein. The differences in crosstalk and specificity may be due to the size/shape of the energy-harvesting “container” and the appropriate “fit” of the energy-transducers. Mutations that change the confirmation of the TMD domain of TonB would cause a loss of TonB interaction with the ExbB protein. While suppressor mutations in the first transmembrane domain of ExbB and TolQ were isolated that restored function for Ser and His mutations of TonB and TolA, respectively (Larsen
et al., 1994, Germon, 1998), these suppressor mutations were not allele specific indicating that the residues mutated in ExbB and TolR were not directly interacting with the Ser and His residues nor did they indicate the interaction between the proteins lay within the transmembrane domains of those proteins. The suppressor mutations that were isolated could have been mutations that slightly changed the known protein/protein interactions of the energy-harvesting complexes (Higgs et al., 1998, Journet et al., 1999), causing a change in the size/shape of that energy-harvesting “container” that would allow the energy-transducer mutant to again fit.

Crosstalk between the TonB and TolA systems is inefficient, suggesting there may be specificity for preferred interaction of the energy-transducing protein with the energy-harvesting complex within the system versus interactions with proteins of the opposite system. Studies in the Higgins laboratory demonstrated that a TolA/TonB chimera protein of Salmonella typhimurium (amino-terminal replacement of the first 32 residues TonB with the first 34 residues of TolA = TolA_{1-34} TonB_{33-239}) had the ability to interact with the various energy-harvesting complexes using a φ80 adsorption assay (Karlsson et al., 1993b). The data suggest the derivative was able to be energized better by the TolQR complex than by the ExbBD complex. While the data was limited, the results do provide a possible role for the first 32 residues of TonB (as well as the first 34 residues of TolA). These other “non-essential” residues may actually be involved in determining the specificity of interaction of the energy-transducer with the energy-harvesting complex (Karlsson et al., 1993b).

The iron transport experiments that examined crosstalk (Chapter III – Figure 37) did not provide much information into the residues involved in proteins interactions of the energy-transducer to interact with the opposite system’s energy-harvesting complex. While the data of this research indicates possible functions for portions of the TonB transmembrane, because this
generic transmembrane of TonB could also be the generic transmembrane domain of TolA, it provides insight into the roles of the TolA protein. Based on the Karlsson et al. (1993b) TolA/TonB chimeric data, a more direct approach to further explore the role of the “non-essential” residues in the transmembrane domain in energy transduction is to create chimeric TMD of the TonB and TolA proteins. This approach could help to identify residues that contribute to the specificity of energization, as well as those involved in protein/protein interactions, especially those involved in interactions with the energy-harvesting complex. As part of this dissertation research two TolA/TonB chimeric proteins were constructed (refer to Appendix A for details of construction) as tools to further study protein interactions and function.

The first TolA/TonB chimeric protein constructed is TolA_1-34, TonB_33-239 (Appendix A – Figure 39), meaning from the amino-terminus through the transmembrane is TolA and the remainder of the protein is TonB. Once tested, this chimeric protein will provide data if indeed the remaining residues in the transmembrane domain, as well as those residues in the very amino terminus, are involved in the specificity of interaction of the energy-transducer and their energy-harvesting complex. To further determine if protein interactions are occurring among the residues of the transmembrane domain, the second chimeric protein constructed was TolA_{1-13}, TonB_{12-239} (Appendix A – Figure 39), and experiments with this chimera will provide insight into the role of the small, amino-terminal portion of the energy-transducers. Experiments to date have always included the very amino-terminus with the predicted transmembrane domains. This chimeric protein will allow the role of those first 13 residues of TolA, and subsequently the first 11 residues of TonB, to be examined. At this time, the third chimeric protein which is TonB_{1-11}, TolA_{14-34}, TonB_{33-239} (Appendix A – Figure 39), has not been constructed. This construct will
help narrow the actual residue involved in interactions. These TolA/TonB chimeric proteins will be examined for their ability to interact and be energized by the two energy-harvesting complexes. By comparing the ability of the chimeras to use either ExbBD or TolQR the importance and function of those particular residues regarding specificity for both proteins can be determined. If interaction of the energy-transducers is occurring through the remaining residues (besides the SH motif), then changing the TMD to TolA should increase the ability of that chimeric protein to utilize TolQR for energization compared to the crosstalk level of wild type TonB protein. If these chimeras are capable of efficient energization by TolQR, then refining this approach and replacing smaller amounts residues of the TMD (such as changing only 12-15) could help evaluate the function of particular TMD residues. This research will provide insight in the functional domains of TolA and aid in our understanding of crosstalk.

While TolA and TonB share a conserved S-X(3)-H-X(6)-LX(3)-S (SHLS) motif (Koebnik, 1993), this dissertation research indicates that only the Ser16-XXX-His20 portion is important for efficient TonB function. TonB mutants with alanyl substitutions at the Leu27-XXX-Ser31 residues maintained almost wild type TonB levels of function. Residues 12-15 in TonB play an undetermined role in iron transport. Colicin data also indicated the importance of residues 12-20. Together, these data indicated the more amino-terminal residues in the transmembrane domain may be important. The role of the first 11 amino residues in TonB to date has not been thoroughly evaluated; however, in light of the importance of residues 12-20 in the transmembrane domain, these residues may also contribute to TonB function. The construct of TolA/TonB chimeric proteins will provide a means to evaluate the role of these amino-terminal residues, as well as providing information about the specificity of certain residues for energization of TonB (and subsequently, TolA) by the energy-harvesting complexes.
REFERENCES


Koster, W., and Braun, V. (1990) Iron (III) hydroxamate transport of *Escherichia coli* – Restoration of iron supply by coexpression of the N-terminal and C-terminal halves of the


Riechmann, L. and Hollinger, P. (1997) The C-terminal domain of TolA is the coreceptor for
filamentous phage infection of *E. coli*. Cell **90**:351-360.


APPENDIX A

THE CONSTRUCTION OF TOLA/TONB TRANSMEMBRANE CHIMERIC PROTEINS

MATERIAL AND METHODS

Media

Bacterial strains and plasmids were maintained on Luria-Bertani (LB) medium (Sambrook et al., 1989), containing ampicillin at 100 μg ml⁻¹ or chloramphenicol at 34 μg ml⁻¹ where required and indicated. Media were also supplemented with L-arabinose as indicated to induce the plasmids. Cells for specific assays were grown on M9 minimal medium (M9 salts supplemented with 0.4% w/v glycerol, 0.8% w/v casamino acids, 40 μg ml⁻¹ tryptophan, 0.4 μg ml⁻¹ thiamine, 10 mM MgSO₄, 0.5 mM CaCl₂, and 1.85 μM iron that was provided as FeCl₃ · 6H₂O). For cloning reactions, SOC medium (2% w/v tryptone, 0.5% w/v yeast extract, 0.05% w/v NaCl, 20 mM glucose, 10 mM MgCl₂, 10 mM MgSO₄) was used as indicated.

Bacteria strains and plasmids

All bacteria are derivatives of Escherichia coli K-12 strain W3110 and the principal bacterial strains used are described in Table 14. The pKP315 plasmid was constructed by R.A. Larsen by inserting the tonB gene from the transcriptional start to past the rho-independent termination site (basepairs 302-1148) into the multiple cloning site of the pBAD18 plasmid. The cloning was performed so that tonB is controlled by the arabinose promoter, with the ribosome binding site of the tonB transcript provided (Larsen et al., 1999). The pRA0004, pRA0023, pRA0024, pRA0025 plasmids were constructed in a similar manner by inserting the gene into the multiple cloning site of the pBAD24 plasmid. All other plasmids are derivatives from pKP325 (Larsen et al., 1999), a construct in which the tonB (including its transcriptional
<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α</td>
<td>F⁻ φ80dlacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17(π-, mπ+) phoA supE44 λ⁻ thi-1 gyrA96 relA1</td>
<td>InVitrogen</td>
</tr>
<tr>
<td>W3110</td>
<td>F⁻, IN(rrnD₁, rrnE₁)</td>
<td>M. Berlyn, <em>E. coli</em> Genetic Stock Center</td>
</tr>
<tr>
<td>KP1406</td>
<td>W3110 ΔtonB::blaM, aroB::Tn10</td>
<td>Larsen <em>et al.</em>, 2003a</td>
</tr>
<tr>
<td>KP1229</td>
<td>W3110 Δ(ana-tonB-trpC)</td>
<td>P. Higgs, WSU</td>
</tr>
<tr>
<td>RA1003</td>
<td>W3110 ΔexbBD::kan (deletion of bases 585-1745 replaced with kan^ gene from pACYC184)</td>
<td>R. Larsen, Present Study</td>
</tr>
<tr>
<td>RA1010</td>
<td>W3110 ΔtonB::blaM, aroB::Tn10, ΔexbBD::kan via P1 transduction from RA1003 into KP1406</td>
<td>Present Study</td>
</tr>
<tr>
<td>RA1020</td>
<td>W3110 ΔexbBD::kan, aroB::Tn10 via P1 transduction from RA1406 into RA1003</td>
<td>Present Study</td>
</tr>
<tr>
<td>RA1023</td>
<td>W3110 aroB::Tn10 via P1 transduction from RA1406 into W3110</td>
<td>Present Study</td>
</tr>
<tr>
<td>RA1037</td>
<td>W3110 ΔtolA::kan</td>
<td>R. Larsen, Present Study</td>
</tr>
<tr>
<td>RA1048</td>
<td>W3110 aroB::Tn10, ΔtolA::kan via P1 transduction from RA1037 into RA0123</td>
<td>Present Study</td>
</tr>
<tr>
<td>RA1049</td>
<td>W3110 ΔtonB::blaM, aroB::Tn10, ΔtolA::kan via P1 transduction from RA1037 into KP1406</td>
<td>Present Study</td>
</tr>
</tbody>
</table>
start and rho-independent terminator) and the *araC* genes flanking a bidirectional *araBAD* promoter from pKP315 was cassetted into pACYC184, disrupting the tetracycline resistance gene (*tetC*). Plasmids encoding TolA/TonB chimeric proteins were generated using a modified sequence overlapping extension (SOEing) polymerase chain reaction (PCR) strategy described below. All plasmids encoding TolA/TonB chimeric proteins were confirmed by DNA sequencing. The plasmids used in this research are summarized in Table 15.

**SOEing PCR**

The SOEing PCR strategy used for the construction of the various TolA/TonB chimeric proteins was modified from methods suggested by Dr. A. Oaryce and described by Lee *et al.*, (2004). The various TolA/TonB chimeric proteins include: TolA₁₋₁₃,TonB₁₂₋₂₃₉; TolA₁₋₁₃,TonB₁₋₁₁,TolA₁₄₋₃₄,TonB₃₃₋₂₃₉ (Figure 39). The TolA₁₋₁₃,TonB₃₃₋₂₃₉; TolA₁₋₁₃,TonB₁₂₋₂₃₉ chimeric proteins were able to be constructed, and the methods for the construction of each are described below. Several attempts were made to construct the TonB₁₋₁₁,TolA₁₄₋₃₄,TonB₃₃₋₂₃₉ chimeric protein (Figure 39); however, to date the construction of this chimeric protein has not been achieved. The sequence alignment of the amino terminal portion of TonB and TolA protein are shown in Figure 40. The basic strategy for the construction of the TolA₁₋₁₃,TonB₃₃₋₂₃₉ and TolA₁₋₁₃,TonB₁₂₋₂₃₉ chimeric proteins is illustrated in Figure 41 and includes two primary polymerase chain reactions and a ligation polymerase chain reaction. Each primary PCR includes the use of an external flanking primary and a SOEing primer that use either the *tolA* or *tonB* genes as a template (Figure 41 – A and B). The SOEing primers were designed to contain an overhang of 9 residues (dashed part of SOEing primer in Figure 41 – B) that is homologous to the opposite gene that is being amplified. The two primary polymerase chain reactions produce a “front” and “back” amplimer containing homologous overlapping regions...
Table 15: Plasmids used in the construction of TolA/TonB chimeric proteins

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Characteristics of Plasmid</th>
<th>Antibiotic Marker</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRA0004</td>
<td>pBAD24 derivative – Encodes wild type <em>tolA</em>, <em>araC</em> regulated by pBAD promoter</td>
<td>Amp</td>
<td>R. Larsen, BGSU</td>
</tr>
<tr>
<td>pKP315</td>
<td>pBAD18 derivative – Encodes wild type <em>tonB</em>, <em>araC</em> regulated by pBAD promoter</td>
<td>Amp</td>
<td>Larsen <em>et al.</em>, 1999</td>
</tr>
<tr>
<td>pKP325</td>
<td>Encodes wild type <em>tonB, araC</em> regulated by pBAD promoter</td>
<td>Cam</td>
<td>Larsen <em>et al.</em>, 2001</td>
</tr>
<tr>
<td>pBAD24</td>
<td>AraBAD promoter, <em>araC</em></td>
<td>Amp</td>
<td>Guzman <em>et al.</em>, 1995</td>
</tr>
<tr>
<td>pKP368</td>
<td>Encodes wild type <em>tonB, araC</em> regulated by pBAD promoter</td>
<td>Cam</td>
<td>Larsen <em>et al.</em>, 2001</td>
</tr>
<tr>
<td>pRA0026</td>
<td>pKP368 derivative – Encodes <em>tolA</em>1-34/<em>tonB</em>33-239 derivative, <em>araC</em> regulated by pBAD promoter</td>
<td>Cam</td>
<td>Present Study</td>
</tr>
<tr>
<td>pKP477</td>
<td>pKP325 Δ<em>tonB</em>, religated with BamHI sites, so still has <em>araC</em> promoter with no <em>tonB</em></td>
<td>Cam</td>
<td>J. Ghosh, WSU</td>
</tr>
</tbody>
</table>
Figure 39: Illustration of the various TolA/TonB transmembrane chimeric proteins. To further investigate the role of the residues in the transmembrane and amino-terminal domains of (A) the TonB and TolA (Bold) proteins, three TolA/TonB transmembrane chimeric proteins were designed. (B) Figures representing the three TolA/TonB transmembrane chimeric proteins including numbers that correspond to the individual nucleotides as well as the numbers that correspond to the amino acids (in parenthesis). Sequences for TonB and TolA are found in Figure 40.
Figure 40: Sequence alignment of the TonB and TolA protein. TolA (Bold) sequences are aligned to the predicted transmembrane domain (residues 12-32) of TonB that is highlighted. The conserved SHLS motif is indicated by rectangles. (A) The alignment of the first 50 amino acid residues of TonB and 52 amino acid residues of TolA. (B) The alignment of coding sequence of TonB and TolA, with the corresponding amino acids. The numbers correspond to the amino acid residue. Underlined and italicized is the FspI restriction site used in the construction of pRA0027. Sequences obtained from UnitProtKB/Swiss-Prot entries.
**Figure 41: SOEing PCR strategy for construction of TolA/TonB transmembrane chimeric proteins.** The TolA<sub>1-34</sub>, TonB<sub>33-239</sub> and TolA<sub>1-13</sub>, TonB<sub>12-239</sub> were constructed using the modified SOEing PCR strategy<sup>a</sup> illustrated in this figure and described in the Material and Methods. Two primary polymerase chain reactions are performed (B) to amplify portions of the two genes using a SOEing primer designed with a 9 residue overhang (dashed line). Amplified products are purified and used in the ligation PCR in which (D) the overlapping sequences are allowed to anneal and elongate in the absence of primers. (E) Adding external primers allows for amplification of the final chimeric PCR product (F).

<sup>a</sup>Figure and method for SOEing PCR are modified from Lee et al., 2004.
The front and back amplimers are purified and using equivalent molar ratios are then used in a ligation PCR (Figure 41 – D, E). Several cycles of PCR are run in the absence of primers but the presence of the polymerase, in order for the overlapping sequence in the two amplimers to anneal and then be elongated (Figure 41 – D). After several rounds, the two flanking primers were added allowing for amplification of the full chimeric gene (Figure 41 – F). Our major modification to previously described approaches was to use a new set of flanking primers, complementary to sites internal to the initial flanking primers, yet still outside the gene. This modification greatly enhanced the yield of chimeric amplimer, facilitating subsequent cloning steps.

Construction of $\text{TolA}_{1-33}/\text{TonB}_{32-239}$ chimeric protein

To generate the front amplimer, a portion of the $\text{tolA}$ gene was amplified by a modified polymerase chain reaction from a pRA0004 template using the SOEing primer oRA0186SOE (Table 16) as well as with the standard flanking primer oKP176 (Table 16 – Larsen et al., 2001) to generate a 551 bp product. To generate the back amplimer, a portion of the $\text{tonB}$ gene was amplified by a modified polymerase chain reaction from a pKP315 template using the SOEing primer oRA0185SOE (Table 16) as well as with the standard flanking primer oKP177 (Table 16 – Larsen et al., 2001) to generate an 835 bp product. The primary polymerase chain reactions used 50 pmol of primers (oRA0186SOE and oKP176 for $\text{tolA}$ amplification; oRA0185 and oKP177 for $\text{tonB}$ amplification) with 200 pmoles of each dNTP, and 2 units of Deep Vent DNA polymerase, in a 1X ThermoPol Reaction buffer (20mM Tris-HCl [pH 8.8 @ 25°C], 10 mM KCl, 10 mM (NH$_4$)$_2$SO$_4$, 2 mM MgSO$_4$, 0.1% Triton X-100) containing additional amounts (0, 1 mM, or 2 mM) of MgSO$_4$. Reaction volumes of 50 μl were subjected to 35 cycles of melting
### Table 16: Primers used in PCR reactions in the construction of TolA/TonB chimeric proteins

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>oRA0185SOE</td>
<td>5’agttcgttcgtacggttatgac3’</td>
</tr>
<tr>
<td>oRA0186 SOE</td>
<td>5’aacctgatgaacgaactccagac3’</td>
</tr>
<tr>
<td>oRA0187 SOE</td>
<td>5’ctcaagccgccgacttttggtc3’</td>
</tr>
<tr>
<td>oRA0188 SOE</td>
<td>5’taacgtcggccgcttgagaactgtg3’</td>
</tr>
<tr>
<td>oRA0192 SOE</td>
<td>5’ttatgcgcaggtggaag3’</td>
</tr>
<tr>
<td>oRA0193 SOE</td>
<td>5’cccctgaggccgaatt3’</td>
</tr>
<tr>
<td>oRA0184</td>
<td>5’acggcgtcacactttgc3’</td>
</tr>
<tr>
<td>oKP175</td>
<td>5’cgactctagaggatccc3’ 1341 1325</td>
</tr>
<tr>
<td>oKP176</td>
<td>5’tcgagataaccgcttggc3’ 877 893</td>
</tr>
<tr>
<td>oKP177</td>
<td>5’atcagaccgctttgtgcg3’ 1433 1416</td>
</tr>
</tbody>
</table>

Sequence of the primers used in PCR reactions. The bold nucleotides represent the nucleotides that correspond to those of the *tolA* gene. Below the primer sequence are numbers that correspond to the numeric amino acid sequence of the TonB and/or TolA protein (oRA0185, oRA0186, oRA0187, oRA0188, oRA0192, oRA0193) or the numbers that correspond to the residue sequence of the pBAD24 plasmid (oKP175, oKP176, oKP177, and oRA0184).
(94°C for 30 seconds), annealing (60°C for 30 seconds), and extension (72°C for 70 seconds).

Reactions were evaluated by electrophoresis in a 1% agarose gel. The remaining PCR samples of the reaction that amplified the most of the predicted bp products (551 for the front amplimer and 835 for the back amplimer) were resolved in a 1% agarose gel and the appropriate size bands were excised and purified using the Qiaquick gel extraction kit (Qiagen). The purified front and back amplimers were evaluated by electrophoresis in a 1% agarose gel and using equivalent molar ratios of front and back amplimers, and ligation polymerase chain reactions were performed. The ligation polymerase chain reactions initiated using ~0.25 pmoles of front and back amplimers, with 200 pmoles of each dNTP, and 2 units of Deep Vent DNA polymerase, in a 1X ThermoPol Reaction buffer additional amounts with additional amounts (0, 1 mM, or 2 mM) of MgSO₄. Reaction volumes of 48 μl were subjected to 3 cycles of melting (94°C for 30 seconds), annealing (60°C for 150 seconds), and extension (72°C for 150 seconds). At the end of the third cycle, the PCR thermocycler was paused once it had reached 94°C for the fourth cycle and 50 pmol of the internal flanking primers, oRA0184 and oKP175 (Table 16) were added. Samples were then subjected to 32 cycles of melting (94°C for 30 seconds), annealing (60°C for 30 seconds), and extension (72°C for 90 seconds). Using pKP315, an additional PCR reaction was performed in the same manner as the ligation polymerase chain reactions. Reactions were evaluated by electrophoresis in 1% agarose gels for the predicted 963 bp product. The ligation reaction with no additional MgSO₄ added successfully amplified the predicted 963 bp product with the fewest amounts of by-products, this and the pKP315 polymerase chain reactions were purified using the Qiaquick PCR purification kit (Qiagen). To confirm that the chimeric gene had been made, restriction mapping was performed using RsaI. Because there is a RsaI restriction site in the amino-terminal portion of the tonB gene if the $tola_{1-33}/tonB_{32-239}$ gene was
produced, that site would no longer exist and the chimeric gene would yield different restriction fragments than the wild type \textit{tonB} gene from pKP315. Once verified, the purified 963 bp TolA$_{1-33}$/TonB$_{32-239}$ amplimer was digested with \textit{BamHI} at 37$^\circ$C for 135 minutes, then resolved on a 1\% agarose gel and the band migrating at \textasciitilde900 bp excised and recovered using the Qiaquick gel extraction kit (Qiagen). Several attempts were made to insert this \textasciitilde900 bp digested fragment into pKP477, pKP325 and pKP368 (Table 16), that had been previously digested with \textit{BamHI} at 37$^\circ$C for 195 minutes and dephosphorylated by incubating with shrimp alkaline phosphatase (SAP) for 15 minutes at 37$^\circ$C (with subsequent inactivation of the SAP by incubating at 70$^\circ$C for 10 minutes). Standard ligation reactions (total volume of 20 \(\mu\)l) containing various combinations of the digested/dephosphorylated pKP325, digested \textit{tolA$_{1-33}$/tonB$_{32-239}$} fragment, and 20 units of T4 ligase, in T4 ligase buffer (50 mM Tris-\(\text{HCl}\) [pH 7.5], 10 mM MgCl$_2$, 10 mM dithiothreitol, 1 mM Adenosine 5\(^\prime\)-triphosphate, 25 \(\mu\)g/ml bovine serum albumin) were incubated at room temperature (~25$^\circ$C) for overnight. Ligation products were recovered by transformation with 2 \(\mu\)l of the ligation reactions added to 50 \(\mu\)l of electro-competent DH5-\(\alpha\) cells in a 0.1 cm electroporation cuvettes and pulsed in an MicroPulser\textsuperscript{TM} Electroporation Apparatus (from BIORAD) using the “Ec1” setting. Cells were recovered in 900 \(\mu\)l SOC medium and transferred to sterile 12 ml culture tubes. Following one hour of incubation at 37$^\circ$C with aeration, transformants were selected by plating 100 \(\mu\)l of recovered cells on LB agar plates supplemented with chloramphenicol at 34 \(\mu\)g ml$^{-1}$, and incubated overnight at 37$^\circ$C. Ligation reactions were also transformed by incubating 2 \(\mu\)l of ligation reactions with 40 \(\mu\)l of chemically competent DH5-\(\alpha\) cells (from InVitrogen) for 30 minutes, heat shocking at 42$^\circ$C for 90 seconds, returning to 4$^\circ$C for 2 minutes, then adding 900 \(\mu\)l SOC medium to the mixture and incubating at 37$^\circ$C for 1 hour. After the 1 hour, 100 \(\mu\)l of the transformation was plated on LB agar plates.
supplemented with chloramphenicol at 34 μg ml⁻¹, and the incubated overnight at 37°C.

Attempts were also made by R. A. Larsen to obtain a $tolA_{1-33}/tonB_{32-239}$ ligation; however, our attempts from both methods were unsuccessful in isolating any $tolA_{1-33}/tonB_{32-239}$ transformants.

Due to the several problems in successfully isolating transformants using the BamHI restriction sites, another cloning approach was used. Purified 963 bp $tolA_{1-33}/tonB_{32-239}$ amplimer was digested with EcoRI and XbaI at 37°C for 120 minutes. Digestion reactions were purified using the Qiaquick PCR purification kit (Qiagen) and then evaluated by electrophoresis in 1% agarose gels for the predicted ~820 bp product. This ~820 bp digested fragment was then inserted into pBAD24 that had been previously digested with EcoRI and XbaI at 37°C for 120 minutes and dephosphorylated by incubating with shrimp alkaline phosphatase (SAP) for 30 minutes at 37°C (with subsequent inactivation of the SAP by incubating at 70°C for 10 minutes).

Standard ligation reactions (total volume of 20 μl) containing various combinations of the digested/dephosphorylated pBAD24, digested $tolA_{1-33}/tonB_{32-239}$ fragment with 20 units of T4 ligase, in T4 ligase buffer (as described above) were incubated at room temperature (~25°C) for overnight. The ligation reactions were transformed by incubating 2 μl of ligation reactions with 40 μl of chemically competent DH5-α cells (from Invitrogen) for 30 minutes, heat shocking at 42°C for 90 seconds, returning to 4°C for 2 minutes, then adding 900 μl SOC medium to the mixture and incubating at 37°C for 1 hour. After the 1 hour, 100 μl of the transformation was plated on LB agar plates supplemented with ampicillin at 100 μg ml⁻¹, and the incubated overnight at 37°C. Eighteen of the colonies obtained in this manner were then screened by growing the colonies overnight in LB containing ampicillin at 100 μg ml⁻¹, taking 1.5 ml of that culture and isolating plasmid DNA by the alkaline lysis method (Sambrook et al., 1989), with recovery of the plasmids in 50 μl of ddH₂O. Using 10 μl of those plasmid preps, digestions with
BamHI were performed to determine which plasmids contained the tolA<sub>1-33</sub>/tonB<sub>32-239</sub> insert (the proper size is a 900 bp fragment). Twelve isolates were confirmed to have the insert and cells containing those plasmids were streaked for isolation and were maintained on LB with ampicillin at 100 μg ml<sup>-1</sup>. The twelve plasmids with the tolA<sub>1-33</sub>/tonB<sub>32-239</sub> insert were transformed into KP1229 cells using TSS transformation (Chung et al., 1989) to verify they could be induced. Overnight cultures of KP1229 cells bearing one of the 12 isolate plasmids were diluted 1:100 into 5 ml of fresh LB supplemented with ampicillin at 100 μg ml<sup>-1</sup>, and in the absence (uninduced) or presence of 0.001% L-arabinose (induced) and grown to an A<sub>550</sub> = 0.4 at which time 500 μl samples were removed and precipitated by adding 500 μl of 20% w/v trichloroacetic acid (TCA). Mixtures were incubated at 4°C for 15 minutes, centrifuged for 5 minutes at ~13,000 x g, and the supernatants removed. Pellets were washed with 1 ml of 80% acetone, centrifuged for 5 minutes at ~13,000 x g, and the supernatants removed. Pellets were suspended in 25 μl 1 M Tris-HCl, pH 8.0 and 25 μl of 2X Laemmli sample buffer (LSB) (Laemmli, 1970) and the samples incubated at 97°C for 5 minutes. The TCA precipitated samples were subjected to electrophoresis on 11% SDS-PA gels. Resolved proteins were electrotransferred to Immunobilon-P polyvinylindene fluoride (PVDF) membranes, and Immunoblot analyses were performed as described previously described in Chapter III using TonB-specific monoclonal antibodies and enhanced chemiluminescence (ECL). All twelve isolates were able to be induced when compared to the pBAD vector and pKP315, the 5 second exposure for four of those isolates is shown in Figure 42. Three of the isolates that were able to be induced from Figure 42 were chosen and the identity of these plasmids was confirmed by sequence determination (by contract with the University of Iowa DNA Facility). One plasmid encoding the TolA<sub>1-33</sub>/TonB<sub>32-239</sub> chimeric protein was chosen and named pRA0023.
Figure 42: Verification the transformants encoding the TolA_{1-34}, TonB_{33-239} chimeric protein are inducible. Expression analysis of the TolA/TonB chimeric protein was performed (as described in Material and Methods) on KP1229 cells harboring either (1 & 2) pBAD24; (3 & 4) pKP315; (5 & 6) isolate #4; (7 & 8) isolate #7, (9 & 10) isolate #11, or (11 & 12) isolate #14. Cells were grown to an A_{550} = 0.4 either in the absence (lanes 1, 3, 5, 7, 9, and 11) or in the presence (lanes 2, 4, 6, 8, 10, and 12) of 0.001% L-arabinose and evaluated using mTonB immunoblot analysis. Following immunoblot analysis, the membrane was stained with Coomassie Blue to ensure protein loads were equivalent (data not shown).
While the method described above solved the problems encountered in the attempts to recover ligated clones with the *tolA/tonB* gene using the *BamHI* restriction sites, the chimeric gene was recovered in a pBAD vector. In order to truly evaluate and compare these TolA/TonB chimeric proteins with the various other TonB derivatives, the *tolA/tonB* gene needed to be cloned into pKP325. Purified pKP368 and pRA0023 plasmids were both digested with *BstEII* at 60°C for 60 minutes then resolved on a 1% agarose gel and the band migrating at ~694 bp from the pRA0023 digestion was excised and recovered using the Qiaquick gel extraction kit (Qiagen), as well as the band corresponding to the digested pKP368. The digested pKP368 plasmid was then dephosphorylated as described above and standard ligation reactions (total volume of 20 μl) containing various combinations of the digested/dephosphorylated pKP368 plasmids and digested *tolA*<sub>1-33</sub>*/tonB*<sub>32-239</sub> fragment with 20 units of T4 ligase, in T4 ligase buffer (as described above) were incubated at room temperature (~25°C) for overnight. Ligation reactions were ethanol precipitated and then suspended in 20 μl of ddH2O. Ligation products were recovered by transformation with 2 μl of the ligation reactions added to 50 μl of electro-competent DH5-α cells and electroporated as described above. Transformants were selected by plating 100 μl of recovered cells on LB agar plates supplemented with chloramphenicol at 34 μg ml<sup>-1</sup> and incubated overnight at 37°C.

The five colonies obtained in this manner were then screened by growing the colonies overnight in LB containing chloramphenicol at 34 μg ml<sup>-1</sup>, taking 1.5 ml of that culture and isolating plasmid DNA by the alkaline lysis method (Sambrook *et al.*, 1989), with recovery of the plasmids in 50 μl of ddH2O. Using 10 μl of those plasmid preps, digestions with *BamHI* were performed to determine which plasmids contained the *tolA*<sub>1-33</sub>*/tonB*<sub>32-239</sub> insert (the proper size is a 900 bp fragment). Four contained the appropriate predicted 900 bp fragment.
Additional plasmid was digested and resolved as above, then recovered using the Qiaquick gel extraction kit (Qiagen) for further restriction mapping. The purified fragments were digested with Rsal and evaluated by electrophoresis in 1% agarose gels for the predicted products. One plasmid encoding the TolA$_{1-33}$/TonB$_{32-239}$ chimeric protein was chosen and named pRA0026.

**Construction of TolA$_{1-13}$/TonB$_{12-239}$ chimeric protein**

The SOEing PCR reactions and recovery into pBAD vector was performed as described above with the following exceptions. The front amplimer (a 488 bp product) and back amplimer (a 898 bp product) were generated using the primers, oRA0188SOE and oKP176 for tolA amplification; and oRA0187 and oKP177 for tonB amplification. Reactions were evaluated, and the appropriate size bands were excised and purified using the Qiaquick gel extraction kit (Qiagen). The purified front and back amplimers were evaluated by electrophoresis in a 1% agarose gel and using equivalent molar ratios of front and back amplimers (~0.08 pmoles) ligation polymerase chain reactions were performed as described above. Reactions were evaluated by electrophoresis in 1% agarose gel for the predicted 963 bp product. The ligation reaction containing the predicted 963 bp product and the fewest amounts of by-products was purified using the Qiaquick PCR purification kit (Qiagen), and digested with EcoRI and XbaI as described above. This digested TolA$_{1-13}$/TonB$_{12-239}$ fragment was ligated into digested/dephosphorylated pBAD24 as described above. The ligation reactions were transformed into chemically competent DH5-α cells and eighteen of the colonies obtained in this manner were then screened by growing the colonies overnight in LB containing ampicillin at 100 µg ml$^{-1}$, taking 1.5 ml of that culture and isolating plasmid DNA by the alkaline lysis method (Sambrook et al., 1989), with recovery of the plasmids in 50 µl of ddH$_2$O. The BamHI digestion
revealed that twelve isolates have the insert and cells containing those plasmids were streaked for isolation and were maintained on LB with ampicillin at 100 μg ml⁻¹.

The twelve plasmids with the \textit{tolA}_{1-13}/\textit{tonB}_{12-239} insert were transformed into KP1229 cells using TSS transformation (Chung \textit{et al}., 1989) and their ability to be induced was verified using Immunoblot analyses as previously described. Three of the isolates that were able to be induced were chosen and the identities of these plasmids were confirmed by sequence determination (by contract with the University of Iowa DNA Facility). Sequence analysis revealed that one of the plasmids encoding the TolA\textsubscript{1,13}/TonB\textsubscript{12,239} chimeric protein carried a Gln107Arg mutation, this plasmid was named pRA0024. The sequence analysis also revealed one of the plasmids encoding the TolA\textsubscript{1,13}/TonB\textsubscript{12,239} chimeric protein contained Ser46Ala and Glu74Lys mutations, this plasmid was named pRA0025. Additional attempts to isolate error free transformants were unsuccessful; as sequence analysis revealed another plasmid encoding the TolA\textsubscript{1,13}/TonB\textsubscript{12,239} chimeric protein had a Gln107Arg mutation. Since it appears this mutation may have possibly occurred in an early round of PCR, meaning several of the isolates could all contain the Gln107Arg mutation, another approach was used to construct the TolA\textsubscript{1,13}/TonB\textsubscript{12,239} chimeric protein.

The Gln107Arg mutation in the TolA\textsubscript{1,13}/TonB\textsubscript{12,239} chimeric protein was the result of nucleotide changes at positions 655, 656, and 657 in the \textit{tonB} gene portion of the chimeric protein. The wild type TonB contains a \textit{FspI} restriction site at position 461, providing an opportunity to “ligate” together an error-free TolA\textsubscript{1,13}/TonB\textsubscript{12,239} chimeric protein. Purified pRA0024 plasmid was digested with \textit{BstEII} at 60°C for 60 minutes then resolved on a 1% agarose gel and the band migrating at ~694 bp was excised and recovered using the Qiaquick gel
extraction kit (Qiagen). The purified ~694 bp fragment was then subject to digestion using FspI for 37°C for 120 minutes.

Digestion reactions were evaluated by electrophoresis in 1.5% agarose gel. The predicted front fragment (~384 bp product) and back fragment (~310 bp) were excised and recovered using the Qiaquick gel extraction kit (Qiagen). The two purified fragments were then inserted into pKP368 that had been previously digested with FspI at 37°C for 120 minutes and dephosphorylated by incubating with SAP as described above. Standard ligation reactions (total volume of 20 μl) containing various combinations of the digested/dephosphorylated pKP368 plasmid, the digested front tolA<sub>1-13</sub>/tonB<sub>12-239</sub> fragment (~384 bp product), the digested back tolA<sub>1-13</sub>/tonB<sub>12-239</sub> fragment (~310 bp) and 20 units of T4 ligase, in T4 ligase buffer were incubated at room temperature (~25°C) for overnight.

The ligation reactions were transformed by incubating 2 μl of ligation reactions with 40 μl of chemically competent DH5-α cells (from InVitrogen) for 30 minutes, heat shocking at 42°C for 90 seconds, returning to 4°C for 2 minutes, then adding 900 μl SOC medium to the mixture and incubating at 37°C for 1 hour. After the 1 hour, 100 μl of the transformation was plated on LB agar plates supplemented with chloramphenicol at 34 μg ml<sup>-1</sup>, and then incubated overnight at 37°C. The eight colonies obtained in this manner were then screened by growing the colonies overnight in LB supplemented with chloramphenicol at 34 μg ml<sup>-1</sup>, taking 1.5 ml of that culture and isolating plasmid DNA by the alkaline lysis method (Sambrook<em> et al.</em>, 1989), with recovery of the plasmids in 50 μl of ddH<sub>2</sub>O. Using 10 μl of those plasmid preps, digestions with BamHI were performed to determine which plasmids contained the tolA<sub>1-13</sub>/tonB<sub>12-239</sub> insert (the proper size is a 900 bp fragment). One isolate was confirmed to have the insert in the proper orientation and cells containing those plasmids were streaked for isolation and were maintained
on LB supplemented with chloramphenicol at 34 μg ml⁻¹. Further restriction mapping on that isolate was performed by digesting with EcoRI to verify the presence of the front portion of the \textit{tola} gene. That plasmid produced bands corresponding to those predicted for a \textit{tola}_1.13/\textit{tonB}_12-239 insert, but not wild type \textit{tonB}. The identity of that plasmid was confirmed by sequence determination (by contract with the University of Iowa DNA Facility). That plasmid encoding the TolA\textsubscript{1-13}/TonB\textsubscript{12-239} chimeric protein was named pRA0027.

\textit{Attempted construction of TonB\textsubscript{1-11}/TolA\textsubscript{14-33}/TonB\textsubscript{32-239} chimeric protein}

Attempts have been made to generate the front and back amplimers required for the SOEing PCR. Amplifying the front portion of the \textit{tonB} gene from a pKP315 template using the SOEing primer oRA0193SOE (Table 16) with the standard flanking primer oKP176 (Table 16) to generate a 486 bp product or amplifying the TolA\textsubscript{14-33}/TonB\textsubscript{32-239} gene from pRA0026 using the SOEing primer oRA0192SOE (Table 16) with the standard flanking primer oKP177 (Table 16) to generate a 900 bp product have been unsuccessful. Changes to the protocol used in the construction of TolA\textsubscript{1-33}/TonB\textsubscript{32-239} chimeric protein and TolA\textsubscript{1-13}/TonB\textsubscript{12-239} chimeric protein include using various melting temperatures and times as well as various annealing temperatures and times have not increased the amount of PCR product produced. The primers that were designed for the amplification in the primary polymerase chain reactions, oRA0192SOE and oRA0193SOE (Table 16), are shorter than the other SOEing primers. These two primers have only a 7 residue overhang, compared to the 9 residue overhangs of the other SOEing primers due to several sequence repeats near the overlapping portion of the two genes. The design of these primers needs to be re-evaluated with one possibility to resolve this current problem being to design and order new SOEing primers that contain longer sequences homologous to the gene being amplified.
REFERENCES


