USING CHIMERAS TO EVALUATE CROSS-TALK, ENERGY TRANSFER, AND PROTEIN-PROTEIN INTERACTIONS IN THE TONB AND TOLA SYSTEMS

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A Dissertation

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The cytoplasmic membrane (CM) protein TonB energizes transport of ferric siderophores and group B colicins across the gram-negative outer membrane, and confers sensitivity to certain bacteriophages. These functions depend upon heteromultimeric protein complexes that couple TonB to the ion electrochemical potential of the CM. This is most efficient when TonB interacts with the ExbB/ExbD complexes; however, the ExbB/ExbD paralogues TolQ/TolR can also support TonB-dependent processes, albeit less efficiently. Strains bearing specific deletions of \textit{exbB}, \textit{exbD}, \textit{tolQ}, and \textit{tolR} were generated and their ability to support TonB function determined. When only ExbB/D was present, activity mirrored that of the wild-type strain, but was diminished when only TolQ/R was present. Low activity was evident for the mixed complex ExbB/TolR, but not for the TolQ/ExbD complex. \textit{In vivo} cross-linking indicated that ExbB interacted with TonB independent of the presence of either ExbD or TolR. TonB stability is greatly decreased in the absence of the ExbB/D complex. Here, ExbB alone, or with TolR, was sufficient for the stability of TonB. TolQ alone, or with ExbD, also stabilized TonB, despite the absence of ExbB. Together, these data suggest that the ExbB/TolQ component of a given complex is sufficient for interaction with TonB, but the ability to couple TonB to the ion gradient of the membrane requires interactions between the ExbB/TolQ and ExbD/TolR components of the complex. These data suggest that instability results from activity and TonB interactions at the CM are dependent upon varying conformational states.

Like TonB, TolA is most efficient when it interacts with its heterologous energy harvesting complex (TolQ/R) and in its absence is less efficiently supported by ExbB/D.
Because TonB and TolA each have a “preferred” energy-harvesting complex, it is clear motifs not shared between TonB and TolA are involved in interactions with energy harvesting complexes. Testing two distinct TolA/TonB chimeric proteins provides a different view of how the transducers in the TolA and TonB systems interact with the energy harvesting complexes. These new data suggest that the transmembrane domains of these two transducers may not be solely responsible for energization.
This body of work is dedicated to my parents, Randy and Marian Brinkman. I love you both so much and I could not have done it without you. Thank you.
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CHAPTER I.

INTRODUCTION TO THE TONB AND TOLA SYSTEMS IN GRAM NEGATIVE BACTERIA

Gram Negative Bacteria

The cell wall of gram negative bacteria is a dual membrane system. The inner membrane, or cytoplasmic membrane (CM), is a standard phospholipid bilayer with a generous number of proteins that aid in essential cellular processes like nutrient transport and energy production (Figure 1). External to this inner membrane is an aqueous compartment (the periplasmic space) that contains the murein sacculus, which gives the cell rigidity and shape. Anchored to this murein corset is a second membrane. This outer membrane (OM) serves as a permeability barrier, excluding hydrophobic substances and hydrophilic molecules larger than about 600 Da due to an outer layer of lipid anchored, anionic oligosaccharides. Entry of small hydrophilic molecules is made possible by diffusion through aqueous channels of porin proteins. Although the OM lacks a local energy source, energy-dependent processes occur at this site. Two specific protein systems harness the ion-electrochemical potential of the CM to support these energy-dependent OM processes.

The OM is not energetically self-sufficient and the presence of porin channels prohibits a major ion gradient from being formed. Ion-electrochemical potential along the CM is directly related to the chemiosmotic hypothesis (Mitchell, 1966). By this now well-established mechanism, certain membranes can generate an electrochemical gradient by pumping protons across the membrane and then using the proton potential to do work. The proton gradient established across a membrane contains both electrical and chemical energy. The electrical energy is created by the charge difference, whereas the chemical energy is a result of the concentration disparity across the membrane for a given ionic species. When it involves protons,
Figure 1. Diagram of gram negative bacterial envelope. An illustration of the gram negative bacterial envelope, consisting of the outer membrane, made of phospholipids and lipopolysaccharides, with an interior peptidoglycan layer, the periplasm, and the cytoplasmic membrane, a phospholipid bilayer. Figure adapted from Dr. Ray Larsen.
this potential energy built up in the electrochemical gradient is known as proton motive force (pmf). The movement of protons across the membrane, done by conductors like solute transporters, ATP synthases, and flagellar motors, couples proton translocation with cellular mechanics.

In the prokaryotic cell membrane, the electron transport chain (ETC) is present, by which a series of redox reactions establishes a proton gradient. Reduced coenzymes (NADH and FADH$_2$) store metabolic energy as electrons. The transfer of these stored electrons to the protein complexes in the ETC, and ultimately to molecular oxygen, results in the transport of protons across the cytoplasmic membrane. ATP synthase can then use this proton gradient to drive ATP synthesis. This gradient is so important that, in the absence of electron transport, ATP synthase can reverse its direction and actually hydrolyze ATP to pump protons towards a higher potential, which are then used for cellular mechanics (Garrett and Grisham, 1999; White, 2000; for review see Boyer, 1997). The proton motive force across the CM in bacteria is then harnessed by translocators that comprise the TonB and Tol systems (for review see Moeck and Coulton, 1998). These two systems harness the energy from the gradient across the CM and transfer it out to the OM. In the case of the TonB system, this energy is used for active transport of specific nutrients across the OM (Frost and Rosenberg, 1975; Hantke and Braun, 1975; Bassford et al., 1976), whereas the TolA system delivers this energy to the OM to aid in OM stability (Bernadac et al., 1998) and invagination during cell division (Gerding et al., 2007).

Protonophores, by collapsing the CM pmf, can block TonB-dependent processes (Hancock and Braun, 1976; Reynolds et al., 1980; Bradbeer, 1993), as does cyanide (by binding to cytochrome $c$ oxidase) in unc strains lacking membrane-bound ATP synthase (Bradbeer, 1993). TonB also undergoes conformational changes in spheroplasts due to disruptions in the
pmf across the CM caused by protonophores or spheroplast lysis (Larsen et al., 1999). This suggests that TonB uses the proton motive force as its energy source and undergoes energy-dependent conformational changes. Likewise, TolA interaction with an OM associated protein (Pal) (Figure 2; Table 1) was also shown to be dependent on proton motive force (Cascales et al., 2000) and the transducer undergoes conformational changes when this energization is disrupted (Germon et al., 2001).

**The Energy Transduction Systems**

Each of these energy transduction systems consists of two distinct components; an energy-harvesting complex and an energy transducer protein (Figure 2). Best studied is the TonB system, where the energy harvesting complex is a heteromultimer predicted to contain 4 to 5 ExbD and 14 to 15 ExbB proteins per complex (Held and Postle, 2002; Higgs et al., 2002a) and the transducer, TonB, presumably a dimer (Sauter et al., 2003; Chang et al., 2001). All told, a single complex may be as large as 520 kDa (Held and Postle, 2002). This system couples the electrochemical gradient of the cytoplasmic membrane to TonB, which transfers it to the OM. In the other system, the energy transducer, TolA, associates with an energy harvesting complex consisting of the ExbB/D paralogues TolQ and TolR, with a presumably similar stoichiometry, although this has not been significantly examined (Cascales et al., 2001). It has also been suggested that the TolQ/R complex may mimic the sodium-dependent flagellar motor complex containing PomA and PomB (Kojima and Blair, 2001).

Energy to drive flagellar motors is coupled to electrochemical gradients through either the proton motive force or the sodium motive force (Imae and Atsumi, 1989; Manson et al., 1977). MotA and MotB, *E.coli* motor proteins, function to form a proton channel in the CM and act as a stator for the proton-driven flagella (Blair et al., 1990; Sharp et al., 1995; Stolz and Berg,
Figure 2. The two energy-harvesting and transducing complexes. On the left is the TonB system consisting of TonB, ExbB, and ExbD (Higgs et al., 2002; Held and Postle, 2002). TonB transfers energy to the iron outer membrane (OM) receptor, FepA (Buchanan et al., 1999), while docking at the OM proteins Lpp (Braun’s Lipoprotein) and OmpA (Outer membrane protein A). On the right is the TolA system consisting of TolA, TolQ, and TolR (Vianney et al., 1996). TolA interacts with TolB and Pal (Peptidoglycan associated lipoprotein) proteins at the outer membrane, which may, in turn, interact with OM receptors such as OmpF (Cowan, 1992), or maintain OM integrity. YbgC, an acyl-coA thiolesterase cytoplasmic protein, and YbgF, a periplasmic protein of unknown function, are transcribed in the same operon with the Tol proteins and are non-essential.
Table 1. Comparisons of proteins in the TonB and TolA systems

<table>
<thead>
<tr>
<th>Protein</th>
<th># a.a.</th>
<th>Mass (kDa)</th>
<th># of TMD</th>
<th>Function</th>
<th>Protein</th>
<th># a.a.</th>
<th>Mass (kDa)</th>
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<tr>
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<td>1</td>
<td>Deliver energy from CM to OM</td>
<td>TolA</td>
<td>421</td>
<td>43</td>
<td>1</td>
<td>Deliver energy from CM to OM</td>
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<tr>
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<td>1</td>
<td>Complexes with ExbB, deliver energy to TonB</td>
<td>TolR</td>
<td>142</td>
<td>16</td>
<td>1</td>
<td>Complexes with TolQ, deliver energy to TolA</td>
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Structural comparisons of the proteins in the TonB system shown in columns 2, 3, and 4 versus the proteins in the TolA system in columns 7, 8, and 9. Functional features are compared in the TonB system in column 5 versus the TolA system in column 10. Similar proteins within the two systems are located in the same row of the table, with TonB and TolA in row 2, ExbB and TolQ in row 3, and ExbD and TolR in row 4.
1991; Tang et al., 1996). Other genera, like Vibrio and Bacillus, have sodium-driven flagella with the PomA and PomB proteins most likely forming the sodium channel (Yorimitsu et al., 1999). PomA and PomB have a 20 to 30% similarity to MotA and MotB and all are integral proteins that comprise the torque generator and form the ion channel. MotA and PomA have four transmembrane domains and can be compared to the TonB/ExbB complex. MotB and PomB have one transmembrane domain and are similar to the ExbD and TolR protein (Saier Jr., 2003). The structural similarities have been used to design a structural model for a potential ExbB/ExbD/TonB cation channel (Figure 3). This model proposes proton transfer along specific residues and water molecules bound to hydroxyl side chains (Zhai et al., 2003). This model may not be correct as a recent study showed that the predicted proton pathways only functioned when water bridges between nonpolar amino acid side groups were formed (Braun and Herrmann, 2004). Even more recent data has shown possible ion channel conductance in four residues found in the TMDs of TolR and TolQ (Goemaer et al., 2007).

Rather than provide a mechanical function, the TonB system instead energizes the transport of ferric siderophores and vitamin B\textsubscript{12} across the outer membrane via specific high-affinity receptors (Frost and Rosenberg, 1975; Hantke and Braun, 1975; Bassford et al., 1976). TonB also confers sensitivity to certain bacteriophage, T1 and \phi\textsubscript{80} (Luria and Delbruck, 1943; Matsushiro, 1963), and group B colicins (Davies and Reeves, 1975), that exploit the system to traverse the OM defense (see Postle and Kadner, 2003 for review). The \textit{tonB} gene is expressed from its own promoter at 28.2 minutes on the \textit{E.coli} K-12 chromosome, whereas \textit{exbB} and \textit{exbD} are expressed together at 67.9 minutes (Berlyn, 1998) with \textit{exbB} being the promoter proximal gene in the operon (Figure 4A). As mentioned previously, \textit{exbB} and \textit{exbD} are expressed at different levels even though they are contained on a single operon, resulting in the stoichiometry
Figure 3. Similarities between the Mot complex and the TonB and TolA complexes. A proton channel is formed by the MotA/MotB complex. MotA has four transmembrane segments which correspond to the single transmembrane domain of TonB or TolA plus the three transmembrane domains of ExbB or TolQ. MotB, ExbD, and TolR have a single transmembrane domain. Figure adapted from Zhai et al., 2003.
Figure 4. Rendering of the TonB and TolA systems operons. *E. coli* K-12 chromosome. (A) *exbB* and *exbD* are expressed together under a single promoter, P1, shown left, at 67.9 minutes. *tonB* is expressed under a separate promoter, P2, at 28.2 minutes (Berlyn, 1998). (B) The Tol system proteins are transcribed from two continuous operons found at 16.7 minutes on the chromosome (Berlyn, 1998). *ybgC, tolQ, tolR*, and *tolA* are all expressed under the first promoter whereas *tolB, pal*, and *ybgF* are expressed together under a second promoter, P2, which may be redundant. Drawings adapted from Lazdunski *et al.*, 1998 and Vianney *et al.*, 1996.
necessary to create a working heteromultimer (Higgs et al., 2002a). Although E.coli only has single tonB, exbB, and exbD genes, and the standard K-12 strain makes a single siderophore called enterochelin, many other species of bacteria have multiple sets of these genes, structurally different operons, and produce more than one siderophore. Vibrio cholerae has two sets of these genes (Seliger et al., 2001), whereas Campylobacter jejuni has three sets of tonB, exbB, and exbD (Crosa et al., 2004). Transcription of tonB is regulated by iron availability and, under aerobic conditions, by the Fur protein (Young and Postle, 1994). There is also oxygen-availability regulation but it is unclear how, exactly, this is mediated. In iron-limited environments, tonB expression is increased whereas, in anaerobic environments, tonB expression is repressed by Fur and an unknown DNA-binding protein. Although this protein is uncharacterized, it is not one of the well-known anaerobic global regulators, ArcA or Fnr (Young and Postle, 1994).

The outer membrane transporters for ferric siderophores (FepA, FhuA, and FecA) and cobalamin (BtuB) of E.coli have been crystallized and share many similarities (Ferguson et al., 1998; 2002; Locher et al., 1998; Buchanan et al., 1999; Chimento et al., 2003). TonB-dependent transporters all contain a 22-stranded transmembrane β-barrel with a small number of residues at the ends of the β-strands located in the periplasm [FecA also has a periplasmically located domain (Garcia-Herrero and Vogel, 2004) involved in transmembrane signaling (Braun et al., 2006)]. Extracellularly, varying loops connect the strands and can close to block the transport channel from extraneous outside material that may try to enter the transporter with the ligand and also help to trap the ligand in the receptor (Held and Postle, 2002; Postle and Kadner, 2003). Within the transmembrane barrel is a highly conserved globular domain that acts as a hatch and
may provide specific substrate recognition. Each of these transporters has a TonB box that is a short, conserved, hydrophobic stretch of 7 residues near their amino terminals.

The TonB box is the only identified site that, when mutated, results in a loss of TonB-dependent functions while ligand binding and membrane insertion still occur (Gudmundsdottir et al., 1989). In TonB, mutations affecting the residues around amino acid 160 can restore function in TonB box mutants (Heller et al., 1988; Bell et al., 1990). Disulfide crosslinking studies have also shown that there is a strong interaction between residues 160, 162, and 163 of TonB and the TonB boxes of BtuB and FecA (Cadieux and Kadner, 1999; Ogierman and Braun, 2003). This is presumably where TonB directly interacts with these OM transporters and transfers the energy harnessed from the CM. Recently the carboxy terminus of TonB was crystallized in a complex with BtuB (Figure 5) (Shultis et al., 2006) and FhuA (Pawelek et al., 2006). Residues 147 – 239 of TonB were shown in a crystallized complex with the OM BtuB receptor and it was found that Arg_{158} of TonB forms a salt bridge with the Asp_{6} of the BtuB TonB box. This interaction, forming an anti-parallel β-sheet between the two proteins, is similar to that of TolA and the g3p phage protein (Figure 6) seen previously (Cadieux and Kadner, 1999; Lubkowsi et al., 1999). When residues 158-235 of TonB were crystallized in complex with the OM receptor FhuA, the receptor’s TonB box forms a parallel β-sheet with the carboxy terminus of TonB. In this study, it was suggested that Gln_{160} of TonB forms a hydrogen bond with Thr_{12} in the FhuA TonB box. There was also evidence of an electrostatic interaction between TonB Arg_{166} and the FhuA hatch residue Glu_{56} (Pawelek et al., 2006). It is believed that ligand binding triggers a series of conformational changes in the OM receptor. The TonB box, normally hidden within the barrel of the empty receptor, extends into the periplasm where it is able to contact the carboxy terminal of TonB (Moeck et al., 1996; Locher et al., 1998; Buchanan et al., 1999).
Figure 5. Crystallized images of TolA with g3p and TonB with BtuB. Structural convergence in TolA and TonB. (A) Structure of the C-terminal domain of TolA and the g3p phage protein (Cadieux and Kadner, 1999). TolA is in yellow, the β-strand of g3p that forms an anti-parallel β-sheet with TolA is in green, and the rest of the g3p protein is in gray. (B) Structure of the C-terminus of TonB (pink) in complex with the TonB box of BtuB. The β-strand of the TonB box that forms an anti-parallel β-sheet with TonB is in blue while the rest of BtuB is in gray. Figure from Shultis et al., 2006.
Figure 6. Crystallized images of TolA, TonB and both structures superimposed. (A) TolA in complex with the filamentous phage protein g3p (Lubkowski et al., 1999). The N1 domain of g3p is shown in blue and the C-terminal of TolA is shown in yellow. (B) Three dimensional structure of a 92-residue carboxy-terminal TonB dimer (Ködding et al., 2005). One molecule is shown in red and the other in blue with aromatic residue clusters shown as a ball-and-stick. One C-terminal β-strand forms a β-sheet with the other TonB C-terminal β-strand. (C) Superimposed TonB C-terminal in red and the C-terminal domain of TolA in blue (Ködding et al., 2005). The N-terminal α-helix in TolA is omitted as it is not found in TonB.
At the OM, *in vivo* cross linking has shown that, besides the TonB-gated transporters mentioned above, TonB also interacts with OmpA and Lpp (Higgs *et al*. 2002b). These proteins most likely serve as a docking point for TonB while it samples for ligand presence in the TonB-gated transporters, as mutations in either OmpA or Lpp have no effect on TonB function and TonB can only transfer energy to ligand-loaded receptors (Larsen *et al*., 1999; Higgs *et al*., 2002b; Moeck *et al*., 1996; 1997; Moeck and Letellier, 2001).

The physiological function of the Tol system is not known but because *tol* strains are hypersensitive to deoxycholate and certain antibiotics, shed outer membrane (Bernadac *et al*., 1998), and leak periplasmic proteins (Nagel *et al*., 1967; Lazzaroni and Portalier, 1981), it is thought that this system is responsible for maintaining outer membrane structure and function. New data has shown that the Tol system appears to play a role in OM invagination during cell division in *E. coli*. It is believed that the Tol-Pal complex forms connections near the septal ring to aid in invagination. TolQRA, Pal, and TolB were shown to aggregate at constriction sites and mutants had retarded OM invagination with blebs forming at the cell poles and constriction sites (Gerding *et al*., 2007).

Like TonB, TolA also confers sensitivity to certain bacteriophage (f1: Smilowitz, 1974) and group A colicins (Nagel *et al*., 1967) that exploit the system to gain access to the cell (see Lazzaroni *et al*., 2002 for review). In *E. coli*, the major proteins involved in the Tol system are encoded by a gene cluster at 16.7 minutes on the *E. coli* chromosome (Berlyn, 1998). Although it has been suggested this represents two operons (Vianney *et al*., 1996), the second promoter can be deleted without any apparent ill effects – thus the transcriptional relationships between these genes remain unknown. *lac* operon fusions show that the first putative operon encodes for YbgC,
TolQ, TolR, and TolA while the second encodes for TolB, PAL, and YbgF (Figure 4B) (Vianney et al., 1996). It is not known how these genes are regulated.

Like TonB, TolA interacts with OmpA and Lpp in the outer membrane, albeit indirectly, through contact with PAL and TolB (Figure 2) (Clavel et al., 1998). Braun’s lipoprotein, or Lpp, plays a major role in maintaining contact between the OM and the peptidoglycan (PG) layer, particularly at constriction sites (Suzuki et al., 1978; Yem and Wu, 1978), contributing to proper invagination during cell division (Hiemstra et al., 1986; 1987). Peptidoglycan-associated lipoprotein, PAL, also directly connects the OM and the PG layers through non-covalent interactions between the carboxy terminal domain and peptides of the PG (Mizuno, 1998; Lazzaroni and Portalier, 1992; Clavel et al., 1998; Bouveret et al., 1999; Parsons et al., 2006). This interaction is likely modulated by TolB, as it competes with PG for binding with PAL (Clavel et al., 1998; Bouveret et al., 1999; Ray et al., 2000; Cascales and Lloubes, 2004). TolB, a 408 residue periplasmic protein, seems to referee certain interactions between TolA, PAL, and the outer membrane (Walburger et al., 2002; Cascales and Lloubes, 2004). The TolB crystal structure has been solved (Abergel et al., 1999) and shows it has a propeller motif that may allow it to interact with both TolA and Pal. A cytoplasmic protein, YbgC, encoded on the first putative Tol operon, is an acyl-coA thiesterase, and a periplasmic protein, YbgF, encoded on the second putative operon, has an unknown function. Two null mutations in these genes do not yield a Tol phenotype (Lazdunski et al., 1998).

The Energy Harvesting Component

Although the TonB and Tol systems have separate and distinct functions, the energy-harvesting components in these two systems have sufficiently similar topologies and amino acid sequences (Figure 7) to consider them members of the same Mot/Exb protein superfamily.
(Cascales et al., 2001; Saier, 2000; Kojima and Blair, 2001). The *E.coli* *exbB* gene encodes a predicted 244 residue protein (Eick-Helmerich et al., 1989) with a membrane topology consisting of a periplasmically exposed amino terminus, three transmembrane segments separated by a large positively charged cytoplasmic domain and a small periplasmic loop, and a cytoplasmically displayed carboxy terminus (Kampfenkel and Braun, 1993; Karlsson et al., 1993). The *E.coli* *tolQ* gene encodes a predicted 230 residue protein (Sun and Webster, 1986) with a membrane topology like that of ExbB (Vianney et al., 1994). ExbB and TolQ share 26.3% amino acid identity and 79.1% amino acid similarity (Figure 8) with a coding sequence homology of 51.2% (Eick-Helmerich et al., 1989). The *E.coli* *exbD* gene encodes a predicted 141 residue protein (Eick-Helmerich et al., 1989) with the membrane topology consisting of a cytoplasmically localized amino terminus followed by a single transmembrane hydrophobic domain, and the polar carboxy terminus that extends into the periplasm (Kampfenkel and Braun, 1992; Muller et al., 1993). Likewise, the *E.coli* *tolR* gene encodes a predicted 142 residue protein with a similar membrane topology. ExbD and TolR share 25% amino acid identity and 70% similarity (Figure 8) with a coding sequence homology of 49.7% (Eick-Helmerich et al., 1989).

It is unknown how the ExbB/D or TolQ/R complexes harvest energy from the proton motive force but their similarities to each other and the Mot/Pom complexes may give some clues. The transmembrane domain (TMD) of ExbD contains a highly conserved residue (Asp25 in *E.coli*) that is present in both MotB and PomB (Zhou et al., 1998), and is important for TonB activity (Braun et al., 1996). TonB loses activity when the seryl and histidyl in its TMD are substituted, or when the Val17, Cys18, or Ile19 are deleted. This loss of activity can be restored by second-site mutations in the first transmembrane domain of ExbB (Larsen et al., 1994, 1999; Larsen and Postle, 2001). Similarly, TolA shares a conserved set of residues with
Figure 7. The TonB and TolA complexes predicted protein topology. The transducer proteins (TonB and TolA) have their amino-terminus located in the cytoplasm, a single transmembrane domain (TMD) spanning the CM, and the majority of the protein and the carboxy-terminus in the periplasm. The energy harvesting complexes consist of ExbB/ExbD and TolQ/TolR. ExbB and TolQ have a periplasmically located amino-terminus, three transmembrane domains spanning the CM, a large cytoplasmic loop between the first and second TMD, a smaller loop in the periplasm between the second and third TMD, and the carboxy-terminus in the cytoplasm. The amino-terminus of ExbD and TolR is located in the cytoplasm, followed by a single TMD spanning the CM, and the bulk of the protein and the carboxy-terminus in the periplasm. Diagram courtesy of Dr. Kathleen Postle.
Figure 8. Sequence comparisons. Sequence alignments of the transmembrane domains (TMD) of the proteins (A) ExbB, TolQ, and MotB (B) ExbD, TolR, and MotB (C) TonB and TolA, in *Escherichia coli*, *Vibrio cholera*, and *Bacillus subtilis*. The predicted TMD residues are indicated by the black bar across the top of each sequence. Shading indicates conserved amino acids. Figure adapted from Postle and Larsen, 2004.
TonB in its TMD, and its activity can be restored by a second-site mutation in the first TMD of TolQ when its corresponding TMD seryl and histidyl residues are substituted (Germon et al., 1998). Because of these suppressor mutations and the high degree of similarity between the ExbB and TolQ first transmembrane domains, it is thought to be a likely site of energy delivery, although there is no hard evidence to support this theory. The second and third transmembrane domains of ExbB and TolQ also share a high degree of similarity and are comparable to the third and fourth transmembrane domains of MotA and PomA. In the second TMD of ExbB and TolQ, there are three glycyl residues shared with MotA/PomA and a prolyl residue that, in MotA may participate in proton conductance (Braun et al., 1999). The third domains of ExbB and TolQ have a hydrophobic face that is shared with MotA and PomA in their fourth TMD. It has also been demonstrated that two prolyl residues and an alanyl in the TMD, or a Thr\textsubscript{139} residue at the extreme carboxy terminus, of TolR are suppressors of alanyl (Ala\textsubscript{177}) mutation in the third TMD of TolQ (Lazzaroni et al., 1995).

Topology, sequence analysis, and comparisons to the MotAB complex have led to structural predictions that suggest possible proton pathways in the ExbBD complex (Zhai et al., 2003). One pathway is formed by the three transmembrane helices in ExbB and the single helix in ExbD (Figure 9A). The other pathway proposes that the TonB TMD is also included (Figure 9B). These proposals do not take into account the predicted stoichiometry of the TonB system (Higgs et al., 2002a) and a study looking at point mutations in the second and third TMDs of ExbB and TolQ did not fully support the proposed proton conductance pathways (Braun and Herrman, 2004). They found that the proposed proton pathways could only occur in the unlikely event that water bridges between nonpolar amino acids were assumed to form. Recent data has
Figure 9. Predicted proton pathways in the TonB/ExbBD complex. Comparisons to the MotAB complex have led to structural predictions that suggest possible proton pathways in the ExbBD complex. Conserved residues are in red. Arrows show the predicted proton pathway from residue to residue. (A) The first predicted pathway uses the three transmembrane helices in ExbB and the single helix in ExbD whereas the other pathway (B) proposes that the TonB TMD is also included. Figure from Zhai et al., 2003.
shown possible ion channel mutations in the TolA system (Goemaer et al., 2007). Specific hydrophilic residues and proline amino acids found in the TMD were targeted due to their energetically unfavorable presence within the membrane. Four residues found in the TMD of TolR and the second and third TMD of TolQ may play a role in ion conductance. This data was interpreted with the assumption that OM integrity requires energy from TolA, whereas colicin uptake, particularly colicins A and E₁, has been believed not to require any energy input (Goemaer et al., 2007). This idea that colicin A or E₁ does not need energy from the TolA system is a broad extension from early, undefinitive studies and conflicts with current experiments and data presented in this Dissertation.

**The Transducer Component**

Unlike the energy harvesting machinery, the energy transducers of these systems are only superficially similar. While their membrane topologies are similar, both have a single transmembrane domain (Hannavy et al., 1990; Roof et al., 1991; Levengood et al., 1991), TonB and TolA share little primary amino acid sequence (Postle and Good, 1983; Levengood and Webster, 1989). The exception to this is in their signal anchors, which share a conserved set of residues, SHLS (residues 16, 20, 27, and 31 in TonB), which occupy one face of the predicted transmembrane α-helix (Figure 10) (Koebnik, 1993). The first seryl residue and the histidyl are essential for TonB and TolA energization in *E.coli* and are conserved throughout a number of species, such as *Yersinia pestis, Haemophilus influenzae*, and *Vibrio cholerae* (Postle and Larsen, 2004). The single transmembrane domain mediates interaction of TonB (and presumably TolA) with cytoplasmic membrane components (Jaskula et al., 1994; Letain and Postle, 1997; Larsen et al., 2003a) while the bulk of the protein occupies the periplasmic space.
**E. coli TonB residues 11-32**

Figure 10. Shared transmembrane residues in TonB and TolA in *E. coli*. A space-filling model of the predicted TonB transmembrane domain. Shared residues of TonB and TolA are highlighted in red. The SHLS motif is found on the same face of the protein. In TonB, the serine, histidine, leucine, serine residues are found at positions 16, 20, 27, and 31. In TolA the same residues are found at positions 18, 22, 30, and 34. Figure from Larsen *et al.*, 1999.
Both the TonB and TolA TMD contain essential seryl and histidyl residues with postulated three residue separation (Germon et al., 1998). When the Ser\textsubscript{16} and His\textsubscript{20} of the TMD are substituted, or the Val\textsubscript{17}, Cys\textsubscript{18}, or Ile\textsubscript{19} deleted, TonB is still able to insert properly into the membrane but function (OM active transport) is lost and pmf-dependent conformational changes do not transpire (Larsen and Postle, 2001). All other residues in the TMD can be changed to alanine without loss of activity, but the spacing between the Ser\textsubscript{16} and His\textsubscript{20} must be maintained (Larsen and Postle, 2001). Recently, data has shown that retention of the Ser\textsubscript{16} and His\textsubscript{20}, among an all-alanyl scaffolding (residues 12-32), was sufficient for maintaining TonB activity (Larsen et al., 2007). Additionally, a Ser\textsubscript{16}Ala mutation, in an otherwise wild-type TonB, had no effect on TonB activity and a His\textsubscript{20}Ala mutation halted TonB activity. These new data have required one to rethink the idea that both seryl and histidyl are necessary for energy transduction of TonB. A Ser\textsubscript{16}Ala mutation on an all-alanyl TMD (with the required His\textsubscript{16} present) did cause loss of activity. This leaves one to conclude that perhaps Ser\textsubscript{16} is not essential but merely an aid in transmembrane orientation or interaction, while the His\textsubscript{20} residue is the actual workhorse in energy transduction (Larsen et al., 2007). When the corresponding Ser and His residues in the TolA transmembrane domain were also substituted, similar results occurred (Germon et al., 1998).

A proline-rich region from residue 66 to 102, consisting of Pro-Glu and Pro-Lys repeats, follows the TMD in *E.coli* TonB (Postle and Good, 1983). This region, and amino acids 32 – 65 before it, can be deleted without impairing TonB activity (Larsen et al., 1993; Vakaria and Postle, in preparation), but expansion of the periplasm via osmotic shock does diminish mutant activity, suggesting that TonB can contact both the OM and CM simultaneously (Larsen et al., 1993). This proline region allows the protein to extend out to the OM. This has also been shown
to occur in *V. cholerae* (Seliger *et al*., 2001). Following the proline-rich region is a suppressor site of TonB box mutations in OM transporters at amino acid 160 (Heller *et al*., 1998; Bell *et al*., 1990; Anton and Heller, 1993). This is followed by the highly ordered strand-exchanged dimer and antiparallel beta-sheets of the carboxy terminal domain, which has been crystallized (Chang *et al*., 2001). A more recent carboxy terminal fragment was crystallized which showed a much less rigid dimerization with only the third β-strands engaging in exchange. This also showed a short α-helical region just before the first β-strand (Ködding *et al*., 2005). Because none of these solved structures used an energy-competent form of TonB, the biological relevance of these structures remains unclear.

The TolA amino terminal domain, containing the SHLS motif in the TMD, is 42 amino acids long and, like TonB, contains signal and stop transfer domains. After residue 34, the remainder of the protein is in the periplasm. The second domain of TolA separates the amino terminus from the carboxy terminus and is almost entirely α-helical in structure. The three domains are separated by short glycine stretches, presumably to allow flexibility (Levengood *et al*., 1991). The carboxy terminal domain runs from amino acid 311 to the final residue at position 421 (Levengood *et al*., 1991) and is the functional domain, interacting with the OM (Levengood and Webster, 1989). This domain has been crystallized in a complex with the g3p protein (Cadieux and Kadner, 1999). The filamentous phage minor coat gene 3 proteins (g3p) are located at one end of the phage particle (Gray *et al*., 1981) and are involved in bacterial infection. Interactions requiring g3p utilize the protein’s three domains (N1, N2, and CT) to support infection. The N2 domain is responsible for the phage binding to the tip of the F pilus (Stengele *et al*., 1990). The N1 domain then interacts with the carboxy terminal domain of TolA in the bacterial envelope, allowing entry into the cell (Riechmann and Holliger, 1997; Click and
Webster, 1997). This complex, the TolA C-terminus and the N1 domain of g3p, was crystallized (Cadieux and Kadner, 1999; Lubkowski et al., 1999). The structure (Figures 5A and 6A) shows residues 333-421 of TolA consist of 3 β-strands and 4 helical motifs (Lubkowski et al., 1999).

The sequence differences between the two transducer proteins are reflected in their OM protein interactions. TonB is known to interact with OM receptors involved in iron (FepA, FhuA, FecA) and cobalamin (BtuB) transport, which contain a conserved TonB box motif (Bell et al., 1990; Gudmundsdottir et al., 1989; Heller et al., 1988), that appears to play a specific role in recognition by TonB (Larsen et al., 1997; Cadieux et al., 2000). TolA has been shown to interact via its carboxy terminal domain with the amino terminus of the periplasmic protein, TolB (Figure 11) (Clavel et al., 1998; Walburger et al., 2002). The carboxy terminal domain of TolA also interacts with PAL in a PMF-dependent manner (Cascales et al., 2000). Recently, a conserved motif in the carboxy terminal domain of PAL was identified to be a type of TolA box (Cascales and Lloubes, 2004), conceptually similar to the better studied TonB box. An SYGK sequence was determined to be an important TolA binding site in PAL. As TolA also interacts with group A colicins (Nagel et al., 1967) and the filamentous phage capsid protein g3p (Lubkowski et al., 1999), these proteins were screened for the TolA box sequence found in PAL. The amino terminal domain of g3p and the TolA binding regions of colicin A and colicin N were found to have similar sequences (Cascales and Lloubes, 2004). It has been shown that in tolB or pal mutants, TolA is stable but in a tolB-pal strain, TolA is degraded (Germon et al., 2001). The dual interaction between the carboxy terminal of TolA with both of these proteins together may somehow stabilize TolA (Cascales and Lloubes, 2004). In yeast two-hybrid screening, TolA has been shown to interact with YbgF (Walburger et al., 2002). Ultimately, recipients of TolA–transduced energy are unknown; however its PMF-dependent interaction with
Figure 11. TolA interactions with the outer membrane. TolA interaction with Pal requires proton motive force via TolQ and TolR (Cascales et al., 2000; 2001). The Tol system maintains OM integrity presumably through interactions (represented by arrows) with TolB and Pal, and Pal with TolB, OmpA and Lpp. Figure from Cascales and Lloubes, 2004.
PAL (Cascales et al., 2000) and new data supporting the Tol system’s role in cell division (Gerding et al., 2007) may start to shed light on the energy recipients.

**Energy Transduction from the CM to the OM**

There are two models that explain how TonB may transduce energy to OM receptors. The mechanical pulling model does not require that TonB have the ability to store energy as a conformational change, whereas the shuttling model does (Chang et al., 2001; Chimento et al., 2005; Larsen et al., 2003a; Postle and Larsen, 2007; Weiner, 2005). TonB cycling is thought to occur as a means of delivering energy from the CM to the OM TonB-dependent transporters (Figure 12). This shuttling model was first suggested when sucrose density gradient fractions showed that TonB was distributed 60% - 40% between the CM and OM fractions, respectively. In the absence of both ExbB/D and TolQ/R, TonB was only associated with the OM (Letain and Postle, 1997).

In the shuttling model, TonB is in complex with ExbB and ExbD at the CM and is in an unenergized conformation. The ExbB/D complex uses the pmf to convert TonB into an energized conformation. The carboxy terminus of TonB contacts the OM and once this occurs, the amino terminus is released from the CM. The amino terminus, in its conformationally charged form, can then interact with OM receptors (Postle and Kadner, 2003). TonB can associate with the OM when a ligand, or even a number of transporters, is not present (Higgs et al., 2002), but can only transduce energy to a transporter when a ligand is bound (Moeck et al., 1996; 1997; Larsen et al., 1999; Moeck and Letellier, 2001; Higgs et al., 2002b). Interaction between the TonB amino acid 160 and the TonB box of the OM transporter is required for ligand transport into the cell (Bell et al., 1990). TonB can be cross-linked to FepA and FhuA in vivo, but this does not occur when a mutation is made in the TonB box (Larsen et al., 1997) and is
Figure 12. The TonB shuttling model. A suggested model for TonB cycling as a means of delivering energy to the OM. (1) TonB is in complex with ExbB and ExbD in an unenergized conformation. (2) The ExbB/D complex uses the pmf to convert TonB into an energized conformation. (3) The C-terminus of TonB contacts the OM via LPP and OmpA and then N-terminus is released from the CM. This conformationally charged form can interact with OM receptors like FepA. (4) Once energy has been transferred to the receptor, TonB is in a discharged conformation. TonB returns to ExbB/D and an unenergized conformation (Postle and Kadner, 2003).
greatly diminished in the absence of transportable substrate (Moeck et al., 1997). This suggests that ligand binding causes a signal transduction cascade that results in presentation of the TonB box which can then signal TonB (Ferguson et al., 1998; 2002; Locher et al., 1998, Fanucci et al., 2003). Once energy has been transferred to the OM receptor, TonB undergoes another conformational change into a discharged conformation, different from the uncharged or unenergized first conformation (Larsen et al., 1999).

This shuttling model has been tested by assaying for the ability of the carboxy terminus and the amino terminus of TonB to be labeled by a probe (Oregon Green® 488 maleimide) for periplasmically accessible cysteine residues. Fractionation showed that TonB was labeled in the CM fraction as well as the OM fraction (Larsen et al., 2003a), suggesting that TonB is, in fact, cycling to the OM to acquire the label and then returning to the CM. Cycling of TonB between the OM and the CM is not dependent upon CM energization as inactive TonB amino terminal mutants cannot change conformation with the pmf but can still associate with the OM (Jaskula et al., 1994; Larsen et al., 1999). The mechanism by which TonB couples CM energy to OM transport is still under some speculation. The recent discovery that His20 in the TonB TMD is the only residue that is essential (Larsen et al., 2007) may lead one to believe that TonB may not, in fact, store energy as a conformational change, although a single change in an amino acid can have a large influence on protein structure.

Data addressing the energetics of the Tol system has not been published due to the lack of quantifiable assays. Because the OM targets of the Tol system are unknown, it is difficult to study. Most studies use the ability of colicins to pirate this system as a means for qualitative analysis and because of this, it has been assumed that the Tol system most likely provides support and reinforcement for the OM rather than aiding in active transport (Lazdunski et al.,
New data suggests that the Tol system plays a role in OM invagination during cell division (Gerding et al., 2007). Although the data are novel and exciting, it reinforces the above idea that active transport cannot be used as a means for testing Tol activity. Additionally, it has only recently been shown that the Tol system most likely acts as an energy transduction apparatus, like the TonB system. TolA also undergoes a pmf-dependent conformational change, as first shown in TonB, when TolQ and TolR are present but the nature of this change is different. Using proteinase K to degrade TolA at different conformations showed that the TolA digestion pattern in a tolQ or tolR strain was the same as seen in a wild-type strain with a dissipated pmf (Germon et al., 2001). This was not observed when done in the TonB system. The pattern of TonB degradation by proteinase K in an exbB or exbD strain was the same as pattern seen in the wild-type strain with an intact pmf (Larsen et al., 1999). The amino terminus of TolA interacts with TolQ at the CM (Derouiche et al., 1995), and its carboxy terminus undergoes a conformational change when interacting with colicin A (Deprez et al., 2002). TolA has also been shown to be unstable in tolB-pal mutants (Germon et al., 2001; Cascales et al., 2001) This may suggest that, like TonB, there are different conformational states of TolA dependent on energy, but there has been no evidence of shuttling and TolA has not been found associated with OM fractions (Journet et al., 1999).

It seems that all evidence points to the coupling of the transmembrane domain of the transducer protein, TonB or TolA, to the energy harvesting complex to gather and deliver energy to the outer membrane. When transcription of the energy harvesting complex is disrupted or prevented, transducer activity is greatly reduced (Guttermann and Dann, 1973; Braun, 1989; Sun and Webster, 1986) and when the transmembrane domain of TonB is replaced with that of TolA, the chimeric protein is more efficient when paired with TolQ and TolR (Karlsson et al., 1993a).
It appears that ExbB is required for TonB and ExbD stability (Fischer et al., 1989; Skare and Postle, 1991). The TMD influences the ability of TonB to be chemically crosslinked with ExbB in vivo (Larsen et al., 1994) and there is evidence of a TonB-ExbD crosslinked complex, but it is unknown whether the TMD or periplasmic domains are involved (Higgs et al., 1998). It has also been seen that TolA/Q and TolA/R heterodimers can be formed via crosslinking and it is believed to involve the TolA transmembrane domain, or perhaps the amino-terminal cytoplasmic domain (Derouiche et al., 1995). TonB and TolA also require their respective harvesting complexes (or in the case of cross-talk, the paralogues) to obtain pmf energy (Larsen et al., 1999; Germon et al., 2001). As mentioned previously, TonB activity is halted when specific residues are substituted or deleted. This loss of activity can be restored by a second-site mutation in the first transmembrane domain of ExbB (Larsen et al., 1994, 1999). Similarly, TolA activity can be restored by a second-site mutation in the first TMD of TolQ when its corresponding TMD Ser and His residues are substituted (Germon et al., 1998). All of this evidence leads one to believe that the TMD is the site where energy is received in the TonB and TolA proteins.

Cumulative evidence indicates the energization of TonB and TolA requires specific interactions between their respective transmembrane domains and components of their energy harvesting apparatus (Germon et al., 1998; Larsen et al., 1994, 1999; Larsen and Postle, 2001). Interestingly, strains that lack one energy harvesting complex remain able to energize both transducers, whereas in strains lacking both energy harvesting complexes neither transducer is energized (Braun, 1989; Braun and Hermann, 1993, Skare and Postle, 1991, Skare et al., 1993).

While there is clearly crosstalk between the systems, it is not efficient. This is consistent with the initial description of exb mutations as conferring “leaky” phenotypes (Gutermann and Dann, 1973) and the observation that sensitivities to group-specific colicins are reduced in both exbBD
and tolQR mutants (Braun and Herrmann, 1993). Quantitative transport assays of TonB-
dependant ligands in exbBD strains indicate that tolQR complexes can support about 10% of the
TonB activity normally achieved using ExbBD complexes (Skare and Postle, 1991; Skare et al.,
1993). Quantitative analysis of the reciprocal circumstance, support of TolA by ExbBD
complexes in the absence of TolQR, has not been reported.

This apparent crosstalk between systems, coupled with the evident homology shared by
the energy harvesting complexes, suggests that the mechanisms of energy harvest and transfer
are similar for the two systems. The lower efficiency of crosstalk suggests that the motifs that
mediate energization of the transducers are different.

Research Goals

The objective of this research was to begin to decipher how the TonB and TolA systems
harvest and transfer energy. The phenomenon of cross-talk provides a way to dissect those
specific structural features that are involved in the interactions between the proteins in these
complexes that facilitate energy transfer. Because cross-talk is inefficient, we can begin by
identifying the characteristics that dictate specificity between transducers and energy harvesting
complexes. My approach was to first generate chimeric energy harvesting complexes (Figure
13), and later proteins, and evaluate changes in cross-talk efficiency. In short, I asked what
features of the ExbBD harvesting complex, the TolQR complex must possess to energize TonB
as efficiently as ExbBD does. This approach allows us to begin to determine which proteins,
domains, and motifs are responsible for energy transfer to the transducer proteins. Further, this
strategy also identifies critical features that do not vary between energy harvesting complexes,
and as such are potentially involved in accessing the ion electrochemical gradient of the
cytoplasmic membrane.
**Hypothesis**: Complex-specific structural motifs are responsible for transducer discrimination of energy-harvesting complexes.

Three specific aims were developed to address this hypothesis:

1. Develop isogenic strain sets and an approach for introducing modified genes that allow for a rational and unambiguous evaluation of energy transduction efficiency.
2. Evaluate mixed (chimeric) energy harvesting complexes (TolQExbD and ExbBTolR) and determine which, if either, complex component plays a dominant role in determining transducer specificity.
3. Identify specific motifs in the transducers that contribute to cross-talk by testing chimeric derivatives created with parts from both homologues.
Figure 13. Chimeric energy harvesting complexes. Strategy to test what features of the ExbBD harvesting complex the TolQR complex must possess to energize TonB efficiently. Rather than ExbB being present in a complex with ExbD, it is paired with TolR. Likewise, TolQ is complexed with ExbD.
CHAPTER II.

AIM ONE: DEVELOPMENT OF TOOLS

Cross-talk allows one to dissect the specific structural features that are involved in facilitating energy transfer due to the interactions between the proteins of the TonB and Tol systems. As mentioned previously, cross-talk is inefficient, and by identifying what protein interactions increase or decrease cross-talk, the characteristics that dictate specificity between transducers and energy harvesting complexes can be determined. My approach is to first generate chimeric energy harvesting complexes and evaluate changes in cross-talk efficiency. This approach will allow me to begin to determine which proteins, domains, and motifs are responsible for energy transfer to the transducer proteins. Further, this strategy will also identify critical features that do not vary between energy harvesting complexes, and as such are potentially involved in accessing the ion electrochemical gradient of the cytoplasmic membrane.

To test the contributions of the specific proteins to energization, each protein needs to be expressed both individually and in combination with other individual proteins of the two systems. This requires strains in which functional genes for each protein are absent or present depending on the phenotype to be tested. I decided to use vehicles that will allow me to reintroduce genes encoding wild-type and modified proteins singly and in various combinations for evaluation.

In a preliminary study, I constructed plasmids encoding individual components of the two energy harvesting complexes, using vectors carrying the $P_{BAD}$ promoter to allow for stringent regulation of expression levels (Table 2). This vector contains $araC$ and the arabinose promoter, allowing control over gene transcription. It also has a multiple cloning site, and ampicillin-resistance cassette, a Shine-Delgarno sequence, and an M13 origin of replication (Guzman et al.,
Table 2. Vectors and Strains used in Chapter Two.

<table>
<thead>
<tr>
<th>Vector / Strain</th>
<th>Relevant Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW25113</td>
<td>$laci^f , rrnB_{T14} , \Delta , lacZ_{W16} , hsdR514 , \Delta araBAD_{AH33} , \Delta , rhaBAD_{LD78}$</td>
<td>Datsenko and Wanner, 2000</td>
</tr>
<tr>
<td>KP1456</td>
<td>$tolQ_{am} , \Delta exbBD$</td>
<td>Larsen et al.,</td>
</tr>
<tr>
<td>W3110</td>
<td>$F1N(rrnD,rrnE)1$</td>
<td>M. Berlyn, <em>E.coli</em> Genetic Stock Center</td>
</tr>
<tr>
<td>pBAD24</td>
<td>AraBAD promoter, AraC, <em>amp</em></td>
<td>Guzman et al., 1995</td>
</tr>
<tr>
<td>pACYC184</td>
<td><em>cat</em>, <em>tet</em></td>
<td>Chang and Cohen, 1978</td>
</tr>
<tr>
<td>pKP325</td>
<td>pBAD promoter in pACYC184; <em>tonB</em></td>
<td>K. Postle</td>
</tr>
<tr>
<td>pKP390</td>
<td>pBAD24; <em>exbBD</em>; <em>amp</em></td>
<td>K. Postle</td>
</tr>
<tr>
<td>pKP392</td>
<td>pBAD24; <em>exbB</em>; <em>amp</em></td>
<td>K. Postle</td>
</tr>
<tr>
<td>pTPS202</td>
<td>pJIH12; <em>tolQRAB</em>; <em>amp</em></td>
<td>Sun and Webster, 1986</td>
</tr>
<tr>
<td>pRA001</td>
<td>pBAD24; <em>tolQ</em>; <em>amp</em></td>
<td>R. Larsen</td>
</tr>
<tr>
<td>pRA002</td>
<td>pBAD24; <em>tolR</em>; <em>amp</em></td>
<td>R. Larsen</td>
</tr>
<tr>
<td>pRA003</td>
<td>pBad24; <em>tolQR</em>; <em>amp</em></td>
<td>R. Larsen</td>
</tr>
<tr>
<td>pRA018</td>
<td>pKP325; <em>tolR</em>; <em>cat</em>, <em>tet</em></td>
<td>K. Brinkman</td>
</tr>
<tr>
<td>pRA017</td>
<td>pKP325; <em>exbD</em>; <em>cat</em>, <em>tet</em></td>
<td>K. Brinkman</td>
</tr>
<tr>
<td>pKD3</td>
<td>pANTSγ derivative; FRT-flanked <em>cat</em></td>
<td>Datsenko and Wanner, 2000</td>
</tr>
<tr>
<td>pKD4</td>
<td>pANTSγ derivative; FRT-flanked <em>kan</em></td>
<td>Datsenko and Wanner, 2000</td>
</tr>
<tr>
<td>pKD46</td>
<td>Red Helper plasmid; pINT-ts derivative, *araC-P$_{arab}$ γ β exo bla repA101ts tL3 terminator, temperature sensitive replication</td>
<td>Datsenko and Wanner, 2000</td>
</tr>
<tr>
<td>pCP20</td>
<td>FLP expression plasmid; <em>amp</em>, temperature sensitive replication and FLP synthesis</td>
<td>Datsenko and Wanner, 2000</td>
</tr>
</tbody>
</table>
To test the functionality of paired transmembrane proteins, plasmids carrying pBAD-regulated tolQ or exbB were paired with plasmids carrying their respective complex partner, i.e. tolR or exbD. Certain cloned plasmids carried the ampicillin resistant gene while others carried a chloramphenicol resistance marker instead. The use of dual antibiotic resistance markers was necessary to confirm and maintain the presence of two different plasmids in the same strain. To retain constant growth conditions, all strains carried a plasmid conferring ampicillin resistance (amp') and a plasmid bestowing chloramphenicol resistance (cm'), regardless of the number of proteins being tested. This was done by using pBAD24 as a negative amp' plasmid or pACYC184 as a negative cm' plasmid. The CoE1 related plasmid, pACYC184 (Figure 15), carries a tetracycline resistance gene as well as the chloramphenicol acetyl transferase, cat, cassette that confers resistance to chloramphenicol (Chang and Cohen, 1978). The plasmids conferring cm' were built by ligating the gene of interest into a plasmid (pKP325) that carries araC, the pBAD promoter, and the MCS from pBAD24 cloned into sites 1517 and 2056 on the pACYC184 plasmid. The two plasmids carrying exbB and exbD were made previously by Dr. Kathleen Postle at Washington State University. All other plasmids carrying tol genes were created in this present study (Table 2).

Plasmids were transformed into an E.coli strain (KP1456: tolQam ΔexbBD), that expresses both wild-type TonB and TolA, but no cytoplasmic membrane energy harvesting complexes [it is important to note that the amber mutation in tolQ is strongly polar on tolR; (Vianney et al., 1996)]. Parent vectors lacking inserts (pBAD24 and pACYC184) served as negative controls whereas positive controls consisted of a plasmid with either the exbB/D genes or the tolQ/R genes as operons under pBAD control. The functionality of each gene product was
Figure 14. Map of pBAD24 vector plasmid. A diagram of pBAD24 plasmid (4542 bp), demonstrating the position of the multiple cloning site (MCS) with various restriction sites noted, the ampicillin resistance cassette (amp), the M13 and pMB1 origin of replication sites, the araC gene, and the arabinose promoter (ParaBAD). Guzman, et al., 1995; Diagram from ©SHIGEN, National Institute of Genetics, 2007.
Figure 15. Map of pACYC184 vector plasmid. A diagram of pACYC184 plasmid (4245 bp), demonstrating the positions various restriction sites, the tetracycline resistance cassette (TcR), the chloramphenicol acetyl transferase cassette (CmR), and the replicon (rep) originating from the p15A plasmid that is responsible for the replication of the plasmid. Chang and Cohen, 1978; Diagram adapted from ©Fermentas, 2006.
confirmed by complementation of relevant strains by testing for restored sensitivity to specific colicins (data not shown).

The ability of wild-type and mixed (chimeric) complexes to energize TolA was scored by examining resistance to erythromycin (a measure of OM integrity) (Rutz et al., 1992) and sensitivity to the TolA dependent colicins, A and E₁. Energization of TonB was scored by examining sensitivity to a TonB-dependent colicin, colicin B.

Erythromycin is an extremely large antibiotic molecule (FW 733.9) that can gain entry into the cell and kill it when the outer membrane is compromised, as occurs with a nonfunctional tol system. Cells were grown in LB broth supplemented with ampicillin and plated onto ampicillin supplemented T plates in T top agar with 100 µg/ml ampicillin, 34 µg/ml chloramphenicol, and 0.1% arabinose. Three sterile filter disks were placed on each plate and 5µl of 50 µg/µl of erythromycin were aliquotted onto each disk. In all cases, the plates were inoculated in triplicate, incubated for 18 h at 37°C, and the size of the clearing zones measured.

As discussed earlier, certain protein toxins can exploit either the TonB or the TolA systems and thus these provide a means to determine transducer activity based on the presence of clearing zones in bacterial lawns. Bacteriocins that are specific to killing E.coli and other related species are called colicins. They can attack nucleic acids, disrupt cytoplasmic membrane transporters, or depolarize the cytoplasmic membrane. To gain entry into the cell, colicins recognize and dock to a specific outer membrane receptor, allowing them to interact with periplasmic proteins to gain access to their target inside the cell (Braun et al., 1994). A centrally located receptor recognition domain binds the OM receptor while the translocation domain at the amino-terminal interacts with the periplasmic proteins. This interaction signals the entry of the cytotoxic carboxy-terminal to enter the cell (Lazdunski et al., 1998). Colicins can be divided into
two main groups depending on which system they exploit; Group A colicins use the TolA system, whereas group B colicins employ the TonB system to gain entry into the cell. In this study, assays were performed with colicin B to determine TonB activity. Colicin B recognizes the FepA receptor in *E.coli* and interacts with TonB and ExbBD via its TonB box to gain entry into the cell and form channels in the cytoplasmic membrane which dissipates the proton motive force (Braun *et al*., 2002). The group A colicins, colicin A and colicin E₁, were used to test TolA activity. Both colicin E₁ and A bind to the BtuB receptor and form pores in the cytoplasmic membrane. They translocate into the cell differently, with colicin E₁ interacting with TolC and TolQRA, whereas colicin A interacts with TolB, TolQRA, and OmpF (Lazzaroni *et al*., 2002).

Colicin spot titer assays were performed as previously described (Larsen *et al*., 1999) with serial five-fold dilutions of Colicin B, Colicin A, and Colicin E₁. Cells were grown in LB broth supplemented with ampicillin and plated onto ampicillin-supplemented T plates in T top agar with 100 µg/ml ampicillin, 34 µg/ml chloramphenicol, and 0.1% arabinose. In all cases, the plates were inoculated in triplicate, incubated for 18 h at 37ºC, and then scored for the highest dilution yielding clearing.

Briefly, our preliminary results suggested that, relative to wild-type function, a TolQ/ExbD chimera had some activity for Tol dependent functions (Table 3; Figure 16). W3110 retained wild-type sensitivity due to the presence of functional chromosomal TonB and TolA systems. The negative control (KP1456 transformed with pBAD24 and pACYC184) was resistant (R) to colicins and sensitive to antibiotics due to the absence of ExbBD and TolQR. The transcription of *exbBD* or *tolQR* from the same promoter on a single plasmid (pKP390 or pRA003) showed higher sensitivity to their respective colicins (colicin B for pKP390 and colicins A and E₁ for pRA003) than did the expression of those genes on separate plasmids.
Table 3. Preliminary Results Testing Functionality of the Tol System

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Relative Phenotype</th>
<th>ColB (5-fold)</th>
<th>Erythro (mm)</th>
<th>ColA (5-fold)</th>
<th>ColE₁ (5-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>W3110 (+)</td>
<td>7</td>
<td>16</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>pACYC184 + pBAD24</td>
<td>(-)</td>
<td>R</td>
<td>30</td>
<td>R*</td>
<td>R</td>
</tr>
<tr>
<td>pKP390 + pACYC184</td>
<td>(BD)</td>
<td>7*</td>
<td>24</td>
<td>2</td>
<td>4*</td>
</tr>
<tr>
<td>pKP392 + pRA017</td>
<td>(B+D)</td>
<td>5</td>
<td>21</td>
<td>R</td>
<td>4</td>
</tr>
<tr>
<td>pRA003 + pACYC184</td>
<td>(QR)</td>
<td>7</td>
<td>20</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>pRA001 + pRA018</td>
<td>(Q+R)</td>
<td>R</td>
<td>25</td>
<td>2</td>
<td>4*</td>
</tr>
<tr>
<td>pRA001 + pACYC184</td>
<td>(Q)</td>
<td>3</td>
<td>28</td>
<td>R</td>
<td>4*</td>
</tr>
<tr>
<td>pRA018 + pBAD24</td>
<td>(R)</td>
<td>R</td>
<td>28</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>pKP392 + pRA018</td>
<td>(B+R)</td>
<td>R</td>
<td>28</td>
<td>R*</td>
<td>R*</td>
</tr>
<tr>
<td>pRA001 + pRA017</td>
<td>(Q+D)</td>
<td>R*</td>
<td>24</td>
<td>2</td>
<td>3*</td>
</tr>
</tbody>
</table>

The ability of the various energy harvesting proteins to support TonB or TolA function was tested using antibiotic and colicin entry into the cell. Colicin B spot titer assays were used to determine TonB activity and energization, Colicin A and Colicin E₁ spot titers were used to determine TolA activity and energization, and Erythromycin filter disk assays were done to determine outer membrane integrity. W3110 is the chromosomal control and pACYC184 + pBAD24 is the negative control. Colicin spot titer assays were performed with serial five-fold dilutions and 5µl of 50 µg/µl of erythromycin was used on the filter disks. Cells were grown in LB broth supplemented with ampicillin and plated onto ampicillin-supplemented T plates in T top agar with 100 µg/ml ampicillin, 34 µg/ml chloramphenicol, and 0.1% arabinose. All assays were performed in triplicate and then scored for clearing out to the highest five-fold dilution for spot titers or the measurement of the clearing around the filter disks for the Erythromycin assays. A * denotes a mixed culture containing small and large colonies formed on the plates. R denotes resistance to toxin and no visible clearings.
Figure 16. Colicin sensitivity of mixed complexes. A graphic representation of colicin data shown in Table 2. Colicin spot titers determine the ability of the various energy harvesting proteins to support TonB or TolA function. Colicin B spot titers were used to determine TonB activity and energization, Colicin A and Colicin E₁ spot titers were used to determine TolA activity and energization. W3110 is the chromosomal control and pACYC184 + pBAD24 is the negative control. Bars represent the highest average five-fold dilution at which cells were visibly sensitive to the colicin in a triplicate experimental set.
Also, the dual genes expressed from a single operon showed evidence of cross-talk, whereas the genes on separate plasmids did not, except in the case of pKP392 + pRA017 (exbB + exbD) showing sensitivity to colicin E1. The TolQ/ExbD chimeric complex had some activity for Tol dependent functions, as evidenced by partial resistance to erythromycin and some sensitivity to group A colicins. Such activity was not evident for the ExbB/TolR chimeric complex, nor did either chimeric complex support detectable TonB activity. Interestingly, tolQ expressed alone did support some activity for both TonB (as substantiated in the ColB assays) and TolA (as demonstrated in the ColE1 assays). This colicin sensitivity may be a result of TolQ and TolR working together, as KP1456 contains an amber mutation on tolQ that is polar on tolR. However this polarity may not be absolute, and this mutation could be “leaky”, allowing for a small amount of tolR to continue to be made as has been suggested in other studies (Held, 2002).

The major obstacle to this approach was the inability of cells carrying certain plasmid combinations to grow uniform lawns. This made it difficult to measure and score the zones of clearing. One likely explanation for this difficulty was the loss of one or more plasmids from individual cells during the course of lawn formation. Several factors may have contributed to this. First, the amount of arabinose used to induce expression was not optimized. For the Tol-encoding plasmids, expression levels could not be monitored (for lack of specific antisera), so I instead selected a level of arabinose (0.1%) at which I was confident all genes would be induced. Second, at this same time, other experiments in our lab indicated that even modest over-expression of TolQ resulted in a cessation of cell growth (Larsen, Brinkman, Keller, and Reed: unpublished observations). Thus it was likely that under our culture conditions there would be some selective pressure against cells retaining functional Tol-encoding plasmids, as these cells produced large, unbalanced amounts of TolQ. Third, plasmid selection was mediated by the
production of enzymes that neutralized the selecting agents (ampicillin and chloramphenicol), creating a circumstance in which cells needn’t carry both plasmids for a lawn to form. Cells were grown on LB agar containing ampicillin and chloramphenicol. If one, or both, of these antibiotics were broken down by a select set of cells, other cells that carried one, or none, of the plasmids could easily overgrow the ‘sickly’ cells that expressed both plasmids. As mentioned briefly, there is the possibility that expression of \textit{tolR} can occur in the mutant host strain. This “leaky” mutation in KP1456 could result in the false positive results demonstrated in the pRA001 + pACYC184 (\textit{tolQ}) experimental results. This expression may also skew the phenotype of the pRA001 + pRA017 (\textit{tolQ} + \textit{exbD}) chimeric complex, as the sensitivity seen in the colA and colE1 assays could be the result of \textit{tolQ} and chromosomal \textit{tolR} working together, not \textit{tolQ} and \textit{exbD} as the results show. Finally, some cells lacking Tol function might indirectly upregulate the production of capsule polysaccharides, adding yet another phenotypic subset of cells to the “lawn” (Clavel \textit{et al.}, 1996). While at least some of these issues could have been resolved, it became clear that we would probably not be able to establish physiologically relevant stoichiometries between the various energy harvesting and transducing components by this strategy.

Expression of individual proteins at chromosomal levels is essential for meaningful interpretation because stoichiometry is vital in the workings of these complexes. The over-expression of TonB results in decreased activity (Heller, 1985; Larsen \textit{et al.}, 2003b). This dominant negative gene dosage effect is most likely to result from the degradation of TonB, which has a greatly decreased stability when over-expressed (Postle and Skare, 1988; Fischer \textit{et al.}, 1989) or in the absence of ExbB (Skare and Postle, 1991). Similarly, ExbD is subject to proteolysis in the absence of sufficient ExbB (Fischer \textit{et al.}, 1989). It has been shown that the
sensitivity of \textit{exb} mutants to colicins can be greatly increased by the amplification of \textit{tonB} copy number (Fischer \textit{et al.}, 1989). The effects of stoichiometric imbalance have not been vigorously examined in the TolA system, but in light of all other similarities between the systems, it is presumed the situation will be similar to that seen with the TonB system.

\textbf{Revised Strategy}

To avoid the potential problems associated with plasmids (i.e. plasmid retention and balancing stoichiometries when genes are present in multiple copies), wherever possible, I decided to work with genes and constructs that have been stably integrated into the chromosome as single copies under the control of their native promoter. A number of new techniques have been adapted to facilitate this approach. First, I needed to create a set of isogeneic strains with individual, defined deletions of each of the genes involved in these systems. The gene knockouts presently in use result from insertions, deletions, and nonsense mutations, some of which may have polar effects on relevant downstream genes, as noted with the \textit{tolQ} am mutation used in KP1456. To construct the strains from which individual genes have been deleted, without disrupting the expression of downstream products, I used the \textit{\lambda} phage Red recombination pathway (Datsenko and Wanner, 2000) (Figure 17). This approach provides for efficient recombination, allowing the incorporation of linear DNA to make chromosomal deletions using relatively short stretches of sequence identity. Briefly, an \textit{E.coli} strain (BW25113) carrying the Red Helper plasmid (pKD46) is used to generate an initial deletion, which can then be moved to a desired strain by generalized transduction. The Red Helper plasmid contains the \textit{gam}, \textit{bet}, and \textit{exo} genes under an \textit{araB} promoter, with plasmid replication controlled by a temperature sensitive \textit{repA} allele, thus the plasmid can be subsequently cleared by growth at the non-permissive (42°C) temperature. The \textit{gam} product inhibits host RecBCD exonuclease V while \textit{bet}
Figure 17. The \( \lambda \) Red system technique used to create chromosomal deletions. A step by step strategy for gene disruption: Step 1. Amplify the FRT sequence from pKD3 or pKD4 with primers that contain nucleotides that are homologous to the flanking region of the gene that is to be deleted (H1 and H2). Step 2. Purify the PCR products and transform them into BW25113 competent cells that contain the pKD46 Red Helper plasmid. Step 3. Plate transformants on antibiotic selective media (LB agar supplemented with 34 µg/ml cm for pKD3 or 30 µg/ml kan for pKD4) to select for gene replacement with the antibiotic resistance cassette. Step 4. To replace the gene of interest in a wild-type strain (W3110) with the FRT flanked antibiotic resistance cassette, perform P1vir transduction and plate on antibiotic selective media. Step 5. To remove the antibiotic cassette, transform strain with pCP20 FLP plasmid and grow at 30°C to allow for replication and removal of the FRT antibiotic resistant cassette. Select colonies and grow at 42°C to lose the FLP plasmid. Final colonies were tested for sensitivity to either chloramphenicol (cm) or kanamycin (kan) (depending on whether pKD3 or pKD4 was used) and sensitivity to amp (100 µg/ml) to determine loss of pCP20. Figure was adapted from Datsenko and Wanner, 2000.
and exo products bind linear DNA ends and promote recombination. Deletions then result by replacement of target sequences with linear DNA by homologous recombination. To target specific genes for deletion, a linear replacement molecule bearing an antibiotic resistance cassette, either chloramphenicol (pKD3) or kanamycin (pKD4) (Figure 18), flanked by FRT sites (FLP recognition sites) is constructed by amplification via standard polymerase chain reaction (PCR). It is optimized with 0 mM to 4 mM MgSO₄, using a set of primers designed with 20 nucleotides that are homologous to sequences flanking the FRT sites and 36-50 nucleotide extensions complementary to sequences bracketing the chromosomal region to be deleted (Table 4).

Amplified PCR fragments are purified and electroporated into the BW25113 pKD46 cells, then selected at 37°C with the appropriate antibiotic. Antibiotic-resistant progeny are screened for the gene deletion via resistance to respective colicins. Once verified, the deletion is moved to the wild type strain (W3110) by general transduction with P1vir bacteriophage (Miller, 1972) with selection for the corresponding antibiotic resistance. Successful transductants are then transformed with the ampicillin resistant FLP plasmid (pCP20), another plasmid with temperature sensitive replication. This plasmid contains a gene that encodes a fungal enzyme that cleaves at the FRT sites flanking the inserted antibiotic cassette, and then ligates the chromosomal DNA back together, leaving a small “scar sequence” in place of the original antibiotic resistance gene. This scar contains a stop codon and a ribosomal binding site (RBS) to minimize disruption of downstream expression. To lose pCP20, the cells were grown at 42°C and tested against ampicillin for loss of pCP20 and chloramphenicol (cm) or kanamycin (kan) for loss of the antibiotic resistant insert.
Figure 18. Template plasmids pKD3 and pKD4. (A) Linear representation of the template plasmids, pKD3 and pKD4. Arrowheads show location and direction of priming sites (see Table 4). (B) Sequences remaining after FLP-mediated removal of the antibiotic resistance genes. Open arrowheads show the FRT site inverted repeats, rbs indicates ribosome binding site, and met indicates methionine start codon. Figure adapted from Datsenko and Wanner, 2000.
Table 4. Sequence of Primers used in PCR reactions.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Gene and Direction</th>
</tr>
</thead>
<tbody>
<tr>
<td>oRA0159RE D</td>
<td>5' gaagccctctgcgtccctcctccaaattatattgctcgaggagtttaagcactggagctgcttc 3'</td>
<td>flanking tolQ + FRT site; forward</td>
</tr>
<tr>
<td>oRA0171RE D</td>
<td>5' ttggaacttgagacgcagacgctcgtcctcctgcttcagcatatgaatatcctcctttag 3'</td>
<td>flanking tolQ + FRT site; reverse</td>
</tr>
<tr>
<td>oRA0172RE D</td>
<td>5' cagctttaccgtagagaggagaaacagttgcatatgaatatcctcctttag 3'</td>
<td>flanking tolR + FRT site; forward</td>
</tr>
<tr>
<td>oRA0173RE D</td>
<td>5' gctgttacccgctcttcttcaagcagcagcagcatatgaatatcctcctttag 3'</td>
<td>flanking tolR + FRT site; reverse</td>
</tr>
<tr>
<td>oRA0189</td>
<td>5' cgagaatcctttcgtga 3'</td>
<td>ybgC (upstream of tolQ)</td>
</tr>
<tr>
<td>oRA0190</td>
<td>5' aagatgacatgcagct 3'</td>
<td>tolA (downstream of tolR)</td>
</tr>
</tbody>
</table>

Plain font denotes gene sequences whereas bold font denotes sequences flanking the FRT site in pKD3 and pKD4. Primers names ending in RED denote use in performing deletions in the λRed recombination system.
Once the primary strains were built (Table 5), I tested the strain phenotypes by complementation studies on RA1035 (ΔtolQR), RA1027 (ΔtolQ), and RA1028 (ΔtolR). Each strain was transformed with pBAD24 as a control or the complementary genes on a pBAD24 background and tested for sensitivity to colicin E1 and colicin Ia (a group B channel-forming colicin that uses the Fe$^{3+}$ OM receptor, Cir) (Braun et al., 2002). When tolQRA (pTPS202) was expressed in RA1035 and tolR (pRA002) was expressed in RA1028, sensitivity to E1 was restored and sensitivity to Ia was unchanged. Unfortunately, when tolQ (pRA001) was expressed in RA1027, sensitivity to E1 was not restored. It was suspected that the deletion of tolQ in RA1027 had polar effects on tolR, prohibiting the activity of TolA in the system. To substantiate this theory, I transformed the strain with pRA002 (tolR) or pTPS202 (tolQRA) and assayed against colE1. When tolR was expressed alone, sensitivity was not restored, but when tolQRA was expressed, sensitivity to colE1 was restored. This confirmed that the tolQ deletion was polar on tolR in RA1027. To rectify this problem, when testing for the effects of a deletion of tolQ, the strain must carry pRA002 to maintain the expression of tolR. Due to these results, I also tested for polar effects of ΔtolQR on the tolA gene. I transformed RA1035 with pRA003 (tolQR) and found that sensitivity to colE1, colA, and colK was restored indicating that the deletion does not block transcription of the downstream tolA gene. As a result of having to use pRA002 in the RA1027 strain, I decided to transform all strains with pBAD24 so that they all carried resistance to ampicillin. This allowed for maintaining uniform growth conditions throughout testing.

To verify the genotype of the deletion strains, I amplified the tolQR operon using standard PCR and primers oRA189 and oRA190 to confirm each deletion on the chromosome (Figure 19B). These primers flank the tolQR operon, homologous to sequences just upstream to tolQ (oRA189) and just downstream tolR (oRA190) (Table 4). When amplified using these
Table 5. Isogeneic strains used to study energy harvesting and transfer to the transducer molecules.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Genotype</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA1003</td>
<td>W3110 Δ<code>exbBD:: kan</code> (deletion of bases 585-1745 replaced with kan^r gene from pACYC184)</td>
<td>Larsen et al., 2007</td>
</tr>
<tr>
<td>RA1015</td>
<td>W3110 Δ<code>exbB; λ</code> Red System</td>
<td>Larsen, Present study</td>
</tr>
<tr>
<td>RA1016</td>
<td>W3110 Δ<code>tolQR; λ</code> Red System</td>
<td>Larsen, Present study</td>
</tr>
<tr>
<td>RA1022</td>
<td>W3110 Δ<code>exbD; λ</code> Red System</td>
<td>Larsen, Present study</td>
</tr>
<tr>
<td>RA1027</td>
<td>W3110 Δ<code>tolQ; λ</code> Red System</td>
<td>Brinkman, Present study</td>
</tr>
<tr>
<td>RA1028</td>
<td>W3110 Δ<code>tolR; λ</code> Red System</td>
<td>Brinkman, Present study</td>
</tr>
<tr>
<td>RA1033</td>
<td>W3110 Δ`exbBD::kan tolQ; P1 Transduction into RA1027</td>
<td>Zimmerman, Present study</td>
</tr>
<tr>
<td>RA1034</td>
<td>W3110 Δ`exbBD::kan tolR; P1 Transduction into RA1028</td>
<td>Zimmerman, Present study</td>
</tr>
<tr>
<td>RA1035</td>
<td>W3110 Δ<code>tolQR; λ</code> Red System</td>
<td>Brinkman, Present study</td>
</tr>
<tr>
<td>RA1044</td>
<td>W3110 Δ`exbBtolR; P1 Transduction into RA1028</td>
<td>Brinkman, Present study</td>
</tr>
<tr>
<td>RA1045</td>
<td>W3110 Δ`exbDtolQR; P1 Transduction into RA1022</td>
<td>Brinkman, Present study</td>
</tr>
<tr>
<td>RA1046</td>
<td>W3110 Δ`exbDtolQR; P1 Transduction into RA1035</td>
<td>Brinkman, Present study</td>
</tr>
<tr>
<td>RA1050</td>
<td>W3110 Δ`exbDtolQ; P1 Transduction into RA1022</td>
<td>Brinkman, Present study</td>
</tr>
<tr>
<td>RA1051</td>
<td>W3110 Δ`exbBDtolQR; P1 Transduction into RA1035</td>
<td>Brinkman, Present study</td>
</tr>
</tbody>
</table>
primers, various sized DNA products will form determined by the absence of each gene (Figure 19A). The amplified product for the wild-type strain was the full 1364 bp due to the presence of both \textit{tolQ} and \textit{tolR} on the chromosome. The strain carrying neither gene produced the smallest gene product at 324 bp, whereas the single deletions produced DNA products of 752 bp for \textit{ΔtolQ} and 1016 bp for the \textit{ΔtolR} strain. A restriction map of the mutants was created using known restriction sites. The NcoI site (Figure 19A), located between \textit{tolQ} and \textit{tolR}, is present in the wild-type operon, but destroyed when either (or both) gene is deleted. This approach was used to create \textit{ΔtolQ}, \textit{ΔtolR}, \textit{ΔtolQRA}, \textit{ΔexbB}, \textit{ΔexbD}, and \textit{ΔtolQR} strains. Additional P1-mediated transductions were used to generate a set of strains bearing these deletions (and a previously made \textit{exbBD::kan} mutation) in a variety of permutations on a common (W3110) genetic background (Table 5).

These combinations allow me to directly compare the ability of various energy transduction complexes to energize either TonB or TolA by using a uniform set of assays. Placing mixed and wild-type operons onto the chromosome may be crucial for achieving an optimal stoichiometric balance of complex components because the second gene, in both cases (\textit{tolR} and \textit{exbD}), does not have its own RBS and requires the ribosome to continue to read through the previous gene and remain on the operon.

These strategies will allow us to make the appropriate protein combinations and introduce protein derivatives to evaluate the contributions of individual proteins and motifs to the transfer of energy from the harvesting complexes to the transducing molecules, as described in the following chapters.
Figure 19. Amplified tolQR operon of strains created using the λRed system. The tolQR operon was amplified with primers oRA0189 and oRA0190 (see Table 4) to verify that the strains had the correct genotype and that the proper deletions did occur. (A) oRA0189 amplifies upstream from tolQ and oRA0190 amplifies downstream of tolR, denoted by the upward facing arrows. The expected DNA amplimers using these primers are shown as black lines below the operon and the deleted sequence is shown as a red dotted line. The NcoI restriction site that is normally present in the operon is destroyed during mutagenesis. (B) PCR products were run on a 0.8% agarose gel and predicted sizes for: (A) W3110 (wild-type), (B) RA1028 (ΔtolR), (C) RA1027 (ΔtolQ), (D) RA1035 (ΔtolQR), are drawn above. Extra bands seen are a result of primers laying down elsewhere on the chromosome.
CHAPTER III.

AIM TWO: EVALUATION OF ENERGY HARVESTING COMPLEXES TO DETERMINE TRANSDUCER SPECIFICITY

Note: This chapter is written in the form of a manuscript for submission to the Journal of Bacteriology. It follows the publication guidelines and format for that journal. As such, some portions of the Methods reiterate and extend aspects of previous chapters.

Introduction

Gram negative bacteria have a dual membrane system. Whereas the outer membrane (OM) is essentially uncharged, the cytoplasmic membrane (CM) maintains a significant electrochemical gradient that supports a variety of energy-dependent processes. *Escherichia coli* and other bacteria have two protein systems that facilitate energy-dependent processes in the OM by transducing energy from the CM. The TonB system consists of two membrane-bound energy harvesting proteins, ExbB and ExbD, and a transducer protein, TonB, which has a single transmembrane domain in the CM and the majority of the protein in the periplasmic space. The TonB system energizes the transport of ferric siderophores and vitamin B$_{12}$ across the outer membrane (Frost and Rosenberg, 1975; Hantke and Braun, 1975; Bassford *et al*., 1976). TonB also confers sensitivity to certain bacteriophage (Hancock and Braun, 1975) and colicins (group B; Davies and Reeves, 1975) that exploit the system to traverse the OM defense (see Postle and Larsen, 2007 for review).

A second system in gram negative bacteria, paralogous to the TonB system, also transfers energy from the CM to the OM. The Tol system contains two CM resident proteins, TolQ, which is paralogous to ExbB, and TolR, which is paralogous to ExbD. The transducer protein in this
system, TolA, is superficially similar to TonB. While their membrane topologies are similar (Hannavy et al., 1990; Roof et al., 1991; Levengood et al., 1991), TonB and TolA share little primary amino acid sequence (Postle and Good, 1983; Levengood and Webster, 1989) with the exception of their signal anchors, which share a conserved set of residues that occupy one face of the predicted transmembrane $\alpha$-helix (Koebnik, 1993). The physiological function of the Tol system is unknown but because tol strains are hypersensitive to deoxycholate and certain antibiotics, shed outer membrane (Bernadac et al., 1998), and leak periplasmic proteins (Nagel et al., 1967; Lazzaroni and Portalier, 1981), it is thought that this system is responsible for maintaining outer membrane structure and function. Recent evidence suggests one potential function is to mediate invagination of the OM as part of septal formation during cell division (Gerding et al., 2007). Like TonB, TolA also confers sensitivity to certain bacteriophage (f1: Smilowitz, 1974) and colicins (Group A; Nagel et al., 1967) that exploit the system to gain access to the cell (see Lazzaroni et al., 2002 for review).

The E.coli exbB gene encodes a predicted 244 residue protein (Eick-Helmerich and Braun, 1989) with a membrane topology consisting of a periplasmically exposed amino terminus, three transmembrane segments separated by a large positively charged cytoplasmic domain and a small periplasmic loop, and a cytoplasmically displayed carboxyl-terminus (Kampfenkel and Braun, 1993; Karlsson et al., 1993). Likewise, tolQ encodes a predicted 230 residue protein (Sun and Webster, 1986) with a membrane topology like that of ExbB (Vianney et al., 1994). The E.coli exbD gene encodes a predicted 141 residue protein (Eick-Helmerich et al., 1989) with the membrane topology consisting of a cytoplasmically localized amino terminus followed by a single transmembrane hydrophobic domain, and the polar carboxyl terminus that extends into the periplasm (Kampfenkel and Braun, 1992). The tolR gene encodes a predicted
142 residue protein (Sun and Webster, 1986) with a membrane topology similar to that of ExbD (Muller et al., 1993).

Cumulative evidence indicates the energization of TonB and TolA requires specific interactions between their respective transmembrane domains and components of their energy harvesting apparatus (Germon et al., 1998; Larsen et al., 1994, 1999; Larsen and Postle, 2001). Interestingly, strains that lack one energy-harvesting complex remain able to energize both transducers, whereas in strains lacking both energy harvesting complexes neither transducer is energized (Braun, 1989; Braun and Hermann, 1993, Skare and Postle, 1991, Skare et al., 1993). While there is clearly crosstalk between the systems, it is not efficient. Quantitative transport assays of TonB-dependent ligands in exbBD strains indicate that tolQR complexes can support about 10% of the TonB activity normally achieved using ExbBD complexes (Skare and Postle, 1991; Skare et al., 1993).

This apparent crosstalk between systems, coupled with the evident homology shared by the energy harvesting complexes, suggests that the mechanisms of energy harvest and transfer are similar for the two systems. The lower efficiency of crosstalk suggests that the motifs that mediate energization of the transducers are different. The phenomenon of cross-talk provides a way to dissect those specific structural features that are involved in the interactions between the proteins in these complexes that facilitate energy transfer. Because cross-talk is inefficient, we can begin by identifying the characteristics that dictate specificity between transducers and energy harvesting complexes. Our approach is to generate chimeric energy harvesting complexes, i.e., pairing energy harvesting proteins from one system with its partner’s homologue from the second system, and evaluate changes in cross-talk efficiency.
To test the contributions of the specific proteins to energization, each protein needs to be expressed both individually and in combination with other individual proteins of the two systems. This requires strains in which functional genes for each protein are absent or present depending on the phenotype to be tested. Isogeneic strains were created with each protein in the energy harvesting complexes (EHC) expressed singly and in combination with the other energy harvesting proteins (see Table 7). The ability of wild-type and mixed (chimeric) complexes to energize TonB and TolA was scored by examining sensitivity to specific colicins and bacteriophage. Energization of TonB was further studied by examining the ability of the different EHC proteins to support TonB-dependent iron uptake and bacteriophage ϕ80 adsorption. EHC-dependent TonB stability and TonB-EHC protein interactions were also determined.

**Materials and Methods**

**Bacterial strains and plasmids.** The bacterial strains and plasmids used in all experiments are listed in Table 7. Plasmids encoding either ExbD or various Tol system proteins were constructed using the polymerase chain reaction (PCR) and specific primers to amplify the desired genes from genomic DNA purified from the *E.coli* strain W3110. Amplimers were then cloned under the regulation of the arabinose promoter of plasmid pBAD24 (Guzman et al., 1995) by standard molecular biology techniques. All constructs were confirmed by sequence analysis. Single and multiple gene deletions were made using the λ phage Red recombination pathway as described by Datsenko and Wanner (2000), which allows a more efficient and enhanced rate of recombination, allowing the incorporation of linear DNA to make chromosomal deletions using relatively short stretches of sequence identity. Briefly, PCR was used to amplify a drug resistance gene that is flanked by a pair of FRT sites, using primers with 5’ identity to sequences
Table 7. *Escherichia coli* strains and plasmids in Chapter III

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant genotype/phenotype</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW25113</td>
<td><em>(lacI, rrnB_{T14}, ΔlacZ_{wJ16}, hsdR514,</em>&lt;br&gt;ΔaraBAD_{AH3}, ΔrhaBAD_{LD78})*</td>
<td>(Dansenko <em>et al.</em> 2000)</td>
</tr>
<tr>
<td>W3110</td>
<td>F– IN(rrnD–rrnE)1</td>
<td>(Hill &amp; Harnish, 1981)</td>
</tr>
<tr>
<td>RA1003</td>
<td>W3110 ΔexbBD::kan</td>
<td>(Larsen <em>et al.</em>, 2007)</td>
</tr>
<tr>
<td>RA1034</td>
<td>W3110 ΔexbBD::kan, ΔtolR</td>
<td>(present study)</td>
</tr>
<tr>
<td>RA1035</td>
<td>W3110 ΔtolQR</td>
<td>(present study)</td>
</tr>
<tr>
<td>RA1044</td>
<td>W3110 ΔexbB, ΔtolR</td>
<td>(present study)</td>
</tr>
<tr>
<td>RA1045</td>
<td>W3110 ΔtolQR, ΔexbD</td>
<td>(present study)</td>
</tr>
<tr>
<td>RA1046</td>
<td>W3110 ΔtolQR, ΔexbB</td>
<td>(present study)</td>
</tr>
<tr>
<td>RA1051</td>
<td>W3110 ΔexbBD::kan, ΔtolQR</td>
<td>(present study)</td>
</tr>
<tr>
<td><strong>Plasmids:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBAD24</td>
<td><em>araBAD</em> promoter, AraC, amp{r}</td>
<td>(Guzman <em>et al.</em>, 1995)</td>
</tr>
<tr>
<td>pRA002</td>
<td>pBAD 24 <em>araBAD</em>-regulated <em>tolR</em></td>
<td>(present study)</td>
</tr>
</tbody>
</table>
that flank the gene targeted for deletion. The resultant amplimers were then electroporated into the *E. coli* strain BW25113, with drug-resistant homogenotes selected. Deletions were then moved to W3110 by generalized transduction using bacteriophage P1\textsubscript{vir} (Miller, 1972). The antibiotic resistance genes were then excised at the FRT sites by a plasmid-encoded FLP recombinase, with removal confirmed by drug-sensitivity testing. This approach was used to create Δ*tolQ*, Δ*tolR*, Δ*tolQRA*, Δ*exbB*, Δ*exbD*, and Δ*tolQR* strains. Additional rounds of P1\textsubscript{vir}-mediated generalized transduction generated further combinations of these deletions, as catalogued in Table 7. Deletions of the *exbB* and *exbD* genes were verified by immunoblot analysis (as described below) of cell lysates, using ExbB-specific and ExbD-specific polyclonal antibodies (Higgs et al., 2002). These analyses demonstrated that not only was the specific gene product (ExbB or ExbD) absent from the corresponding deletion strain, but also that the deletions did not have polar effects – i.e. the deletion on one did not alter the expression of the other member of this gene pair (data not shown). These observations were confirmed by colicin-sensitivity assays to determine loss of function, followed by complementation experiments in which individual deletion strains were transformed with plasmids encoding either ExbB or ExbD which, in every case – restored wild-type levels of function (data not shown). Because specific antibodies are not available for proteins of the Tol system, an alternative strategy was used to verify deletions. First, the absence of the each gene or gene set was confirmed by PCR, using genomic DNA isolated from each strain, and primers that flank the *tolQRA* operon. The identity of resultant amplimers (and the deletions evident therein) was confirmed by mapping with restriction endonucleases (data not shown). Tol deletions were confirmed by colicin-sensitivity assays to determine loss of function, followed by complementation experiments in which individual deletion strains were transformed with plasmids carrying either the *tolQRA* gene
cluster, *tolQR*, or the individual genes. Interestingly, while the introduction of *tolQRA* bearing plasmids restored activity to all deletions tested, complementation studies with individual genes in their corresponding deletions did identify polar effects. Specifically, deletion of *tolQ* was polar on *tolR*, such that function could only be restored in these strains with plasmids encoding both TolQ and TolR proteins (data not shown). Thus, for strains in which we desired the absence of TolQ, but the presence of TolR, it was necessary to provide TolR *in trans* from the plasmid pRA002.

**Media.** All bacterial strains and plasmids were maintained on Luria-Bertani (LB) agar plates supplemented with ampicillin at 100 µg ml⁻¹ where appropriate. Colicin and phage sensitivity assays were performed in T-top agar overlays on T plates supplemented with ampicillin where appropriate. Cells for all experiments were grown at 37°C with aeration in broth (either LB- or T-broth, as indicated) with antibiotic supplement as appropriate.

**Chemicals and reagents.** Colicin B and colicin A were produced as described previously (Larsen *et al.*, 2003) with cell membranes being disrupted via passage through a French pressure cell at 20,000 psi. The TonB-specific monoclonal antibody 4F1 (Larsen *et al.*, 1996) was kindly provided Dr. Kathleen Postle (Department of Biochemistry and Molecular Biology, The Pennsylvania State University) and used at 1:1,000 dilution, whereas goat-anti-mouse immunoglobulin G was purchased from Caltag Laboratories and used at 1:2,500 dilution. ⁵⁵Fe (4.5 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences. Iron-free ferrichrome was purchased from Sigma.

**TonB stability.** All strains were grown to an A₅₅₀ of 0.4 (with a path length of 1.5 cm) in LB broth supplemented with ampicillin. Chloramphenicol was then added to a final concentration of 100 µg ml⁻¹ to halt protein synthesis. Samples were taken at 0 min, 15 min, 30
min, 60 min, and 120 min and precipitated in 10% w/v trichloroacetic acid (TCA). Samples were washed in 100 mM Tris-Cl (pH 8.0), then suspended in 25 µl of Laemmli sample buffer (Laemmli, 1970), incubated at 98°C for 5 min, then stored at -20°C for later immunoblot analysis.

**Phage and colicin assays.** Phage and colicin spot titer assays were performed as previously described (Larsen et al., 2003) with serial five-fold dilutions of colicin B and colicin A and ten-fold dilutions of bacteriophage φ80. Cells were grown in LB broth supplemented with ampicillin and plated onto ampicillin supplemented T plates in T top agar containing ampicillin and 0.01% arabinose. In all cases, the plates were inoculated in triplicate, incubated for 18 h at 37°C, and then scored for clearing or plaque number out to the highest dilution.

**Transport of radiolabeled ligands.** Ferrichrome transport assays were performed with [⁵⁵Fe]-loaded ferrichrome as previously described (Larsen and Postle, 2001; Koster and Braun, 1990). Strains were grown to an A₅₅₀ of 0.4 in T-broth with ampicillin, at which time 10 ml were harvested and spun down. The pellets were resuspended in 10 ml of M9 salts with 0.1 mM nitrilotriacetate and 4% w/v D-glucose. Cell suspensions were incubated for 5 min at 30°C and a 0.5 ml sample was removed and TCA precipitated prior to performing the transport assay. These samples were later immunoblotted to verify equal sample amounts. Transport began by adding 10 µl of 150 pmol [⁵⁵Fe]-loaded ferrichrome to the cell culture and shaking for 2, 6, 10, and 14 min at 30°C. At each time point, 500 µl of cells were removed and deposited in the center of a Whatman GF/C filter in a vacuum manifold. This was done in triplicate and the remaining cells were placed back into the incubator until the next time point. Each filter was rinsed thrice with 100 mM LiCl to wash away any [⁵⁵Fe]-ferrichrome not taken up by the cells. Filters were removed and air-dried. This was repeated for each time point. Once all filters were dry, they
were placed in plastic scintillation tubes and 2.5 ml of CytoScint was added. The tubes were incubated overnight at room temperature. Incorporated $[^{55}\text{Fe}]$ was determined as counts per minute (cpm) using a Beckman LS 3801 liquid scintillation counter.

**Irreversible phage adsorption assay.** The φ80 adsorption assays were performed as previously described (Larsen *et al.*, 1993). Strains were grown in LB supplemented with ampicillin overnight, diluted 1:100 and grown to an $A_{550}$ of 0.4, at which time 1 ml samples were taken, spun down, and resuspended in LB with 5 μM CaCl$_2$. Cells were incubated for 5 min at 37°C and 10μl of φ80 dilution was added. Cell-phage mixtures were incubated again and at 0, 5, 10, and 20 min 20 μl samples were harvested in LB with 5% v/v CHCl$_3$. Residual phage for each sample were titered on W3110 and the number of phage plaques present on the bacterial lawns was determined. The experiment was performed in quadruplicate for each strain, except strains RA1045 + pRA002 and pRA1035 + pBAD24, which were performed in triplicate.

**In vivo chemical cross-linking.** Cells were grown in T-broth supplemented with ampicillin to an $A_{550}$ of 0.4, harvested in 1.0 ml aliquots, centrifuged and suspended in 938 μl of 100 mM phosphate buffer (pH6.8). Then 62 μl of 16% para-formaldehyde was added and suspensions were incubated at room temperature for 15 min, centrifuged and suspended in 25 μl of Laemmli sample buffer. Samples were incubated at 60°C for 5 min and stored at -20°C for later immunoblot analysis.

**Immunoblot analysis of TonB protein.** Samples prepared as described above were resolved on SDS 11% polyacrylamide gels (Laemmli, 1970), immunoblotted using TonB-specific monoclonal antibodies (Larsen *et al.*, 1996), and visualized by enhanced chemiluminescence as previously described (Skare *et al.*, 1993). Following immunoblot
analysis, membranes were stained for total protein with Coomassie blue and visually examined to confirm equivalent sample loading of all lanes.

Results and Discussion

To test the contributions of the specific proteins to transducer energization, each protein needs to be expressed both individually and in combination with other individual proteins of the two systems. This requires strains in which functional genes for each protein are absent and vehicles that will allow us to reintroduce genes encoding wild-type proteins singly and in various combinations for evaluation. Expression of individual proteins at chromosomal levels is essential for meaningful interpretation because stoichiometry is vital in the workings of these complexes. Over-expression of TonB results in decreased activity (Heller et al., 1985; Larsen et al., 2003). This dominant negative gene dosage effect probably results from the degradation of TonB, which has a greatly decreased stability when over-expressed (Postle and Skare, 1988; Fischer et al., 1989) and in the absence of ExbB and ExbD (Skare and Postle, 1991). To avoid the potential problems associated with plasmids (i.e. plasmid retention and balancing stoichiometries when genes are present in multiple copies) we decided to, wherever possible, work with genes and constructs that have been stably integrated into the chromosome as single copies. Due to problems with polarity an active tolR was replaced on a pBAD24 background. Because this plasmid (pRA002) contains an ampicillin-resistance cassette, it was necessary to introduce an ampicillin resistance plasmid into all strains. Using pBAD24, we were able to maintain as little variation between strains as possible.

Activity of chimeric energy-harvesting complexes. The functional state of various energy-harvesting complexes was determined by examining their ability to support TonB- and
TolA-specific processes. Because the actual function of the Tol system remains unknown, most assays of Tol function are indirect and qualitative (for review see Lazzaroni et al., 2002). The most readily quantifiable measure of Tol system activity is in sensitivity to group A colicins --- protein toxins that exploit the Tol system to traverse the OM barrier of *E. coli*. In the present study, sensitivity to group A colicins was evaluated by spot titer assays (Table 8). Wild-type cells (B+D+Q+R+) were sensitive to colicin A at a 78,125–fold dilution (5^7), with similar sensitivities in B’D’Q’R+ cells, confirming the primacy of the TolQ/R complex in energizing the TolA transducer protein. Conversely, little activity was evident in B’D’Q’R- cells, suggesting that ExbB/D complexes only poorly supported the ability of TolA. No activity was evident for partial complexes, or for chimeric complexes (B’D’Q’R- or B’D’Q’R+) by this assay (Table 8). Similar results were obtained with the additional group A colicins K and N (data not shown).

Much as group A colicins exploit the Tol system, group B colicins target the TonB system – providing a similar assay for TonB function. Here, the group B colicins, B (Table 8) and Ia (data not shown), were used to evaluate the ability of various energy-harvesting complexes to energize the TonB transducer protein. As seen for the Tol system, the cognate transducer system (ExbB/D) supported TonB activity much more efficiently than the paralogous system (TolQ/R), with no activity detected for either the partial or the chimeric complexes (Table 8).

Much like group B colicins, the bacteriophage φ80 is also dependent upon the TonB system to traverse the OM. However, because a single phage is sufficient to produce cell death, φ80-dependent killing provides a much more sensitive assay of TonB activity than colicin-mediated killing, detecting as little as one functional copy of TonB protein per cell (Larsen et al., 2003). In this study, wild-type cells (B’D’Q’R+) were sensitive to φ80 dilutions as high as
<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Sensitivitya</th>
<th>Sensitivityb</th>
<th>Sensitivityc</th>
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<tr>
<td>B⁺D⁺Q⁺R⁺</td>
<td>7 7 7</td>
<td>7 7 7</td>
<td>8 8 8</td>
</tr>
<tr>
<td>B⁺D⁺Q⁻R⁻</td>
<td>R R R</td>
<td>R R R</td>
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<td>7 8 7</td>
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<td>B⁺D⁻Q⁻R⁻</td>
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<td>R R R</td>
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aScored as the highest dilution (five-fold for colicins, ten-fold for φ80) of agent that provided an evident zone of clearing on a cell lawn. “R” indicates resistance (i.e. no clearing) with the undiluted colicin or phage stock. The values of three platings are presented for each strain/plasmid and agent pairing.
bStrain/plasmid combinations were plated in T medium supplemented with 0.01% w/v L-arabinose.
pR designates that tolR is expressed from a plasmid rather than chromosomally.
100,000,000-fold ($10^{-8}$), with similar sensitivities seen with cells that express only the cognate energy-harvesting complex ($B^+D^+Q^-R^-$), and cells expressing only the paralogous energy-harvesting complex ($B'D'Q'^+R'^-$) sensitive to a one-log lower $\phi$80 dilution (Table 8). This assay also detected some activity for $B^+D'Q^-pR^+$, but not $B'D^+Q'^+R^-$ cells, indicating that chimeric complexes comprised of ExbB and TolR can, at low efficiency, energize TonB. No activity was detected for partial complexes (Table 8).

**Siderophore transport.** Unlike the Tol system, a specific function for the TonB system is known – to transport iron-bearing siderophores. This provides a biologically relevant assay for evaluating TonB function – and the ability of energy-harvesting complexes to support that function. Here, the rate of uptake of ferrichrome loaded with $^{55}$Fe was measured in several different strains, including those strains bearing chimeric complexes. High levels of transport were evident for both the wild-type strain and the strain bearing only the cognate energy-harvesting complex (Figure 20A). Much lower levels of transport were detected for the strain bearing only the paralogous energy-harvesting complex, with no evidence of transport in strains bearing chimeric energy harvesting complexes (Figure 20B). Because the overall sensitivity of siderophore transport is similar to that of group B colicin sensitivity, it is not unexpected that activity was not evident for chimeric complexes – nevertheless these results confirm that mixed complexes, if they occur in wild-type cells, are unlikely to function at biologically relevant levels.

**Phage adsorption.** Irreversible phage adsorption is a liquid-based transport assay that has been shown to distinguish between chromosomal levels and up to 1/200-fold levels of TonB (Larsen *et al.*, 2003). Such a sensitive assay can be useful in correlating TonB function to the
**Figure 20.** [⁵⁵Fe]-ferrichrome transport assay. Various strains were subjected to siderophore transport as described in Materials and Methods. Strains tested were as follows: W3110 + pBAD24 (B⁺D⁺Q⁺R⁺), RA1051 + pBAD24 (B⁻D⁻Q⁻R⁻), RA1035 + pBAD24 (B⁻D⁻Q⁺R⁻), RA1003 + pBAD24 (B⁻D⁺Q⁻R⁻), RA1045 + pRA002 (B⁺R⁺), RA1044 + pBAD24 (B⁻D⁺Q⁻R⁻). Graphed is the average counts per minute (cpm) of three experiments for each strain at each of the 2 min through 14 min time point samples. (A) All six strains graphed, showing wild-type with the greatest transport ability, followed by the B⁺D⁺ strain. (B) Transport for all other strains shown on a smaller axis of cpm counts.
amount of TonB available. Cells that are able to energize TonB will uptake phage efficiently, leaving less phage present in the media. Scoring the amount of residual phage allows for a quantitative determination of TonB energization. The wild-type strain (B\(^{+}\)D\(^{+}\)Q\(^{+}\)R\(^{+}\)) and the strain containing only ExbB/D are both able to sufficiently energize TonB, with less than 10% of residual phage found in the media (Figure 21). This was to be expected as the appropriate energy-harvesting complex was paired with the transducer, TonB. Crosstalk levels, pairing Q\(^{+}\)R\(^{+}\) with TonB, showed previously reported phage adsorption at about 15%. Interestingly, the chimeric complex consisting of ExbB and TolR exhibited phage adsorption levels at or near those of crosstalk levels. This is consistent with results shown in the \(\varphi80\) spot titer assays but not with those shown in Fe-transport or colicin B titers, as these assays are far less sensitive.

**TonB physically interacts with partial and chimeric energy-harvesting complexes.**

The wild-type TonB protein can be chemically cross-linked *in vivo* with other proteins using formaldehyde to form a specific set of complexes that migrate with characteristic molecular masses (Skare *et al.*, 1993). One such complex, migrating with an apparent molecular mass of 59 kDa, is a heterodimer consisting of TonB and ExbB (Skare *et al.*, 1993, Larsen *et al.*, 1994, Higgs *et al.*, 1998). Because the ability to form this cross-linked complex correlates with the productive energization of TonB (Larsen *et al.*, 1994, 1999; Larsen and Postle, 2001), it is likely that the ability to form such complexes reflects close protein – protein interactions between TonB and its energization complex. In the present study, we examined the ability of TonB to form the 59 kDa heteromultimeric complex with ExbB in partial and chimeric energy-harvesting complexes (Figure 22). In the absence of ExbB, a corresponding complex was not evident, consistent with the apparent inability of TonB to form such a complex with TolQ, which would theoretically run at about the same mass. Interestingly, the ability of an energy-harvesting
Figure 21. Irreversible phage adsorption assay. Various strains were subjected to irreversible phage assays as described in Materials and Methods. Strains tested were as follows: W3110 + pBAD24 (B⁺D⁺Q⁺R⁺), RA1051 + pBAD24 (B⁻D⁻Q⁻R⁻), RA1035 + pBAD24 (B⁺D⁻Q⁻R⁻), RA1003 + pBAD24 (B⁻D⁻Q⁺R⁺), RA1045 + pRA002 (B⁺D⁺Q⁻R⁺), RA1044 + pBAD24 (B⁻D⁻Q⁻R⁻). Phage measurements were taken and the percent phage remaining calculated. Graphed is the average of four experiments. The number of plaques at time points 0, 5, 10, and 20 min were divided by the number of plaques at time 0 and multiplied by 100 to determine percentage of plaques remaining.
Figure 22. *In vivo chemical cross-linking*. The various strains were chemically crosslinked using formaldehyde as described in Material and Methods. The immunoblot analysis using α-TonB (4F1) shows wild-type TonB (36 kDa) as well as other TonB-protein complexes. Lanes (A) W3110 + pBAD24 (B tá+Q Q R), (B) RA1051 + pBAD24 (B tá+Q Q R), (C) RA1035 + pBAD24 (B tá+Q Q R), (D) RA1003 + pBAD24 (B tá+Q Q R), (E) RA1044 + pBAD24 (B tá+Q Q R), (F) RA1045 + pRA002 (B tá+Q Q R), (G) RA1045 + pBAD24 (B tá+Q Q R). The position of mass standards (in kilodaltons) and the positions of monomeric TonB, as well as heterodimers of TonB-Lpp and TonB-ExbB, are indicated at the left of the panel.
complex to support TonB activity was not essential for \textit{in vivo} chemical cross-linking between TonB and ExbB – as both strains with a partial complex consisting of ExbB alone and strains with chimeric complexes of ExbB and TolR formed the characteristic 59 kDa complex (Figure 22).

\textbf{TonB stability.} Beyond energizing TonB, the ExbB/D energy-harvesting complex is also important for the stability of TonB. While stable in wild-type cells, in the absence of ExbB/D the chemical stability of TonB is greatly reduced (Skare and Postle, 1991). It is presumed that this stabilization involves physical interactions between TonB and ExbB/D. The ability of ExbB to cross-link with TonB regardless of the presence or nature of the second component of the energy-harvesting complex suggested that perhaps ExbB was alone sufficient to stabilize TonB. To test this hypothesis, the stability of TonB in wild-type and mutant strains was examined (Figure 23). In the wild-type strain, TonB had a chemical half-life of about 90 min, while in the absence of ExbB and ExbD the chemical half life of TonB was less than 15 min, consistent with previous studies (Skare and Postle, 1991). Consistent with the \textit{in vivo} chemical cross-linking results, the presence of ExbB alone, or with TolR was sufficient for the stability of TonB. Surprisingly, TolQ alone, or with ExbD, was also able to stabilize TonB, despite the absence of ExbB. Also interesting is that TolQ alone can stabilize TonB better than when it is complexed with TolR.

\textit{Summary and Prospectus}

Spot titers using \textit{\phi}80 bacteriophage show that chimeric complexes composed of ExbB and TolR energize TonB at a low efficiency. ExbB or TolR alone is unable to support \textit{\phi}80 sensitivity, supporting the idea that transducer activity is dependent upon both energy harvesting
Figure 23. TonB stability. Exposure times were selected from immunoblots of the various strains (A) W3110 + pBAD24 (B’D’Q’R’), (B) RA1051 + pBAD24 (B’D’Q’R’), (C) RA1003 + pBAD24 (B’D’Q’R’), (D) RA1045 + pRA002 (B’D’Q’R’), (E) RA1044 + pBAD24 (B’D’Q’R’), (F) RA1045 + pBAD24 (B’D’Q’R’), (G) RA1034 + pBAD24 (B’D’Q’R’). Exposure times were 1 min (B, G), 5 min (A, D, E), 10 min (F), and overnight (G). Size standards in kilodaltons indicated at the right of figure. Lower bands seen are TonB degradation products.
proteins working in concert. Because ExbB alone can cross-link with TonB, yet ExbB alone cannot energize TonB, the protein may help to stabilize and properly configure the transducer (and this would also explain the question of TolQ alone maintaining TonB stability) while ExbD energizes TonB. This also supports the idea that cross-linking and activity are independent from one another.

The ExbB-TolR complex is able to sufficiently energize TonB enough for \( \phi 80 \) to gain entry into the cell but could not energize it enough for colicin B to parasitize the system. The iron transport assay shows that the chimeric complexes’ ability to energize TonB is insufficient to quantify with radioactively loaded ferrichrome. \( \phi 80 \) sensitivity is a more sensitive assay (Larsen et al., 2003) and by performing \( \phi 80 \) adsorption, quantification of this activity was possible, showing that when ExbB is paired with TolR, TonB can be energized to about the same efficiency as in crosstalk. Considering these results, it is possible that perhaps the TolQ/ExbD complex can, in fact, energize TolA but function cannot be determined due to an insufficiently sensitive assay.

TonB half-life experiments show that ExbB alone or in combination with ExbD or TolR can stabilize TonB. Surprisingly, TolQ alone, or with ExbD, can stabilize TonB better than when TolQ is in complex with TolR. This may show that TolQ and ExbB are mainly responsible for transducer recognition and interaction while ExbD and TolR may play a role in energization. This would also explain why the ExbB-TolR complex is able to sufficiently energize TonB, whereas ExbB alone could not.

When all the data are considered – TonB activity, TonB interactions, and TonB stability – they provide some insight into what may be TonB conformational changes and the varying stability of these conformational variations. There is some evidence to suggest that TonB exists
in three distinct conformations (Letain and Postle, 1997; Larsen et al., 1999; 2003). The TonB “shuttling” model discussed in Chapter one (Figure 12), compiling previously published data, suggests that TonB stores and releases energy through conformational changes while shuttling between membranes. The above data strengthen this theory by showing that instability is a product of activity and that TonB interactions at the CM are dependent upon activation, i.e. conformational, states. When TonB is in an uncharged conformation, it is stable. TonB is not activated by ExbB alone, TolQ alone, or TolQExbD, yet it has a stable half-life in the presence of these three protein combinations. If TonB is charged (by ExbBD, ExbBTolR, or TolQR), its conformation is altered to store potential energy. TonB must interact with one of these three protein complexes to accept the energy transfer, thus stabilizing the protein (Figure 23). Once TonB releases this energy, this discharged conformation can only be recaptured and stabilized by ExbB in complex with either ExbD or TolR (Figure 24). This idea that energization, and thus conformational, states differ in TonB would be greatly enhanced by further studies. Performing membrane fractionation or TonB proteolysis in cells with the single CM proteins or the chimeric complexes could deliver more insight into this phenomenon.

In the future it would be beneficial to continue to look at what roles ExbB and TolR play in interacting and energizing TonB. Because a chimeric complex of ExbB and TolR will support energization of TonB, this suggests that ExbB may be the protein that is responsible for transducer discrimination. It seems as though ExbB, in complex with another CM protein is largely responsible for the recapture of the discharged TonB, after the transducer has delivered energy to the OM. If all of this is true, TolQ is most likely playing the same role in the Tol system. Unfortunately, as of yet this cannot be tested due to a lack of Tol antibodies and assays sufficiently sensitive to test activity.
Figure 24. **TonB stability as a product of activity.** A diagram of the three putative conformational states of TonB and the protein interactions that occur at the cytoplasmic membrane. (1) TonB is not energized by ExbB, TolQ or the TolQExbD complex and thus remains in an uncharged, stable state. (2) TonB is energized and stabilized by ExbBExbD, ExbBTolR, and TolQTolR and is in a charged conformational state. (3) Once TonB delivers the potential energy to the outer membrane, it changes into a discharged conformational state that can only be recaptured by ExbBExbD and ExbBTolR (in red).
The next logical step would be to determine which domains of the ExbB protein are responsible for transducer recognition. Replacement of specific ExbB transmembrane domains with those of TolQ and then pairing these chimeric proteins with ExbD or TolR to evaluate function could be helpful in pinpointing specific regions of the energy harvesting proteins that are interacting with transducer molecules. There are several reasons for swapping transmembrane domains: First, mutations that suppress TonB and TolA mutations occur in this domain (Larsen et al., 1994, 1999; Germon et al., 1998). Second, sequence comparisons of ExbB and TolQ with MotA suggest that transmembrane domains two and three are involved in the proton channel (Zhou et al., 1998; Sato and Homma, 2000), whereas the first domain bears no homology to other proton translocators. Third, the degree of conservation between ExbB and TolQ is less in the first than in the other two domains, making it a more likely candidate for discriminating between the very different energy transducers.
CHAPTER IV.

TONB/TOL A AMINO TERMINAL DOMAIN MODELING

Kimberly L. Keller, Kerry K. Brinkman, & Ray A. Larsen

Note: The following document contained in the first part of this chapter is a manuscript that has been submitted to, and accepted by, Methods in Enzymology. The manuscript, in its entirety, is found on the following pages and describes techniques used to create specific mutants in the TonB and TolA systems. The manuscript was written by Dr. Ray Larsen, construction and testing of mutants was provided by Dr. Kim Keller, and the author of this dissertation was responsible for the transport data. Following this document is an appendix (Chapter V) that describes additional data generated after the document was submitted using the mutants created and described in the manuscript.
Introduction

A dual membrane envelope forms the interface between gram-negative bacteria and their surroundings. The inner layer of this envelope provides a permeability barrier in the form of a cytoplasmic membrane (CM) – a phospholipid bilayer rich in proteins that create and harvest ion gradients to drive essential transport processes. While enclosing the cell proper, the CM is itself enfolded by an aqueous compartment, the periplasmic space. A viscous mix of osmoregulatory polysaccharides and proteins that support nutrient transport and envelope biogenesis, the periplasm also houses a thin corset of peptidoglycan – a concentric mesh of oligopeptide-crosslinked glycan that confers rigidity to the cell. Trussed to this network and enclosing the periplasm is a final layer – a diffusion barrier in the form of an outer membrane (OM). This membrane is uniquely asymmetric, with an inner phospholipid face and an external surface rich in anionic lipid-anchored oligopolysaccharides. Small hydrophilic nutrients can passively traverse this barrier via the aqueous channels of resident porin proteins; however, the polar surface of the OM hinders the passage of various detergents and other hydrophobic toxins that commonly corrupt bacterial surroundings.

The barrier functions of the OM allow gram-negative bacteria to compete in a variety of niches, yet this fortification also poses complications. One major consequence of envelope architecture is that the OM is spatially removed from any significant source of energy. Specifically, the aqueous channels that afford the entry of hydrophilic nutrients also preclude the formation of energetically useful ion gradients; further, the hydrolytic resident proteins of the periplasmic space are not compatible with those phosphorylated molecules that cells favor as energy currency. Thus, any energy-dependent processes that occur at the OM must rely upon imported energy.
An obvious energy need of the OM is simply to support upkeep. It is thermodynamically unlikely that a structure as complex as the OM can be maintained without an input of energy. How such energy is actually used remains ill-defined, indeed the details of OM biogenesis are themselves far from resolved (Ruiz et al., 2006). One set of proteins implicated in OM maintenance is the Tol system. The core components of this system include two cytoplasmic membrane proteins (TolQ and TolR) that appear to form a heteromultimeric complex with a third protein (TolA) that spans the periplasmic space to interact with a fourth protein (TolB) that is peripherally associated with the OM (reviewed in Lazzaroni et al., 1999). Tol mutations disrupt OM integrity, with resultant hypersensitivity to bile salts, detergents and certain antibiotics, leakage of periplasmic contents (Lazzaroni et al., 1989; Lloubes et al., 2001) and shedding of OM vesicles (Bernadac et al., 1998). The possibility that the Tol system provides for the transfer of energy to the OM was first inferred from the ability of TolQ and TolR to partially replace two paralogous proteins (ExbB and ExbD, respectively) from a system known to support energy dependent OM processes – the TonB system (Braun and Herrrmann, 1993).

The TonB system supports a different; more well defined OM energy need, facilitating the active transport of specific nutrients across the outer membrane. Primarily serving to fuel high-affinity transporters of iron siderophores and host iron sequestration proteins (reviewed in Postle and Kadner, 2003; Perkins-Balding et al., 2004; Weiner, 2005), TonB also energizes the high affinity transport of cobalamin, with recent evidence indicating that TonB might support the transport of a more diverse set of ligands in some species (Neugebauer et al., 2005). Owing in part to its well-defined function and relative amenability to quantitative analysis, the TonB system provides the paradigm for the transduction of CM energy to the OM.
Like the Tol system, the core components of the TonB system include two cytoplasmic membrane proteins (ExbB and ExbD) that form a heteromultimeric complex with a third protein (TonB) that traverses the periplasmic space (Skare et al., 1993; Higgs et al., 1998; Held and Postle, 2002). Lacking a TolB analog, TonB instead interacts directly with TonB-dependent OM receptors, facilitating the transport of ligand into the periplasmic space (reviewed in Weiner, 2005). TonB-dependent transport can be blocked by protonophores (Hancock and Braun, 1976; Reynolds et al., 1980) and, in unc strains (which lack CM-bound ATP synthase) by cyanide (Bradbeer, 1993), suggesting that the proton gradient of the CM (i.e. proton motive force: pmf) is the energy source. Subsequent demonstration that TonB undergoes pmf-dependent conformational changes and that this phenomenon requires the ability to interact with ExbB and ExbD suggests that ExbB and ExbD together function to harvest the potential energy of the CM proton gradient, which is then stored conformationally in TonB (Larsen et al., 1999). Further support for a role as the energy-harvesting complex comes from the apparent homology of ExbB and ExbD (and their paralogs TolQ and TolR) with the proton-harvesting MotA and MotB proteins of the bacterial flagellar motor (Kojima and Blair, 2001).

The preponderance of data suggest that the ability of TonB to interact productively with the ExbB/D energy-harvesting complex is dependent upon the amino-terminal region of TonB. Originally predicted to contain a single transmembrane domain (residues 12-32) on the basis of hydrophobicity (Postle and Good, 1983), this region was subsequently shown to mediate the Sec-dependent partitioning of TonB to the CM (Postle and Skare, 1988). Topological analyses indicate the bulk of TonB localizes to the periplasmic space, confirming a single amino-terminal anchorage in the CM (Hannavy et al., 1990; Roof et al., 1991). Essential for function (Karlsson et al., 1993, Jaskula et al., 1994), specific interactions between this signal anchor domain and the
energy-harvesting ExbB/ExbD complex provide for the conversion of TonB to the energized state (Larsen et al., 1999). In the absence of ExbB and ExbD, TonB function is impaired, but not absent (Eick-Helmerich and Braun, 1989) – with the residual TonB activity reflecting the ability of the ExbB/D paralogs TolQ/R to energize TonB (Braun and Herrmann, 1993).

When modeled as an α-helix, the TonB signal anchor presents a face of four residues (Ser$_{16}$, His$_{20}$, Leu$_{27}$, & Ser$_{31}$) shared by the TonB paralog TolA (Koebnik, 1993) and conserved amongst various gram-negative enteric bacteria (reviewed in Postle and Larsen, 2004). Because this motif marks the sole sequence identity between TonB and TolA, it is likely that these residues contribute to the ability of these energy transducers to (albeit imperfectly) be energized by each other’s energy-harvesting complexes. Clearly two of the residues in this motif are important for the activity of TonB (Ser$_{16}$ and His$_{20}$) and TolA (Ser$_{18}$ & His$_{22}$), with substitutions of either residue (and, at least in the case of TonB, alterations in the spacing between the two residues) rendering the proteins unable to support OM functions (Larsen et al., 1994, 1999; Germon et al., 1998; Larsen and Postle, 2001). In the case of TonB, the mutant phenotype can be suppressed by second site mutations that map to the gene encoding the energy-harvesting complex protein ExbB (Larsen et al., 1994, 1999); similar suppressors of TolA mutations map to the gene encoding the ExbB paralog TolQ (Germon et al., 1998). Interestingly, in the presence of extragenic suppression, these TonB mutants are unstable, with significantly shorted physical half-lives – but only in cells exposed to transportable ligand. This suggests substitutions at Ser$_{16}$ and His$_{20}$ alter features required for efficient recycling of TonB following the release of conformationally stored energy (Larsen et al., 1999).

Thus, beyond simply tethering the protein to the energy source, the amino termini of TonB and TolA make specific contributions to their function as energy transducers. First, this
region participates in the acquisition of energy from the energy-harvesting complex (ExbB/D or TolQ/R). Second, at least in the case of TonB, it contributes to the efficient recycling of TonB following the transfer of energy to an OM recipient. It is likely that each of these roles involve the specific recognition of structural motifs by components of the energy-harvesting complexes. As noted above, the Ser-His couple appears to contribute one such motif. Scanning single-residue deletion mutagenesis of the amino-terminal portion of the TonB signal anchor (residues 10-24) found only residues 16-20 crucial for function. In that study, the replacement of the residues at positions 17-19 by three consecutive alanyl residues did not alter TonB function, indicating that these residues served solely to maintain the relative positioning of Ser16 to His20 (Larsen and Postle, 2001).

Mutagenesis studies involving single-residue changes have not provided for the identification of additional regions that participate in the interaction of TonB and TolA with their respective energy-harvesting complexes. However, because the efficiency with which TonB and TolA are energized varies depending upon which energy-harvesting complex is available, there must be features in addition to the shared Ser-His motif that dictate this specificity. To identify such amino-terminal regions, we have adopted a strategy wherein blocks, rather than individual residues, are altered. Specifically, we have used two approaches. In the first approach, consecutive alanyl residues replace blocks of wild type residues, creating a set of TonB derivatives with an increasingly “generic” signal anchor. In the second approach, specific TolA sections are exchanged for the corresponding TonB region, producing a set of chimeric proteins for evaluation. Both sets of derivatives are generated by adaptations of polymerase chain reaction (PCR), as described and illustrated with the examples that follow.
Alanyl replacement

Two previously constructed plasmids (Larsen *et al.*, 1999) provide the foundation for constructing *tonB* derivatives that encode proteins with multiple alanyl substitutions. The first of these is pKP315, a pBAD18-based construct (Guzman *et al.*, 1995) in which the *tonB* gene (including its transcriptional start and rho-independent terminator) is placed under control of the bidirectional *araBAD* promoter, providing for the arabinose-regulated expression of wild-type TonB. The second is pKP325, in which this *tonB* assemblage and the flanking *araC* gene have been moved into the plasmid pACYC184. In both plasmids, a pair of *BamHI* restriction sites flanks the *tonB* gene to facilitate the recovery of engineered derivatives (Figure 25A). Site-directed mutagenesis is performed by a modification of a method described by Michael (1994) in which a third, 5’-phosphorylated oligonucleotide primer carrying the desired base changes is included in a modified polymerase chain reaction that uses a thermostable ligase to incorporate this mutagenic primer in the extending strand (Larsen and Postle, 2001). Using non-mutagenic primers that flank the *BamHI* sites, an amplimer is generated that, upon restriction with *BamHI*, can be inserted as a cassette into a similarly restricted parent vector. By designing mutagenic primers to include (or, as possible, exclude) a given restriction site, products bearing the desired mutations can be identified by restriction mapping, with identities then verified by nucleotide sequence determination. Initially used to make silent mutations that introduce restriction sites and to make single-residue substitutions and deletions, we ultimately used this approach to generate a larger mutation, replacing TonB residues 17-19 with alanyl residues (Larsen and Postle, 2001). We have subsequently used this approach to replace blocks of four to seven residues with alanyl residues. To illustrate this approach, the replacement of TonB residues 12-15 by four consecutive alanyl residues is described.
Figure 25: Three primer site-directed mutagenesis. Panel A: The template portion of plasmids pKP315 and pKP325 is depicted, including the *araBAD* promoter (*pBAD*) and the *tonB* gene. Primers used for amplification are indicated at each edge of the region, and the relative position of the 5' phosphorylated mutagenic primer is shown. The *BamHI* sites used for cloning are indicated. Panel B: The mutagenic primer and corresponding wild type coding strand are shown. Substitutions introduced by the mutagenic primer are indicated in bold, with the introduced *PvuII* site used for screening indicated by the underlined sequence.
To replace TonB residues 12-15, a mutagenic primer was designed (Figure 25B) in which the four-codon change was nested between five upstream and four downstream codons identical to the wild-type coding strand. The mutagenic codons also introduced a *PvuII* restriction site (CAGCTG – as underlined in Figure 25B) for screening purposes. Reactions were set up in volumes of 50 µl as follows:

1.) 50 pmol of each flanking primer.

2.) 5 pmol of mutagenic primer.

3.) 200 pmol each dNTP.

4.) 5 µl 10 X *Taq* DNA thermoligase buffer (200 mM Tris-Cl [pH 7.6], 250 mM potassium acetate, 100 mM magnesium acetate, 100 mM dithiolthritol, 10 mM NAD, 1% triton X-100; New England Biolabs, Inc., Beverly, MA).

5.) 10 units *Taq* DNA ligase (New England Biolabs, Inc.).

6.) 2 units Deep Vent *Taq* DNA polymerase (New England Biolabs, Inc.).

7.) 0.01 µg pKP315 (as template).

Controls lacking either the mutagenic primer or the template were included. Mixtures were subjected to 35 cycles of melting (94°C for 30 sec), annealing (52°C for 30 sec), and extension (67°C for 240 sec). Note that extensions are allowed to run longer than for normal polymerase chain reactions because our reaction conditions, optimized for the ligation reaction, are not optimal for the polymerase itself. Also note that these reactions will generate three products – two will be full length (in this case 963 bp), the majority of which will have incorporated the mutagenic primer and are distinguished from the wild type by having a new *PvuII* site; and one will be shorter (in this case 780 bp), produced from the mutagenic and downstream primers. If the mutagenic primer is used in a 1:1 molar ratio with the flanking primers, virtually all of the
final product will be present in this shorter form – thus we use one-tenth the concentration of mutagenic primer relative to the flanking primers; and under these circumstances the full length amplimers are generally the dominant reaction products.

Resultant products were purified using a Qiaquick PCR purification kit (Qiagen Inc., Valencia, CA), then digested with *BamHI* and resolved on a 1% agarose gel. In this example, digestion of the full-length amplimer (963 bp) resulted in a band of about 900 bp, readily distinguished from the secondary, ~780 bp band generated by the mutagenic and the downstream primers. The full length amplimer was excised from the gel and recovered using a Qiagen gel extraction kit (Qiagen, Inc.), then inserted into a *BamHI* restricted, dephosphorylated pKP325 vector in a standard ligation reaction. Products were electroporated into *E. coli* DH5α cells and selected on standard LB plates with 34 µg ml⁻¹ chloramphenicol. Plasmids were recovered from transformants by alkaline lysis and restriction mapped for the presence and orientation of insert, as well as the presence of a new *PvuII* site (indicative of the mutation). In this case, two of the twelve transformants evaluated met all of the above criteria, with subsequent sequence determination confirming the identity of each.

Having generated the mutation, it is important to verify that the construct will indeed make protein. Thus, the two derivative-bearing plasmids created above were transformed into the ΔtonB strain KP1344. These transformants and an isogenic strain encoding wild type TonB from the native chromosomal promoter were grown with aeration at 37°C in supplemented M9 minimal salts containing 34 µg ml⁻¹ chloramphenicol and various levels of L-arabinose. Cells were harvested at an *A₅₅₀* of 0.4 (as determined with a Spectronic 20 spectrophotometer with a path length of 1.5 cm) by precipitation with 10% w/v trichloroacetic acid (TCA), resolved on SDS 11% polyacrylamide gels, immunoblotted, and visualized as previously described (Larsen...
et al., 1999). Visual comparisons of the ECL results identified concentrations of L-arabinose that provided levels of full-length TonB for each derivative similar to that of chromosomally encoded TonB. For these particular constructs, a concentration of 0.001% L-arabinose produced TonB levels similar to that of wild type cells grown under the same conditions. One of the derivatives was thus selected for further characterization and named “pRA022”.

To determine the relative stability of this derivative, cells bearing either pRA022 or the wild type pKP325 plasmid were grown as above in supplemented M9 minimal salts containing 34 µg ml⁻¹ chloramphenicol and 0.001% L-arabinose. When cells reached an A₅₅₀ of 0.4, the synthesis of new TonB was halted by the addition of D-fucose and D-glucose-6-phosphate (to 12 and 7 mM, respectively) to repress the araBAD promoter, and spectinomycin (to 50 µg ml⁻¹) to halt protein synthesis. Samples were harvested at 0, 15, 30, 60 and 120 minutes following additions, precipitated in 10% v/w TCA and processed as above for immunoblot analysis.

Consistent with previous studies (Skare and Postle, 1991), the chemical half-life of wild type TonB under these culture conditions is greater than one hour (Figure 26A). A similar degree of stability is evident for the TonB derivative encoded by pRA022 (Figure 26B), indicating that the replacement of four residues in the amino-terminal portion of the signal anchor does not significantly alter the stability of this protein. Further, in vivo chemical cross-linking studies indicate that this derivative is properly trafficked to the CM, indicating that the substitutions have not disrupted signal function; similarly this derivative is able to support iron transport, indicating this region is not essential for energy transduction (data not shown).

Using this approach we have made alanyl replacements throughout the signal anchor of TonB, with second- and third-generation derivatives created using plasmids that already bear substitutions as templates. Characterization of these derivatives is ongoing.
Figure 26: Alanyl substitution of TonB residues 12-15 does not reduce the chemical stability of the protein. A ΔtonB strain expressing either wild type (Panel A) or the alanyl-substituted TonB (Panel B) were grown, with the chemical stability of individual derivatives monitored by sampling cultures for immunoblot analysis at the indicated time points (in minutes) following the cessation of protein synthesis, as described in the text. The position of molecular mass standards, and their apparent mass (in kilo Daltons) is indicated at the right of each panel.
**TonB/TolA chimeras**

Early studies in the Higgins laboratory (Karlsson et al., 1993) suggested that the amino terminal regions of TonB and TolA contributed to the ability of these energy transducers to discriminate between their respective energy-harvesting complexes. Specifically, they found that replacement of the first 32 residues of TonB by the first 34 residues of TolA produced a derivative that retained activity (as measured by sensitivity to the TonB-dependent bacteriophage φ80), and appeared to be more active in the presence of TolQ/R than in the presence of ExbB/D. Because these regions bear little similarity save for the shared Ser/His motif (Figure 27), these findings suggest that a similar strategy, focusing on smaller portions of the amino-terminal region, might identify motifs that contribute to the recognition of specific energy-harvesting complexes. To build such chimeras, we have adapted a sequence overlap extension (SOEing) PCR procedure (Lee et al., 2004) to replace specific regions of TonB with the analogous regions of TolA. Here we describe the production of one such construct, in which the cytoplasmic domain of the extreme amino terminus of TolA replaces the corresponding region of TonB.

The basic approach for assembling chimeric genes is depicted in Figure 28. To provide a source of *tolA*, the plasmid pRA004 was constructed by inserting a PCR-derived *tolA* amplimer into a pBAD24 plasmid (Guzman et al., 1995) under control of the bidirectional *araBAD* promoter, using the plasmid-provided ribosome binding site and start codon. To either side of this insert are a number of unique restriction sites, including a 5’ located *EcoRI* site and a 3’ located *XbaI* site (the latter of which is also present in pKP315) that allow for the subsequent cassetting of PCR-derived constructs into an expression vector (much as *BamHI* sites were used in recovering alanyl-substituted derivatives above). As with the production of alanyl-substituted derivatives, the plasmid pKP315 served as a template for *tonB* DNA. Primary PCR reactions
**Figure 27: The amino-terminal regions of TonB and TolA.** An alignment of the coding strands of TonB and TolA (in bold), with the corresponding amino acid residues and codon numbers indicated. The predicted signal anchor-encoding region (TonB codons 12-32) is highlighted. Boxes identify the conserved Ser and His residues. The *FspI* restriction site used in the construction of pRA0027 is underlined.
Figure 28: Strategy for construction of TolA/TonB chimeric proteins. Graphic summary of the sequence overlap extension (SOEing) PCR strategy as described in the text. The beginning templates and the relative positions of restriction sites used in subsequent cloning of products are depicted in (A). Two primary polymerase chain reactions are performed (B) to amplify portions of the two genes using a standard flanking primer and a SOEing primer designed with a 9 residue overhang (dashed line). Amplified products are purified (C) and used in the ligation PCR in which (D) the overlapping sequences are allowed to anneal and elongate in the absence of primers. A pair of flanking primers is then added (E) and the reaction continued to produce the final chimeric PCR product (F).
were run on these templates (Figure 28B), each pairing a normal flanking primer with an internal “SOEing” primer – designed to contain a nine-base overhang (the dotted portion of the primers in Figure 28B) complementary to a region of the gene to which the fusion will ultimately be made. Thus, the two primary PCR runs produce a “front” and a “back” amplimer, each containing an 18 base homologous region consisting of the nine residues from each gene that will flank the eventual fusion site (Figure 28C). In a subsequent “ligation” PCR, the front and back amplimers are paired, with the homologous regions annealing to provide for an extension reaction (Figure 28D) that produces a fused template that can now be amplified by conventional PCR to produce a final fused product (Figure 28E-F). It should be noted that initial experiments using the same flanking primers as used to generate the initial “front” and “back” amplimers yielded very little full length product in this final amplification – this problem was readily resolved by using a second, recessed set of flanking primers.

To construct a fusion in which the first 11 codons of the tonB gene are replaced by the first 13 codons of the tolA gene, reactions were performed as follows:
The front amplimer (a 488 bp product), produced from the tolA template pRA004; and back amplimer (a 898 bp product), produced from the tonB template pKP315 were generated in standard 50 µl reactions:

1.)  50 pmol of each flanking primer.
2.)  200 pmol of each dNTP.
3.)  5 µl 10 X ThermoPol Reaction buffer (200 mM Tris-HCl [pH 8.8 @ 25°C], 100 mM potassium chloride, 100 mM ammonium sulfate, 20 mM magnesium sulfate, 1% Triton X-100; New England Biolabs, Inc.).
4.)  2 units Deep Vent Taq DNA polymerase (New England Biolabs, Inc.).
5.) 0.01 µg pKP315 or pRA004 (as template).

Mixtures were subjected to 35 cycles of melting (94°C for 30 sec), annealing (60°C for 30 sec), and extension (72°C for 70 sec). Products were resolved on 1% agarose gels, with the appropriate products excised and recovered using a gel extraction kit (Qiagen Inc.). Purified front and back amplimers were then used for ligation PCR as follows:

Reactions were initiated in a 48 µl volume using ~0.08 pmol each of front and back amplimers, with 200 pmol of each dNTP and 2 units of Deep Vent DNA polymerase, in a 1X ThermoPol Reaction buffer with additional amounts (0, 1 mM, or 2 mM) of magnesium sulfate. Mixes were subjected to 3 cycles of melting (94°C for 30 sec), annealing (60°C for 150 sec), and extension (72°C for 150 sec). Following the third cycle, products were heated to 94°C and 50 pmol of each recessed flanking primer was added. Samples were then subjected to 32 cycles of melting (94°C for 30 sec), annealing (60°C for 30 sec), and extension (72°C for 90 sec).

Reactions were resolved on 1% agarose gels and examined for the presence of the predicted 963 bp product. The ligation reaction containing the predicted 963 bp product and the least by-products (in this case the reaction not supplemented with additional magnesium sulfate) was purified with a Qiaquick PCR purification kit (Qiagen, Inc.). The purified amplimer was then digested with *EcoRI* and *XbaI* and inserted into a similarly restricted (and additionally dephosphorylated) pRA004 in a standard ligation reaction. Products were transformed into chemically competent DH5α cells and selected on standard LB plates with 100 µg ml⁻¹ ampicillin. Plasmids were recovered from transformants by alkaline lysis and restriction mapped for the presence of insert. Twelve plasmids with putative *tolA1-13/tonB12-239* inserts were transformed into the ΔtonB strain KP1229, induced with 0.0001% L-arabinose, and their ability to encode a protein detectible by a TonB-specific monoclonal antibody was subsequently
verified by immunoblot analysis (data not shown). Three positive isolates were chosen and the identity of each construct was confirmed by sequence determination. Interestingly, each of the selected constructs had an unintended point mutation downstream from the fusion site that resulted in a residue substitution (Ser$_{46}$Ala, Glu$_{74}$Lys, and Gln$_{107}$Arg). To obtain a derivative free of unintended substitutions, an FspI site (at codons 41-43; underlined in Figure 27) and a BstEII site in the vector 5’ to the construct (not shown) were used to move the fusion region into pKP325, replacing the corresponding 5’ portion of $tonB$ with the tolA/tonB fusion. The resultant construct was recovered as above, screened by restriction mapping, confirmed by sequence analysis, and named “pRA027”.

The strategy used to screen the initial fusion products included an immunoblot verification that confirmed these constructs did encode protein. The final derivative, now on a pKP325 framework similar to that used for the alanyl substitution derivatives, was similarly shown to express a product upon induction with L-arabinose, the chemical half life of which was similar to that of a wild type TonB (data not shown). To determine if this chimeric protein retained activity, its ability to support siderophore-mediated iron transport was examined. Iron transport assays were performed using aroB, $\Delta tonB$ strains as previously described (Larsen and Postle, 2001), here bearing plasmids either encoding wild-type TonB (pKP368 – a pKP325 derivative carrying a silent mutation that introduces a novel restriction site irrelevant to the current experiment), the TolA/TonB fusion (pRA027), or a vector control (pACYC184). In these preliminary studies the TolA/TonB fusion protein provided for the transport of $[^{55}\text{Fe}]$-ferrichrome at rates only slightly less than those achieved with wild type TonB (Figure 29).

In the above experiment, both the ExbB/D and the TolQ/R energy-harvesting complexes are present. Future studies with this and additional chimeric constructs will use iron transport
Figure 29: The TolA/TonB chimera supports TonB-dependent transport of (FeIII)-siderophores. The ΔtonB, aroB strain RA1023 carrying plasmids that either express no TonB (A), wild type TonB (B), or the TolA/TonB chimera (C) were grown and assayed for the uptake of $^{55}\text{Fe}$-ferrichrome as described below. To verify levels of TonB expression, samples were harvested at the onset of the experiment (i.e. at 0 min) and processed for immunoblot analysis (inset). Briefly, cells were grown with aeration at 37°C in supplemented M9 minimal salts (containing 34 μg ml$^{-1}$ chloramphenicol and 0.001% L-arabinose) to an $A_{550}$ of 0.4, centrifuged and then suspended to $2 \times 10^8$ colony-forming units ml$^{-1}$ in M9 minimal salts containing 0.1 mM nitrilotriacetate and 0.2% w/v $D$-glucose. Cells were equilibrated for 5 min at 30°C, then transport initiated with the addition of 150 pmol of $^{55}\text{Fe}$-ferrichrome. Samples were harvested at indicated time points by filtration onto Whatman GF/C filters, washed three times with 5 ml of 0.1 M LiCl, then air dried. Incorporated $^{55}\text{Fe}$ was determined by liquid scintillation counting, and recorded as dpm per $10^8$ colony-forming units. All experiments were performed in triplicate.
and other assays with cells lacking one or the other energy-harvesting complex to determine which regions of TonB and TolA provide for specific interactions with the energy-harvesting complexes. Together with the ongoing characterization of alanyl-substituted TonB derivatives, these studies will afford dissection of the molecular interactions that provide for the coupling of the CM electrochemical potential to the activation of these energy transduction proteins.
CHAPTER V.

AIM THREE: IDENTIFYING SPECIFIC MOTIFS IN THE TRANSDUCER PROTEINS THAT CONTRIBUTE TO CROSS-TALK

Note: This section is written as an appendix to the submitted publication presented above. As such, this document provides additional data not generated until recently, after the manuscript was submitted. Some portions of the Methods reiterate aspects of previous chapters.

Introduction

Construction of TolA/TonB transmembrane chimeric proteins was done by Dr. Kim Keller as part of her dissertation work as described in the manuscript above. Plasmids encoding TolA/TonB chimeric proteins were generated using a modified sequence overlapping extension (SOEing) polymerase chain reaction (PCR) strategy (Keller, PhD Dissertation, 2007; Lee et al., 2004) and all plasmids encoding TolA/TonB chimeric proteins were confirmed by DNA sequencing. The fusion proteins allow for the use of TonB-dependent assays while testing TolA transmembrane domain (TMD) interactions. Because the TolA system does not have sensitive or quantitative assays to test for activity, these chimeras allow for this possibility. These fusions also allow for the testing of transducer – energy harvesting complex (EHC) interactions, narrowing down specific domains within the transducer that engage with the cytoplasmic membrane proteins. The two TolA/TonB chimeric proteins created were TolA1-34,TonB33-239 and TolA1-13,TonB12-239. The former fusion protein contains the full amino-terminus of TolA (the cytoplasmic tail and the TMD) in place of the amino-terminus of TonB, whereas the latter fusion protein contains a swap of the cytoplasmic tail of TonB for that of TolA (Figure 30). After

\begin{align*}
\text{TolA} & \quad \text{and} \quad \text{TonB}
\end{align*}
Figure 30. Illustration of the TolA/TonB chimeric proteins. (A) Simple drawing of wild-type TonB (blue) and TolA (purple) with the N-terminus in the cytoplasm, a single transmembrane domain (TMD), and the majority of the protein in the periplasm. (B) Figures representing the two TolA/TonB chimeras, with tolA residues in purple and tonB residues in blue. The left panel represents the protein expressed from pRA026, with residues 1-34 from tolA present; the right panel represents the protein expressed from pRA027, with residues 1-13 from tolA present. Figure from Keller, PhD Dissertation, 2007.
creating the fusion genes, they were digested and ligated into pBAD24 to provide regulation under the control of the arabinose promoter (see methods described in above manuscript). To properly compare the activity of TolA/TonB chimeras with the activity of wild-type TonB, the \( \text{tolA/tonB} \) gene was cloned into pKP368, a pACYC184 derivative with wild-type \( \text{tonB} \) expression dependent upon the pBAD promoter (Table 9). The chimeric genes replaced the \( \text{tonB} \) gene in that vector, in an effort to isogeneic mutants.

To evaluate the activity of these fusion proteins, they were transformed into \( \Delta \text{tonB} \) strains that allowed for testing of the chimeric proteins’ interaction with either the ExbBD complex or the TolQR complex (Table 10). To determine which part of the TolA amino terminus is responsible for energization and protein interaction, transport of \( [^{55}\text{Fe}] \)-loaded ferrichrome was studied.

**Materials and Methods**

**Media.** All bacterial strains and plasmids were maintained on Luria-Bertani (LB) agar plates supplemented with chloramphenicol at 34 \( \mu \text{g ml}^{-1} \) where appropriate. Cells for all experiments were grown at 37°C with aeration in broth (either LB- or T-broth, as indicated) with antibiotic supplement as appropriate.

**Transport of radiolabeled ligands.** Ferrichrome transport assays were performed with \( [^{55}\text{Fe}] \)-loaded ferrichrome as per Chapter Three and as previously described (Larsen and Postle, 2001; Koster and Braun, 1990). Strains were grown to an \( \text{A}_{550} \) of 0.4 in T-broth, supplemented with chloramphenicol. The experiments were performed in triplicate for each strain.

**Immunoblot analysis of TonB protein.** Samples prepared as described above were resolved on SDS 11% polyacrylamide gels (Laemmli, 1970), immunoblotted using TonB-
Table 9. Plasmids used in Chapter V

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Genotype/Phenotype</th>
<th>Source</th>
</tr>
</thead>
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<tr>
<td>pACYC184</td>
<td>cat</td>
<td>Chang and Cohen, 1978</td>
</tr>
<tr>
<td>pKP325</td>
<td>pBAD promoter in pACYC184; tonB, cat</td>
<td>Larsen et al., 1999</td>
</tr>
<tr>
<td>pKP368</td>
<td>pBAD promoter; wild-type tonB with unique NcoI site in TMD, cat</td>
<td>Larsen et al., 2001</td>
</tr>
<tr>
<td>pRA023</td>
<td>pBAD derivative, tolA\textsubscript{1-34},tonB\textsubscript{33-239} via SOEing PCR</td>
<td>Keller, BGSU</td>
</tr>
<tr>
<td>pRA024</td>
<td>pBAD derivative, tolA\textsubscript{1-13},tonB\textsubscript{12-239} via SOEing PCR</td>
<td>Keller, BGSU</td>
</tr>
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<td>pRA026</td>
<td>pKP368 derivative, tolA\textsubscript{1-34},tonB\textsubscript{33-239} ligation of BstEII pRA023 into pKP368; cat</td>
<td>Keller, BGSU</td>
</tr>
<tr>
<td>pRA027</td>
<td>pKP368 derivative, tolA\textsubscript{1-13},tonB\textsubscript{12-239} ligation of BstEII-FspI pRA024 into pKP368; cat</td>
<td>Keller, BGSU</td>
</tr>
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</table>
Table 10. *Escherichia coli* strains used in Chapter V

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>KP1344</td>
<td>W3110 $\Delta$tonB::blaM</td>
<td>Larsen <em>et al.</em>, 1999</td>
</tr>
<tr>
<td>RA1003</td>
<td>W3110 $\Delta$exbBD::kan (deletion of bases 585-1745 replaced with kan&lt;sup&gt;r&lt;/sup&gt; gene from pACYC184)</td>
<td>Larsen, BGSU</td>
</tr>
<tr>
<td>RA1006</td>
<td>W3110 $\Delta$tonB::blaM, $\Delta$exbBD::kan via P1 transduction of RA1032 into KP1344</td>
<td>Keller, BGSU</td>
</tr>
<tr>
<td>RA1017</td>
<td>W3110 $\Delta$exbBD::kan, $\Delta$tolQRA</td>
<td>Larsen, BGSU</td>
</tr>
<tr>
<td>RA1024</td>
<td>W3110 $\Delta$tonB::blaM, $\Delta$exbBD::kan, $\Delta$tolQRA via P1 transduction of KP1344 into RA1017</td>
<td>Keller, BGSU</td>
</tr>
<tr>
<td>RA1032</td>
<td>W3110 $\Delta$tolQR::kan</td>
<td>Brinkman, present Study</td>
</tr>
<tr>
<td>RA1052</td>
<td>W3110 $\Delta$tonB::blaM, $\Delta$tolQR::kan via P1 transduction of RA1032 into KP1344</td>
<td>Larsen, present study</td>
</tr>
</tbody>
</table>
specific monoclonal antibodies (Larsen et al., 1996), and visualized by enhanced chemiluminescence as previously described (Skare et al., 1993) to determine similar TonB expression levels were maintained. Following immunoblot analysis, membranes were stained for total protein with Coomassie blue and visually examined to confirm equivalent sample loading of all lanes.

**Results and Discussion**

To test the contributions of the transducer proteins, TonB and TolA, to energy harvesting and transport, strains with a deletion of *tonB*, in combination with a deletion of one of the EHCs (Table 10), were used to determine cross-talk interactions between systems. By using fusion proteins of TolA and TonB, specific domains and motifs within each transducer can be tested for the ability to interact with its cross-talk EHC pair and become energized.

TonB has been shown to display dominant-negative gene dosage effects (Mann et al., 1986; Dorman et al., 1988) where TonB-dependent activity decreases as *tonB* expression levels increase. Because of this phenomenon, it was important to maintain as close to wild-type expression levels as possible. Expression levels of *tonB* derivatives were compared to wild-type levels by adding 0.001%, 0.0001%, or 0.00001% arabinose to cells and comparing plasmid expression to that of wild-type via Western Blots (data not shown). This was done to confirm that any low TonB activity seen is not due to overexpression. For all transport experiments, the determined arabinose levels that showed wild-type expression levels for each plasmid, was used.

**Siderophore transport.** Unlike the Tol system, a specific function for the TonB system is known – to transport iron-bearing siderophores. This provides a biologically relevant assay for evaluating TonB function – and the ability of energy-harvesting complexes to support that function. Here, the uptake of ferrichrome loaded with [$^{55}$Fe] was measured in several different
strains bearing no transducer, TonB, or one of the two chimeric TonB/TolA proteins. Baseline levels of transport were detected in all strains lacking the \textit{ton}B gene (Figure 31). The small amounts that were detected are a result of less efficient iron transport systems in \textit{E. coli} that are not dependent on TonB. High levels of transport were evident for the strain with both EHC pairs intact bearing wild-type TonB and the strain bearing only the cognate EHC (ExbBD) paired with wild-type TonB (Figure 32).

Not surprisingly, the fusion protein TolA_{1-34},TonB_{33-239}, when coupled with both EHC pairs, has high levels of transport. This same fusion protein, when paired with ExbBD, had comparable levels of transport to the wild-type protein (Figure 33). This was interesting due to the fact that this fusion protein carries a complete amino terminal swap of TonB with that of TolA. When paired with TolQR, the fusion of TolA_{1-34},TonB_{33-239} registered only low levels of transport (Figure 33).

Much lower levels of transport were detected for the strain bearing only the ExbBD energy harvesting complex paired with the fusion protein of TolA_{1-13},TonB_{12-239}. There was no evidence of transport in the strain bearing TolQR (Figure 34). This fusion protein that carried the cytoplasmic tail of TolA in place of the cytoplasmic tail of TonB, when paired with both EHC pairs, had high levels of transport.

\textit{Summary and Prospectus}

Previous literature has examined the role of the amino terminal signal-like sequence of the TonB and TolA proteins (Karlsson \textit{et al.}, 1993). These experiments, performed in the Higgins laboratory, looked at sequence-specific interactions of TonB/TolA fusion proteins with the ExbBD and TolQR proteins. The fusion protein used was a TonB protein with the first 32 residues replaced with the first 35 residues of TolA, closely resembling a chimeric
Figure 31. $^{55}$Fe-ferrichrome transport assay using pACYC184. Various strains were subjected to siderophore transport as described in Materials and Methods. All strains tested were transformed with the negative control plasmid, pACYC184. Strains tested have a deletion of tonB and the following additional phenotypes: KP1344 (BD+QRA+), RA1024 (BD-DR-), RA1006 (BD-DR+A+), KP1052 (BD+QRA-). Graphed is the average counts per minute (cpm) of three experiments for each strain at each of the 2 min through 14 min time point samples. All strains graphed show no TonB transport of $^{55}$Fe-ferrichrome.
Figure 32. $^{55}$Fe-ferrichrome transport assay using pKP325. Various strains were subjected to siderophore transport as described in Materials and Methods. All strains tested were transformed with the positive control plasmid, pKP325, which carries a wild-type $\text{tonB}$. Strains tested have a deletion of $\text{tonB}$ and the following additional phenotypes: KP1344 (B$^+$D$^+$Q$^+$R$^+$A$^+$), RA1024 (B$^-$D$^-$Q$^-$R$^-$), RA1006 (B$^-$D$^-$Q$^+$R$^+$A$^+$), KP1052 (B$^-$D$^+$Q$^+$R$^-$A$^-$). Graphed is the average counts per minute (cpm) of three experiments for each strain at each of the 2 min through 14 min time point samples. KP1344 + TonB showed wild-type transport levels of $^{55}$Fe-ferrichrome and RA1052 + TonB showed near wild-type levels of transport.
**Figure 33.** $^{55}$Fe-ferrichrome transport assay using pRA026. Various strains were subjected to siderophore transport as described in Materials and Methods. All strains tested were transformed with the chimeric plasmid, pRA026, which carries the fusion $tolA_{1-34}$,$tonB_{33-239}$. Strains tested have a deletion of $tonB$ and the following additional phenotypes: KP1344 ($B^+D^+Q^+R^+A^+$), RA1024 ($B^-D^-Q^-R^-A^-$), RA1006 ($B^-D^-Q^+R^+A^+$), KP1052 ($B^-D^-Q^-R^-A^-$). Graphed is the average counts per minute (cpm) of three experiments for each strain at each of the 2 min through 14 min time point samples. KP1344 + TolA$_{1-34}$/TonB$_{33-239}$ showed wild-type transport levels of $^{55}$Fe-ferrichrome and RA1052 + TolA$_{1-34}$/TonB$_{33-239}$ also showed wild-type levels of transport. RA1006 + TolA$_{1-34}$/TonB$_{33-239}$ showed low levels of transport.
Figure 34. $^{55}\text{Fe}$-ferrichrome transport assay using pRA027. Various strains were subjected to siderophore transport as described in Materials and Methods. All strains tested were transformed with the chimeric plasmid, pRA027, which carries the fusion $\text{tolA}_{1-13}/\text{tonB}_{12-239}$. Strains tested have a deletion of $\text{tonB}$ and the following additional phenotypes: KP1344 ($\text{B}^+\text{D}^+\text{Q}^+\text{R}^+\text{A}^+$), RA1024 ($\text{B}^-\text{D}^-\text{Q}^-\text{R}^-\text{A}^-$), RA1006 ($\text{B}^-\text{D}^-\text{Q}^-\text{R}^+\text{A}^+$), KP1052 ($\text{B}^-\text{D}^+\text{Q}^-\text{R}^-$). Graphed is the average counts per minute (cpm) of three experiments for each strain at each of the 2 min through 14 min time point samples. KP1344 + TolA$_{1-13}$/TonB$_{12-239}$ showed wild-type transport levels of $^{55}\text{Fe}$-ferrichrome and RA1052 + TolA$_{1-13}$/TonB$_{12-239}$ only showed low levels of transport.
protein we have created in the above experiments (we have replaced first 32 residues replaced with the first 34 residues of TolA). The Higgins chimera was constructed by creating specific restriction sites within each gene and then digesting and ligating the two gene fragments together in an expression vector. Activity was assayed by φ80 adsorption and plaque assays in ΔtonB strains. They found that the chimera had high levels of activity when in the presence of both EHC pairs together. They tested chimeric activity in strains with transposon insertions in either exbB, tolQ, or both genes and found greater than cross-talk levels (TonB working with TolQR) of activity in the exbB mutant and in the tolQ mutant. They interpreted adsorption graphs as showing that the chimera worked better with TolQR than with ExbBD. The data we have obtained with a similar chimera is in direct conflict with the conclusions from the Higgins laboratory.

TonB with the first 32 residues replaced with the first 34 residues of TolA is able to support activity as efficiently as wild-type TonB (expressed from a plasmid) when interacting with ExbBD. Because the protein carries the TolA TMD, previous literature would lead one to believe that it would be energized more efficiently when paired with TolQR, but this was not the case. When only the cytoplasmic tail of TonB was replaced with that of TolA, cross-talk levels of activity were seen in the strain bearing only ExbBD. This may suggest that one specific domain is not responsible for energization, but the interaction of these two domains (the cytoplasmic tail and the TMD) spacially with respect to each other and with the different EHCs determines activity.

The conflicting results found in this study with those from a previous study in the Higgins laboratory (Karlsson et al., 1993) may be a result of several experimental design discrepancies. First, the previous study’s TolA_{1-35}/TonB_{33-239} chimera has a single residue
difference from the TolA_{1-34}/TonB_{33-239} chimera in the present study. The presence of Asp_{35} in the Higgins chimera, which is predicted to be the first residue exposed to the periplasm (Levengood et al, 1991), is doubtful in playing a major role in energy transduction or transducer specificity, although an aspartyl has the ability to be protonated and deprotonated and could hypothetically play a role in proton transport. Second, in the previous study the strains used to test the chimeric activity carried insertion mutations in specific EHC proteins, whereas the current study tested activity in strains carrying clean deletions of genes. This too, is not a convincing reason for such discrepancies in results between the two studies. Finally, and most importantly, the data published to support the Higgins interpretations were provided on a graph expressed in log scale which proves difficult in viewing the difference between a small or large change in activity between the strains. They interpreted a minor difference in adsorption levels to be significant, but without quantifying this data, this proves unreliable and perhaps even inaccurate. Also, error bars for this data were not provided and the small difference between the activity of the chimera with ExbBD and the chimera with TolQR could not be properly interpreted.

The results found in the present study may provide a different view of previously assumed ideas of how the transducers in the TolA and TonB systems accept energy from and interact with the energy harvesting complexes. What once was thought to be specifically transmembrane domain interactions may now be ambiguous at best and misleading at most. This new data suggests that TMD alone may not responsible for energization, but its interaction with other domains may determine activity. Further experiments need to be performed to verify the present results are accurate.
CHAPTER VI.
SUMMARY AND GENERAL CONCLUSIONS

The cytoplasmic membrane protein TonB provides for transport of ferric siderophores and group B colicins across the gram-negative outer membrane, and confers sensitivity to T1 and φ80 bacteriophages. This function requires interaction with a heteromultimeric protein complex that couples TonB to the ion electrochemical potential of the cytoplasmic membrane. This process is most efficient when TonB interacts with complexes consisting of ExbB and ExbD; however, in their absence complexes comprised of the ExbB and ExbD paralogues TolQ and TolR can support TonB-dependent processes, albeit less efficiently. This dissertation was designed to study how the proteins within these two systems interact with one another and the mechanisms by which the energy is transferred from the energy harvesting complexes to the transducers, particularly during cross-talk.

A portion of this research involved developing vectors in which each protein in the systems could be studied alone or in concert with any of the other proteins in the systems. Plasmids carrying each gene were created and tested, resulting in ambiguous and flawed data. A decision was made to work with these genes and their deletions on the chromosome rather than plasmid vectors. The majority of these deletions were created by the author as detailed in Chapter Two.

Strains bearing two or more precise deletions of the genes encoding ExbB, ExbD, TolQ, and TolR were generated and their ability to support TonB function determined. Once these isogeneic strains were created, they were tested for each protein’s ability to energize TonB or TolA (using colicins), physically interact with TonB, and stabilize TonB. As expected, TonB activity in the strain expressing only ExbB/D mirrored that of the wild-type strain, and was
diminished (but not absent) in the strain expressing only TolQ and TolR. Interestingly, low levels of activity were evident in the strain expressing ExbB and TolR, but not in the strain expressing the inverse combination – TolQ and ExbD. In Chapter Three, data suggests that chimeric complexes comprised of ExbB and TolR energize TonB at a low efficiency. ExbB or TolR alone is unable to support φ80 sensitivity, supporting the idea that transducer activity is dependent upon both energy harvesting proteins working in concert. Considering these results, it is possible that perhaps the TolQ/ExbD complex can, in fact, energize TolA but function cannot be determined due to an insufficiently sensitive assay. In vivo chemical cross-linking experiments indicated that ExbB engaged in specific interactions with TonB independent of the presence of either ExbD or TolR. Because ExbB alone can cross-link with TonB, yet ExbB alone cannot energize TonB, the protein may help to stabilize and properly configure the transducer while ExbD energizes TonB. This also supports the idea that cross-linking and activity are independent from one another.

The chemical stability of TonB is greatly decreased in the absence of ExbB/D and has been presumed that TonB stability requires interactions with ExbB/D. We have shown that the presence of ExbB alone, or with TolR was sufficient for the stability of TonB. Surprisingly, TolQ alone, or with ExbD, also stabilizes TonB, despite the absence of ExbB. When TolQ is in complex with TolR, TonB is energized but is not stabilized. Together, these data suggest that the ExbB/TolQ component of a given heteromultimeric complex is sufficient for interaction with TonB, but the ability of this interaction to couple TonB to the ion electrochemical potential of the membrane is dependent upon interactions that occur between the ExbB/TolQ and ExbD/TolR components of the complex. Further, these data suggest that instability is the result of activity, i.e., TonB interactions at the CM are dependent upon varying conformational states.
When all the data are considered – TonB activity, TonB interactions, and TonB stability – they provide some insight into what may be TonB conformational changes and the varying stability of these conformational variations. The data in the Chapter Three show that instability is a product of activity and that TonB interactions at the CM are dependent upon varying conformational states. When TonB is in an uncharged conformation, it is stable. TonB is not activated by ExbB alone, TolQ alone, or TolQExbD, yet it has a stable half-life in the presence of these three protein combinations. If TonB is charged (by ExbBD, ExbBToIR, or TolQR), its conformation is altered to store potential energy. TonB must interact with these three protein conformations to accept the energy transfer, thus showing a stable protein. Once TonB releases this energy, this discharged conformation can only be recaptured and stabilized by ExbB in complex with either ExbD or ToIR.

These data discussed in Chapter Three may stress the importance of complex interactions rather than singular interactions. These suggest that the interactions of the energy harvesting complex proteins with one another are more important to the system’s function as a whole rather than the interaction between an individual protein in the EHC with the transducer. It also seems that the ExbB/TolQ protein is most important in the stability of the transducer. This could potentially be one explanation of the stoichiometry of these systems. In the TonB system, the energy harvesting complex is a heteromultimer predicted to contain 4 to 5 ExbD and 14 to 15 ExbB proteins per complex (Held and Postle, 2002; Higgs et al., 2002a) and the transducer is presumably a dimer (Sauter et al., 2003; Chang et al., 2001). This design has raised questions as to why the energy harvesting complex is so large and has a skewed ratio of ExbB proteins to ExbD proteins. If ExbB is the primary protein that stabilizes TonB and, in complex with either ExbD or ToIR, can recapture a recycling TonB, it would explain the reason for the large number
of ExbB proteins in the complex. ExbB (and presumably TolQ) in complex with another EHC protein, ExbD or TolR, is the only way TonB (or TolA) can be energized. Alone, none of the EHC proteins is able to activate TonB, yet it does not matter which protein is paired with ExbB, it becomes an energizing complex when paired with a partner protein.

The iron transport data show that the chimeric complexes’ ability to energize TonB is insufficient to quantify with radioactively loaded ferrichrome uptake. Unlike cross-talk complexes that are able to energize their counterpart’s transducer at quantifiable and biologically relevant levels, the chimeric complexes, if they occur naturally, do not function at biologically relevant levels.

This idea that energization, and thus conformational, states differ in TonB would be greatly enhanced by further studies. Performing membrane fractionation or TonB proteolysis in cells with the single CM proteins or the chimeric complexes could deliver more insight into this phenomenon. This assay would allow for comparison of different degradation products as a result of varying conformational states. In the future it would be beneficial to continue to look at what roles ExbB and TolR play in interacting and energizing TonB. Because a chimeric complex of ExbB and TolR will support energization of TonB, this suggests that ExbB may be the protein that is responsible for transducer discrimination. It seems as though ExbB, in complex with another CM protein is largely responsible for the recapture of the discharged TonB, after the transducer has delivered energy to the OM. If all of this is true, TolQ is most likely playing the same role in the Tol system. All of the conclusions made for the TonB system can be presumed for the TolA system because of the EHC high degree of similarity but, as of yet this cannot be tested due to a lack of Tol antibodies and assays sufficiently sensitive to test TolA activity.
would be a direction in which this research would be beneficial to the field as the Tol system has not been studied as in depth as the TonB system.

The next logical step in this research would be to determine which domains of the ExbB protein are responsible for transducer recognition. Replacement of specific ExbB transmembrane domains with those of TolQ and then pairing these chimeric proteins with ExbD or TolR to evaluate function could be helpful in pinpointing specific regions of the energy harvesting proteins that are interacting with transducer molecules. There are several reasons for swapping transmembrane domains: First, mutations that suppress TonB and TolA mutations occur in this domain (Larsen et al., 1994, 1999; Germon et al., 1998). Second, sequence comparisons of ExbB and TolQ with MotA suggest that transmembrane domains two and three are involved in the proton channel (Zhou et al., 1998; Sato and Homma, 2000), whereas the first domain bears no homology to other proton translocators. There have been some recent data to confirm the possibility of a proton conductance channel in the TolQ/R system (Goemaer et al., 2007) but this idea has been refuted in the ExbB/D complex (Braun and Herrman, 2004). Third, the degree of conservation between ExbB and TolQ is less in the first than in the other two domains, making it a more likely candidate for discriminating between the very different energy transducers.

Like TonB, TolA is most efficient when it interacts with its heterologous energy harvesting complex (TolQ/R) and in its absence can be less efficiently supported by ExbB/D during cross-talk. Because TonB and TolA each have a “preferred” energy-harvesting complex, it is clear motifs not shared between TonB and TolA are involved in interactions with energy harvesting complexes. We have adopted a strategy of examining derivatives with multiple-residue substitutions to identify such regions. This involves the characterization of specific TonB derivatives generated by the exchange of short regions between TonB and TolA. In this study,
testing two distinct TolA/TonB chimeric proteins provides a different view of previously assumed ideas of how the transducers in the TolA and TonB systems accept energy from, and interact with, the energy harvesting complexes.

In Chapter Five, iron transport was used to determine the activity of both chimeras, TolA\textsubscript{1-34}TonB\textsubscript{33-239} and TolA\textsubscript{1-13}TonB\textsubscript{12-239} (created in Chapter Four), paired with each energy harvesting complex. TonB with the first 32 residues replaced with the first 34 residues of TolA was shown to support activity as efficiently as wild-type TonB (expressed from a plasmid promoter) when interacting with ExbBD. Because the protein carries the TolA transmembrane domain (TMD), it was suspected that it would be energized most efficiently when paired with TolQR, but rather, ExbBD was most efficient. When only the cytoplasmic tail of TonB was replaced with that of TolA, cross-talk levels of activity were seen in the strain bearing only ExbBD. These data together may suggest that one specific domain is not responsible for energization, but the interaction of these two domains (the cytoplasmic tail and the TMD) with respect to each other and with the energy harvesting complexes determines activity. The results found in Chapter Five provide a different view of previously assumed ideas of how the transducers in the TolA and TonB systems accept energy from and interact with the energy harvesting complexes. It once was thought that the transmembrane domain specifically interacts with the EHC to receive energy. These results may now suggest that the TMD alone may not be responsible for energization, but its interaction with other domains may determine activity.

Further study is needed to confirm results found in Chapter Five. As discussed previously, iron transport is not the most sensitive assay to quantify TonB activity, thus the chimeric activities should also be determined via \( \phi \)80 adsorption. These experiments are currently being done by the author. As a result of the findings in Chapter Three, the TolA/TonB
chimeras should undergo half-life studies and formaldehyde crosslinking assay to determine the role of either the TMD or the cytoplasmic tail of the transducer in protein stability and protein-protein interactions. Finally, it would be beneficial to consider these chimeras in the different strains built in Chapter Two. It may be interesting to see activity, crosslinking, and stability of the chimeras in the presence of only one EHC protein or a chimeric energy harvesting complex, like ExbBToIR. Unfortunately, these strains all carry TonB and TolA and it would be necessary to delete these genes in each strain before proceeding.
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