CONFORMATIONAL DYNAMICS OF THE ESCHERICHIA COLI TONB PROTEIN
AND IMPLICATIONS FOR ENERGY TRANSDUCTION

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by
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ABSTRACT

To cross the outer membrane of Gram-negative bacteria such as *Escherichia coli*, large, scarce, and important nutrients such as iron or vitamin B12 first bind to specific outer membrane transporters. A complex of the cytoplasmic membrane proteins ExbB, ExbD, and TonB transduces energy derived from the cytoplasmic membrane protonmotive force (PMF) to permit ligand release into the periplasm, with TonB physically contacting the outer membrane transporter. Although no individual sidechain in the *E. coli* TonB is irreplaceable, TonB consists of two essential domains: a transmembrane domain (TMD) that interacts with ExbB and ExbD and a periplasmic carboxy-terminal domain that interacts with outer membrane transporters.

The mechanisms by which TonB facilitates ligand transport across the outer membrane have been a matter of considerable debate. In one model, the TonB TMD delivers energy to transporters by “shuttling” between cytoplasmic and outer membranes, a process that would require the TMD to be removed from the cytoplasmic membrane. Alternatively, several mechanical models suggest that TonB always remains anchored in the cytoplasmic membrane during energy transduction. The ideal test of the shuttle model is to fuse a stable protein domain to the amino terminus of TonB to prevent it from shuttling and determine if the fusion retains activity. In this study, a ToxR-TonB fusion was proteolytically stable, retained the ability to form PMF-independent and PMF-dependent interactions with ExbD and exhibited wild-type ferrichrome transport rates. Therefore, TonB does not shuttle *in vivo*.
The observation that the TonB TMD did not shuttle suggested that its primary role was to mediate assembly of TonB with ExbB, ExbD, or another TonB. TonB was previously known to dimerize in vivo, yet the contributions of TMD sidechains (e.g. H20) to dimerization were relatively unexplored. Here, an inactive TonB H20A substitution categorically reduced dimerization through the TonB carboxy terminus. TonB H20A also prevents association between TonB and ExbD. Since ExbD forms homodimers before associating with TonB, the results are consistent with the idea that TonB forms homodimers before forming TonB-ExbD heterodimers. Disulfide-linked TonB dimers formed through TonB F125C, located in a disordered region of the periplasmic domain, could fractionate with the outer membrane whereas dimers formed within TonB carboxy-terminal residues 186-230 were unable to associate with the outer membrane. This suggests that dimers formed through TonB residues 186-230 need to be resolved for this region to associate with the outer membrane, which is consistent with the suggestion that the dimers form early in an energy transduction cycle.

Finally, this work identified sites of functionally significant interaction between TonB and the FepA cork domain using in vivo photocrosslinking with the photoreactive amino acid p-benzoyl-l-phenylalanine (pBpa). Each of the pBpa substitutions that could crosslink to TonB are located in regions of FepA that are exposed to the periplasm in the FepA crystal structure, suggesting that TonB does not contact other areas of the cork in vivo. The presence of enterochelin strengthened crosslinking through some positions but diminished crosslinking through FepA I14pBpa, V28pBpa and T32pBpa. This suggests that TonB binds more efficiently to these three sidechains in the absence of ligand, and once ligand binds, the transporter undergoes a conformational rearrangement to bind
different sites. Deletion of the TonB box did not reduce crosslinking through FepA T32pBpa or I145pBpa, suggesting that formation of these complexes does not require a functional transporter.

Overall, this work extended knowledge of how the TonB TMD and carboxy-terminal domain participate in early and late stages of energy transduction and provided more insight into TonB-transporter interactions beyond the characterized TonB-TonB box interaction.
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CHAPTER 1

INTRODUCTION TO THE TONB SYSTEM
Chapter 1

Introduction

Nutrient transport across the cell envelope of Gram-negative bacteria

The cell envelope of Gram-negative bacteria consists of three main compartments: a cytoplasmic membrane, periplasm and an outer membrane. The cytoplasmic membrane is a symmetric phospholipid bilayer and contains proteins which contribute to generation of protonmotive force (PMF) and ATP synthesis by oxidative phosphorylation. Other proteins embedded within the cytoplasmic membrane facilitate nutrient transport into the cytoplasm or enable export of proteins to the periplasm or outer membrane. The periplasm is an aqueous space between the cytoplasmic and outer membranes which contains a thin layer of peptidoglycan that provides structural rigidity to the cell (Nikaido, 1993). The periplasm is an oxidizing environment which contains many hydrolytic and degradative enzymes as well as nutrient-binding proteins that deliver incoming sugars, amino acids and vitamins to nutrient transport proteins in the cytoplasmic membrane. In contrast to the cytoplasmic membrane, the outer membrane is an asymmetric bilayer, with phospholipids comprising the inner leaflet and lipopolysaccharide (LPS) occupying the outer leaflet. The outer membrane serves as a protective barrier against, detergents, bile salts and other toxic compounds, but it still permits diffusion of small (<600 Da) hydrophilic nutrients through beta-barrel proteins known as porins (Nikaido, 1993).
Iron is an important element for most living organisms, as it is a cofactor for proteins involved in processes such as ribonucleotide synthesis and cellular respiration. In anaerobic environments, iron is predominantly found in the soluble ferrous form that can diffuse through the outer membrane porins. Once in the periplasmic space, ferrous iron binds to the FeoABC complex in the cytoplasmic membrane, and upon ATP hydrolysis, is translocated across the cytoplasmic membrane into the cytoplasm (Faraldo-Gomez and Sansom, 2003).

In aerobic environments or mammalian hosts, however, iron is not as easy to acquire. At neutral pH in the presence of oxygen, iron predominantly exists in the form of insoluble (10^{-18}M) ferric hydroxides (Andrews et al. 2003). Moreover, iron-binding proteins such as transferrin and lactoferrin chelate much of the free ferric iron in mammalian cells (Faraldo-Gomez and Sansom, 2003). However, bacteria need significantly higher iron concentrations of approximately 10^{-8}M to support growth (Thulasiraman et al. 1998), so bacteria must utilize high-affinity iron retrieval mechanisms to acquire sufficient iron.

Bacteria can efficiently acquire iron by secreting different types of iron-chelating compounds. Siderophores represent one type of high-affinity, iron-binding compound produced by Gram-negative bacteria (Neilands, 1995). Enterochelin, the sole native siderophore produced by standard lab strains of *Escherichia coli* K-12, is a cyclic trimer of 2,3-dihydroxy-N-benzoyl-L-serine (Raymond et al. 2003). However, in mammals, enterochelin can be bound by host proteins such as serum albumin (Konopka and Neilands, 1984) which will prevent its return to the bacterial cell. Some pathogenic *E. coli* strains can produce other siderophores such as aerobactin (Konopka et al. 1982; de
Lorenzo and Martinez, 1988) which retrieve iron and deliver it to cells without being bound by serum albumin or chemically modified by host proteins. Hemophores, such as HasA produced by *Serratia marcescens*, are another class of iron-scavenging compounds which are involved in the uptake of heme (Arnoux *et al.* 1999; Letoffe *et al.* 2004).

However, iron-siderophore complexes such as ferric-enterochelin (716Da) (Liu *et al.* 1993) or other important nutrients such as vitamin B12 (1350 Da) (Trainor and Silverman, 1982) are too large and scarce to passively diffuse through porins. Gram-negative bacteria overcome this obstacle by synthesizing outer membrane transporters that can bind ligands with subnanomolar affinities (Figs. 1-1; 1-5). Each transporter consists of a 22-strand, antiparallel beta-barrel and a globular domain referred to as a “cork” or “plug” that resides in the lumen of the barrel (Chimento *et al.* 2005; Noinaj *et al.* 2010). However, the outer membrane lacks energy sources such as electrochemical gradients or ATP that would enable active transport of iron-siderophore complexes or vitamin B12 across the outer membrane. Instead, the necessary energy for transport is provided by the cytoplasmic membrane PMF. Treatment of cells with protonophores prevented transport of vitamin B12 through BtuB (Bradbeer, 1993) and lysis by bacteriophages T1 and ϕ80 which bind to the outer membrane transporter FhuA (Hancock and Braun, 1976). To energize transport at the outer membrane, the cytoplasmic membrane proteins ExbB and ExbD harness protonmotive force and transmit this energy to TonB (Fig. 1-1). TonB directly contacts outer membrane transporters (now referred to as TonB-gated transporters) and, through an unknown mechanism, promotes dissociation of ligand. After translocation into the periplasmic
space, the iron-siderophore complex will be bound by a periplasmic binding protein which will deliver it to a dedicated cytoplasmic membrane ATP-binding cassette transport complex (Raymond et al. 2003). ATP hydrolysis will then release the iron-siderophore complex into the cytoplasm. Once in the cytoplasm, iron is removed from most siderophores by cytoplasmic reductases that reduce tightly-bound ferric iron to the ferrous form, which will detach from the siderophore (Andrews et al. 2003). In the case of enterochelin, the esterase Fes will hydrolyze enterochelin and free the ferric ion (Brickman and McIntosh, 1992).

**Roles of TonB in other Gram-negative bacteria**

Although *E. coli* has only one copy of the *tonB* gene that is dedicated to uptake of iron and vitamin B$_{12}$, other species of Gram-negative bacteria are known to have two or more copies of *tonB* genes (Chu et al. 2007). The resultant proteins of these genes can potentially impact a variety of cellular functions. For example, *Vibrio cholerae* has two TonB proteins, named TonB1 and TonB2. Both are involved in uptake of the siderophore vibriobactin, but only TonB2 can mediate uptake of enterochelin (Seliger et al. 2001). Interestingly, both *V. cholerae* TonB1 and TonB2 could support hemin uptake under conditions of normal osmolarity but only the longer TonB1 could support hemin uptake in high salt conditions that expand the size of the periplasmic space (Seliger et al. 2001). One of the three TonB proteins in *Pseudomonas aeruginosa*, TonB3, is required for proper localization of type IV pili and the flagellum (Cowles et al. 2013).

*Pseudomonas syringae* pv. *syringae* B728a features nine different TonB proteins, the
most by any Gram-negative bacterium thus far (Chu et al. 2007), although the precise functions of each TonB are unknown.

Gram-negative bacteria also utilize the TonB system to support uptake of a variety of nutrients besides iron and vitamin B12. For example, Helicobacter pylori is involved in uptake of nickel (Schauer et al. 2007; de Reuse et al. 2013). Nickel is an important cofactor for urease, and a defective urease enzyme will render \textit{H. pylori} susceptible to acidic conditions and unable to cause stomach ulcers. \textit{Neisseria meningitidis} can extract zinc from calprotectin, a chelator that limits the concentration of zinc in host tissues, and utilize the TonB system for zinc uptake (Stork et al. 2013). For some Gram-negative bacteria, TonB is involved in the uptake of carbohydrates. The plant pathogen \textit{Xanthomonas campestris} synthesizes outer membrane transporters that are dedicated to the uptake of xylans (Dejean et al. 2013) and sucrose (Blanvillain et al. 2007; Schauer et al. 2008). The oligotrophic bacterium \textit{Caulobacter crescentus} uses TonB to support transport of maltodextrins (Neugebauer et al. 2005) and cellobiose (Presley et al. 2014).

\textit{Characteristics of ExbB, ExbD and TonB}

The cytoplasmic membrane components of the TonB system consist of three proteins: ExbB, ExbD and TonB (Fig. 1-2). ExbB is a 26 kDa protein that contains 3 TMDs, a significant cytoplasmic loop domain linking TMDs 1 and 2, and a C-terminal cytoplasmic extension (Kampfenkel and Braun, 1993). ExbB is important for the stability of both ExbD and TonB (Fischer et al. 1989; Karlsson et al. 1993; Ahmer et al. 1995) and serves as a scaffold in the energy-transducing complex (Pramanik et al. 2013).
Because PMF is required for TonB function, the TMDs of ExbB likely played a central role in a proton pathway formed by sidechains in ExbB, ExbD, and TonB (Zhai et al. 2003; Braun and Herrmann, 2004). Recently, however, it has been shown that residues in the ExbB TMDs do not participate in proton translocation yet are still important for TonB activity (Baker and Postle, 2013). The ExbB cytoplasmic loop and cytoplasmic tail are also important for activity, and certain mutations in the cytoplasmic tail eliminate PMF-dependent interaction between ExbD and TonB (Jana et al. 2011; Bulathsinghala et al. 2013). ExbD is a 15.5 kDa protein with 1 TMD and the majority of the protein residing in the periplasmic space (Kampfenkel and Braun, 1992; Held and Postle, 2002). The conserved ExbD TMD sidechain D25 is essential for TonB activity (Braun et al. 1996) and important for PMF-dependent interaction with TonB (Ollis et al. 2009). Of all the TMD sidechains in ExbB, ExbD, and TonB, only ExbD D25 is thought to bind protons (Baker and Postle, 2013). ExbB and ExbD are paralogues of TolQ and TolR, respectively, of the Tol system which is important for maintaining outer membrane integrity in Gram-negative bacteria (Germon et al. 1998). Interestingly, TolQ and TolR can weakly complement a deletion of both ExbB and ExbD and support approximately 10% of wild-type TonB activity (Braun and Herrmann, 1993; Brinkman and Larsen, 2008). MotA and MotB, which form the stator of the bacterial flagellum, are also paralogues of ExbB and ExbD, respectively (Zhai et al. 2003). Chapter 3 of this dissertation provides some evidence suggesting that MotA and MotB may contribute to TonB activity.

TonB consists of three main domains: an amino-terminal TMD (residues 12-32), proline-rich region (residues 70-102) and a periplasmic carboxy-terminal domain
(residues 150-239). Residues 33-69 connect the TMD to the proline-rich domain and are predicted to be disordered (Larsen et al. 2007), and residues 103-151 are disordered in a NMR structure of the TonB carboxy terminus (Peacock et al. 2005). Deletions of residues 33-64 and 103-149 substantially reduce but do not eliminate uptake of $^{55}$Fe-ferrichrome (Postle et al. 2010). The TonB TMD is essential for activity and is required for interaction with ExbB and ExbD (Larsen et al. 1994a; Jaskula et al. 1994; Swayne and Postle, 2011). Deletion of the proline-rich domain does not reduce TonB activity except under high salt conditions that expand the distance of the periplasm (Larsen et al. 1994b; Seliger et al. 2001). The TonB carboxy terminus (150-239) interacts with the periplasmic domain of ExbD (Ollis et al. 2009; Ollis and Postle, 2012) and OM transporters (Larsen et al. 1997; Higgs et al. 2002b; Larsen et al. 2003; Ghosh and Postle, 2004; Khursigara et al. 2004; Pawelek et al. 2006; Shultis et al. 2006). No single sidechain in TonB is essential for activity (Postle et al. 2010). The ExbB:ExbD:TonB copy number ratio per cell is 7:2:1, but the exact composition of an in vivo energy-transducing complex is unknown (Higgs et al. 2002a). Recently, a complex of 4 ExbB and 2 ExbD proteins was purified (Sverzhinsky et al. 2014), and a complex ratio of 2:1 was also observed for MotA to MotB (Kojima and Blair, 2004) and proposed for TolQ to TolR (Cascales et al. 2001). It is clear that ExbB/TolQ/MotA are produced in excess over ExbD/TolR/MotB, although the reasons for this overproduction are unclear.

**Role of the TonB TMD in energy transduction**

TonB can deliver energy to TonB-gated transporters according to two sets of models: mechanical and shuttle (Fig. 1-3). The mechanical models include a propeller
model in which TonB rotates to allow ligand uptake (Chang et al. 2001), a periplasmic-binding protein model where TonB binds to periplasmic-binding proteins and orients them to receive ligands through TonB-gated transporters (Carter et al. 2006b; James et al. 2009) and a pulling model which suggests that TonB unfolds the cork domain by applying a force perpendicular to the cork (Chimento et al. 2005; Gumbart et al. 2007).

In each of the mechanical models, the TonB TMD will remain attached to the cytoplasmic membrane complex of ExbB and ExbD while the carboxy-terminal domain simultaneously contacts the ligand-loaded transporter to allow ligand transport. After a transport event, the TonB carboxy-terminal domain will disengage from the transporter and “re-set” for another activation cycle, with the TMD remaining anchored in the cytoplasmic membrane. The shuttle model suggests that active TonB will contact a ligand-loaded transporter and then the TMD will disengage from the cytoplasmic membrane, leaving TonB completely associated with the outer membrane (Letain and Postle, 1997; Larsen et al. 2003). After ligand transport, the TonB TMD will re-associate with ExbB and ExbD, and the cycle can begin again. The presence of full-length wild-type TonB in outer membrane fractions of sucrose density gradients (70:30 cytoplasmic:outer membrane ratio) suggested that TonB could detach from the cytoplasmic membrane (Letain and Postle, 1997). In further support of the shuttle model, TonB L3C, located in the cytoplasmic amino-terminal tail, could only be labeled by the “membrane-impermeable” probe Oregon Green Maleimide (OGM) in the context of active, full-length TonB but not in a truncated construct missing carboxy-terminal residues 169-239 (Larsen et al. 2003). Because residues 169-239 are involved in transporter recognition (Larsen et al. 1997), this suggested that full-length TonB had to
first interact with a TonB-gated transporter before L3C could become exposed to the periplasm and labeled by OGM. L3C would not be labeled in a truncated TonB construct because it would not have the ability to contact TonB-gated transporters and subsequently enter the periplasm. Chapter 2 of this work indicates that TonB does not shuttle in vivo.

Although the TonB TMD is essential for activity (Jaskula et al. 1994), histidine 20 (H20) is the only sidechain in the TMD that renders TonB inactive when substituted with alanine (Larsen et al. 2007). E. coli TolA also has a conserved, important histidine at position 22 in its TMD (Germon et al. 1998). Given that PMF is needed for TonB activity and conformational changes (Larsen et al. 1999), it was once thought that TonB could respond directly to PMF with H20 serving as part of a proton pathway. However, replacement of H20 with asparagine retains wild-type activity, suggesting that H20 is not directly involved in harnessing PMF (Swayne and Postle, 2011). Prior to the studies in this dissertation (Chapter 3), the precise role of H20 was unknown.

**TonB dimerization in vitro and in vivo**

Purified TonB periplasmic domains can exist either as a dimer or monomer, but shorter fragments tend to dimerize while longer fragments tend to remain as monomers. Two solved crystal structures of TonB carboxy-terminal fragments show that the isolated domain can dimerize. One structure (Fig. 1-4A) solved by Chang et al. (2001) shows that two fragments of TonB 164-239 (76 residues) form a highly-intertwined dimer with 6258 Å² of buried surface area (I. Moustafa, personal communication). Another structure (Fig. 1-4B) solved by Kodding et al. (2005) shows that two subunits of TonB 148-239 (92 residues) dimerize, but the dimerization interface is smaller.
compared to the Chang et al. (2001) structure. This structure features 4602 Å² of buried surface area (I. Moustafa, personal communication). The high amounts of buried surface area in both structures suggests that they are very rigid and unlikely to be flexible enough to undergo significant conformational changes. These structures, however, do not represent in vivo conformations of TonB (Postle et al. 2010). In contrast to the crystal structures, longer TonB fragments consisting of residues 32-239 (Moeck and Letellier, 2001; Khursigara et al. 2004) or residues 103-239 (Peacock et al. 2005) exist as monomers in solution. This suggests that TonB residues 103-147, which are disordered (Peacock et al. 2005), reduce dimerization in vitro.

Full-length TonB dimerizes in vivo when fused to the V. cholerae ToxR transcriptional activator domain (Sauter et al. 2003). In this reporter system, dimerization of TonB resulted in the dimerization of the ToxR transcriptional activator domains which activates transcription of reporter genes (e.g. lacZ) placed downstream of the P_{ctx} promoter. Interestingly, while ToxR fused to full-length TonB (ToxR-TonB) dimerizes, ToxR-TonB 33-239 did not, suggesting that the TonB TMD contributes to dimerization of full-length TonB in vivo. ToxR-TonB 164-239, which features the same TonB carboxy-terminal residues observed in the Chang et al (2001) crystal structure, also dimerizes. However, dimerization of ToxR-TonB was dependent on the presence of ExbB and ExbD whereas the dimerization of ToxR-TonB 164-239 was unaffected by the absence of ExbB and ExbD. Therefore, it was suggested that ExbB and ExbD contribute to TonB dimerization in vivo.

Site-directed mutagenesis of each residue in the TonB carboxy-terminal domain suggests that the aromatic residues in the carboxy terminus could be involved in
interacting with TonB-gated transporters and also forming part of a TonB dimeric interface. Of all 90 residues in the TonB carboxy-terminal domain from 150-239, only 7 (Y163, F180, G186, F202, W213, Y215, and F230) are important for activity (Ghosh and Postle, 2004; Ghosh and Postle, 2005; Postle et al. 2010). When individually substituted with alanine or cysteine, each of the 7 important sidechains gives rise to “idiosyncratic phenotypic profiles,” meaning that they support activity for some ligands which utilize certain transporters but do not support transport for other ligand/transporter combinations. For example, cells expressing TonB F180A are fully sensitive to colicins B and D, which utilize the transporter FepA, yet are only able to support ~17% of the wild-type ferrichrome uptake rate through the transporter FhuA (Ghosh and Postle, 2004). In contrast, cells expressing F202A are less sensitive to killing by colicin B and completely tolerant to colicin D but can transport ferrichrome at the same rate as wild-type (Ghosh and Postle, 2004). Still, no individual substitution was completely inactive in all TonB-dependent assays tested. However, when any 2 of the 7 sidechains are simultaneously substituted with alanine, TonB activity is abolished, suggesting that the 7 important sidechains exhibit synergism and interact with one another in vivo (Ghosh and Postle, 2004; Postle et al. 2010). All but one of the sidechains is aromatic, suggesting that these residues could form an “aromatic cluster” (Ghosh and Postle, 2004; Ghosh and Postle, 2005).

In fact, cysteine substitutions at 5 of the 7 residues (G186C, F202C, W213C, Y215C and F230C) spontaneously form disulfide-linked TonB dimers, suggesting that the aromatic sidechains are directly interact with the corresponding residue in another TonB (Ghosh and Postle, 2005; Postle et al. 2010). The dimers appear as a set of three
higher-molecular weight complexes on immunoblots of non-reducing SDS-polyacrylamide gels and will now be referred to as “triplet dimers” (Ghosh and Postle, 2005; Postle et al. 2010). Triplet dimer formation is functionally relevant because the triplet dimers do not form efficiently if ExbB/ExbD and TolQ/TolR are absent, and the inactivating TonB ΔVal17 mutation also reduces dimer formation (Ghosh and Postle, 2005). Furthermore, the absence of ferric-enterochelin does not change the intensity of TonB triplet dimer formation. Since the TonB system does not transduce energy to the outer membrane when ferric-enterochelin is absent (Larsen et al. 1999), this result indicates that the complexes form before energy is transduced to the outer membrane (Ghosh and Postle, 2005). The triplet dimers formed through F202C, W213C, or Y215C localize strictly to the cytoplasmic membrane in sucrose density gradient fractionations whereas the uncrosslinked monomer localizes to both cytoplasmic and outer membranes in a 70:30 cytoplasmic:outer membrane ratio similar to wild-type TonB (Letain and Postle, 1997). An inability to interact with the outer membrane suggests that the dimers formed through F202C, W213C, and Y215C need to be resolved so that TonB can continue its progression through an energy transduction cycle.

**Stages of ExbD-TonB interaction**

TonB interacts with ExbD in both the presence (Ollis et al. 2009) and absence (Ollis and Postle, 2012) of PMF. Given the central roles of ExbD and PMF in TonB activity, a three-stage model was suggested describing the nature and sequence of characterized interactions between ExbD and TonB (Ollis and Postle, 2012). An adapted version of this model is presented in Chapter 3 (Fig. 3-5). In Stage I, TonB and ExbD do
not detectably interact. Inactivating substitutions such as TonB H20A or ExbD L132Q prevent TonB-ExbD interaction and presumably stall TonB in Stage I. In Stage II, ExbB facilitates association of ExbD and TonB such that, in the absence of PMF, ExbD protects the amino-terminal 2/3 of TonB from cleavage by proteinase K in spheroplasts (Held and Postle, 2002; Ollis and Postle, 2012). The inactivating substitution ExbD D25N also stalls TonB in Stage II. PMF acts as a “toggle switch” between Stages II and III because removal of protonophore restores PMF and enables TonB to become fully susceptible to proteinase K and enables TonB to form a PMF-dependent formaldehyde crosslink to ExbD in Stage III (Ollis and Postle, 2012). The periplasmic domain of ExbD regulates conformational changes in the TonB carboxy-terminal domain, so PMF-dependent interactions between ExbD and TonB influence the ability of TonB to productively associate with TonB-gated transporters (Ollis et al. 2009).

**TonB-gated transporters**

There are seven TonB-gated transporters in *E. coli* K-12 (Fig. 1-5). BtuB transports vitamin B12, but most of the TonB-gated transporters in *E. coli* are dedicated to iron uptake. In addition to transport of enterochelin, its native siderophore, through FepA, *E. coli* can also transport the enterochelin precursor dihydroxybenzoylserine through Cir and FiuA (Hantke, 1990). *E. coli* also synthesizes transporters for import of siderophores produced by other organisms. For example, ferrichrome (*Ustilago sphaerogena*) and ferrioxamine B (*Streptomyces pilosus*) can be transported through FhuA (Schoffler and Braun, 1989) and FhuE (Sauer et al. 1990), respectively. FecA serves to transport ferric dicitrate (Zimmermann et al. 1984). Thus far, *Bacteroides*
\textit{thetaiotaomicron} has the most predicted TonB-gated transporters of any Gram-negative bacterium to date with 120 (Schauer \textit{et al.} 2008).

Pathogenic strains of Gram-negative bacteria enhance virulence by synthesizing TonB-gated transporters involved in retrieval of iron from heme, transferrin, or lactoferrin. \textit{E. coli} synthesizes the transporter ChuA which is dedicated to heme uptake (Hoffmann \textit{et al.} 2001). In \textit{Neisseria} species, the outer-membrane protein TbpB extracts iron from transferrin or lactoferrin, and energy from TonB is utilized to transport the iron through the transporter TbpA (Noto and Cornelissen, 2008).

Each TonB-gated transporter consists of a 22-strand antiparallel \( \beta \)-barrel and an amino-terminal domain referred to as a cork or plug that occludes the barrel lumen (Fig. 1-5). The strands comprising the barrel are linked by ligand-binding loops on the extracellular face of the barrel and shorter turns on the periplasmic face (Noinaj \textit{et al.} 2010). Whereas the cork and barrel domains have several conserved motifs, residues in the ligand-binding loops are poorly conserved, likely because each transporter binds a different set of ligands (Chimento \textit{et al.} 2005; Noinaj \textit{et al.} 2010). During ligand transport, the cork domain remains in the barrel but either undergoes a large conformational rearrangement to open up a channel or exits the barrel completely. Cysteine substitutions tethering the cork to the barrel did not prevent ferricrocin transport through FhuA (Eisenhauer \textit{et al.} 2005), suggesting that the cork domain did not need to be completely removed from the barrel in order for transport to occur. Unfortunately, it was not possible to fully crosslink the cork and barrel domains, so it is unclear to what extent the uncrosslinked species contributed to transport activity. Interestingly, the cork may come out of the barrel completely because separately expressed cork and barrel
domains can be reconstituted into a functional transporter in vivo (Vakharia and Postle, 2002; Braun et al. 2003). Also, the cork presumably exits the barrel to allow large ligands, such as colicin B, across the outer membrane (Devanathan and Postle, 2007). Although the energy barrier for removing the cork would be higher than if it were to rearrange inside the barrel, the presence of interfacial water molecules lining the periphery of the cork would help reduce the energy barrier required for cork expulsion (Chimento et al. 2005; Gumbart et al. 2007).

**Interactions between the transporters and TonB**

The TonB box is a conserved sequence located near the extreme amino terminus of each TonB-gated transporter that is essential for ligand transport but not ligand binding (Schoffler and Braun, 1989; Kadner, 1990). Individual sidechains in the TonB box can generally be substituted with other amino acids without losing significant activity, but substitutions with proline or glycine (e.g. BtuB L8P or FepA I14P) tend to inactivate the transporter (Gudmundsdottir et al. 1989). Suppressor mutations in the TonB carboxy-terminal domain (e.g. Q160K or Q160L) weakly restore the cobalamin-defective transport associated with inactivating glycine or proline substitutions, suggesting that TonB Q160 potentially contacts the BtuB TonB box (Heller et al. 1988; Bell et al. 1990). It was later shown that TonB Q160C, as well as Q162C and Y163C, could spontaneously form disulfide crosslinks with cysteine substitutions in the TonB boxes of BtuB (Cadieux and Kadner, 1999) and FecA (Ogierman and Braun, 2003), indicating a direct interaction.
Interestingly, the ability of TonB to interact with the TonB box is not dependent on an active TonB system. Some of the BtuB TonB box cysteine substitutions were still able to crosslink to cysteine substitutions surrounding residue Q160 in the presence of the inactivating BtuB L8P substitution, but the combinations of residues forming TonB-BtuB crosslinks changed (Cadieux and Kadner, 1999; Cadieux et al. 2000). Moreover, TonB cysteine substitutions Q160C, Q162C and Y163C were still able to crosslink to cysteine substitutions in the FecA TonB box when the inactivating ExbD D25N substitution was present (Ogierman and Braun, 2003). These observations suggest that both TonB and the TonB box must interact in a specific conformation for transport to occur.

*In vitro* site-directed spin labeling reveals that, in the absence of ligand, the BtuB TonB box exists in a folded conformation within the beta-barrel (Fanucci et al. 2003). Upon binding of vitamin B12, the TonB box of BtuB becomes flexible and highly mobile (Merianos et al. 2000; Fanucci et al. 2003). Incorporation of the inactivating proline substitutions L8P and V10P maintained the TonB box in a constitutively disordered state regardless of the presence of vitamin B12, suggesting that the TonB box needs to transition between a “docked/folded” conformation in the absence of ligand and an “undocked/flexible” conformation in the presence of ligand (Coggshall et al. 2001; Fanucci et al. 2003). These *in vitro* studies support the observation that TonB does not transduce energy to TonB-gated transporters *in vivo* unless ligand is bound (Larsen et al. 1999).

Whereas the site-directed spin labeling studies suggest that addition of ligand alone exposes the TonB box to the periplasm, *in vivo* labeling suggests that both ligand
and TonB are required for the movement of the TonB box. Cysteine substitutions were engineered into the FepA cork domain, and the ability of these substitutions to become labeled by the thiol-specific probe 1-biotinamido-4-[4′-(maleimidomethyl) cyclohexanecarboxamido] butane (BMCC) was determined (Devanathan and Postle, 2007). Because BMCC gains access to the periplasm by diffusing through porins, increases in BMCC labelling correlate with increased exposure to the periplasm. One substitution, T13C, was located in the TonB box, and maximal labelling of T13C could only occur when both colicin B and TonB were present. Interestingly, the absence of TonB did not result in significant labelling of T13C even when colicin B was added. These results suggest that, in order for the TonB box to be exposed to the periplasm, TonB likely binds to other areas of the transporter before the TonB box becomes accessible to the periplasm.

The nature of TonB-transporter interactions outside the TonB box is not thoroughly characterized. Crystal structures of TonB carboxy terminal domain fragments interacting with BtuB (Shultis et al. 2006) or FhuA (Pawelek et al. 2006) show that TonB residues 225-232 interact with the TonB box through β-strand complementation. TonB residues R166 and R204 were able to interact with periplasmic turns in both structures, but the arginines in the TonB carboxy terminus are not essential for TonB activity (Vakharia-Rao et al. 2007). In the case of the TonB-FhuA structure (Fig. 1-6), TonB R166 interacted with the FhuA switch helix which links the TonB box to the rest of the cork domain, but this secondary structure element is not essential for FhuA activity (Endriss et al. 2003) and is absent in FepA. Using phage display, an isolated TonB periplasmic domain (residues 33-239) interacts with peptides corresponding to the TonB
box, switch helix, the short loop connecting the cork domain to the \( \beta \)-barrel and a periplasmic turn (Carter et al. 2006a). Peptides shown to bind sequences on FhuA were then analyzed for potential binding to other transporters such as BtuB, FepA, and FecA. TonB was able to bind the TonB box and some of the periplasmic turns in each of the transporters analyzed, but no interactions were predicted between the TonB-selected peptides and areas of the FepA cork domain beyond the TonB box (Carter et al. 2006a).

Overall, this dissertation provides mechanistic insight into how the TonB TMD and periplasmic carboxy-terminal domain contribute to TonB activity. TonB was once considered to function by shuttling between membranes and utilizing its TMD to deliver energy to TonB-gated transporters. Interestingly, the work in Chapter 2 demonstrates conclusively that the TonB TMD remains anchored in the cytoplasmic membrane during energy transduction. This finding disproves the shuttle model of TonB-dependent energy transduction and suggests that TonB operates according to one of several mechanical models. Although TonB dimerizes \textit{in vivo}, the correlations between dimerization and TonB function and localization was unknown. In Chapter 3, TonB H20A reduces the \textit{in vivo} dimerization of cysteine substitutions in the TonB carboxy terminus. Moreover, sucrose density gradient fractionation reveals that disulfide-linked TonB dimers can interact with the outer membrane but only when the crosslink is formed through a region outside residues 186-239. The findings in this chapter suggest that dimerization is an early event in the energy transduction cycle, likely prior to formation of PMF-dependent ExbD-TonB heterodimers. In Chapter 4, \textit{in vivo} photocrosslinking demonstrates that TonB interacts with several areas of the FepA cork domain outside the TonB box during enterochelin transport. This is the first \textit{in vivo} demonstration that TonB binds to other
areas of the cork domain besides the TonB box. This study also analyzed which residues of FepA could interact with TonB in the presence or absence of ligand and how a deletion of the TonB box, which inactivates FepA, influenced those interactions. The presence of enterochelin either enhances or diminishes the formation of these interactions, and most contacts require the TonB box for optimal detection. These results suggest that the trapped complexes have functional significance with respect to the transport of enterochelin in vivo. The overall significance of the findings and directions for future research are discussed.
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**Figure Legends:**

**Fig. 1-1:** TonB-gated transport of iron-siderophores across the outer membrane of Gram-negative bacteria. A cytoplasmic membrane complex of ExbB/ExbD/TonB harnesses cytoplasmic membrane PMF to allow transport of nutrients across the outer membrane. FepA (Buchanan *et al.* 1999; Protein Data Bank: 1fep) is depicted as an example of an outer membrane TonB-gated transporter. Enterochelin is shown as an example of an iron-siderophore with the ferric iron depicted as a red circle. Adapted from (Prescott *et al.* 1996).

**Fig. 1-2:** Topologies of the ExbB, ExbD and TonB proteins. ExbD and TonB share a similar topology with a single TMD and the majority of the protein occupying the periplasm. TonB TMD sidechain H20 is the only individually important sidechain in the TonB TMD. ExbB has 3 TMDs and significant cytoplasmic regions. The positions of the periplasm, cytoplasmic membrane (C.M.), cytoplasm and TonB H20 are indicated.

**Fig. 1-3:** Models for TonB energy transduction. In the mechanical models, unenergized TonB (blue) is anchored in the cytoplasmic membrane (CM) complex of ExbB (dark green) and ExbD (light green). Energy derived from CM protonmotive force drives conformational changes to “activate” TonB (now colored orange). While remaining attached to the CM, active TonB will contact outer membrane (OM) transporters such as FepA (Buchanan *et al.* 1999; Protein Data Bank: 1fep) to allow ligand transport, though precise sites of contact outside the TonB box are unknown (?). However, work shown in Chapter 4 identified several regions of TonB-FepA interaction beyond the TonB box. In the shuttle model, energized TonB first contacts an OM transporter while remaining attached to the CM and then the TonB amino terminus detaches from the cytoplasmic
membrane to deliver conformationally stored potential energy to the OM transporter.

After an activity event, the amino terminus will re-attach to the CM.

**Fig. 1-4:** Crystal structures of TonB carboxy-terminal fragments. A) Structure of TonB residues 164-239 (Protein Data Bank: 1ihr) solved by Chang *et al.* (2001). Image is from Ghosh and Postle (2004) *Mol Microbiol* **51**: 203-213. Reprinted with the permission of John Wiley and Sons via the RightsLink® Copyright Clearance Center. B) Structure of TonB residues 148-239 (Protein Data Bank: 1u07). Image is from [Kodding *et al.* (2005) *J Biol Chem* **280**: 3022-3028] and was reprinted with permission from the American Society of Biochemistry and Molecular Biology (ASBMB) via the RightsLink® Copyright Clearance Center. In both structures, the aromatic sidechains in each subunit are buried.

**Fig. 1-5:** TonB-gated outer membrane (OM) transporters in *E. coli* and their ligands.

Solved crystal structures have been incorporated into the figure to represent FepA (Buchanan *et al.*, 1999), FhuA (Ferguson *et al.* 2000), BtuB (Chimento *et al.* 2003), FecA (Ferguson *et al.* 2002), and Cir (Buchanan *et al.* 2007). With the exception of BtuB, each of the transporters is involved in the uptake of iron siderophores or other iron-containing compounds. ChuA and IutA are only found in pathogenic strains of *E. coli*.

**Fig. 1-6:** Solved crystal structure of the TonB carboxy-terminal domain with FhuA.

Figures taken from Pawelek *et al.* (2006) *Science* **312**: 1399-1402, and reprinted with permission from the American Association for the Advancement of Science (AAAS) via the RightsLink® Copyright Clearance Center. A) The TonB carboxy terminus (yellow) interacts with the BtuB TonB box (blue strand outside barrel) through beta-strand-exchange. Similar interactions were demonstrated for the TonB-BtuB crystal structure
(Shultis et al. 2006). The remainder of the FhuA cork domain is colored green while the beta-barrel is shown in blue. B) TonB R166 interacts with the indicated residues in the FhuA barrel (Asn594, Ala591) and cork domain (Ala26, Glu56). The 4 beta-strands comprising the interior of the FhuA cork domain are labeled β1-β4.
Fig. 1-1
Fig. 1-2
Fig. 1-3
A. 

Fig. 1

1:Phe^{180}, 2:Phe^{202}, 3:Trp^{213}, 4:Tyr^{215}, 5:Phe^{230}

B. 

Fig. 1-4
Fig. 1-5
A.

B.

Fig. 1-6
CHAPTER 2

DEATH OF THE TONB SHUTTLE HYPOTHESIS

This chapter was published as an open-access article in *Frontiers in Microbiology*.
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MGG is the first author and performed all experiments relating to the ToxR-TonB fusion. MIS performed OGM labeling experiments and visualized cells with electron microscopy. RAL characterized the localization of TonB L3C ΔTMD. AAO determined steady-state levels of GFP-TonB. KP and MGG wrote the paper. To comply with publication regulations, the organization / layout of this chapter is somewhat different from other chapters.
Death of the TonB shuttle hypothesis

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Running title: TonB does not shuttle

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Abstract

A complex of ExbB, ExbD, and TonB couples cytoplasmic membrane (CM) proton motive force (pmf) to the active transport of large, scarce, or important nutrients across the outer membrane (OM). TonB interacts with OM transporters to enable ligand transport. Several mechanical models and a shuttle model explain how TonB might work. In the mechanical models, TonB remains attached to the CM during energy transduction, while in the shuttle model the TonB N terminus leaves the CM to deliver conformationally stored potential energy to OM transporters. Previous studies suggested that TonB did not shuttle based on the activity of a GFP-TonB fusion that was anchored in the CM by the GFP moiety. When we recreated the GFP-TonB fusion to extend those studies, in our hands it was proteolytically unstable, giving rise to potentially shuttleable degradation products. Recently, we discovered that a fusion of the *Vibrio cholerae* ToxR cytoplasmic domain to the N terminus of TonB was proteolytically stable. ToxR-TonB was able to be completely converted into a proteinase K-resistant conformation in response to loss of pmf in spheroplasts and exhibited an ability to form a pmf-dependent formaldehyde crosslink to ExbD, both indicators of its location in the CM. Most importantly, ToxR-TonB had the same relative specific activity as wild-type TonB. Taken together, these results provide conclusive evidence that TonB does not shuttle during energy transduction. We had previously concluded that TonB shuttles based on the use of an Oregon Green® 488 maleimide probe to assess periplasmic accessibility of N-terminal TonB. Here we show that the probe was permeant to the CM, thus permitting the labeling of the TonB N terminus. These former results are reinterpreted in the context that TonB does not shuttle, and suggest the existence of a signal transduction pathway from OM to cytoplasm.
Introduction:

The TonB system energizes the transport of large, scarce, and important nutrients such as vitamin B12 and siderophores complexed with iron across the outer membrane (OM) of *Escherichia coli*. A complex of the (CM) proteins ExbB and ExbD harnesses energy derived from the proton motive force (pmf) and transmits this energy to TonB. TonB then physically contacts a ligand-loaded TonB-gated transporter (TGT) in the OM, thus enabling release of the ligand into the periplasmic space. The per-cell copy number ratio of the TonB:ExbB:ExbD proteins is 1:7:2, although the precise ratio in an energy-transducing complex is unknown (Higgs *et al.*, 2002). In *E. coli*, one set of tonB-exbB-exbD genes supports transport for multiple TGTs. But in certain other species, logistics are more complex; for example, *Xanthomonas campestris* has 8 tonB genes and genes for 48 different transporters (Schauer *et al.*, 2008).

*E. coli* TonB has a topology consistent with that of an inter-membrane energy transducer: a short cytoplasmic region (residues 1-11), an N-terminal transmembrane domain (TMD, residues 12-32), a periplasmic linker region from 33-149 and a C terminus (residues 150-239) capable of interacting with TGTs. The TMD is essential for localization and maintaining interactions with integral CM proteins ExbB/D (Karlsson *et al.*, 1993; Larsen *et al.*, 1999; Ollis *et al.*, 2009). The periplasmic linker domain is predicted to be largely disordered and contains a non-essential proline-rich domain from 70-102 (Larsen *et al.*, 1993; Peacock *et al.*, 2005; Larsen *et al.*, 2007). The C terminus is essential for interaction with OM transporters, but none of the 90 residues in the C terminus are individually essential for function (Postle *et al.*, 2010). The molecular mechanism by which TonB transduces energy from the CM to OM transporters and facilitates subsequent ligand transport is largely unknown.

The question of how TonB participates in energy transduction has been fertile ground for researchers’ imaginations since the idea of energy transduction from the CM pmf to the OM first
emerged (Hancock and Braun, 1976). In 1980, the Bradbeer laboratory suggested the possibility that TonB might synthesize a mobile messenger that would diffuse from CM to OM (Reynolds et al., 1980). In 1982, Wookey proposed that TonB might be a periplasmic permease (Wookey, 1982). In 1990, Kadner proposed the idea that TonB might shuttle in a review article (Kadner, 1990). Subsequent evidence for the shuttle model came from sucrose density gradient fractionation experiments where our lab showed TonB to localize in a CM:OM ratio of 60:40%, and exclusively at the OM in conditions in which ExbB/D and TolQ/R were absent (Letain and Postle, 1997). These initial findings were supported by in vivo demonstrations that a cyst substitution in the extreme TonB N terminus, L3C, could be labeled with the probe Oregon Green® 488 maleimide (OGM) (Larsen et al., 2003). As a control, truncated TonB L3C, consisting of the N-terminal 168 amino acids, was unable to interact with TGTs and could not be labeled although it formed a normal complex with ExbB. The degree of labeling for TonB L3C also increased substantially in the absence of the CM proteins ExbB/D and TolQ/R. Taken together, the results suggested that the extreme N terminus of TonB was labeled in the periplasm after it had departed from the CM. We suggested that energy transduction involved the physical cycling between the CM and OM compartments, a process called “shuttling.”

Alternative models have arisen since that time, including a propeller model, a pulling model, and a PBP-assisted model [reviewed in (Krewulak and Vogel, 2011)]. What the later models have in common is that the TonB TMD never leaves the CM while accomplishing its functions. None of the models for TonB-dependent energy transduction has been widely embraced or ruled out, because of a lack of conclusive supporting evidence.

Here we show that a GFP-TonB fusion was too proteolytically unstable to allow meaningful interpretation of the data. In contrast, a fusion of the cytoplasmic domain of Vibrio cholerae ToxR (aa 1-181) to the N terminus of TonB was proteolytically stable, localized to the CM and had a relative specific activity that was essentially the same as wild-type TonB. These
data indicated that TonB does not shuttle. We also discovered that our previous interpretation that TonB shuttled had been compromised by the fact, shown in these studies, that OGM could leak across the CM and label a cys substitution in a cytoplasmically confined TonB derivative. In light of these findings, our previous data are reinterpreted here.

Materials and methods

Media, culture conditions

Saturated overnight cultures grown in Luria-Bertani (LB) broth were subcultured 1:100 into M9 minimal medium or MOPS medium with chloramphenicol added to 34 µg/ml as necessary to maintain plasmids (Shedlovsky and Brenner, 1963; Miller, 1972; Neidhardt et al., 1974). For M9 minimal medium, M9 salts were supplemented with 0.5% glycerol, 0.2% casamino acids (wt/vol), 40 µg/ml tryptophan, 0.4 µg/ml thiamine, 1 mM MgSO₄, 0.5 mM CaCl₂, and 1.85 µM FeCl₃ (Postle, 2007). When necessary, L-arabinose or sodium propionate was added to induce expression of the TonB derivatives to normal chromosomal levels. For all assays, cells were grown to mid-exponential phase of 0.4-0.5 A₅₅₀ units as measured on a Spectronic 20 spectrophotometer using a 1.5 cm pathway. A sample of each culture was precipitated with an equal volume of ice cold 20% w/v trichloro acetic acid (TCA) and protein levels were visualized on immunoblots of SDS-polyacrylamide gels.

Strains and construction of plasmids

E. coli K-12 strains W3110 (F⁻ IN(rrnD-rrnE)1) (Hill and Harnish, 1981), KP1344 (W3110 ΔtonB::blaM) (Larsen et al., 1999), KP1440 (W3110 ΔtonB::blaM exbB::Tn10, tolQam) (Larsen et al., 2003), KP1035 (W3110 entA::mini-kan), KP1229 (W3110 Δ(ana-tonB-trpC) (Ahmer et al., 1995) and KP1231 [W3110 Δ(ana-tonB-trpC) entA::mini-kan] were the principal strains used in this study. KP1231 was constructed by sequential P1vir transduction of the
entA::mini-kan cassette from MK3, a generous gift from Mark McIntosh, into W3110 to generate KP1035 and finally from KP1035 into KP1229 to yield KP1231 (Ahmer et al. 1995). Plasmids used in this study are shown in Table 1. Attempts were made to replicate construction of a GFP-TonB fusion protein previously described (Kaserer 2008), where sggfp is connected to tonB by a BamHI site. The plasmid encoding the exact GFP variant (sggfp) used in that study was no longer available (discontinued by the manufacturer), so a gfpmut2 variant was used (encoded on pDR107B, a generous gift from P. de Boer). sggfp and gfpmut2 both encode derivatives of Aequorea victoria green fluorescent protein. Each has three substitutions to increase or enhance GFP fluorescence, but the substitutions are different—for sggfp, F64L, S65C, and I167T (Kaserer et al., 2008) and for gfpmut2, S65A, V68L, and S72A (Cormack et al., 1996) gfpmut2-tonB was constructed in three steps. First, tonB including the native RBS, from pKP325, was cloned into pPro33 using a 5’ KpnI site and 3’ SphI site, creating pKP1141 (primer sequences available upon request). Digestion of pPro33 with these restriction enzymes excised the unique BamHI site in the pPro33 MCS, since a unique BamHI site was needed in the final construct, as described above. In the second step, a BamHI site was added immediately before the tonB start codon in pKP1141, creating pKP1159. Forward and reverse primers were designed with the desired insertion flanked on both sides by 12-15 homologous bases. DpnI digestion was used to remove the template plasmid. Clones were screened for the presence of the BamHI site. In the third step, gfpmut2 from pDR107B was amplified, from the start codon through the final codon for Lys238 (no stop codon) and introducing flanking BamHI sites. This was cloned into the BamHI site of pKP1159, creating pKP1160 (gfpmut2-tonB). Clones were screened for insert orientation with NcoI. With the exception of the GFP modifications, this construct was the same as pGT from Kaserer et al. (Kaserer et al., 2008).

Plasmid pKP1529 was generated by cloning PCR-amplified ToxR-TonB (including the ToxR Shine-Dalgarno sequences) from pASAToxRTonB1 (Sauter et al., 2003) into the unique
XbaI site in pBAD33 (Guzman et al., 1995). As a result, ToxR-TonB expression was now regulated by the P_{BAD} promoter. All other plasmids are derivatives of pKP325 (Larsen et al., 2007). Plasmid pKP544 was constructed by extra-long PCR, resulting in the removal of TonB residues 12-32. Plasmid DNA sequences were verified at the Penn State Genomics Core Facility – University Park, PA.

**In vivo formaldehyde crosslinking**

Formaldehyde crosslinking assays were performed essentially as described previously (Postle, 2007; Ollis et al. 2009). Mid-exponential phase cells were resuspended in sodium phosphate buffer pH 6.8. A portion of each culture served as a control for TonB expression levels. To examine crosslinking of ToxR-TonB in the absence of pmf, carbonylcyanide m-chlorophenylhydrazone (CCCP) was added to a final concentration of 50µM. As a control for solvent addition, dimethyl sulphoxide (DMSO) was added to each sample that was not treated with CCCP. Samples were incubated at 37°C for 5 min. and treated with 16% paraformaldehyde (final concentration 1%) for 15 minutes at room temperature. After centrifugation for 5 min. at room temperature, crosslinked samples were solubilized in sample buffer (Laemmli, 1970) at 60°C for 5 min., and uncrosslinked samples were boiled at 95°C for 7 min. Complexes were visualized on immunoblots with the anti-TonB monoclonal (4F1) and anti-ExbD polyclonal antibodies (Larsen et al., 1996; Higgs et al., 2002).

**[^55 Fe]ferrichrome transport**

Mid-exponential phase cells were harvested, suspended in buffer and initial rates of[^55 Fe]ferrichrome transport were determined in triplicate as described (Postle, 2007). A sample of each culture was TCA precipitated immediately prior to assay to determine TonB expression levels. Proteins were visualized on immunoblots of SDS-PAGE gels with the anti-TonB
monoclonal antibody 4F1. This experiment was performed independently three times and the same results obtained.

**Proteinase K accessibility**

Strains expressing TonB derivatives were converted to either intact or lysed spheroplasts and were treated with or without proteinase K and with or without carbonylcyanide-\(m\)-chlorophenylhydrazone (CCCP) as described previously (Larsen et al., 1999). Proteinase K-resistant forms were visualized on immunoblots with the monoclonal antibody 4F1.

**Electron microscopy preparations**

Plasmid-bearing mid-exponential phase KP1344 cells were pelleted and fixed with glutaraldehyde and paraformaldehyde at concentrations of 2% w/v, each according to standard protocols from the Franceschi Microscopy and Imaging Center, Washington State University, Pullman, WA. Samples were dehydrated in an ethanol series and then embedded in LR-White resin. Cured samples were thin sectioned and adhered to nickel grids. Immunolabeling was performed with 1:100 4F1, followed by 1:100 goat anti-mouse antibody conjugated with 20 nm gold particles. Grids were stained with uranyl acetate and lead citrate and visualized with a JEOL transmission electron microscope (JEOL USA Inc.) with images captured with a MegaViewIII Digital camera at the Franceschi Microscopy and Imaging Center.

**Oregon Green® maleimide labeling**

*In vivo* labeling was performed as described previously (Larsen et al., 2003). Briefly, 75 ml cells were grown in LB broth with 34 µg/ml chloramphenicol to an \(A_{550}\) of 0.50. Buffer A (50 mM KPO\(_4\), 100 mM K\(_2\)SO\(_4\), pH 7.0) was added to 25 ml of cells and OGM in DMSO was added to a final concentration of 172 µM. Samples were incubated for 1 hour on ice with shaking and were quenched with 2-mercapoethanol. After washing 3 times with Buffer A, samples were suspended in Tris-LSB, boiled for 10 minutes and frozen at -30°C.
In vitro labeling was performed in a manner similar to the in vivo labeling protocol but differed in that RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 1% SDS in 150mM NaCl 50 mM Tris-acetate pH 7.6) was added to harvested cells, the cells were passed twice through a French Pressure cell, and lysate was collected. Samples were incubated with OGM and frozen as above except that no LSB-Tris was added prior to freezing. The results did not change for either in vivo or in vitro labeling regardless of whether or not 2-mercaptoethanol was added to quench the reaction.

Immunoprecipitation

In vivo and in vitro samples were thawed, and TonB was immunoprecipitated with a cocktail of 4F1 and 4H4 monoclonal antibody as described previously (Larsen et al. 2003). Samples for GroEL analysis were prepared the same way as the TonB samples but immunoprecipitated with anti-GroEL antibody (Stressgen Bioreagents, Victoria, B.C., Canada). Samples were divided, resolved on SDS-polyacrylamide gels and immunoblots were subsequently developed with either anti-TonB or anti-GroEL antibodies and anti-OGM (Pierce) antibodies.

Results

GFP-TonB can give rise to “shuttleable” degradation products

A recent study fused GFP from A. victoria to the N terminus of TonB and asked whether or not this GFP-TonB construct was active (Kaserer et al., 2008). The fusion of the TonB cytoplasmic N terminus to a large cytoplasmically localized protein should prevent potential shuttling by keeping TonB retained in the CM. The GFP-TonB construct supported TonB activity and the authors concluded that TonB did not shuttle. To extend this work, we reconstructed a GFP-TonB fusion with the same linker sequence between GFP and TonB as was used in that
study and used TCA to precipitate bacteria immediately upon harvest to prevent the degradation of GFP-TonB (and presumably GFP-TonB fragments) that could occur in gel sample buffer at 95°C (Skare and Postle, 1991). This allowed for accurate determination of the steady-state levels of all TonB-specific peptides. In our hands, expression of GFP-TonB at chromosomal levels resulted in the detection of several prominent degradation products (Fig. 2-1). The results did not change regardless of whether M9 or MOPS medium was used, nor if GFP-TonB was expressed in the tonB entA strain KP1231 (data not shown). The degradation products that were the size of full-length TonB or slightly larger might support TonB activity by shuttling. Because of the challenges and problems with proteolysis and because the mechanism of TonB energy transduction is still considered an open question (Krewulak and Vogel, 2011), we wanted to identify a system that could answer the question of shuttling more definitely.

**ToxR (1-181)-TonB is stable and fully active**

During the course of studies on TonB dimerization, we identified a fusion of full-length TonB to the cytoplasmic domain (aa 1-181) of *V. cholerae* ToxR (Miller *et al.*, 1987), that was extremely stable as expressed from pASAToxRTonB1 and analyzed by immunoblot (data not shown). This plasmid was a kind gift of V. Braun from earlier studies on TonB dimerization where the expression levels and activities of the ToxR-TonB constructs were not reported (Sauter *et al.*, 2003). Because ToxR-TonB was overexpressed from pASAToxRTonB1, we cloned toxR-tonB, including its putative Shine-Dalgarno sequences into pBAD33 so that its expression could be regulated by the P_{BAD} promoter (Guzman *et al.*, 1995).

Like wild-type TonB, virtually all of the ToxR-TonB could be converted into a proteinase K-resistant conformation by collapse of the pmf due to CCCP addition (Fig. 2-2). Because ExbB and ExbD are required for this proteinase K-resistant conformation (Larsen *et al.*, 1999), it suggested that ToxR-TonB was located in the CM. The pmf is also required for TonB and ExbD
to formaldehyde crosslink in vivo through their periplasmic domains (Ollis et al., 2009).
Likewise, ToxR-TonB was also able to form this functionally significant crosslink with ExbD (Fig. 2-3), and this complex was undetected when pmf was collapsed with CCCP. Thus, ToxR-TonB could be energized in the same way as wild-type TonB.

Finally, $^{55}\text{Fe}$ferrichrome transport assays revealed that ToxR-TonB, when expressed to chromosomal levels, supported ~100% of the wild-type plasmid-encoded TonB ferrichrome transport rate (Fig. 2-4). ToxR-TonB supported 84% and 92% of wild-type activity in additional triplicate trials (data not shown). The key observation was that this activity could only have arisen from full-length ToxR-TonB. The amount of TonB in the uninduced sample (pTonB no ara) far exceeded the amount of the only detectable degradation product that derived from ToxR-TonB and supported only 12% activity (Fig. 2-4). Thus, ToxR-TonB had the same relative specific activity as chromosomally encoded wild-type TonB.

**Membrane permeability of OGM explains previous results**

The strongest prior evidence for in vivo shuttling was the demonstration that a cys substitution predicted to be cytoplasmically localized could be labeled by OGM in full-length TonB but not in a truncated TonB derivative missing much of its C terminus and incapable of interaction with the OM (Larsen et al., 2003). OGM has been considered a “generally membrane impermeant” probe, and other groups have used this reagent to discriminate between free cysteines in the periplasm or cytoplasm of Gram-negative bacteria (Ye et al., 2001; Ye and Maloney, 2002; Yang et al., 2005; Liu et al., 2007; Nanatani et al., 2007; Nanatani et al., 2009). Given the contrast in findings between the ToxR fusions and the OGM labeling, we tested the possibility that OGM leakage across the CM could account for our previous findings (Larsen et al., 2003).
To assess the potential for OGM to leak across the CM, we constructed a TonB variant lacking its transmembrane domain but retaining the L3C substitution of the original OGM labeling study. TonB L3C ΔTMD expressed from pKP544 should be cytoplasmically localized (Karlsson et al., 1993). The cytoplasmic localization of the TonB L3C ΔTMD construct was tested by two methods: proteinase K accessibility assays in intact spheroplasts and transmission electron microscopy immunolocalization. Whereas wild-type TonB (TonB L3C C18G) is sensitive to proteinase K in intact spheroplasts, the TonB L3C ΔTMD was sequestered from proteinase K (Fig. 2-5A). When spheroplasts were lysed, both TonB L3C C18G and TonB L3C ΔTMD were sensitive to proteinase K, indicating that TonB L3C ΔTMD was cytoplasmically localized (Fig. 2-5A). The same results were also obtained in KP1344 (data not shown).

Consistent with that result, electron microscopy revealed that the TonB L3C ΔTMD was confined to the cytoplasm whereas gold particles detecting wild-type TonB localized to the envelope (Fig. 2-5B).

OGM was able to label the cytoplasmically localized TonB L3C ΔTMD both in vitro and in vivo. This showed that in our previous experiments, OGM was indeed capable of crossing the CM and TonB L3C did not need to shuttle to become labeled by it (Fig. 2-6). In addition, OGM labeled the cytoplasmic chaperone GroEL in vitro and in vivo, confirming its ability to leak across the CM (Fig. 2-6).

Discussion

The fate of the TonB N terminus during energy transduction has been speculated about and investigated for many years. Today, the primary models that explain its role include several mechanical models where TonB remains continuously associated with the CM and the shuttle model, where TonB cyclically dissociates from the CM and shuttles to the OM. The mechanical
models suggest direct use of the pmf in energizing TGTs with TonB as a bridge between the two. The shuttle model suggests that TonB somehow stores potential energy from the pmf prior to shuttling to the OM. The TonB TMD was proposed to store the potential energy through response of residues S16 and H20 to the pmf (Larsen and Postle, 2001). Because these two models have different consequences and make different predictions, it is important to determine which is correct.

Since the emergence of the shuttle model, we have learned that the TonB TMD is highly tolerant to mutation. In particular, the only sidechain that cannot be replaced by ala and retain activity is H20, yet H20 can be replaced by asn and retain full activity, suggesting that the TonB TMD is not on a proton translocation pathway [(Larsen et al., 2007; Swayne and Postle, 2011). These results cast doubt on the shuttle hypothesis by eliminating the TMD as the region of TonB that needed to be delivered to the OM. Here we demonstrate that, in contrast to GFP-TonB, a fusion of the cytoplasmic domain of ToxR (1-181) to TonB produced virtually no degradation products when expressed at levels characteristic of native TonB. ToxR-TonB appeared to be anchored in the CM at all times because it could be fully converted to a proteinase K-resistant form in de-energized cells. ToxR-TonB could also form a pmf-dependent crosslink with ExbD and was fully active in ferrichrome transport assays. This eliminated the possibility that somehow the entire ~180 residue folded cytoplasmic domain of ToxR could be pulled out of the CM to shuttle. Even if the ToxR domain could be pulled out of the CM, it would not be possible for it to recross back into the cytoplasm and allow the TonB portion to assume its rightful relationship with ExbB and ExbD such that it had 100% activity. Because ToxR-TonB had an equivalent relative specific activity to TonB, we conclude that TonB does not shuttle. These results confirm and expand the conclusions of Kaserer et al. (2008).

The permeability of OGM to the CM explained the apparent contradiction between the ToxR-TonB results, and the previous ones suggesting that TonB shuttles in vivo (Larsen et al.,
2003). The data from our 2003 study can be reinterpreted in the light of this new information. The labeling seen for full-length TonB L3C can be explained by the observation that OGM diffuses into the cytoplasm. A more interesting explanation may pertain to the observation that TonB\textsubscript{am168} L3C did not become labeled with OGM, even though it still retained the ability to respond conformationally to pmf in spheroplasts and formaldehyde crosslink to ExbB, indicating that the truncation did not radically interfere with assembly with ExbB/D. The reinterpretation of the data raises the question of whether there is signal transduction occurring from the OM to the CM ExbB/D complex through TonB. It could be that when full-length TonB is present, the cytoplasmic domains of ExbB allow access of OGM to the extreme N terminus of TonB. In this model, when the C terminus of TonB is deleted and TonB does not associate with the OM, then the cytoplasmic domains of ExbB are conformationally rearranged such that the TonB L3C residue is sequestered. Consistent with that idea, TonB L3C was not labeled by OGM in the inside-out vesicles arising from French-pressed cells that not only lacked pmf but also any connection to the OM (data not shown).

The observation that TonB remains attached to the CM during energy transduction has ramifications for the meaning of the solved TonB-FhuA and TonB-BtuB crystal structures (Pawelek \textit{et al.}, 2006; Shultis \textit{et al.}, 2006). The conformation of the TonB C terminal domain solved in complex with the TGTs accounts only for ~25Å, but arises from ~ 1/3 of the length of TonB (residues 153-233, with 239 being the terminal residue) (Fig. 2-7). While the proline-rich domain can span up to 100 Å of periplasmic space, it is non-essential (Evans \textit{et al.}, 1986; Larsen \textit{et al.}, 1993; Seliger \textit{et al.}, 2001). Based on the assembled structure of the periplasmic-spanning AcrAB / TolC complex, the distance between the CM and OM appears to be approximately 180Å (Symmons \textit{et al.}, 2009). This suggests that the C terminus of TonB must be more disordered than is represented by the solved structures as it spans the periplasm and identifies a ligand-loaded transporter. Consistent with that, the TonB primary amino acid sequence predicts a highly
disordered protein, and the C terminus is conformationally dynamic in vivo (Larsen et al., 1999; Larsen et al., 2007; Postle et al., 2010). If TonB must be disordered in order to span the periplasm, then it is likely that TonB C terminus participates in an energy transduction cycle of association and dissociation from the TGTs rather than being constantly associated with the OM. In support of this idea, the number of OM transporters in a given cell exceeds the number of available TonB molecules (Kadner and Heller, 1995; Higgs et al., 2002; Postle et al., 2010).

In a detailed analysis of four TGT structures, Chimento et al. noted that the β-strands of the cork domain that fills the TGT barrel are mostly parallel to the periplasmic space. They propose that only a modest perpendicular force supplied by TonB would be necessary to unfold the cork (Chimento et al., 2005). Now that we know that TonB does not shuttle, and that it may contact the TGT in a disordered conformation, it suggests that the mere folding of TonB upon contact might be sufficient to release the cork from the barrel and allow transport.

These new data also raised the question of what the 30-40% of TonB that fractionates with the OM represents. It could possibly represent the fraction of TonB that is strongly associated with the OM transporters at any one point in the energy transduction cycle. Alternatively it could represent TonB that has temporarily dissociated from the ExbB/D complex while its C terminus is associated with the OM. In either case, it must mean that TonB is being artifactually pulled out of the CM because ExbB and ExbD remain associated with the CM (Letain and Postle, 1997).

A remaining mystery is why, in the absence of ExbB/D and TolQ/R, nearly all of the TonB is found associated with the OM. It could be that the conformation of the TonB C terminus is such that its default interaction is at the OM. Alternatively it may be that the action of ExbB/D is primarily required to assist the TonB C terminus in dissociating from an OM transporter after an energy transduction event. The demonstration that TonB does not shuttle eliminates debate concerning how energy would be used to physically remove TonB from a CM complex of
ExbB/D, and also how the N terminus would somehow target TonB back to an ExbB/D complex at the CM (Larsen et al., 2003; Postle and Kadner, 2003).

**Conflict of interest statement**

The authors are aware of no personal or monetary issues which arose during preparation of this manuscript that could represent a conflict of interest.

**Acknowledgements**

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Escherichia coli TonB-dependent energy transduction system: TonB, ExbB, ExbD, and


Figure legends:

**Fig. 2-1:** GFP-TonB exhibits prominent degradation *in vivo*. Steady-state levels were determined as described in Materials and Methods with the following strains: cTonB (W3110 expressing chromosomally encoded TonB); ΔTonB (KP1344); pPro33-TonB (KP1344 / pKP1159) and pPro33 GFP-TonB (KP1344 / pKP1160). Sodium propionate (*) was added to induce expression of plasmid-encoded TonB or GFP-TonB as follows: 0, 1, 5, 10 mM. TonB expression levels were visualized on immunoblots of 11% SDS-polyacrylamide gels with the TonB monoclonal antibody 4F1. Mass standards (kDa) are shown on the left. Full-length TonB migrates at 36 kDa, and the position of full-length GFP-TonB is indicated by the arrow. The most likely proteins to serve as the source of activity if TonB shuttles are indicated by the bracket.

**Fig. 2-2:** ToxR-TonB is conformationally responsive to pmf. Strains were converted to spheroplasts and treated with or without proteinase K and CCCP as described in Materials and Methods with the following strains: cTonB (W3110 expressing chromosomally encoded TonB) and pToxRTonB [KP1344 (∆tonB) / pKP1529]. L-arabinose was added to induce expression of ToxR-TonB to chromosomal levels (0.00046%). Samples were resolved on 13% SDS-polyacrylamide gels, and immunoblotting was performed with the α-TonB monoclonal antibody 4F1. Mass standards are shown on the left. Abbreviations: WC = whole cells, Sph = spheroplasts, PKRF = proteinase K resistant fragment

**Fig. 2-3:** ToxR-TonB makes the pmf-dependent crosslink with ExbD. Crosslinking was performed as described in Materials and Methods with the following strains: cTonB (W3110 expressing chromosomally encoded TonB and ExbD); ΔTonB (KP1344); pTonB (KP1344 / pKP325); pToxR-TonB (KP1344 / pKP1529) and pToxR-TonB CCCP (KP1344 / pKP1529 treated with 50 µM CCCP). L-arabinose was added to induce expression of plasmid-encoded
TonB as follows: 0.00075% for pTonB and 0.00046% for pToxR-TonB. Complexes were detected on immunoblots of 13% SDS-polyacrylamide gels using ExbD polyclonal antibodies (upper two panels) and the monoclonal anti-TonB antibody 4F1 (bottom panel). The molecular mass standards (kDa) are shown on the left.

**Fig. 2-4:** ToxR-TonB supports as much activity as plasmid-encoded wild-type TonB.

Ferrichrome transport assays were performed as described in Materials and Methods with the following strains: cTonB (W3110 expressing chromosomally encoded TonB); ΔTonB (KP1344); pTonB (KP1344 / pKP325); pTonB no ara (KP1344 / pKP325 uninduced) and pToxR-TonB (KP1344 / pKP1529). L-arabinose was added to induce expression of plasmid-encoded TonB as follows: 0.00075% for pTonB and 0.00045% for pToxR-TonB. Initial ferrichrome transport rates are as follows, expressed as counts per minute (cpm) / 0.05 A550 ml cells / minute: cTonB 453±31; pTonB 326 ±13 (standard to 100%); pTonB no ara 39±7 (12%); pToxR-TonB 333±15 (102%); ΔTonB -4±4 (0%). TonB expression levels were visualized on immunoblots of 11% SDS-polyacrylamide gels with anti-TonB monoclonal antibody 4F1. A longer exposure is shown to provide a comparison between uninduced pKP325 and the lack of full-length degradation product from pKP1529. Mass standards are shown on the right. Degradation products are marked with an asterisk (*).

**Fig. 2-5:** TonB L3C ΔTMD is cytoplasmically localized. (A) TonB L3C ΔTMD is resistant to proteinase K in intact spheroplasts but sensitive to proteinase K in lysed spheroplasts. Strains pTonB L3C ΔTMD (KP1440 (∆tonB, exbB::Tn10, tolQun) / pKP544) and pTonB L3C C18G (KP1440 / pKP478) were converted to either intact or lysed spheroplasts and treated with or without proteinase K as described in Materials and Methods. Proteins were resolved on 11% SDS-polyacrylamide gels and immunoblotted with anti-TonB 4F1 antibodies. Abbreviations: WC= whole cells, Sph = spheroplast, Lys = lysed spheroplast. (B) Electron micrographs of strains cTonB (W3110 expressing chromosomally encoded TonB) or pTonB L3C ΔTMD
Cells were fixed and labeled with immunogold particles as described in Materials and Methods. Examples of labeled TonB derivatives are indicated with arrows. Size bars are included at the bottom.

**Fig. 2-6**: Oregon Green maleimide labels cytoplasmic proteins. OGM labeling and subsequent immunoprecipitation were performed as described in Materials and Methods with the following strains: cGroEL (W3110 expressing chromosomally encoded GroEL) and pTonB L3C ΔTMD (KP1344 / pKP544). Samples of cells were also lysed by passage through a French pressure cell and labeled *in vitro*; these served as a standard for 100% labeling. L-arabinose was added to induce expression of TonB L3C ΔTMD (0.002%). Proteins were also harvested by treatment with TCA to serve as a total protein control. Samples were divided and resolved on 11% SDS-polyacrylamide gels and immunoblotted with anti-TonB or anti-GroEL antibodies for half of the samples and with anti-OGM antibodies for the other half. Positions of TonB L3C ΔTMD and GroEL are indicated. Molecular mass standards are shown in the center. Abbreviation: IP = immunoprecipitation.

**Fig. 2-7**: TonB cannot reach OM transporters with the conformation depicted by crystal structures of its C terminus. TonB residues 33-69 and 103-152, considered to be disordered regions, are depicted as yellow rectangles. The span of the periplasmic space was calculated based on crystal structure reconstructions of the AcrA/B/TolC complex (Symmons *et al.*, 2009). Abbreviations: CM = cytoplasmic membrane, OM = outer membrane
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<tr>
<td>pDR107B</td>
<td>pET21B derivative encoding GFPmut 2-T7tag-his fusion protein; Amp&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>pASAToxRTonB1</td>
<td>pHK toxR(1–181)-tonB(1–239) Cam&lt;sup&gt;+&lt;/sup&gt;</td>
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**cTonB**  **pToxR-TonB**

![Image of gel electrophoresis with protein bands](image)

- 55 kDa
- 36 kDa
- 28 kDa

- ToxR-TonB
- ToxR-TonB PKRF
- TonB
- TonB PKRF

Fig. 2-2, Gresock et al.
Fig. 2-3, Gresock et al.
Fig. 2-4, Gresock et al.
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Fig. 2-7, Gresock et al.
CHAPTER 3

H20 IN THE TONB TRANSMEMBRANE DOMAIN

MEDIATES AN EARLY HOMODIMERIZATION STEP IN

ENERGY TRANSDUCTION

This chapter was submitted to the Journal of Bacteriology in November 2013. MGG is the first author of this work and characterized the effect of H20A on TonB dimerization. KAK determined the localization of dimers formed through TonB F125C, G186C and F230C. KP and MGG wrote the paper. To comply with publication regulations, the format of this chapter differs from the other chapters.
H20 in the TonB transmembrane domain mediates an early homodimerization step in energy transduction

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Running title: H20 mediates TonB dimerization

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Abstract

The TonB system couples the cytoplasmic membrane protonmotive force to outer membrane TonB-gated transporters for active transport of nutrients into the periplasm of virtually all Gram-negative bacteria. Mutagenic studies indicate that residue H20 in the amino terminal TonB transmembrane domain is important for TonB function, but does not participate in proton translocation. Here we show that H20 is important for homodimerization of TonB. The H20A mutant inhibited dimerization of a fusion of the ToxR cytoplasmic domain to wild-type TonB (ToxR-TonB). The H20A mutant also inhibited spontaneous homodimer formation by cysteine substitutions in the extreme periplasmic carboxy terminus of TonB (residues 150-239). A sub-region of cysteine substitutions in residues 186-230 was identified where efficient trapping of TonB as a disulfide-linked homodimer prevented its natural ability to associate with the outer membrane. In contrast, when TonB was crosslinked through a site amino-terminal to that sub-region, F125C, the majority of homodimers associated with the outer membrane. These data suggest that correct H20-mediated dimerization of the TonB carboxy terminus is an important step prior to interaction of TonB with outer membrane proteins. Results are presented within the context of a model for initial events in TonB energization.
Introduction:

The outer membrane of Gram-negative bacteria protects the cells from various environmental agents such as degradative proteins that target peptidoglycan, detergents and many antibiotics. Most nutrients cross the outer membrane by diffusion through porin proteins. Other nutrients, such as iron-siderophore complexes and vitamin B12, in the case of Escherichia coli, are actively transported across the outer membrane through seven different transporters. Integral cytoplasmic membrane proteins TonB/ExbB/ExbD couple the protonmotive force (PMF) energy of the cytoplasmic membrane to that process. ExbB/D convert PMF into conformational changes in the carboxy terminus of TonB, which directly contacts the ligand-loaded TonB-gated outer membrane transporters and enables passage of nutrients into the periplasmic space [for reviews see (1-3)]. Upon entering the periplasmic space, the iron-siderophores or vitamin B12 encounter binding proteins that deliver them to cytoplasmic membrane permeases for transport into the cytoplasm (4). E. coli K12 TonB-gated transporters also serve as receptors for toxins known as colicsins and bacteriophage such as $\phi$80 (5). Measurement of iron transport rates and sensitivity to colicsins or bacteriophage constitute a comprehensive set of phenotypic assays for TonB system activity (6).

E. coli TonB protein is divided into two functional domains, an amino terminal transmembrane domain (TMD, residues 12-32) and a periplasmic carboxy terminus (residues 103-239). Residues 103-239 can be further subdivided into two regions: a confirmed unstructured region from 103-149 (7), and a region from 150-239 (referred to here as the extreme carboxy terminus) that is essential for interaction with TonB-gated transporters (8-10). The proline-rich domain (residues 70-102) between amino and carboxy termini is important for extending the reach of the TonB carboxy terminus across the periplasm, but is not essential [(11, 12) and data not shown].
The TonB carboxy terminal residues from 150-239 have been extensively characterized both \textit{in vitro} and \textit{in vivo}. \textit{In vitro}, two crystal structures of homodimeric carboxy terminal domains (~160-239) have been solved which are rigid strand-exchanged structures with buried surface areas of over 4500 angstroms$^2$ [(13-15) and unpublished data]. \textit{In vivo}, TonB forms homodimers through the same domain, but with properties that are inconsistent with the homodimeric crystal structures (6).

A cysteine scanning analysis of 91 TonB carboxy-terminal residues (F125 and consecutively through residues 150-239) identifies only 7 functionally important residues: G186, and 6 aromatic residues, Y163, F180, F202, W213, Y215 and F230. Although not really part of the extreme carboxy terminus, TonB F125C, was included in that study as the sole remaining aromatic residue in the carboxy terminal domain from residues 103-239(6).

Ala (or Cys) substitutions at these 7 sites form a unique group in which all members exhibit idiosyncratic phenotypic profiles in four different phenotypic assays. For example, TonB F202A/C is fully sensitive to colicin Ia and has much reduced sensitivity to colicin B; for TonB F215C, the situation is reversed. No single Ala (or Cys) substitution of the 7 residues is inactive in all TonB-dependent activity assays. The idiosyncratic profiles suggest that the residues in question are important for discrimination amongst the various TonB-gated transporters. These data are inconsistent with the crystal structures because the 7 functionally important residues are buried in those structures. In addition, replacement of each surface-exposed residue in the crystal structures with Cys has at most only minor effects on TonB phenotypes (6, 16, 17).

In the dimeric crystal structures, residues G186, W213, Y215, and F230 are too far apart to form homodimers if substituted with Cys. \textit{In vivo} however, G186C, F202C, W213C, Y215C and F230C, each efficiently form a set of three homodimeric disulfide-linked complexes, henceforth referred to as “triplet dimers” (6, 17). Each of the homodimeric complexes represent a distinct conformation of TonB in which a given Cys substitution is sufficiently close to crosslink,
but results in 3 different mobilities on SDS-polyacrylamide gels (17). TonB F125C, which is fully active, also efficiently forms triplet dimers.

TonB protein is the limiting factor in energy transduction, suggesting that rather than have one TonB dedicated to each TonB-gated transporter, there must be cyclic reversible interactions (18, 19), which presupposes conformational changes. Similar to other proteins that function in molecular recognition (20), TonB is predicted to contain disordered regions (21), which if present could allow TonB to bind transporters with the high specificity and low reversible affinity in vivo that characterizes signal transduction proteins (22).

Initial events in TonB energy transduction involve sequential in vivo conformational changes in its extreme carboxy terminus that are modulated by PMF and the periplasmic domain of ExbD(17, 23). In vitro, purified periplasmic domains of TonB and ExbD do not interact (24). An intact TonB TMD, ExbB/ExbD, and the PMF are required to detect TonB-ExbD interaction which is a reflection of TonB activity (25).

The amino-terminal TonB TMD is essential for proper localization to the cytoplasmic membrane and proper interaction with ExbB and ExbD. It cannot be separated from the periplasmic domain or substituted with a non-cognate TMD without loss of activity (21, 23, 25-29).

TonB TMD residue H20 is the only residue in full-length TonB (239 residues) that cannot be either deleted, or substituted with Ala or Cys, and still preserve TonB function when expressed at chromosomal levels (6, 21, 30). Although the histidinyl sidechain is protonatable, H20 participation in a proton translocation pathway has been ruled out because H20 can be substituted with the non-protonatable Asn and remain fully functional (31). The role of H20 thus remains unknown.

Here we show that H20 in the TMD mediates homodimerization through the extreme TonB carboxy terminus. A TonB H20A mutation decreased the dimerization of a ToxR-TonB
fusion protein and reduced disulfide-linked triplet dimer formation within the extreme carboxy terminus. Consistent with a role for H20 in mediating dimerization, no new complexes or increases in dimerization were observed for any of the 91 carboxy-terminal Cys substitutions analyzed in an H20A mutant. Unlike wild-type TonB, disulfide-linked TonB triplet dimers formed through the extreme carboxy terminus were largely unable to associate with outer membrane fractions, suggesting that the dimerization was an important but transitional step on the energy transduction pathway. The requirement for TonB homodimerization mediated through H20 supports our previous model for initial events in TonB energy transduction and explains why TonB H20A is inactive (23).

Materials and Methods

Bacterial strains and plasmids

Bacterial strains used in this study are described in Table 1. DH5α was used as the host strain for constructed plasmids. KP1560 (FHK12 ΔtonB::kan) was created by P1 transduction of the ΔtonB::kan cassette from KP1477 into FHK12. The resultant TonB deletion was confirmed by immunoblot (data not shown).

The plasmids used in this study are listed in Table 1. The C18G H20A substitutions from pKP879 were cloned into the set of preexisting plasmids expressing TonB Cys substitutions [pKP568 and derivatives; (6)] by BstEII digestion. First, the C18G substitution was created in pKP381 (TonB H20A) by 30-cycle extra-long PCR (to amplify entire plasmid) using forward and reverse primers encoding the C18G substitution flanked by 12bp of complementary sequence to create pKP879. After digestion of both pKP879 and the plasmids expressing TonB Cys substitutions with BstEII, the 650bp BstEII fragment of pKP879 containing the C18G H20A substitution was ligated with the 5232 bp BstEII-digested fragment containing the unique Cys substitutions. Thus, each resultant C18G H20A substitution combined with a carboxy-terminal
Cys substitution was expressed under the control of the $P_{BAD}$ promoter. The H20A substitution was introduced into ToxR-TonB expressed from plasmid pKP1529 via site-directed mutagenesis, resulting in pKP1530. Plasmids were sequenced and verified at the Penn State Genomics Core Facility – University Park, PA. Restriction enzymes were purchased from New England Biolabs, and primers for mutagenesis were ordered from Invitrogen.

**Media and culture conditions**

Strains were maintained on Luria-Bertani (LB) agar plates supplemented with 34 µg/ml chloramphenicol (32). For assays, strains were grown overnight at 37°C with shaking in Luria-Bertani (LB) broth supplemented with 34 µg/ml chloramphenicol. T-broth was made as described previously, and M9 minimal salts were supplemented with 0.5% glycerol, 0.2% casamino acids (wt/vol), 40 µg/ml tryptophan, 0.4 µg/ml thiamine, 1 mM MgSO$_4$, 0.5 mM CaCl$_2$, and 1.85 µM FeCl$_3$ (33). Chloramphenicol was added to maintain plasmids, and, when appropriate, arabinose was added to induce TonB expression to approximate chromosomal levels. All cultures were grown aerobically at 37°C at 275 rpm shaking. Cells were grown to mid-exponential phase to an $A_{550}$ of 0.4-0.5 as measured on a Spectronic 20 spectrophotometer using a 1.5 cm pathway.

**Disulfide Crosslinking of TonB Cys substitutions**

All TonB Cys substitutions were coexpressed with a C18G substitution to prevent any intramolecular crosslinking between the native TonB C18 and the engineered carboxy-terminal Cys residue (17). TonB Cys substitutions with a wild type or H20A TMD were analyzed for disulfide crosslinking in KP1344. Briefly, cells were diluted 1:100 into fresh T-broth supplemented with 34 µg/ml chloramphenicol and various concentrations of arabinose to induce TonB expression to near chromosomal levels. 0.2 $A_{550}$ ml cells were harvested when the $A_{550}$ of the culture reached 0.40-0.50, and proteins were precipitated with an equivalent volume of 0.2%
trichloroacetic acid (TCA). An equivalent sample was harvested and TCA precipitated as a control for total protein levels. Pellets were resuspended in Laemmli Sample Buffer (LSB) supplemented with 50mM iodoacetamide (to analyze crosslinked species) or LSB with 2-mercaptoethanol (to analyze total protein levels) and boiled for 10 minutes at 95°C. Crosslinks were detected as the presence of higher molecular weight complexes on immunoblots of 11% non-reducing SDS-polyacrylamide gels.

β-galactosidase activity of ToxR-TonB derivatives

Strains FHK12 and KP1560 feature a chromosomally encoded lacZ gene under the control of the P_{ctx} promoter. Dimerization of full-length TonB fused to the cytoplasmic domain of ToxR (ToxR-TonB) enables the ToxR cytoplasmic domain to dimerize and activate transcription from the P_{ctx} promoter (34). To determine relative levels of ToxR dimerization, plasmids encoding ToxR-TonB and ToxR-TonB H20A under control of the arabinose promoter were transformed into both strains, were grown overnight in T-broth with 34 µg/ml chloramphenicol, and subcultured 1:100 into T-broth containing chloramphenicol and L-arabinose to induce expression of TonB derivatives. Cells were grown to an A_{600} of 0.45-0.50, and β-galactosidase assays were performed in triplicate as described in (32). A 0.2 A_{600} ml portion of each culture was treated with 0.2% TCA and served as a total protein control. Proteins were resuspended in LSB, boiled 10 min. at 95°C and visualized on immunoblots of 11% SDS-polyacrylamide gels.

Sucrose Density Gradient Fractionation

Strains expressing wild-type or plasmid-encoded TonB derivatives were subcultured 1:100 into supplemented M9 with antibiotics, and when necessary, arabinose to achieve expression to near chromosomal levels. Cells were grown to an A_{550} of 0.50, pelleted by centrifugation, and suspended in 10mM HEPES buffer, pH 7.8. Concentrated cells were then passed through a French press at 20,000 psi three times (or until a clear lysate was obtained).
Samples were then loaded onto a sucrose gradient consisting of 25%, 30%, 35%, 40%, 45%, 50% and 56% sucrose supplemented with 50mM iodoacetamide and centrifuged at 35000 for 15 hr. Fractions were collected, and a portion of each fraction was TCA-precipitated. Proteins were visualized on immunoblots of non-reducing and reducing SDS-polyacrylamide gels with anti-TonB antibodies. Immunoblots were also probed with anti-ExbB, and/or anti-CorA antibodies as markers for cytoplasmic membrane fractions; stained membranes served to identify outer membrane fractions (35).

**Results**

*TonB H20A inhibits dimerization of ToxR-TonB*

We tested the hypothesis that the TonB H20 sidechain plays a role in homodimerization, using the ToxR reporter system from *Vibrio cholerae*. *V. cholerae* ToxR consists of a cytoplasmic domain, a single TMD and a periplasmic domain. When ToxR dimerizes through its periplasmic domain, it brings the two cytoplasmic domains into proximity such that they together activate transcription of the Pctx promoter. Thus, fusions of the ToxR cytoplasmic domain to the TMD and periplasmic domain of any membrane protein can be used to assess its dimerization potential. The extent of dimerization is correlated with the degree of β-galactosidase activity from transcription of genes (e.g. *lacZ*) engineered downstream of the Pctx promoter (36, 37).

The amino terminus of TonB is localized in the cytoplasm, followed by the TMD with the rest of TonB extending into the periplasm. A fusion of the cytoplasmic domain of ToxR to the amino terminus of full-length TonB (referred to here as ToxR-TonB) induces the β-galactosidase activity indicative of dimerization in vivo (34), and has the same relative specific activity as wild-type TonB (38).

Here, we used the same ToxR-TonB fusion protein to address the effect of H20A on TonB dimerization. The H20A substitution was engineered into ToxR-TonB, rendering it, like
TonB H20A, insensitive to colicins. The β-galactosidase activities of strains expressing either
ToxR-TonB or ToxR-TonB H20A were measured in the tonB+ strain FHK12. Expression of
wild-type ToxR-TonB induced significant levels of β-galactosidase activity, confirming that
dimerization occurred (Fig. 3-1). ToxR-TonB H20A dimerization induced 10-fold less β-
galactosidase activity compared to wild-type ToxR-TonB (Fig. 3-1).

If ToxR-TonB was indeed dimerizing, the chromosomally encoded TonB from parent
strain FHK12 was also dimerizing with ToxR-TonB and thus competitively inhibiting
dimerization of the ToxR-TonB derivatives. To test that idea, ToxR-TonB and ToxR-TonB
H20A were expressed in a ΔtonB derivative of FHK12 (KP1560) (Fig. 3-1A). This led to
increased β-galactosidase activities for both constructs, confirming that TonB dimerization was
occurring. In the ΔtonB background, the dimerization of ToxR-TonB H20A increased to the
point where it constituted ~20% of the activity induced by wild-type ToxR-TonB in the same
strain (Fig. 3-1A).

*TonB H20A inhibits formation of TonB carboxy-terminal homodimers*

Full-length TonB homodimerizes through engineered Cys substitutions in its extreme
carboxy terminus *in vivo* (6, 17). To analyze the influence of H20A, the set of 91 previously
characterized carboxy-terminal Cys substitutions was combined with the H20A substitution,
expressed to near chromosomal levels, and assayed for their ability to form triplet dimers
compared to all 91 Cys substitutions with a wild-type TMD (6).

When combined with a wild-type TonB TMD, six Cys substitutions (F125C, G186C,
F202C, W213C, Y215C and F230C) supported characteristic triplet dimer formation as seen
previously--graded intensities of the homodimers from top to bottom (e.g. TonB G186C), with
the top complex being the most abundant (17). When these Cys substitutions were combined
with H20A, the levels of triplet dimers were strongly reduced (Fig. 3-2). TonB Y163C or F180C
could support low-level formation of the triplet dimers that was visible only on long exposures and characteristic of the remaining 85 Cys substitutions (Fig. 3-2 and data not shown). The effect of the H20A mutation on all these substitutions was to further reduce the low levels of triplet dimers to undetectable levels. Taken together these data showed that the amino-terminal TMD residue H20 played an important role in homodimerization through the TonB carboxy terminus.

*Conformational constraint of TonB homodimers through disulfide bonds prevents outer membrane association*

Although no single residue in the TonB carboxy terminus is essential for function, the carboxy terminus as a whole is required for association with the outer membrane (6, 8, 10). When wild-type cells are lysed by French press and fractionated on sucrose density gradients, approximately 1/3 of the TonB is found in outer membrane fractions. Because TonB remains associated with the cytoplasmic membrane throughout its energy transduction cycle in whole cells, the TonB found in the outer membrane of fractionated cells must represent a species so tightly bound to the outer membrane that it is pulled out of the cytoplasmic membrane during the fractionation process (38). In contrast, the triplet dimers formed by TonB W213C, F202C (Y215A) and Y215C (F202A) are found associated entirely with the cytoplasmic membrane following lysis and sucrose density gradient fractionation, indicating that they were unable to interact with the outer membrane (17).

To determine if the triplet dimers associated with TonB F230C, G186C and F125C also appeared exclusively in cytoplasmic membrane fractions, strains expressing the Cys substitutions were fractionated on sucrose density gradients, and dimers were detected on immunoblots of non-reducing SDS-polyacrylamide gels (Fig. 3-3). Like the previously observed triplet dimers formed by TonB F202C, W213C and Y215C, those formed by F230C were exclusively confined to cytoplasmic membrane fractions (Fig. 3-3). When the disulfide bonds were formed through
G186C, TonB triplet dimers were similarly almost exclusively confined to the cytoplasmic membrane (Fig. 3-3).

In contrast, triplet dimers formed by F125C, which is outside the extreme carboxy terminal region where the 7 functionally important residues reside, strongly associated with outer membrane fractions, indicating that the triplet dimers retained conformation(s) necessary for interaction with the outer membrane (Fig. 3-4). These results also showed that TonB dimerization per se did not interfere with outer membrane association. TonB F125C triplet dimers associated with the outer membrane in a higher proportion than expected and gave rise to high levels of proteolytic degradation products, possibly because they were somewhat deficient in outer membrane dissociation as the energy transduction cycle (29).

Discussion

*TonB H20 plays an important role in homodimerization of the TonB carboxy terminus.*

We investigated the effect of the H20A substitution on TonB homodimerization as assayed through the ToxR transcriptional reporter system (37). This reporter system measures the ability of a membrane protein fused to the ToxR cytoplasmic domain to homodimerize. Previously it was shown that ToxR-TonB dimerizes, whereas ToxR-TonB (33-239) lacking the TonB TMD does not (34), indicating that the TMD is involved in mediating dimerization in some way. In our studies here, the H20A substitution in the TonB TMD decreased ToxR-TonB dimerization ~5-fold, the same relative degree as observed previously for ToxR-TonB (33-239) in which the TonB TMD was replaced by the ToxR TMD (34). Because H20A inactivates TonB, these results suggested that TonB homodimerization, mediated by H20, was important. This assay was unable to discriminate between homodimerization through the TMD and through the carboxy terminus.
However, the assay of disulfide-linked dimer formation was specific for the TonB carboxy terminus. The effect of TonB H20A on triplet dimer formation through all 91 carboxy terminal Cys substitutions was unanticipated. We began these studies with the hypothesis that the H20A substitution would influence dimerization in a residue-specific manner: decreasing dimerization among a subset of the Cys substitutions known to crosslink efficiently and identifying new carboxy-terminal Cys substitutions through which novel triplet dimers would efficiently form. Had this been observed, it would have suggested that H20 and the TonB TMD influenced conformational changes at its carboxy terminus without significantly inhibiting interactions with ExbB, ExbD or another TonB TMD (29).

Instead, the H20A substitution uniformly decreased the degree of triplet dimer formation amongst all the substitutions, even the 85 Cys substitutions that crosslinked only inefficiently. This result also confirmed that the triplet dimers are biologically relevant because they form most efficiently when the wild-type TonB TMD is present, as well as when ExbB/D are present (17).

*TonB homodimerization is an initial event in an energy transduction cycle*

Taken together, our results here show that TonB H20 mediates dimerization of TonB through its periplasmic domain. We previously demonstrated that ExbD forms homodimers in *vivo* through its periplasmic domain and uses virtually the same set of residues to subsequently interact with the TonB periplasmic domain (23, 41). Because we also previously knew that the H20A mutation does not interact with ExbD and thus acts early in the initial events of TonB energization (23, 25), we can now propose that TonB homodimerization as well as ExbD homodimerization occur in Stage I (Fig. 3-5). The idea of homodimers as required intermediates for subsequent formation of heterodimers has been proposed for the homodimeric yeast copper chaperone yCCS and its interaction with homodimeric superoxide dismutase, SOD1. Interaction between the two homodimers results in the activation of the heterodimeric yCCS-SOD1 complex.
(42, 43). By blocking TonB homodimerization and interaction with ExbD, the H20A mutation prevents progression of TonB to Stages II and III TonB-ExbD heterodimers (23).

The conformation of TonB determines its ability to interact with the outer membrane

Both energized and unenergized forms of TonB with wild-type carboxy termini are found in outer membrane fractions following sucrose density gradient fractionation. As an example, when TonB cannot be “energized” due to absence of ExbB/D and paralogues TolQ/R, it fractionates entirely with the outer membrane (35). Likewise if the amino terminal TonB TMD has been substituted with the first TMD of TetA protein, the inactive TetA-TonB does not crosslink to ExbB or ExbD in vivo and fractionates entirely with the outer membrane (27). In both cases, the uncleaved TMDs should theoretically retain the TonBs in the cytoplasmic membrane. The association of, for example, TetA-TonB with the outer membrane must therefore be so strong (and permanent) that the TMDs are pulled out of the cytoplasmic membrane during the fractionation process.

The fractionation of unenergized TonB with the outer membrane may reflect its capacity, when unmodulated by ExbB/D, to bind indiscriminately to a variety of outer membrane proteins. In vivo, inactive TonB variants bind to outer membrane proteins FepA, Lpp and OmpA(17, 44). However, mutations in lpp and ompA have little effect on TonB activity either separately or in combination (17, 44). In vitro, the purified TonB carboxy terminus binds to peptidoglycan, lysozyme, and TonB-gated transporters in various configurations (45-48), but it does not bind to the purified ExbD periplasmic domain (24).

In the context of wild-type TonB carboxy terminal interactions, the observation that several forms of disulfide-linked Cys substitutions in TonB could not associate with the outer membrane was noteworthy. The TonB carboxy terminal residues in the region from 150-239 that interact with ExbD are also required for TonB interaction at the outer membrane (8, 49, 50). Thus
when extreme carboxy termini were trapped as a disulfide-linked dimer, they were trapped in stage I, unable to undergo further conformational changes that would allow for proper interaction with ExbD and, then subsequently by definition, the outer membrane (Fig. 3-5). Disulfide formation through TonB F125C left the extreme carboxy terminus free to homodimerize at Stage I and assume a conformation that presumably allowed interaction with ExbD homodimers in stages II and III as a prerequisite to its observed interaction with the outer membrane.

Why did TonB H20A retain slight but detectable activity?

Despite its inactivity when expressed at chromosomal levels, the H20A mutation was not 100% effective in blocking dimerization in the disulfide crosslinking and ToxR-TonB assays, both of which represent accumulations over time rather than snapshots of activity.

One possibility for detection of H20A activity is that the only purpose of ExbB and ExbD is to shift the proportion of TonB carboxy-terminal conformations from mostly inactive to mostly active. This would mean that by itself, TonB is capable of transducing energy to the outer membrane transporters, at least once per newly synthesized TonB. Possibly by overexpressing TonB, a very low level of “one hit” activity can be detected. In support of that idea, when overexpressed in the absence of ExbB/D and paralogues TolQ/R that are required for outer membrane integrity (51), TonB supports low level sensitivity to bacteriophages ϕ80 and T1 (52).

In support of that idea, TonB H20A exhibits sensitivity to undiluted ϕ80 (~10^{11} PFU/ml) only when overexpressed (data not shown). In this case, perhaps some few H20A molecules adopt the proper conformation necessary to ultimately support transport. If so, then the amount of TonB H20A dimer detected when expressed to chromosomal levels is insufficient to ultimately promote ligand transport.

A second possibility that could explain why H20A dimerizes weakly is cross-talk. There are three different paralogues in what is called the ExbB/TolQ/MotA, ExbD/TolR/MotB family,
with MotA/B required for flagellar rotation (53). All members of the family use PMF as an
energy source. W3110 motA::Tn10 is 100-fold less sensitive to bacteriophage ph80 than W3110
metC::Tn10 (Ollis and Postle, data not shown). One interpretation of these data is that paralogues
MotA and MotB can energize TonB (and presumably promote low-level dimerization) without a
stringent requirement for histidine at position 20 in the amino acid sequence in the TonB TMD.

The oligomeric state of TonB when it interacts with a transporter in vivo is unresolved

In co-crystal structures with BtuB or FhuA, the TonB carboxy terminus is a monomer,
even though there is theoretically enough surface area on the periplasmic face of a TonB-gated
transporter to bind dimeric TonB (47, 48). Other in vitro studies suggest that the purified TonB
carboxy terminus is dimeric when it interacts with a purified TonB-gated transporter (46, 54, 55).

Recently it has been shown that the periplasmic domain of TonB (residues 33-239) binds
to TonB-gated transporters BtuB and FhuA as a dimer in vitro and, once bound, converts to a
monomer with a binding affinity of 10^-8 M (56). Such tight binding would enable TonB to be
pulled out of the cytoplasmic membrane during the fractionation process in whole cells. It may
represent TonB at a native point in its energy transduction cycle. Alternatively, since it occurs in
the absence of the TonB TMD, ExbD and ExbB, it may represent TonB that has reached a dead
end. Although binding of TonB carboxy terminal variants to TonB-gated transporters has been
repeatedly characterized, in no instance has transport through a TonB-gated transporter been
demonstrated in vitro. Thus the oligomeric state of active TonB during energy transduction has
yet to be identified.

While the ability of TonB F125C dimers to fractionate with the outer membrane suggests
that TonB interacted with the transporters as a dimer, it seems equally possible that only one
TonB F125C protein interacted with a transporter, and its disulfide-linked partner was just along
for the ride. It is important to note, however, that transporters themselves may form functionally
significant oligomers. Dimerization of Cir has been recently shown to be necessary for colicin Ia uptake, so it is possible that other TonB-gated transporters could also function as dimers (57). FepA is known to crosslink both as a dimer and a trimer in vivo (8, 58, 59), although it is not known if this multimerization is necessary for function.

Perhaps the as-yet-unknown oligomeric state of TonB during its interactions with TonB-gated transporters in vivo holds a clue to the differences between the energized and non-energized TonB contacts at the outer membrane. In any case, identification of potential differences between the two forms of TonB will be crucial to our understanding of the mechanism of energy transduction.

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Figure Legends:

**Fig. 3-1:** ToxR-TonB H20A reduces ToxR-mediated β-galactosidase activity compared to ToxR-TonB. (A) β-galactosidase assays were performed as described in Materials and Methods with 3 different subcultures of each of the following strains: *tonB*<sup>+</sup> (chromosomal TonB, FHK12/pBAD33); *tonB*<sup>+</sup> WT (FKH12/pKP1529); *tonB*<sup>+</sup> H20A (FKH12/pKP1530); Δ*tonB* WT (KP1560/pKP1529); Δ*tonB* H20A (KP1560/pKP1530). Background activity from FHK12 or KP1560 was subtracted as appropriate from each reported average. Average β-galactosidase activities were measured as follows [standard deviation (SD) in parentheses]: *tonB*<sup>+</sup> WT 100 (SD=15); *tonB*<sup>+</sup> H20A 10 (SD=5); Δ*tonB* WT 245 (SD=30); Δ*tonB* H20A 45 (SD=2). (B) Expression levels of ToxR-TonB derivatives from A) in *tonB*<sup>+</sup> (FKH12) or Δ*tonB* (KP1560). Each sample was visualized on an immunoblot of an 11% SDS-polyacrylamide gel with the anti-TonB monoclonal antibody 4F1 (63). The figure is a composite of two separate immunoblots. Abbreviations: WT = Wild Type, cTonB = chromosomal TonB from strain FHK12.

**Fig. 3-2:** TonB H20A reduces crosslinking intensity of TonB C-terminal Cys substitutions. Disulfide crosslinking was performed as described in Materials and Methods. cTonB = chromosomal TonB, W3110/pKP477. Plasmid-encoded Cys substitutions were combined with a wild-type (-) TMD (6) or H20A (+) TMD (this study) and expressed in KP1344 (W3110, Δ*tonB*) with L-arabinose to induce expression of TonB derivatives as follows: F125C (0.00045%); H20A F125C (0.0005%); Y163C (0.0004%); H20A Y163C (0.00056%); F180C (0.0005%); H20A F180C (0.00055%); G186C (0.00075%); H20A G186C (0.0008%); F202C (0.00045%); H20A F202C (0.00055%); W213C (0.00036%); H20A W213C (0.00055%); Y215C (0.00045%); H20A Y215C (0.0005%); F230C (0.0005%); H20A F230C (0.0006%). Samples were divided and resuspended in either non-reducing or reducing Laemmli sample buffer (LSB) and loaded onto separate 11% SDS-polyacrylamide gels and probed with the anti-TonB monoclonal antibody 4H4 (63). The figure is a composite of samples from two separate experiments (divided by space in
figure). In some cases, it was necessary to dilute overexpressed samples prior to loading so that the total amounts of TonB monomer in the reducing gels were approximately equal for all samples. The same proportion of each sample was loaded on both gels. Disulfide-crosslinked triplet dimers are labeled with asterisks. Abbreviations: deg. products = endogenous proteolysis degradation products.

**Fig. 3-3:** Disulfide-linked triplet dimers formed through F230C and G186C exhibit little or no association with the outer membrane. KP1344 cells expressing TonB C18G F230C (pKP570) and TonB C18G G186C (pKP612) were fractionated as described in Materials and Methods. Disulfide-linked dimers (*) were visualized on immunoblots of non-reducing SDS-polyacrylamide gels with the TonB monoclonal antibody 4F1. Abbreviations: SP = soluble proteins, CM = cytoplasmic membrane, OM = outer membrane

**Fig. 3-4:** TonB F125C dimers fractionate with the outer membrane. (A) KP1344 cells expressing TonB C18G F125C (pKP1070) were fractionated as described in Materials and Methods, and TonB F125C was visualized on immunoblots of non-reducing SDS-polyacrylamide gels with the TonB monoclonal antibody 4H4. F125C monomer, homodimers (*), and deg products are indicated on the right. An immunoblot of the same samples on a reducing SDS-polyacrylamide gel is shown beneath. (B) Because the presence of iodoacetamide in the sucrose solutions (to prevent disulfide formation during the fractionation procedure) interfered with NADH oxidase activity determinations, we evaluated the efficiency of membrane separation by immunoblots with antibodies directed against cytoplasmic membrane proteins ExbB and CorA. (C) The coomassie-stained PVDF membrane confirmed that outer membrane proteins FhuA, OmpF and OmpA localized to the outer membrane fraction. Panels A, B, and C all derived from the same fractions. Abbreviations: deg products = degradation products, SP = soluble proteins, CM = cytoplasmic membrane, OM = outer membrane
Fig. 3-5: Model for the role of TonB H20-mediated dimerization in energy transduction, adapted from Ollis and Postle (23). In stage I, TonB and ExbD each form homodimers through their periplasmic domains, but do not detectably interact with one another. H20 is important for TonB dimerization. In stage II, ExbB facilitates TonB-ExbD heterodimer formation through their periplasmic domains. Stage II not dependent on the presence of PMF (23, 64). Since TonB H20A also does not interact with ExbD at this stage (23, 25), the results suggest that TonB dimerization is a prerequisite for proper interaction with ExbD in Stage II. In the presence of the PMF, the Stage II TonB-ExbD heterodimer undergoes a conformational rearrangement such that the two periplasmic domains can now be formaldehyde crosslinked into the TonB-ExbD complex that characterizes Stage III. TonB H20A also blocks Stage III, as expected if it blocks stage II. The presence or absence of PMF acts as a toggle switch between Stage II and Stage III (23).
Table 3-1: Strains and plasmids used in this study

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**Plasmids**

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a Plasmids below the line are listed in the order of the TonB C18G H20A substitution they express (in parentheses). All plasmids are derivatives of pKP879.

b Plasmids expressing the set of 91 carboxy-terminal Cys substitutions in the presence of a wild-type TMD (pKP568 derivatives) were also used in this study and are described in (6).
Fig. 3-1, Gresock et al.
Fig. 3-2, Gresock et al.
Fig. 3-3, Gresock et al.
Fig. 3-4, Gresock et al.
I. TonB and ExbD homodimers

H20 mediates TonB dimerization
H20A reduces TonB dimerization and eliminates TonB-ExbD interaction

Constrained TonB carboxy terminus unable to interact with ExbD and OM e.g. disulfide-linked TonB F230C

II. PMF-independent TonB-ExbD heterodimer

ExbB mediates TonB/ExbD homodimer-heterodimer transition

III. PMF-dependent TonB-ExbD heterodimer

TonB-ExbD formaldehyde crosslink ★

Unconstrained TonB carboxy terminus interacts with ExbD and OM e.g. disulfide-linked TonB F125C

Fig. 3-5, Gresock et al.
CHAPTER 4
DEFINING FUNCTIONALLY SIGNIFICANT TONB-FEPA
INTERACTIONS IN ESCHERICHIA COLI USING IN VIVO
SITE-DIRECTED PHOTOCROSSLINKING

This chapter is in preparation for submission. MGG performed the work. MGG and KP designed experiments. MGG wrote the paper.
Defining functionally significant TonB-FepA interactions in *Escherichia coli* using *in vivo* site-directed photocrosslinking

Michael G. Gresock and Kathleen Postle*

Running title: *In vivo* TonB-FepA interactions

**Keywords:** TonB, FepA, cork domain, p-benzoyl-l-phenylalanine, enterochelin, TonB box, energy transduction

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Abstract

In Gram-negative bacteria, a complex of the cytoplasmic membrane proteins ExbB, ExbD, and TonB harnesses energy derived from protonmotive force to facilitate transport of important nutrients through outer membrane TonB-gated transporters. Each TonB-gated transporter consists of two domains: a 22-stranded beta barrel and a cork domain which fills the barrel lumen and contains a conserved sequence known as the TonB box. The TonB box is an established site of interaction between TonB and TonB-gated transporters, but interactions between TonB and other sites on the transporters have not been characterized in vivo. In this study, we individually substituted 26 sidechains in the FepA cork domain with the photoreactive amino acid p-benzoyl-l-phenylalanine (pBpa) and determined the ability of each substitution to crosslink to TonB. We found that the majority of crosslinkable sites were located in the amino-terminal 51 residues of FepA, and only two substitutions outside this region, E120pBpa and I145pBpa, crosslinked to TonB. The absence of enterochelin diminished crosslinking intensity at most positions in the TonB box and elsewhere. However, crosslinks formed through FepA I14pBpa, V28pBpa, and T32pBpa were enhanced in the absence of enterochelin, suggesting that TonB docks at these sites prior to ligand binding or transport. Deletion of the FepA TonB box, which inactivates the transporter, did not reduce crosslinking efficiency through FepA T32pBpa or I145pBpa. Taken together, the results suggest that TonB contacts several areas of the FepA cork domain, and the interactions are influenced by enterochelin and the presence or absence of the TonB box.
Introduction

Bacteria require iron concentrations of 10^{-6} to 10^{-8}M to support growth (Thulasiraman et al. 1998), but iron is often difficult to acquire. In oxic environments at neutral pH, iron is sequestered in insoluble ferric hydroxides and much of the free iron in mammalian hosts is chelated by proteins such as transferrin or lactoferrin. To obtain iron, bacteria secrete siderophores which bind iron with high affinities and synthesize outer membrane transporters that bind iron-siderophore complexes with subnanomolar affinity (Andrews et al. 2003). However, energy is needed to release the tightly-bound iron-siderophore complexes into the periplasm, yet the outer membrane lacks energy sources such as ATP or electrochemical gradients. For iron-siderophore transport, energy is provided by the cytoplasmic membrane protonmotive force with the aid of ExbB, ExbD, and TonB. ExbB and ExbD drive conformational changes in TonB in response to protonmotive force (Ollis et al. 2009; Jana et al. 2011; Ollis et al. 2012a), and activated TonB will directly contact outer membrane transporters (now referred to as TonB-gated transporters) to permit ligand transport.

TonB has two essential domains: a transmembrane domain (TMD, residues 12-32) that anchors the protein in the cytoplasmic membrane throughout an energy transduction cycle (Jaskula et al. 1994; Gresock et al. 2011) and a periplasmic carboxy terminus (residues 150-239) that interacts with the periplasmic domain of ExbD (Ollis et al. 2009; Ollis and Postle 2012b) and TonB-gated transporters (Larsen et al. 1997; Letain et al. 1997; Ghosh and Postle, 2004; Khursigara et al. 2004; Khursigara et al. 2005a, Khursigara et al. 2005b; Shultis et al. 2006; Pawelek et al. 2006). Three unessential
regions are located between the TMD and carboxy terminus: a proline-rich domain (residues 70-102), a predicted disordered region from residues 33-69 (Larsen et al. 2007) and a known disordered region from 103-149 (Peacock et al. 2005).

Each of the known TonB-gated transporters consists of two domains: a 22-strand antiparallel beta-barrel and an essential globular domain referred to as a cork that completely blocks the lumen of the barrel. During ligand transport, the cork is thought to either completely exit the barrel or remain inside but undergo a conformational rearrangement that allows ligand passage into the periplasm (Endriss et al. 2003; Eisenhauer et al. 2005; Ma et al. 2007; Devanathan and Postle, 2007; Smallwood et al. 2009).

The TonB box is a short, conserved sequence located near the amino terminus of the cork domain and is essential for ligand transport (Gudmundsdottir et al. 1989; Bell et al. 1990). The TonB box was confirmed as a site of interaction with TonB when cysteine substitutions in the TonB box of BtuB (Cadieux and Kadner, 1999; Cadieux et al. 2000) and FhuA (Ogierman and Braun, 2003) formed spontaneous disulfide crosslinks with TonB substitutions R158C-P164C. Recently, it has been shown that mutations in the TonB box drastically weakened the binding affinity of TonB 33-239 for BtuB as shown by fluorescence anisotropy, suggesting that the TonB box mediates high-affinity binding between TonB and the transporters (Freed et al. 2013). Despite its apparent sequence conservation, the individual sidechains comprising the TonB box are relatively tolerant to mutation (Gudmundsdottir et al. 1989). Therefore, recognition of this sequence by TonB is likely to be based on secondary structure rather than the specific identity of individual side chains (Gudmundsdottir et al. 1989; Bell et al. 1990; Vakharia-Rao et al. 2007).
vitro, binding of ligand to TonB-gated transporters has been shown to induce an order-to-disorder transition in the TonB box that increases its aqueous exposure (Merianos et al. 2000; Fanucci et al. 2003; Xu et al. 2006; Kim et al. 2007).

To monitor similar conformational changes in vivo, a cysteine substitution was engineered in the FepA TonB box (T13C) and examined for its ability to be labeled by 1-Biotinamido-4-[4'-(maleimidomethyl) cyclohexanecarboxamido]butane (BMCC) upon addition of the FepA ligand colicin B (Devanathan et al. 2007). In the presence of TonB, addition of a four-fold excess of colicin B increased the BMCC labeling efficiency of FepA T13C from ~12% to 100%, but labeling did not increase when TonB was absent. This shows that exposure of the FepA box to the periplasm is dependent on TonB, and this contrasts with previous demonstrations where addition of ligand alone increased the aqueous exposure of the BtuB TonB box (Merianos et al. 2000; Fanucci et al. 2003; Xu et al. 2006; Kim et al. 2007). It was then suggested that TonB interacted first with other areas of the transporter in order for the TonB box to become exposed to the periplasm. Devanathan et al. (2007) also showed that cysteine substitutions within the amino-terminal 51 residues of the FepA cork domain were highly susceptible to BMCC labeling, with colicin B, TonB, and the FepA TonB box required for all labeling increases. In contrast, cysteine substitutions within cork residues 91-142 were sequestered from BMCC labeling. These residues were not labeled because they either remained in the barrel or were shielded from labeling by another protein such as colicin B or TonB.

Although TonB has been shown to bind transporters with high affinity (Khursigara et al. 2004; Freed et al. 2013), specific sites of interaction outside the TonB box have not been identified in vivo. Using phage display, a TonB fragment consisting of
residues 32-239 bound to the FhuA TonB box, the short loop linking the C-terminal part of the cork domain to the beta barrel and several periplasmic turns in the beta barrel (Carter et al. 2006). A solved crystal structure of FhuA bound to TonB carboxy-terminal residues 158-235 revealed that TonB-FhuA interactions are mostly limited to the TonB box and several periplasmic barrel loops (Pawelek et al. 2006). Few contacts were observed between the TonB fragment and other sites in the cork domain. Another solved crystal structure between TonB carboxy-terminal residues 147 to 239 and BtuB also showed binding to the TonB box and barrel turns (Shultis et al. 2006). However, the in vivo relevance of the phage display and crystallography findings is unknown because the interactions were observed in the absence of the essential TonB transmembrane domain, ExbB, ExbD and protonmotive force.

Here, we replaced 26 residues in the FepA cork domain with p-benzoyl-l-phenylalanine and identified novel in vivo TonB-FepA interactions through photocrosslinking. Each of the FepA pBpa substitutions that could crosslink to TonB were located in areas that are exposed to the periplasm in the FepA crystal structure. These include substitutions within the amino-terminal 51 residues of FepA and also at positions E120 and I145. The equivalent sites to FepA E120 and I145 in the transporters BtuB and FhuA did not interact with the TonB carboxy-terminal domain in solved crystal structures (Shultis et al. 2006; Pawelek et al. 2006). The presence of enterochelin enhanced crosslinking for most substitutions in the FepA TonB box as well as A33pBpa and E120pBpa, suggesting that detected interactions formed through these sites reflect steps in the transport of ligand. Because the presence of enterochelin diminished crosslinking through I14pBpa, V28pBpa and T32pBpa, TonB binds these sites more
strongly prior to ligand reception and less so when ligand becomes available. Deletion of the TonB box reduced crosslinking through V28pBpa and E120pBpa, suggesting that these contacts formed optimally only when TonB initially recognized the TonB box. However, TonB-FepA crosslinking through T32pBpa and I145pBpa was essentially unaffected by the TonB box deletion; therefore, these interactions are not dependent on FepA function. Taken together, the results suggest that in vivo interactions between TonB and FepA are limited to regions of the transporter exposed to the periplasm and that the core beta sheet within the FepA cork domain plays a role in TonB recognition.

Materials and Methods

Strains and Plasmids

All strains and plasmids used in this study are listed in Table 1. Strain KP1487 was constructed by replacing the W3110 fepA gene with a kan^r gene by the Wanner method (Datsenko and Wanner, 2000). The kan^r gene was then excised from KP1487 with FLP-recombinase encoded from plasmid pCP20 (Datsenko and Wanner, 2000) to create KP1489.

To construct plasmid-encoded FepA derivatives, the wild-type fepA gene from pKP515 was amplified by PCR using primers with incorporated XbaI sites. Both the pPro24 vector and the PCR-amplified fepA gene were then digested with XbaI and ligated together with T4 DNA ligase to generate pKP1302. A His_6 tag was incorporated between residues 393 and 394 of the mature FepA protein to create pKP1693. Amber codons were introduced into the fepA gene in pKP1693 by site-directed mutagenesis using
primers with at least 12 base pairs on both sides of the introduced TAG sequence. TonB box deletion derivatives, lacking residues 12-16 of mature FepA, were constructed by extra-long PCR using phosphorylated primers and ligation of PCR products with T4 DNA ligase.

\textit{55}\textsuperscript{Fe}-enterochelin transport

Transport assays were performed in the enterochelin-deficient (\textit{aroB}) strain KP1490 to eliminate competition between native enterochelin and \textit{55}\textsuperscript{Fe}-enterochelin for binding to FepA. Plasmid-bearing strains expressing the FepA pBpa derivatives and orthogonal pBpa tRNA/tRNA synthetase from pEVOL-\textit{BpF} were grown in M9 minimal medium supplemented with 0.4\% glycerol, 0.2\% casamino acids, 40\mu g/ml tryptophan, 4\mu g/ml vitamin B1, 1mM MgSO\textsubscript{4}, 0.5mM CaCl\textsubscript{2} and 1.85\mu M FeCl\textsubscript{3}. Tyrosine and phenylalanine were also added to a final concentration of 40\mu g/ml each to support growth of \textit{aroB} strains which are unable to synthesize aromatic amino acids. Ampicillin (100\mu g/ml) and chloramphenicol (34\mu g/ml) were added to maintain plasmids. Cells expressing chromosomal FepA (KP1270/pPro24/pEVOL), wild type His-tagged FepA (KP1490/pKP1693/pEVOL), and no FepA (KP1490/pPro24/pEVOL) served as controls. Once strains reached an A\textsubscript{550} of 0.20, pBpa (Bachem) was added to a final concentration of 0.4mM, and sodium propionate was added to a final concentration of 2-3mM to induce expression of FepA pBpa substitutions. Cells were harvested at an A\textsubscript{550} 0.45-0.50 and assayed for \textit{55}\textsuperscript{Fe}-enterochelin uptake as described previously for \textit{55}\textsuperscript{Fe}-ferrichrome transport (Postle, 2007). Protein samples were collected by precipitation with
trichloroacetic acid (TCA), and steady-state levels of FepA were visualized on immunoblots with polyclonal anti-FepA antibodies.

**Spot titer assays**

Strain KP1490 was co-transformed with pEVOL-BpF and plasmids encoding FepA pBpa substitutions. Saturated overnight cultures were diluted 1:200 in T-broth supplemented with 34µg/ml chloramphenicol and 100µg/ml ampicillin. Once the culture reached an $A_{550}$ of 0.20, pBpa was added to a final concentration of 0.4mM. When the $A_{550}$ reached 0.40, cells were spread on T-plates containing 0.4mM pBpa, chloramphenicol and ampicillin. Five-fold dilutions of colicin B were spotted onto the T-plates and incubated overnight at 37°C.

**In vivo photocrosslinking**

Strains KP1489 or KP1490 expressing FepA pBpa derivatives were grown in supplemented M9 as described for $^{55}$Fe-enterochelin transport assays along with 34 µg/ml chloramphenicol and 100 µg/ml ampicillin to maintain plasmids. Once cells reached an $A_{550}$ of 0.20, pBpa was added to 0.4 mM to all cultures, and expression of FepA pBpa substitutions was induced by adding sodium propionate to a final concentration of 2-4mM. Cells were grown further to mid-exponential phase ($A_{550}$ of 0.50) and 1.0 $A_{550}$ ml cells (e.g. 2 ml of cells for $A_{550} = 0.50$) were removed and resuspended in supplemented M9 lacking antibiotics, pBpa and propionate. Cells were then incubated at 37°C with shaking for 20 min. to allow cells to equilibrate and were then transferred to a multi-well plate and immediately irradiated with 365nm light at a distance of 2.5 cm for 30 min. on
ice. After irradiation, proteins were harvested by TCA precipitation, resuspended in Laemmli Sample Buffer with freshly added 2-mercaptoethanol and heated for 10 min. at 95°C. TonB-FepA photocrosslinks and TonB steady-state levels were visualized on immunoblots of 9% SDS-polyacrylamide gels with the TonB monoclonal antibody 4F1. Steady-state levels of FepA were visualized on immunoblots of 9% SDS-polyacrylamide gels with polyclonal FepA antibodies.

**Results**

Unnatural amino acids such as p-benzoyl-l-phenylalanine (pBpa) can be incorporated into proteins of interest through amber codon (UAG) suppression using a plasmid-encoded orthogonal suppressor tRNA and tRNA synthetase (Chin et al. 2002; Young et al. 2010). Upon excitation with near-UV light at 365nm, pBpa can crosslink to aliphatic side chains within 3.0Å of its benzophenone group (Farrell et al. 2005). Thus, to trap an interaction between two proteins using pBpa-mediated photocrosslinking, only of one of the two proteins needs to be substituted with pBpa. This system has been used to study in vivo protein-protein interactions between membrane proteins in *E. coli* (Farrell et al. 2005; Freinkman et al. 2011; Okuda et al. 2012; Yu et al. 2013).

**Rationale for FepA pBpa incorporations**

To examine interactions between TonB and FepA, pBpa was incorporated at 26 positions in the FepA cork domain (Fig. 4-1). FepA was chosen for mutagenesis because TonB-gated transporters are relatively tolerant to mutation (Endriss et al. 2003), so FepA might be able to accommodate the relatively bulky pBpa sidechain more effectively than
TonB. Five consecutive substitutions were located in the FepA TonB box from residues 12-16 and served as crosslinking standards as known sites of FepA-TonB interaction (Cadieux and Kadner, 1999; Ogierman and Braun, 2003).

The FepA cork domain contains a four-stranded β-sheet [denoted here as β1 (residues 27-33), β2 (residues 82-87), β3 (residues 118-127) and β4 (residues 139-147)] that is a common feature among TonB-gated transporters (Buchanan et al. 1999; Chimento et al. 2005). Substitutions were generated at FepA residues 28, 29, 32, and 33 to test the interaction of TonB with strand β1, the strand that is most accessible to the periplasm in the FepA crystal structure (Fig. 4-5B). Sidechains T82 and L85 are located on opposite sides of strand β2, and L85 is part of the conserved LIDG motif whose function is unknown (Chimento et al. 2005). Substitutions were also made in place of E120 and V124 in strand β3 and V142 and I145 on strand β4.

Additional substitutions were created to test whether sites accessible or sequestered from the periplasm could crosslink to TonB (Fig. 4-1). Devanathan et al. (2007) showed that FepA substitutions A42C, S46C and T51C could be labeled by BMCC in a TonB-dependent manner, so it is possible that these sites, when replaced by pBpa, would crosslink to TonB by virtue of being accessible to the periplasmic space during ligand transport. In contrast, FepA V91C, S92C, S112C and A131C are sequestered from labeling by BMCC and thus were either not accessible to the periplasm in any circumstance or shielded from the label by another protein such as TonB (Devanathan et al. 2007). Cork domain sidechains R75 and R126 are sequestered from the periplasm in the crystal structure and form part of the conserved “lock region” that aligns the cork to the barrel wall (Chakraborty et al. 2003; Endriss et al. 2003).
Mutations at these residues interfere with ligand transport but not ligand binding (Barnard et al. 2001; Chakraborty et al. 2003), suggesting they play a role in transport and potentially contacting TonB. Y133 is a conserved sidechain in TonB-gated transporters (Chimento et al. 2005), and is positioned above the TonB box in the sequestered portion of the cork domain. FepA S63pBpa, located at the apex of the cork domain, was engineered to test if the cork domain fully exits the barrel during transport.

**FepA pBpa substitutions retain activity**

To examine whether or not the FepA pBpa substitutions retained activity, we first tested the ability of each substitution to transport $^{55}$Fe-enterochelin. Wild-type, His-tagged FepA expressed from pKP1693 was able to support full $^{55}$Fe-enterochelin uptake compared to chromosomal FepA and served as the standard for 100% uptake. A majority of the pBpa substitutions, especially those found in the amino-terminal 51 residues, were able to transport $^{55}$Fe-enterochelin between 65-100% of the pFepA (pKP1693) uptake rate, indicating that they retained essentially wild-type function (Fig. 4-2). However, the TonB box substitutions T13pBpa and V16pBpa (Fig. 4-2A) and several substitutions within FepA residues 63-145 (Fig. 4-2B) supported $^{55}$Fe-enterochelin uptake rates of less than 40% of the wild type. Substitutions R75pBpa, T82pBpa and V142pBpa were unable to transport $^{55}$Fe-enterochelin (Fig. 4-2B). Each of the FepA pBpa derivatives analyzed were expressed to roughly the same level as the pFepA (pKP1693) control.

The pBpa substitutions which showed no or weak enterochelin transport activity were then examined for sensitivity to colicin B, a more sensitive assay that is useful for detecting low levels of TonB activity (Larsen et al. 2003). Each of the inactive or nearly
inactive pBpa derivatives exhibited reduced sensitivities to colicin B compared to wild
type FepA (Table 4-S1); therefore, the pBpa substitutions did not completely abolish
transporter activity.

*TonB crosslinks to pBpa substitutions in the FepA TonB box*

The TonB box (residues 12-16) is a known site of interaction between TonB and
various TonB-gated transporters (Gudmundsdottir *et al.* 1989; Bell *et al.* 1990; Cadieux
and Kadner, 2000; Ogierman and Braun, 2003; Shultis *et al.* 2006; Pawelek *et al.* 2006).
Likewise, each pBpa substitution in the TonB box could form a UV-dependent, ~117
kDa complex with TonB when expressed to chromosomal levels (Fig. 4-3). The TonB-
FepA complex was not detected with less sensitive polyclonal anti-FepA antibodies (data
not shown). However, chromosomal and plasmid-encoded wild-type FepA both lack
pBpa substitutions and could not crosslink to TonB. Therefore, pBpa-mediated
photocrosslinking is suitable for trapping interactions between TonB and FepA. When
cells were unable to produce enterochelin, crosslinks formed through D12pBpa,
T13pBpa, V15pBpa and V16pBpa were diminished, supporting previous demonstrations
that interactions between TonB and TonB-gated transporters are enhanced in the presence
of ligand (Higgs *et al.* 2002; Cadieux and Kadner, 1999; Ogierman and Braun, 2003). In
contrast, I14pBpa was able to crosslink more strongly in the absence of enterochelin than
when enterochelin was present. Relative to the other substitutions, V15pBpa crosslinked
strongly to TonB and also maintained ~80% of the wild-type enterochelin transport rate
(Fig. 4-2), so it was chosen to serve as a crosslinking standard for future experiments.
Interestingly, a UV-dependent crosslink migrating at ~55 kDa was detected on TonB
immunoblots for all strains examined, even for those that did not contain a FepA pBpa substitution (Fig. 4-3). Therefore, the 55 kDa complex is likely formed between TonB and an unidentified protein that has pBpa incorporated at its native amber codon. The absence of enterochelin also enhanced formation of this complex, suggesting that TonB is more likely to interact with this unidentified protein when not involved in ligand transport. The identity of this complex remains unknown.

*TonB interacts with FepA pBpa substitutions in residues 28-51*

The amino-terminal 51 residues of FepA are accessible to the periplasm when colicin B, TonB, and the FepA TonB box were present (Devanathan and Postle, 2007). Residues 28-33 comprise a beta strand that is exposed to the periplasm in the FepA crystal structure (Fig. 4-4B; Buchanan *et al.* 1999). Of the pBpa substitutions generated in this strand, V28pBpa, T32pBpa, and A33pBpa were able to crosslink to TonB in the presence of enterochelin; however, crosslinking was not as strong as the TonB box V15pBpa control (Fig. 4-4A). TonB-FepA photocrosslinks formed through substitutions S29pBpa, A42pBpa, S46pBpa and T51pBpa were visible only on the longest exposures (Figs. 4-4; 4-S1) and were thus not considered to be significant. As observed with the TonB box substitutions, the absence of enterochelin influenced crosslinking through residues 28-33 in a residue-specific manner. In the absence of enterochelin, A33pBpa-TonB crosslink formation was reduced, but detection of the T32pBpa-TonB complex was significantly enhanced without ligand present (Fig. 4-4A). The V28pBpa-TonB complex was somewhat enhanced in the absence of enterochelin. To examine how inactivating mutations in FepA influenced TonB-FepA interactions, photocrosslinking was examined
in the absence of the essential TonB box. Deletion of the TonB box did not influence
formation of the T32pBpa-TonB complex but severely diminished crosslinking of TonB
to V28pBpa and A33pBpa (Fig. 4-4A). No TonB-FepA complexes were detected for the
remaining substitutions in the absence of the TonB box (Figs. 4-4; 4-S1).

*TonB makes novel contacts with FepA residues E120pBpa and I145pBpa*

Interestingly, of all the substitutions analyzed between FepA residues S63 and
I145, only E120pBpa and I145pBpa were able to crosslink to TonB (Figs. 4-5; 4-S2). Both of these pBpa substitutions also retained essentially full activity in $^{55}$Fe-enterochelin
uptake assays (Fig. 4-2). The absence of enterochelin diminished the crosslinking
efficiency of E120pBpa and I145pBpa and did not result in increased crosslinking
elsewhere (Figs. 4-5; 4-S2). Because E120pBpa and I145pBpa were the only
substitutions within residues 63-145 that could interact with TonB, the effect of a TonB
box deletion on photocrosslinking was only examined for these two sites. Deletion of the
TonB box reduced crosslinking formed through E120pBpa but increased crosslinking
through I145pBpa (Fig. 4-5).

**Discussion**

Each TonB-gated transporter features a cork domain which is important for ligand
transport (Vakharia and Postle, 2002; Braun et al. 2003). TonB is known to interact with
the cork domains of several TonB-gated transporters *in vitro* (Peacock et al. 2006;
Pawelek et al. 2006; Shultis et al. 2006) and *in vivo* (Cadieux and Kadner, 1999;
Ogierman and Braun, 2003; Devanathan et al. 2007), but surprisingly little is known
about how TonB interacts with the cork domain outside the TonB box and whether or not these contacts are functionally relevant.

In this study, we utilized the photoreactive amino acid pBpa to identify novel sites of interaction between TonB and the FepA cork domain in vivo. Each substitution in the FepA TonB box was able to crosslink to TonB. Interestingly, FepA I14pBpa retained significant enterochelin uptake activity and could crosslink to TonB whereas FepA I14P is inactive and does not crosslink to TonB in the presence of formaldehyde (Larsen et al. 1997). Two pBpa substitutions, T13pBpa and V16pBpa, retained the ability to interact with TonB despite exhibiting substantially reduced $^{55}$Fe-enterochelin transport rates. T13pBpa and V16pBpa likely altered the conformation of the TonB box in a manner that interfered with proper energy transduction. This is consistent with previous findings showing that TonB can still interact with the BtuB TonB box even when the TonB box contains an inactivating proline substitution (Cadieux and Kadner, 1999).

**FepA residues that are accessible to the periplasm crosslink to TonB**

For substitutions located outside the TonB box, there was a strong correlation between exposure to the periplasm, as predicted by the FepA crystal structure and BMCC labeling profiles, and ability to be crosslinked to TonB. The amino-terminal 51 residues become exposed to the periplasm during colicin B transport (Devanathan et al. 2007), and many of the pBpa substitutions in this region retained nearly wild-type enterochelin transport rates. TonB interacts strongly with the TonB box and less so through V28pBpa, T32pBpa and A33pBpa. Furthermore, crosslinks were only weakly detected for A42pBpa, S46pBpa and T51pBpa which are located further away from the periplasm.
(Fig. 4-4B). Out of the 14 substitutions analyzed within FepA residues 63-145, only E120pBpa and I145pBpa crosslinked to TonB, and the sidechains of E120 and I145 project downward toward the periplasmic space in the FepA crystal structure. Although the sidechains of S63, R75, L85, V91, S112, R126, A131, and Y133 also project outward from the cork domain, they are not exposed to the periplasmic space in the crystal structure and do not crosslink to TonB when substituted with pBpa. It is thus unlikely that FepA residues 91-142 were sequestered from BMCC labeling because TonB was shielding these sites from BMCC (Devanathan et al. 2007). Together, the results suggest that TonB does not interact with cork domain residues located deep within the barrels of TonB-gated transporters as was previously suggested (Barnard et al. 2001).

Functional significance of detected interactions

The presence of ligand has been known to enhance the ability of TonB to interact with TonB-gated transporters (Cadieux and Kadner, 1999; Ogierman and Braun, 2003; Higgs et al. 2002). In this study, enterochelin enhanced TonB-FepA crosslinking for some substitutions and reduced crosslinking through other positions. Sidechains V28, T32, and A33 are located in the cork β1 strand, with V28 and T32 oriented toward the periplasm and A33 projecting toward the interior of the cork (Fig. 4-4B). Since binding of TonB to V28pBpa and T32pBpa was strengthened in the absence of enterochelin, TonB likely interacts with V28 and T32 prior to ligand binding. When enterochelin binds, TonB undergoes a conformational transition that reduces interaction with V28 and T32, explaining why crosslinking was reduced for these substitutions in the presence of enterochelin. Because crosslinking through A33pBpa, on the opposite side of the same
beta strand, was enhanced in the presence of enterochelin, it is possible that TonB interacts more strongly with the other side of the cork β1 strand during ligand transport. Similarly, TonB was able to interact optimally with substitutions E120pBpa (cork strand β3) and I145pBpa (cork strand β4) when enterochelin was present. These portions of the core beta sheet may signal to TonB that ligand is bound.

The functional relevance of the detected photocrosslinks was further examined by analyzing crosslink formation in the absence of the TonB box. We hypothesized that if TonB interacted with the TonB box prior to interacting with a specific pBpa substitution elsewhere, then deletion of the TonB box would reduce if not eliminate crosslinking to the pBpa sidechain. Perhaps deletion of the TonB box also alters the structure of certain regions of the cork such that interactions with TonB are reduced. If deletion of the TonB box did not hinder crosslinking, then the crosslink likely forms in a manner independent of TonB box recognition. In the absence of the TonB box, crosslinking was strongly reduced for substitutions V28pBpa, A33pBpa and E120pBpa. This suggests that interaction with the TonB box is needed in order for TonB to bind optimally to these sidechains. Crosslinking may not have been completely eliminated in the absence of the TonB box because TonB still might transiently recognize certain accessible areas of the transporter, though the transporter will not be in the right conformation to allow transport without the TonB box.

Interestingly, a TonB box deletion did not alter crosslinking through T32pBpa and slightly increased the efficiency of the FepA I145pBpa-TonB crosslink. Given that TonB crosslinked optimally to T32pBpa in the absence of enterochelin and that the TonB box did not reduce the level of crosslinking, T32 may be a site that TonB recognizes prior to
ligand transport. The observations that the I145pBpa-TonB crosslink was enhanced by enterochelin and formed more efficiently in the absence of the TonB box also suggest that this contact is formed when TonB is “searching” for a ligand-loaded transporter. Devanathan et al. (2007) proposed that TonB initially binds areas of the cork outside the TonB box prior to maximal exposure of the TonB box to the periplasm. Of the sidechains examined in this study, T32 and I145 are the most likely candidates to be such sites of interaction.

*Comparison of findings to in vitro studies*

In several instances, our *in vivo* crosslinking results are consistent with the TonB-transporter contacts observed in the TonB-FhuA crystal structure (Pawelek *et al.* 2006) and phage display analyses (Carter *et al.* 2006). Like the crosslinking findings presented here, both structures show that the interface between TonB and the transporter cork domain is limited to areas exposed to the periplasm. In the TonB-FhuA crystal structure, most of the TonB interaction sites were located at residues in the FhuA TonB box and periplasmic barrel loops, but TonB R166 contacts the FhuA cork domain sidechain E56. A structure-based sequence alignment of TonB-gated transporters shows that FhuA E56, which aligns with FepA D34, is a conserved residue in the TEE motif of TonB-gated transporters (Chimoto *et al.* 2005). In FepA, residues T32 and A33 are also within the TEE motif and crosslink to TonB. In addition to the TonB box, phage display identified FhuA residues 153-161 as an interaction site with TonB residues 33-239 (Carter *et al.* 2006). In FhuA, these residues encompass the loop connecting the cork domain to the
beta barrel (Pawelek et al. 2006). In this study, FepA I145pBpa crosslinks to TonB, and the equivalent sidechain in FhuA, V152, is located in a beta strand adjacent to this loop.

However, there are several important differences between our in vivo photocrosslinking data and the results from the in vitro studies. In the TonB-FhuA crystal structure, only interactions with the first strand of the core beta sheet were observed, yet TonB was able to crosslink to 3 out of 4 strands comprising the core beta sheet in FepA (Pawelek et al. 2006). While phage display detected an interaction with FhuA similar to the FepA I145 contact observed here, it did not detect an interaction with FhuA E127, the equivalent residue to FepA E120. Hence, the FepA E120pBpa crosslink, which was dependent on both enterochelin and the FepA TonB box for optimum formation, represents a novel contact site between TonB and a TonB-gated transporter. Carter et al. (2006) also found that FhuA residues 143-149, located deep within the cork domain, bound TonB when fused to maltose binding protein. However, pBpa substitutions in the FepA cork domain could not trap an interaction between TonB and FepA through any site located far from the periplasmic face of the transporter.

The cork likely remains in the barrel during energy transduction

During ligand transport, the cork domain either exits the barrel or remains in the barrel and undergoes a conformational change to allow passage of ligand into the periplasm (Eisenhauer et al. 2005; Devanathan and Postle, 2007; Ma et al. 2007; Noinaj et al. 2010). In this study, we demonstrated that pBpa substitutions exposed to the periplasm were able to crosslink to TonB. In contrast, no contacts were detected for pBpa substitutions located in the interior or apex of the cork domain. If the cork indeed
exited the barrel during enterochelin transport, then the binding interface between TonB and FepA would have presumably been more extensive. Therefore, our results are consistent with the idea that the cork likely remains in the barrel but changes conformation to allow passage of enterochelin, with the extreme amino terminus of the cork domain (~51 residues for FepA) potentially being removed from the cork to open up a channel. In support of this idea, the amino-terminal 67 residues of FhuA become susceptible to cleavage by trypsin in the presence of urea (Udho et al. 2012). Electron-paramagnetic resonance showed that the amino-terminal 30 amino acids of BtuB become denatured at lower urea concentrations than cork residues which are sequestered from solvent, and that the residues refold back into the barrel when urea is removed (Flores-Jimenez and Cafiso, 2012). Molecular dynamics simulations with the TonB-BtuB crystal structure suggest that only a portion of the cork domain of BtuB becomes unfolded while TonB applies mechanical force during ligand transport (Gumbart et al. 2007). It is important to note, however, that the molecular mass of enterochelin is 716 Da (Kaserer et al. 2008) while colicin B is 55kDa (Hilsenbeck et al. 2004), so ligand size may ultimately determine whether or not the cork remains in the barrel or exits the barrel completely during transport.

This study identified several areas of interaction between TonB and FepA outside the TonB box. Our in vivo results extend the in vitro analyses by analyzing the effects of enterochelin and the presence/absence of the TonB box on crosslinking. It will be important to identify the TonB residues involved in forming the TonB-FepA photocrosslinks. As TonB is thought to interact with each TonB-gated transporter in a slightly different manner (Ghosh and Postle, 2004), it will also be important to determine
if similar crosslinking patterns are observed between TonB and other TonB-gated transporters.

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**Figure Legends**

**Fig. 4-1.** *Locations of FepA pBpa substitutions used for TonB-FepA photocrosslinking analysis mapped onto the FepA crystal structure.* Swiss-Pdb viewer (Guex and Pietsch, 1997) was used to create images of the FepA crystal structure (Protein Data Bank: 1fep).

A) Positions of pBpa substitutions generated within the amino-terminal 51 residues of FepA. This region has been shown to become accessible to the thiol-specific probe BMCC *in vivo* during transport of colicin B (Devanathan et al. 2007). The FepA beta barrel is colored black. The polypeptide backbone for cork domain residues is shown as a yellow space-filling model, and the positions of the diagnostic pBpa substitutions are colored red. The periplasm corresponds to the area underneath the beta barrel. B) Positions of pBpa substitutions generated within FepA residues 63-145. The FepA beta barrel and cork polypeptide backbone is presented in the same manner as A), but the polypeptide backbone residues are now colored blue to reflect the observation that many of these sidechains are sequestered from BMCC when substituted with cysteine (Devanathan et al. 2007). Positions 85, 91, 92, and 133 were not visible (NV) when presented as shown in B), but approximate positions are labeled.

**Fig. 4-2.** *55Fe-enterochelin transport rates for FepA pBpa substitutions.* A) Initial rates of transport and FepA expression levels for D12pBpa-T51pBpa and B) Initial rates of transport and FepA expression levels for S63pBpa-I145pBpa. Substitutions were expressed in strain KP1490 (W3110 ∆fepA aroB) to approximately the same levels as uninduced plasmid-encoded wild-type FepA [KP1490/pFepA (pKP1693)/pEVOL-BpF]. Strain KP1270 (W3110 aroB) transformed with pEVOL-BpF (cFepA/pEVOL) and ∆FepA/pEVOL served as uptake controls. Initial rates of *55Fe-enterochelin transport*
were determined as described in Materials and Methods. At an A550 of 0.20 (early exponential phase), all samples were treated with 0.4mM pBpa, and expression of pBpa substitutions was induced by the addition of sodium propionate. Expression of T13pBpa, V16pBpa and R75pBpa required 3mM sodium propionate, and all other substitutions were induced with 2mM sodium propionate. Samples were processed in triplicate, and linear regression was used to determine initial enterochelin uptake rates. Resulting slopes were normalized to the rate observed for pFepA/pEVOL. Average normalized activities of at least two independent experiments are shown. This figure is a composite of several separate experiments.

**Fig. 4-3.** *pBpa substitutions in the FepA TonB box crosslink to TonB in vivo.* FepA pBpa substitutions were expressed in the enterochelin-producing strain KP1489 (Fe-Ent +) or the enterochelin-deficient strain KP1490 (Fe-Ent -). Substitutions were induced with the same concentrations of sodium propionate in both KP1489 and KP1490 as follows: D12pBpa (2mM), T13pBpa (3mM), I14pBpa (2mM), V15pBpa (2mM), and V16pBpa (3mM). Strain W3110/pPro24/pEVOL expresses FepA from the chromosome (CHR). Plasmid-encoded wild-type FepA (WT) is KP1489/pKP1693/pEVOL and was not expressed with sodium propionate. Photocrosslinking was performed as described in Materials and Methods, and samples were either exposed to UV (+) or not (-). Proteins were precipitated with TCA, and TonB-FepA crosslinks were visualized on immunoblots of 9% SDS-polyacrylamide gels with anti-TonB antibodies. Membranes were re-probed with anti-FepA antibodies to determine steady-state levels of FepA. Positions of the FepA pBpa substitutions are indicated at the top. Positions of the TonB and FepA
monomer, TonB-FepA photocrosslink, and the TonB-unknown protein photocrosslink are indicated on the right-hand side.

**Fig. 4-4.** *FepA pBpa substitutions in residues 28-33 crosslink to TonB in vivo.* A) Strains expressing FepA pBpa substitutions in the presence (+) or the absence (-) of ferric-enterochelin (Fe-Ent) or the TonB box were treated with UV (+) as described in Materials and Methods. During early exponential phase, pBpa was added to all strains at a final concentration of 0.4mM. All pBpa substitutions expressed in the presence of a wild-type TonB box were induced with 2mM sodium propionate, but substitutions combined with a TonB box deletion were induced with 4mM sodium propionate. Crosslinks were observed as higher-molecular weight complexes on TonB immunoblots of 9% SDS-polyacrylamide gels. Steady-state levels of TonB and FepA (re-probe of TonB immunoblot) are shown underneath. Positions of photocrosslinked complexes and TonB and FepA monomer are shown on the right, and the identity of each pBpa substitution is listed at the top. B) Position of residues 28-33 in the crystal structure of FepA (Protein Data Bank: 1fep) relative to the TonB box and residues A42, S46 and T51. This region of FepA becomes exposed to the periplasm in the presence of colicin B and TonB (Devanathan *et al.* 2007). The image was created with Swiss-Pdb viewer (Guex and Pietsch, 1997). Residues 28-33 occupy a beta strand exposed to the periplasm. Sidechains of V28 and T32 are colored green and are oriented toward the periplasm. Sidechains S29 and A33 are colored blue and are oriented toward the interior of the cork. The TonB box is colored red, and the sidechains A42, S46, and T51 are colored black. The position of the periplasm is also indicated.
Fig. 4-5. *FepA* E120pBpa and I145pBpa crosslink to *TonB* in vivo. A) Photocrosslinking was performed as described in Materials and Methods. Strains expressing pBpa substitutions were analyzed for photocrosslink formation as described in the legend to Fig. 4-4A. Sodium propionate was used to express the FepA pBpa substitutions as follows: 2mM for all substitutions in the presence or absence of enterochelin as long as the TonB box was present and 4mM when the TonB box was deleted. As before, proteins were precipitated with TCA, and TonB-FepA crosslinks and steady-state levels of TonB and FepA were visualized on immunoblots with anti-TonB and anti-FepA antibodies. Positions of monomeric TonB and FepA as well as crosslinked species are indicated. B) Positions of cork domain sidechains E120 and I145 in the crystal structure of FepA (PDB 1fep; Buchanan *et al.* 1999). E120 is colored blue and is located on the cork β3 strand, and I145 is colored green and is located on the cork β4 strand. The sidechains of both E120 and I145 are oriented toward the periplasm. The TonB box is colored red. The image was generated with Swiss-Pdb viewer (Guex and Pietsch, 1997).

**Fig. 4-S1:** *FepA* pBpa substitutions A42pBpa, S46pBpa and T51pBpa do not crosslink to *TonB*. Photocrosslinking was performed as described in Materials and Methods. Strains expressing the pBpa substitutions were induced with 2mM sodium propionate in the presence of the TonB box (regardless of Fe-Ent status) or with 4mM sodium propionate when the TonB box was absent. Crosslinks and steady-state levels of FepA and TonB were visualized on immunoblots with anti-FepA or anti-TonB antibodies.

**Fig. 4-S2:** *FepA* pBpa substitutions within *FepA* cork domain residues 63-112 do not crosslink to *TonB*. Strains expressing FepA pBpa substitutions in the presence or absence
of enterochelin were induced with 2mM sodium propionate. Steady-state levels of FepA and TonB were visualized with anti-FepA and anti-TonB antibodies, respectively. The position of the TonB-unknown complex is indicated.
Table 4-1: Strains and Plasmids Used in This Study

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<td>Life Technologies</td>
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</tr>
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<td>KP1490</td>
<td>W3110 ΔfepA ar0B</td>
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<table>
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<td>pCP20</td>
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<td>pKD4</td>
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<td>Wild-type FepA with internal His6 tag between FepA residues 393&amp;394; pKP1302 derivative</td>
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All plasmids listed below encode FepA amber codons (AM) and are derivatives of pKP1693

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**Table 4-S1.** FepA pBpa substitutions compromised with respect to $^{55}\text{Fe}$-enterochelin uptake retain the ability to transport colicin B.

<table>
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<td>142</td>
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</table>

$^a$ Spot titre assays were performed as described in Materials and Methods. Scores indicate the last 5-fold dilution at which sensitivity to colicin B was evident on a cell lawn. The results were the same for two separate experiments. cFepA refers to chromosomal FepA expressed in the enterochelin-deficient strain KP1270 (W3110 aroB).
Fig. 4-1, Gresock and Postle
Fig. 4-2, Gresock and Postle
Fig. 4-3, Gresock and Postle
Fig. 4-4, Gresock and Postle
A.

Fig. 4-5, Gresock and Postle

B.

Fig. 4-5, Gresock and Postle
Fig. 4-S1, Gresock and Postle
Fig. 4-S2, Gresock and Postle
CHAPTER 5

DISCUSSION
Chapter 5

Discussion

The TonB system is found in virtually all Gram-negative bacteria and is involved in high-affinity transport of iron and other important compounds. Although the role of TonB in iron uptake is well established, several underlying mechanisms by which TonB promotes ligand uptake at the outer membrane are less understood. First, although the TonB TMD is essential for activity, it was unclear as to whether or not the TMD always remained attached to a cytoplasmic membrane complex of ExbB and ExbD during energy transduction (mechanical model) or disengaged from the cytoplasmic membrane to deliver energy to TonB-gated transporters (shuttle model). Second, full-length TonB was known to dimerize in vivo (Sauter et al. 2003; Ghosh and Postle, 2005), but the role of H2O in influencing TonB dimerization and the relationship of TonB dimerization to ExbD-TonB interactions was unknown. Finally, regions of the FepA cork domain outside the essential TonB box were known to interact with TonB in vivo (Devanathan et al. 2007), but the precise sites of interaction as well as their functional significance was unknown. Collectively, this work utilized in vivo methodologies to provide new insights into each of these aforementioned mechanisms and strengthen understanding of TonB-dependent energy transduction in Escherichia coli.

Models for TonB function

TonB was once believed to shuttle because a cysteine substitution located in the cytoplasmic tail of full-length TonB was labeled by the “membrane-impermeable” probe
Oregon Green maleimide whereas the same site was not labeled in an inactive, truncated TonB that could not associate with the outer membrane (Larsen et al. 2003). The results presented in Chapter 2 of this study show that a fusion of the ToxR cytoplasmic domain to full-length TonB, ToxR-TonB, is proteolytically stable and can support full $^{55}$Fe-ferrichrome transport activity compared to wild-type TonB. Because ToxR-TonB retained activity and the ToxR domain (181 residues) would be unlikely to be pulled out of the cytoplasm and properly reinserted, TonB does not shuttle during energy transduction. Because Chapter 2 showed that Oregon Green maleimide is permeable to the cytoplasmic membrane, the labeling observed for the TonB amino-terminal cysteine substitution in Larsen et al. (2003) occurred in the cytoplasm and not in the periplasm. The cysteine substitution was likely not labeled in a truncated TonB derivative missing its carboxy-terminal domain becauseTonB would be unable to receive signals from the outer membrane or ExbD in the absence of the carboxy terminus. Consequently, an inability to receive signals perhaps maintains TonB residues 1-11 in a conformation that prevents labeling of L3C. Now that the shuttle model is removed from consideration, there are three remaining mechanical models that can explain TonB function: propeller, periplasmic-binding protein, and pulling (Krewulak and Vogel, 2011).

*The Propeller Model.* The propeller model was originally conceived by Chang et al. (2001) upon solving the crystal structure of TonB 164-239 and noting structural and functional similarities between ExbB/ExbD and their paralogues MotA/MotB of the flagellar motor. While MotA and MotB convert energy from protonmotive force (PMF) into mechanical torque (Braun et al. 1999; Zhai et al. 2003), they do so by remaining stationed in ~11 complexes around the periphery of the flagellar base-plate that rotates
with respect to them. Rotation of MotA and MotB together or with respect to one another has never been described. Hence the idea that ExbB and ExbD would similarly harness PMF and promote rotation of dimeric TonB seems based on a misinterpretation of the function of MotA/B. Chang et al. suggested that, acting as a propeller, the rotating dimeric TonB would interact with the TonB box of the transporters to allow ligand transport. A recent study showed that a GFP-TonB fusion exhibited increased anisotropy, or less molecular motion, in the presence of protonophores compared to when PMF was present, suggesting that PMF drives conformational changes in TonB (Jordan et al. 2013). The observed motion of GFP-TonB occurred in a time frame consistent with rotation as opposed to other types of conformational changes and was thus interpreted by the authors as representing rotation of TonB.

The propeller model also has other drawbacks. First, a peptidoglycan-binding motif enables MotB to serve as an appropriate stator, but this motif is absent in ExbD (Ollis et al. 2012). With ExbD unable to serve as a stator, ExbB, ExbD, and TonB would likely rotate together as a complex, unless another protein served as a stator for ExbB and ExbD. Another problem is that the dimeric TonB structure solved by Chang et al. (2001) does not represent a dimeric conformation that the carboxy-terminal domain achieves in vivo (Ghosh and Postle, 2005; Appendix A).

The Periplasmic Binding Protein Model. The periplasmic binding protein (PBP) model suggests that TonB interacts with periplasmic binding proteins and positions them so that they can receive incoming ligands through TonB-gated transporters (Carter et al. 2006b; James et al. 2009; Krewulak and Vogel, 2011). The TonB periplasmic domain interacts with peptides corresponding to the PBPs FhuD (Carter et al. 2006b) and BtuF
(James et al. 2009) in phage display experiments, and FhuD has been shown to associate with a complex of TonB and FhuA in surface plasmon resonance analyses (Carter et al. 2006b). If TonB interacts with PBPs, then it may be possible that TonB also directs the PBP to an inner membrane ABC transporter complex for transport of the ligand across the cytoplasmic membrane (Carter et al. 2006b; Krewulak and Vogel, 2011). However, PBPs have not been shown to interact with TonB or TonB-gated transporters \textit{in vivo}. TonB does not crosslink to FepB, the PBP for enterochelin, in the presence of formaldehyde (Higgs et al. 2002), and \textit{in vivo} photocrosslinking did not capture complexes between FepA pBpa substitutions and FepB (Chapter 4 and data not shown).

\textit{The Pulling Model}. Of the three mechanical models, the pulling model is the most consistent with the results from \textit{in vivo} analyses, but conclusive \textit{in vivo} evidence for this model is also lacking. Based on molecular dynamics simulations, the pulling model suggests that TonB interacts with the TonB box and applies a force perpendicular to the 4-stranded beta sheet in the cork domain (Chimento et al. 2005; Gumbart et al. 2007). As a result, the cork domain will become partially unfolded, creating a channel for ligand transport. When the BtuB cork domain became unfolded enough to form such a channel (i.e. \sim 200 \AA of extension), the first beta strand ($\beta_1$) of the BtuB cork domain was the only strand comprising the 4-stranded beta sheet that became accessible to the periplasm (Gumbart et al. 2007). This is consistent with the \textit{in vivo} BMCC labeling profile of cysteine substitutions within the FepA cork domain upon binding of colicin B: sites within FepA strand $\beta_1$ (residues 28-33) were susceptible to labeling in the periplasm whereas sites located deep into the cork were sequestered from BMCC (Devanathan et al. 2007). However, the pulling model does not adequately address how interactions
between TonB and residues in TonB-gated transporters outside the TonB box may influence conformational changes in the cork domain.

**Relationship of TonB dimerization to energy transduction cycle**

The TonB carboxy-terminal domain dimerizes both *in vitro* (Chang *et al.* 2001; Kodding *et al.* 2005) and *in vivo* (Sauter *et al.* 2003; Ghosh and Postle, 2005). TonB TMD sidechain H20 is known to be important for activity, yet its potential role as part of a proton pathway was recently ruled out (Swayne and Postle, 2011). To further investigate the functional relevance of dimerization *in vivo* and also gain insight into the role of H20, this work analyzed how an inactivating H20A substitution affected dimerization. As described in Chapter 3, an inactivating TonB H20A substitution significantly reduced dimerization of a ToxR-TonB fusion and universally reduced the levels of disulfide-linked dimers formed through TonB residues 125 and 150-239. This was important because it suggested that dimerization is a functionally relevant step in the energy transduction cycle and that H20 plays a role in dimer assembly. It is unlikely that H20 directly mediates TonB homodimerization but rather is responsible for correct fit of TonB with ExbB or ExbD such that homodimerization is achieved.

This work also suggests how TonB dimerization fits into the “bigger picture” of ExbD-TonB interactions (Fig. 5-1). ExbD forms homodimers before formation of TonB-ExbD heterodimers (Ollis and Postle, 2011). Likewise, it is possible that TonB homodimers could form prior to formation of TonB-ExbD heterodimers. Because H20A eliminates detectable interaction between TonB and ExbD (Ollis *et al.* 2009; Ollis and
Postle, 2012) as well as reduces TonB dimerization (Chapter 3), it is likely that TonB dimerizes in Stage I, prior to the formation of the TonB-ExbD heterodimer in Stage II (Figs. 3-5, 5-1). To more definitively test the idea that TonB dimerizes during Stage I, before TonB associates with ExbD in stage II, dimer formation could be assayed in the presence of the inactivating ExbD substitution D25N. ExbD D25 is a conserved TMD sidechain that is thought to participate on a proton pathway (Zhai et al. 2003; Baker and Postle, 2013) but does not inhibit TonB progression to Stage II. If TonB dimerization occurs in Stage I, prior to interaction with ExbD, then the presence of ExbD D25N will likely not have an impact on the ability of TonB to dimerize. However, if TonB dimerization is either enhanced or reduced in the presence of ExbD D25N, it suggests that TonB dimerization is not necessarily limited to Stage I and that TonB can exist as a dimer in Stage II.

The precise molecular mechanism by which TonB H20 influences dimerization through the carboxy-terminal domain remains unclear, but it is conceivable that H20 may influence dimerization of TonB through assembly/fit with another TonB TMD, as histidines and similar sidechains such as asparagine have been shown to mediate TMD dimerization in the context of an oligo-leucine TMD (Herrmann et al. 2009). To determine how the status of H20 directly influences dimerization through the TonB TMD, cysteines could be engineered in the TonB TMD and assayed for disulfide crosslinking in the context of wild type and H20A TMDs. Addition of an oxidative catalyst such as iodine would be necessary to detect dimer formation within the cytoplasmic membrane. It would also be interesting to see if substitutions on the same face of the TMD helix as H20 could form disulfide-linked dimers whereas those on the
opposite face would not. Such a result would reinforce the idea that H20 is part of a TMD dimerization interface. If H20A reduces or abolishes crosslinking formed through the TMD cysteine substitutions, then it would suggest that the observed dimerization is functionally relevant. Moreover, since H20A also reduces crosslinking through the TonB carboxy-terminal domain (Chapter 3), reductions in TMD dimerization might prevent subsequent dimerization through the TonB carboxy terminus. If TMD dimerization is unaffected by the presence of H20A, then it would suggest that H20 does not play a role in directly mediating TMD-TMD interactions. Such a result would also suggest that TonB H20A reduces dimerization through the TonB periplasmic domain via an indirect mechanism (i.e. possibly altering the ability of TonB to interact with ExbD).

TonB residues 103-149 are disordered in the monomeric NMR structure of the TonB periplasmic domain (Peacock et al. 2005). In contrast, shorter TonB carboxy-terminal fragments consisting of only residues 148-239 or 164-239 crystallize as dimers (Chang et al. 2001; Kodding et al. 2005), raising the possibility that residues 103-149 do not dimerize and, in fact, may prevent dimerization of residues 150-239. However, this study (Chapter 3) shows that a cysteine substitution located within this region, F125C, formed disulfide-linked dimers and that crosslinking was diminished in the presence of TonB H20A, indicating that the dimers are functionally relevant. Moreover, the dimers formed through F125C could associate with the outer membrane whereas dimers formed through cysteines in residues 186-230 only fractionated with the cytoplasmic membrane. This suggests that dimerization through residues 103-149 does not prevent TonB residues 186-230 from undergoing conformational changes that permit interaction with ExbD, TonB-gated transporters, or other outer membrane proteins. Cysteine substitutions have
not been engineered at other locations within this disordered region other than F125C, so it is possible that some sites may form disulfide-linked dimers more efficiently than F125C. If a given cysteine substitution can form nearly 100% dimer, then it may be possible to determine if the crosslinked TonB can support full activity in ferrichrome or enterochelin transport assays. If the tether does not inhibit TonB activity compared to wild-type TonB, then it may suggest that TonB is functional as a dimer.

*In vivo characterization of TonB-FepA interactions*

Both active and inactive forms of TonB interact with FepA, but, prior to this work, the manner in which active TonB contacts TonB-gated transporters at regions outside the TonB box *in vivo* was unknown. Although the TonB box is an important site of contact between TonB and TonB-gated transporters (Gudmundsdottir *et al.* 1989; Cadieux and Kadner, 1999), the results in this study suggest that TonB also contacts sidechains within the FepA cork domain’s 4-stranded beta sheet (β1-β4). These can be considered novel interactions because TonB fragments did not interact extensively with this beta sheet in the solved TonB-transporter crystal structures with BtuB (Shultis *et al.* 2006) and FhuA (Pawelek *et al.* 2006). Although phage panning identified an interaction between the TonB periplasmic domain and FhuA residues 146-149, which form part of the FhuA β4 strand, other interactions between TonB and the 4-stranded beta sheet were not detected (Carter *et al.* 2006a).

It will be important to ultimately identify which regions in TonB are trapped in the TonB-FepA photocrosslinks. Currently, only TonB residues R158-R166 are the only sidechains in the periplasmic domain that have been demonstrated to interact with TonB-
gated transporters in vivo (Cadieux and Kadner, 1999; Cadieux et al. 2000; Ogierman and Braun, 2003). Other candidates for transporter recognition include six aromatic sidechains (Y163, F180, F202, W213, Y215 and F230) because they, along with G186, are the only sidechains within periplasmic residues 150-239 that reduce TonB-dependent transport of siderophores, colicins, or bacteriophage when individually substituted with alanine or cysteine (Ghosh and Postle, 2004; Ghosh and Postle, 2005; Postle et al. 2010). Sidechains F202, W213 and Y215 are located within a putative amphipathic helix (residues 199-216) that may be important for recognizing TonB-gated transporters (Larsen et al. 1997).

To identify the TonB residues trapped in the photocrosslinked TonB-FepA complexes, the complexes could be purified through nickel-affinity chromatography and analyzed by mass spectrometry. In Chapter 4, each of the FepA pBpa substitutions has an internal His6 tag fused between amino acids 393 and 394 of the mature FepA protein to aid in subsequent purification. It will be especially important to determine the identity of TonB residues in the FepA E120pBpa-TonB crosslink because its formation is dependent on both enterochelin and the TonB box, and it was the most intense crosslink detected outside the TonB box. Complexes formed between FepA TonB box pBpa substitutions and TonB could serve as useful controls since TonB Q160C is known to crosslink to cysteines in the BtuB and FecA TonB boxes (Cadieux and Kadner, 1999; Ogierman and Braun, 2003). Another method to identify crosslinking sites in TonB would be to introduce cysteine substitutions in both TonB and FepA and determine which sites can form disulfide-linked dimers. Preliminary data suggest that cysteine substitutions such as TonB Q160C and within the TonB amphipathic helix (residues 199-
crosslink to cysteine substitutions within the amino-terminal 51 residues of FepA but can only crosslink inefficiently to other areas in the cork domain (Kho and Gresock, unpublished). Therefore, both *in vivo* photocrosslinking and disulfide crosslinking have identified the amino-terminal 51 residues of FepA as being important for interaction with TonB.

To further explore the functional relevance of the detected TonB-FepA photocrosslinks, crosslinking could be examined in the presence of the inactivating TonB H20A or ExbD D25N substitutions. It was previously shown that, in the absence of ligand, ExbD D25N did not significantly impact crosslinking of full-length TonB Q160C, Q162C or Y163C to cysteine substitutions in the TonB box of FecA (Ogierman and Braun, 2003). However, the steady-state levels of TonB, FecA, and ExbD were not shown, so it is unclear if overexpression of one or more TonB system proteins distorted the crosslinking profile. The FecA ligand sodium citrate was not added when crosslinking was performed, so the impact of ligand on crosslinking was also unknown. If detection of a ligand-dependent TonB-FepA crosslink is reduced or abolished in the presence of either TonB H20A or ExbD D25N, then it suggests that the contact requires active TonB for optimal formation. If the inactivating substitutions do not affect the level of TonB-FepA crosslinking through a given site, then it would mean that the contact is not essential for ligand transport.

It remains unclear if the cork domain remains within the barrel or exits the barrel completely during ligand transport of ferric-enterochelin. In Chapter 4, no TonB-FepA contacts were detected for pBpa substitutions sequestered from the periplasm, consistent with the idea that the cork remains inside the barrel. Alternatively, the entire cork
domain could be expelled from the barrel during enterochelin transport, but some pBpa substitutions may have been too far away to crosslink to TonB. It would be interesting to determine how addition of a larger ligand, such as colicin B, would change the crosslinking profile compared to enterochelin. Perhaps addition of colicin B would expel the cork into the periplasm and facilitate detection of new TonB-FepA contacts not seen with enterochelin. To obtain the most relevant results, a derivative of colicin B lacking its carboxy-terminal killing domain should be used; otherwise, full-length colicin B will kill cells and disrupt homeostasis. Another way to examine the accessibility of the cork domain is to utilize a derivative of colicin B that can bind to FepA but not translocate through it, such as colicin B ΔTonB box (Devanathan et al. 2007). Binding of colicin B ΔTonB box to FepA would maintain the transporter in a ligand-bound state that would not be able to undergo further conformational transitions related to ligand transport. Photocrosslinking could then be performed to identify regions that TonB contacts during the early stages of ligand transport.

The ultimate goal for understanding TonB-dependent energy transduction is to design an antibiotic that can inhibit the TonB system and render pathogens unable to acquire iron from their hosts. The results in this study, namely the identification of novel TonB-FepA contacts outside the TonB box, may provide new target sites for small-molecule inhibitors that can inhibit TonB system function. A recent study utilized high-throughput screening to identify compounds that appeared to abrogate TonB function, though the molecular targets of these compounds were not identified (Yep et al. 2014). Perhaps the compounds target residues that form the TonB-FepA interactions identified in this study. The effectiveness of any compound disrupting TonB-FepA interactions
would be enhanced if TonB interacts with other transporters through similar locations, expanding the repertoire of transporters that could be targeted by the compound.
References


Figure Legend

Fig. 5-1:  *Summary of in vivo protein-protein interactions characterized in this study.*

Because TonB H20A eliminates interaction with ExbD (Stage I of Fig. 3-5) and reduces TonB dimerization, TonB dimerization represents an early stage in TonB energy transduction. Guided by ExbB and protonmotive force (PMF), TonB homodimers and ExbD homodimers interact and ultimately form PMF-dependent TonB-ExbD heterodimers. In this conformation, the periplasmic domain of ExbD regulates the conformation of the TonB carboxy-terminal domain so that TonB can interact optimally with ligand-loaded TonB-gated transporters such as FepA (Buchanan *et al.* 1999) without detaching from a cytoplasmic membrane complex of ExbB/D (Chapter 2). For the enterochelin transporter FepA, TonB bound most strongly to pBpa substitutions located within the TonB box, but significant contacts were also observed through other periplasm-exposed regions of the cork domain (Chapter 4). Several of these contacts were dependent on enterochelin and the TonB box for optimal formation, indicating that they are functionally relevant.
Fig. 5-1

TonB homodimers

+ExbB
+ExbD homodimers
+PMF

TonB H20A reduces dimerization
Dimers need to be resolved for OM association to occur

Formation of ExbD-TonB heterodimers
TonB does not shuttle
APPENDIX

The TonB dimeric crystal structures do not exist in vivo

This appendix was published in mBio and is reprinted with permission from the RightsLink® Copyright Clearance Center. MGG contributed several findings to this work. First, MGG examined disulfide crosslinking for TonB cysteine substitutions L229C-N233C in the presence and absence of enterochelin (Fig. A-1). This experiment showed that the disulfide crosslinking patterns observed in vivo do not match predictions based on the crystal structures of the TonB carboxy-terminal domain. Moreover, MGG verified that double alanine substitutions of aromatic residues in the TonB carboxy-terminal domain localized properly (Fig. A-S2) and that some of the inactive double alanine substitutions could form a PMF-dependent formaldehyde crosslink with ExbD (Fig. A-S3). Furthermore, MGG assisted KP with editing the manuscript. KP directed research and wrote the paper. Experimental contributions of other authors: KAK characterized TonB carboxy-terminal cysteine substitutions through disulfide-crosslinking, spot titre and ferrichrome transport assays. JG constructed many of the TonB carboxy-terminal cysteine substitutions and conducted disulfide crosslinking analyses. CS performed spot titres for TonB double alanine substitutions.
The TonB Dimeric Crystal Structures Do Not Exist *In Vivo*.

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Running title: TonB crystal structure does not exist in vivo.

Keywords: TonB, iron transport, membrane protein, crystal structure, cysteine scanning

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Abstract:

The TonB system energizes transport of nutrients across the outer membrane of *Escherichia coli* using cytoplasmic membrane proton motive force (PMF) for energy. Integral cytoplasmic membrane proteins ExbB and ExbD appear to harvest PMF and transduce it to TonB. The carboxy terminus of TonB then physically interacts with outer membrane transporters to allow translocation of ligands into the periplasmic space. The structure of the TonB carboxy terminus (residues ~150 to 239) has been solved several times with similar results. Our previous results hinted that *in vitro* structures might not mimic the dimeric conformations that characterize TonB *in vivo*. To test structural predictions and to identify irreplaceable residues, the entire carboxy terminus of TonB was scanned with Cys substitutions. TonB I232C and N233C, predicted to efficiently form disulfide-linked dimers in the crystal structures, did not do so. In contrast, Cys substitutions positioned at large distances from one another in the crystal structures efficiently formed dimers. Cys scanning identified seven functionally important residues. However, no single residue was irreplaceable. The phenotypes conferred by changes of the seven residues depended on both the specific assay used and the residue substituted. All seven residues were synergistic with one another. The buried nature of the residues in the structures was also inconsistent with these properties. Taken together, these results indicate that the solved dimeric crystal structures of TonB do not exist. The most likely explanation for the aberrant structures is that they were obtained in the absence of the TonB transmembrane domain, ExbB, ExbD, and/or the PMF.
Importance:

The TonB system of Gram-negative bacteria is an attractive target for development of novel antibiotics because of its importance in iron acquisition and virulence. Logically, therefore, the structure of TonB must be accurately understood. TonB functions as a dimer \textit{in vivo}, and two different but similar crystal structures of the dimeric carboxy-terminal ~90 amino acids gave rise to mechanistic models. Here we demonstrate that the crystal structures, and therefore the models based on them, are not biologically relevant. The idiosyncratic phenotypes conferred by substitutions at the only seven functionally important residues in the carboxy terminus suggest that similar to interaction of cytochromes P450 with numerous substrates, these residues allow TonB to differentially interact with different outer membrane transporters. Taken together, data suggest that TonB is maintained poised between order and disorder by ExbB, ExbD, and the proton motive force (PMF) before energy transduction to the outer membrane transporters.

Introduction

The outer membrane (OM) of Gram-negative \textit{Escherichia coli} is a diffusion barrier to the transport of nutrients greater than 600 Da through porin proteins. This common limitation is circumvented by the TonB system, which is present in virtually all Gram-negative bacteria (1). A TonB system includes a characteristic OM active transporter specific for one transport substrate or a few transport substrates (22-stranded $\beta$-barrel with N-terminal cork that fills the lumen, known as a TonB-gated transporter)
and three integral cytoplasmic proteins, ExbB, ExbD, and TonB. ExbB and ExbD appear to harvest the proton motive force (PMF) of the cytoplasmic membrane, which is then transmitted to TonB. In turn, the energized TonB physically interacts with TonB-gated transporters. TonB is capable of interaction with multiple OM transporters to enable transport of diverse substrates across the OM. For example, *Xanthomonas campestris* has over 70 predicted TonB-gated transporters and six sets of *tonB-exbB-exbD* genes. In addition, TonB-dependent transport of maltodextrins, nickel, sucrose, and potentially sulfate has been characterized (2–6). Thus, iron and vitamin B₁₂ acquisition may represent only the “tip of the iceberg” (7, 8). Clues about the role of a given TonB system can arise from understanding the regulation of each *tonB* gene. In *E. coli*, *tonB* expression is regulated by iron availability (9).

In *E. coli*, the 239-residue cytoplasmic membrane protein TonB consists of an amino-terminal signal anchor with a single important residue, His20. The bulk of TonB occupies the periplasmic space (10). The topology of the 141-residue ExbD protein is similar to that of TonB, and ExbD is proposed to be a chaperone for TonB conformational changes *in vivo* (11). The three transmembrane domains of ExbB share homology with MotA, a protein that translocates protons to energize flagellar rotation (12). Since the TonB system requires the PMF, it is likely that ExbB also translocates protons.

Full-length TonB fractionates with both the cytoplasmic membrane and the OM and is a stable protein. Since it is much less abundant than the various OM transporters it serves, TonB must undergo cyclic contact and disengagement from the TonB-gated
transporters during energy transduction (9, 13, 14). During the energy transduction cycle, TonB undergoes conformational changes both before and after an energy transduction event; however, if transport ligands are not present, energy is not transduced (15, 16). It is clear that TonB directly interacts with a conserved region at the extreme amino terminus of the transporters known as the TonB box (17, 18).

The carboxy-terminal ~90 amino acids of TonB have been cocrystallized with the BtuB and FhuA transporters (19, 20). The structure of monomeric TonB in the complexes is similar to the crystal structures of dimeric TonB and to a monomeric configuration from nuclear magnetic resonance (NMR) studies (21–24). In the TonB-BtuB structure, the BtuB TonB box interacts with TonB residues 225 to 232 and residues 158 to 171, suggesting that the residues in those domains would prove to be important for TonB function. In the TonB-FhuA structure, the TonB box was not strictly visible.

Using a genetic approach, we had previously identified 5 out of 20 residues assayed in the TonB carboxy terminus as being functionally important. In that case, “functionally important” meant that a substitution mutation resulted in a nearly complete loss of function in at least one of the several available assays for TonB activity (26). To identify additional important residues and to test whether the dimeric crystal structures reflect actual conformations of TonB, the remaining 70 amino acids were replaced with cysteinyI residues and the resulting phenotypes in a panel of TonB-dependent assays and their ability to form disulfide-linked dimers were analyzed. For all identified functionally important residues, their ability to synergize with one another was also tested as discussed below.
Our resulting data support two conclusions: first, and most importantly, neither of the two dimeric crystal structures represents the conformation of the TonB carboxy terminus, and second, while there are functionally important residues in TonB, no single residue is irreplaceable.

**Results and Discussion**

*TonB conformations represented by dimeric crystal structures do not exist in vivo.*

The dimeric crystal structures, which represent stable forms by their very nature, are the most relevant, since TonB is known to function as a dimer *in vivo* (15, 25). Previous work with a limited set of 20 Cys substitutions in the TonB carboxy terminus suggested the possibility that the dimeric crystal structures might not represent *in vivo* conformations. Four Cys substitutions, each distantly located from the cognate partner in the dimeric crystal structures, spontaneously formed a set of three disulfide-linked dimers *in vivo*. These so-called triplet dimers did not form if TonB was not energized, indicating that they were biologically relevant (15, 26). Since the crystal and NMR structures represent only a snapshot of each protein’s conformation, it is possible that the stable conformation of TonB depicted in the crystal structures was only one of at least three conformations that TonB is known to inhabit (16).

To further examine the relevance of the crystal structures, predicted residues at the dimeric interface, close enough to form disulfide bonds, were identified. In both of the existing crystal structures, Cys substitutions at I232 and N233 would be diagnostic.
These substitutions were individually engineered in TonB, along with nearby L229C, F230C (known to cross-link strongly), and K231C and examined for their ability to spontaneously form disulfide-linked dimers. The maximum distance between α-carbons of Cys residues that can form disulfide-linked dimers is ~7 Å (27). TonB L229C and K231C did not form cross-linked dimers and would not be predicted to form them on the basis of the distances between their α-carbons (Fig. A-1 and Table A-1). However, the distances between I232 α-carbons in structures 1IHR and 1U07 are 4.4 and 6.5 Å, respectively, and the distances between sulfur atoms of optimized rotamers of TonB I232C are 3.7 and 2.7 Å, respectively. If the conformations represented by the crystal structures existed, TonB I232C would dimerize efficiently. In contrast, virtually no disulfide-linked dimers of TonB I232C were detected except on extended overnight exposure of the immunoblots, circumstances under which detectable levels of the largest of the triplet dimers can be observed in a majority of the 90 TonB proteins with Cys substitutions (data not shown). As seen previously, the absence of the sole TonB-dependent ligand produced by *E. coli* K-12, enterochelin, did not affect the extent of cross-linking in the *aroB* strain, KP1406 (Fig. A-1). Similarly, TonB N233C residues, while slightly more distant in 1IHR (8.1 Å) than in 1U07 (5.7 Å) would be predicted to form dimers strongly and formed them only weakly.

As shown previously, several substituted cysteines spontaneously cross-link efficiently but are distantly located from their cognate partners in the crystal structures and would not be predicted to do so: F202C, W213C, Y215C, and F230C. To this list, TonB G186C (characterized below) can be added (Fig. A-2). It cross-linked efficiently,
yet according to the crystal structures, these residues are >50 Å apart (Table A-1). Thus, we conclude that none of the conformations TonB assumes is represented by the dimeric crystal structures.

There are 7 functionally important, but not irreplaceable, residues in the TonB carboxy terminus.

We had previously identified 5 functionally important aromatic residues (F180, F202, W213, Y215, and F230) in the region of the TonB carboxy terminus for which a crystal structure existed (21). Those aromatic amino acids appeared to play a role in discriminating among the many ligand/transporter combinations that TonB contacts (15, 26). Substitution of each residue with either Ala or Cys residues resulted in idiosyncratic profiles of sensitivities to various colicins and phage and ability to transport ferrichrome. Thus, each TonB protein with a specific substitution was often fully active with respect to one agent but virtually inactive with respect to another agent. Sensitivity to different agents varied with each different substitution. Any pair-wise combination of the 5 Ala substitutions in a double mutant cycle analysis resulted in a single synergistically inactive phenotype with respect to all assays (28). It was proposed that the aromatic amino acids all worked together in some fashion, most likely in receptor/colicin recognition (15, 26). This suggests that these aromatic residues are relatively close to each other. Cys substitutions supported this hypothesis. In 4 of the 5 Cys substitutions at these aromatic residues, disulfide-linked triplet dimers were demonstrated. The biological relevance of the disulfide-linked dimers was demonstrated by the fact that dimers did not form if the transmembrane domain carried the inactivating tonBΔV17 mutation or if the ExbB/D and
TolQ/R functions were absent (15). These studies did not, however, preclude the existence of more mechanistically important, irreplaceable residues that could overturn that hypothesis.

Indeed, many residues in the TonB carboxy terminus are highly conserved, suggesting that they could represent irreplaceable residues (1). To identify such residues, 70 previously untested residues from residues 150 to 239, and the sole remaining aromatic residue in the entire carboxy terminus, F125, were substituted with Cys residues in a plasmid expressing TonB C18G, which lacks the sole naturally occurring Cys residue. The 71 individual substitutions were expressed at chromosomally encoded levels in a ΔtonB strain, and each Cys substitution was assayed for colicin B, Ia, and M sensitivity and for the ability to energize [55Fe]ferrichrome transport. This combination of assays reflected interactions with three different transporters, each with their different TonB boxes (FepA, Cir, and FhuA, respectively), and three colicins, each of which also has a different TonB box. Sensitivity to colicins can register as little as 1 molecule of TonB per cell, while ferrichrome transport can discriminate in the range between 100% TonB activity (340 molecules per cell in media containing high levels of iron [9]) and 10% TonB activity (29). Because these comprehensive assays represent a spectrum of sensitivities and TonB recognition of different transporters and their colicin ligands, they ensured that few, if any, important mutant phenotypes were overlooked.

The results indicated that no single residue in the TonB carboxy terminus was essential for activity. Of the 71 Cys substitutions, 69 supported essentially full activity with respect to the colicin assays and from 60 to 100% [55Fe]ferrichrome transport,
including F125C (see Fig. A-S1 in the supplemental material). Because Cys side chains could functionally substitute for Ser or Thr side chains, the TonB S151, S157, T183, S195, S222, T235, and T236 residues were individually replaced with Ala residues and assayed. Like the Cys substitutions, each of the Ala substitutions retained wild-type activity, confirming that the Ser and Thr residues were not important for TonB activity (data not shown).

The only two previously undiscovered Cys substitutions that did not support nearly full activity in all assays were Y163C and G186C. Interestingly, these also exhibited idiosyncratic phenotypic profiles (Table A-2). TonB Y163C exhibited full sensitivity to colicin Ia and the ability to energize $[^{55}\text{Fe}]$ferrichrome transport at ~60% of the rate of normal wild-type levels. However, it exhibited tolerance (insensitivity) to both colicins B and M. TonB G186C exhibited a different profile of activities, being only slightly impaired in colicin B and Ia sensitivity, completely tolerant to colicin M, and capable of energizing <10% of the initial rate of normal wild-type $[^{55}\text{Fe}]$ferrichrome transport.

The full set of 91 Cys substitutions from this and previous work was also assayed for spontaneous dimer formation. Other than the 4 cross-linkable Cys substitutions previously identified, only two residues, F125 and G186, formed triplet dimers slightly less efficiently than F230C, the most efficient dimer former (Fig. A-2 and data not shown). Weak formation of the dimers of highest mass in the triplet was observed for several residues from amino acids (aa) 150 to 170. This region immediately follows an unstructured region in the NMR structure (aa 103 to 149) (23). There were essentially no
cross-linked complexes observed from aa 171 to 239 with the exception of weak formation of the dimers of highest mass by P184C, D185C, P198C, and N233C (data not shown). These results indicate that the only 7 residues important for TonB activity share the characteristic of idiosyncratic phenotypic profiles and a subset of these participate in triplet dimer formation.

(i) Conservation does not correlate with functional importance in the TonB carboxy terminus.

Interestingly, there was only partial correlation between the ~30 highly conserved residues in the TonB carboxy terminus and the functionally important residues. As an example, Chu et al. identified TonB Y163 and P164 as constituting a highly conserved motif (1). Consistent with that, TonB Y163 showed 82% conservation and was one of the newly identified functionally important residues in this study. In contrast, P164 exhibited comparable conservation at 86% conservation, but TonB P164C showed only minor decreases in activity (see Fig. A-S1 in the supplemental material). TonB G186 is highly conserved at 96% and was also functionally important. In contrast, the remaining 5 previously identified functionally important residues are much less well conserved: F180 (45%), F202 (53%), W213 (26%), Y215 (23%), and F230 (22%). These residues are, however, highly conserved among enteric bacteria (26). (The overall percentages of conservation of residues in this discussion were generously provided by B. Chu and H. Vogel [personal communication].)
The 7 functionally important amino acids play roles in recognition.

On the basis of their individual idiosyncratic phenotypic profiles and the lack of any irreplaceable residues in the carboxy terminus, it seems clear that TonB uses the 7 residues identified as functionally relevant to discriminate among the various TonB-gated transporters and their colicin ligands. Aromatic residues often constitute part of a protein-protein binding interface (30). Precedence for idiosyncratic phenotypic profiles indicating differential binding interactions can be found in the cytochrome P450s, which adopt different conformations to interact with different substrates (31). These results are also consistent with an earlier hypothesis by Sauter et al. (25) based on different phenotypes conferred by Y163C and F230V substitutions in various assays. The fact that the side chains of these 7 functionally important residues are buried in the crystal structures was consistent with the finding that those structures did not exist.

Perhaps, based on their high levels of sequence conservation, Y163 and G186 reflect residues with more intrinsic mechanistic function in transporter recognition. The relatively low overall level of sequence conservation of TonB F202C, W213C, Y215C, or F230C, coupled with a high level of conservation among closely related bacteria, suggests that the residues directly involved in recognition have evolved as the transporter sequences evolved.

The relationship of these 7 residues to the TonB box remains to be determined. TonB residues 158 to 162, virtually all of the region through which TonB interactions with TonB boxes occur, can be deleted and still retain 60% ferrichrome transport activity,
indicating that additional amino acids must be involved in interaction with some transporters (32). Furthermore, it has been demonstrated that TonB interacts \textit{in vivo} with sites other than the transporter TonB boxes before interacting with the TonB boxes themselves (33). In addition, phage display experiments have identified regions of \textit{in vitro} interactions between the TonB carboxy terminus and outer membrane (OM) transporters outside the TonB box (34).

\textit{Each of the functionally important residues exhibits synergy in a double mutant cycle analysis.}

Like the previously identified functionally important residues, a double mutant cycle analysis expanded to include Y163A and G186A indicated that they also exhibited synergy, showing complete insensitivity to colicins B, D, M, and Ia (see Table A-S1 in the supplemental material). Trivial explanations for the inactivity of the double mutants were ruled out. The double Ala substitutions were efficiently exported as determined by their proteinase K accessibility in spheroplasts (see Fig. A-S2 in the supplemental material). They displayed the ability to form abundant and apparently random formaldehyde-mediated cross-links as described previously for a subset of the double mutants (26) (see Fig. A-S3, far left, middle, in the supplemental material). Two of the TonB double Ala substitutions showed reduced cross-linking to ExbD, while the remainder were too unstable to survive 15 min at room temperature during the formaldehyde cross-linking assay but did not otherwise affect the cross-linking profile of ExbD (see Fig. A-S3, far right, in the supplemental material).
The concept of synergy has been classically applied to enzymatic reaction mechanisms where measurements of ΔΔG are feasible. The interpretation in those cases is that the two wild-type residues in question either interact directly or they facilitate the same non-rate-limiting step, such that when each is mutated, a new slower rate-limiting step is imposed (28, 35). While all possible combinations of the mutations (912) were not assayed to develop a synergy matrix, selected combinations of residues whose mutation had no effect on function were examined, and no synergy was detected. When TonB F125A was combined with each of the other Ala substitutions in this study, the phenotypes exhibited additivity but not synergy: for example, TonB F125A F180A exhibited the phenotype that is characteristic of TonB F180A (see Table A-S1 in the supplemental material). Likewise, the pairing of M201C and E216C, each at a residue next to a Cys substitution that exhibited synergy, exhibited only additivity (data not shown). While mechanistic interpretations of these data were not yet possible, they did indicate that the set of 7 amino acids all have special properties when considered within the context of carboxy-terminal residues and, most importantly, that they all functioned as a group. Their interaction and functional importance would not be predicted to occur if current crystal structures represented the structure of TonB.

There are no irreplaceable residues in TonB.

The TonB protein has unusual properties. Approximately 17% of its amino acid residues are prolines compared to the average E. coli TonB protein that contains ~4% proline (36). While many of the proline residues are located in the proline-rich region
from residues 70 to 102 (42% proline), there are 7 prolines in the carboxy-terminal 90 amino acids (~8%) and still higher proportions in the rest of the protein (Fig. A-3). In addition, the TonB carboxy terminus from residues 102 to 239 is highly basic, with an overall calculated pI of 10.9.

Another unusual feature of TonB is that there are no irreplaceable residues in the entire protein. Residues 1 to 12 preceding the uncleaved signal sequence are replaceable with the first 25 residues of the soluble protein TrpC (37). The only important residue in the transmembrane domain (aa 12 to 32) is the highly conserved H20 residue (10). Nonetheless, overexpression of TonB H20A or other inactivating mutations in the transmembrane domain restores low-level activity (16, 38; unpublished data). The periplasmic region proximal to the membrane (aa 33 to 64) can be deleted without inactivating TonB entirely. Moderate sensitivity to colicins B and Ia and ~2% $[^{55}\text{Fe}]$ferrichrome transport are retained (this study). The triple proline motif from residues 63 to 65 can be deleted without significant effect (this study); the proline-rich domain from residues 66 to 100 (or residues 70 to 102 [this study]) can be deleted without effect on TonB activities unless the periplasmic space is temporarily expanded, resulting in ~50% decrease in TonB activity (39, 40). Consistent with the nonessentiality of residue F125, the region from residues 103 to 149 can be deleted with retention of nearly full colicin sensitivity and an initial rate of $[^{55}\text{Fe}]$ferrichrome transport of 14% of the wild-type rate (this study). This study showed that 83 of the 90 residues from residues 150 to 239 are replaceable. The importance of the remaining 7 residues depends entirely on the assay, and no single mutation inactivates TonB completely. By comparison,
complete Cys scanning of *E. coli* lactose permease yielded 6 irreplaceable residues out of 417 (41). We are unaware of any proteins that have proven to be as tolerant of mutation as TonB.

**Final thoughts.**

It might not be surprising that *in vivo* TonB conformations are not represented by the dimeric crystal structures. First, the crystal structures were solved in the absence of the TonB transmembrane domain, ExbB, ExbD, and the PMF—components all known to influence its conformation *in vivo* (11, 15, 16, 42). Furthermore, while interactions could not be detected between the purified periplasmic domains of TonB and ExbD, PMF-dependent interactions were detected *in vivo* (11, 43). Second, the TonB carboxy terminus appears poised between predicted structured and unstructured tendencies (Fig. A-3), bearing many of the hallmarks that characterize other intrinsically unstructured proteins (44), such as the fact that TonB is modular with two independent functional domains, the amino terminus and the carboxy terminus. Like other such proteins, part of the linker region that separates these two functional domains is rich in proline and can assume a rigid extended conformation (45). Such a linker region often serves to allow an efficient search for the correct target, which in this case would be ligand-bound transporters. For intrinsically unstructured proteins, coupled folding and binding often result in high specificity with low affinity to allow cyclic binding and release. TonB, as the limiting protein in the system, must interact with the numerous available transporters with high specificity to ensure uptake of needed nutrients (13). It must, however, also
have low affinity to allow rapid cyclic binding and release from target transporters, of which there are 7 in *E. coli* K-12 (9, 14, 46). A model that accommodates the data is one where TonB identifies and binds to ligand-bound transporters by means of conformational sampling.

**Materials and Methods**

*Bacterial strains and plasmids.*

*Escherichia coli* strains W3110, KP1270 (W3110 *aroB*), KP1344 (W3110 *tonB::blaM*), and KP1406 (W3110 *aroB tonB::blaM*) were used in this study (16). The plasmid strains used in this study are all derivatives of pKP325 (16), where the *araBAD* promoter regulates TonB expression. Cysteinylation codon substitutions were created in the *tonB* gene by two different methods as previously described and are listed in Table A-S2 in the supplemental material (26, 32). For each mutant, the entire *tonB* open reading frame was sequenced to rule out unintended base changes (Penn State Genomics Core Facility, University Park, PA).

*Media and culture conditions.*

Luria-Bertani (LB), tryptone (T), and M9 minimal salts were prepared as previously described (47). Liquid cultures, agar plates, and tryptone top agar were supplemented with 34 µg ml⁻¹ chloramphenicol and/or 100 µg ml⁻¹ ampicillin and plasmid-specific levels of l-arabinose as needed for expression of plasmid-encoded TonB
variants at chromosomally encoded levels, determined by immunoblot analysis. M9 salts were supplemented with 0.5% glycerol (wt/vol), 0.4 µg ml\(^{-1}\) thiamine, 1 mM MgSO\(_4\), 0.5 mM CaCl\(_2\), 0.2% Casamino Acids (wt/vol), 40 µg ml\(^{-1}\) tryptophan, and 1.85 µM FeCl\(_3\). Cultures were grown with aeration at 37°C. Adapted M9 medium for growth of \(aroB\) strains contained additional aromatic amino acids (0.4% each of tyrosine, phenylalanine, and tryptophan) and iron (37 µM FeCl\(_3\)).

Activity assays.

Spot titer assays were performed in triplicate as previously described (29, 48). The initial rates of \([^{55}\text{Fe}]\)ferrichrome uptake were determined as described previously (48).

In vivo disulfide cross-linking assay.

Saturated overnight cultures of strains carrying plasmids encoding TonB substitutions were subcultured 1:100 in T broth containing chloramphenicol and supplemented with l-arabinose. Cultures were harvested in mid-exponential phase and precipitated with trichloroacetic acid (TCA). Cell pellets were suspended in nonreducing Laemmli sample buffer containing 50 mM iodoacetamide, as previously described (15). Samples were resolved on 11% nonreducing SDS-polyacrylamide gels and evaluated by immunoblot analysis using anti-TonB monoclonal antibody 4F1.
In vivo formaldehyde cross-linking.

Saturated overnight cultures were subcultured 1:100 into M9 minimal medium (above) supplemented with arabinose, and when the cultures reached mid-exponential phase, they were treated with formaldehyde as previously described (48). Cross-linked complexes were detected by immunoblotting with monoclonal TonB 4F1 antiserum or ExbD-specific polyclonal antibodies (9).

Proteinase K accessibility assays.

Spheroplasts of strains expressing TonB double Ala mutants at chromosomally encoded levels were prepared and treated with proteinase K as described previously (16). Samples were visualized on immunoblots of 11% SDS-polyacrylamide gels with anti-TonB monoclonal antibody 4F1 (49).
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178:1363–1373.
Figure Legends:

Fig. A-1: *In vivo*, TonB does not exhibit the crystal structure-predicted disulfide cross-link at residue I232C. TonB proteins were expressed at chromosomal levels from plasmids carrying genes encoding wild-type (W.T.) TonB or TonB Cys substitutions in pKP632 (L229C), pKP570 (F230C), pKP628 (K231C), pKP629 (I232C), and pKP630 (N233C) and processed in nonreducing sample buffer containing iodoacetamide as described in Materials and Methods. *E. coli* strain KP1344 (W3110 ΔtonB::blaM) produced enterochelin (+), while strain KP1406 (W3110 aroB ΔtonB::blaM) did not (−). Samples were resolved on an 11% nonreducing SDS-polyacrylamide gel and immunoblotted with TonB-specific monoclonal antibodies and are labeled according to the Cys substitution being examined. The positions of disulfide-linked dimers are indicated by asterisks to the right of the gel. The positions of degradation products (deg products) are also indicated to the right of the gel. The levels of total TonB protein in the nonreducing samples were determined on a reducing gel shown at the bottom of the figure.

Fig. A-2: Six of the 91 Cys substitutions can efficiently and spontaneously form disulfide-linked dimers *in vivo*. *E. coli* strain KP1344 (W3110 ΔtonB) carrying plasmids (see Table A-S2 in the supplemental material) expressing TonB Cys substitutions at chromosomal levels were processed in nonreducing sample buffer containing iodoacetamide as described in Materials and Methods. Samples of TonB proteins encoded by genes carried on plasmids pKP1070 (F125C), pKP588 (Q160C), pKP586 (Y163C), pKP569 (F180C), pKP612 (G186C), pKP415 (F202C), pKP472 (W213C),
pKP474 (Y215C), and pKP570 (F230C) were resolved on an 11% nonreducing SDS-polyacrylamide gel and immunoblotted with TonB-specific monoclonal antibodies and are labeled according to the Cys substitution being examined. TonB Q160C is included as an example of a Cys substitution at a nonaromatic amino acid that does not cross-link efficiently. The positions of disulfide-linked dimers are indicated by asterisks to the right of the gel. The positions of degradation products (deg products) are also indicated to the right of the gel. The identities of the intermediate bands between asterisks are not known. The levels of total protein in the nonreducing samples were determined on a reducing gel (bottom gel) and compared to chromosomally encoded TonB (c).

**Fig. A-3:** Features of the TonB primary amino acid sequence. The top line displays some of the key features of the TonB primary amino acid sequence that have been studied by mutational analysis. The TonB sequence has been divided into 4 regions: (i) residues 1 to 69, containing the essential amino-terminal signal anchor and showing the position of the H20 residue within it; (ii) residues 70 to 102, constituting the proline-rich region; (iii) residues 103 to 149; and (iv) residues 150 to 239, the only region for which structures have been determined thus far (CTD, C-terminal domain). The next line displays the calculated pI for each of the 4 regions. The calculated percentage of prolyl residues found in each region is shown on the next line. At the bottom is a prediction of unstructured regions in TonB protein determined by the PONDR program, with the corresponding amino acid numbers on the x axis. Predicted unstructured regions are shown above the line, and structured regions are shown below the line. A slightly different view of the PONDR graph for TonB is shown in reference 10.
Fig. A-S1: Activities of the TonB proteins with Cys substitutions expressed at chromosomally encoded levels. Each bar graph presents the relative levels of activity conferred by the ΔtonB aroB parental strain KP1406, the chromosomally encoded copy of tonB in strain W3110 (Chro), the plasmid-encoded TonB in pKP568 expressed at chromosomally encoded levels (wild type [wt]), TonB F125C, and strains with TonB and the remaining 90 substitutions from residues 150 to 239 (indicated below the bars of each graph; plasmids listed in Table A-S2 in the supplemental material). Sensitivities to colicins B, Ia, and M (from top to bottom) and the initial rates of [55Fe]ferrichrome transport performed in strain KP1344 (ΔtonB) are shown. The values are means plus standard errors (error bars). For residues 172 to 239, a slightly less potent preparation of colicin M was used than for the other combinations of strain KP1406 and plasmids. Data for residues 180, 199 to 216, and 230 are from supplemental reference 1 (see Table A-S2).

Fig. A-S2: Inactive TonB aromatic-to-Ala double substitutions are exported from the cytoplasm. Double Ala substitutions encoding TonB Y163A F202A (pKP1106), TonB Y163A W213A (pKP1115), and Y163A F180A (pKP1083) were expressed in strain KP1344; strain W3110 expressed chromosomally encoded TonB. Whole cells (WC) and spheroplasts (Sph) were prepared and treated with proteinase K (+) or not treated with proteinase K (−) as described in Materials and Methods. Proteins were precipitated with TCA, resolved on 11% polyacrylamide gels, and detected on immunoblots with the anti-TonB monoclonal antibody 4F1. The Y163A F180A samples are from a different immunoblot than the other samples. The same results, sensitivity to proteinase K in spheroplasts, were obtained for all inactive double substitutions.
Fig. A-S3: TonB double Ala substitutions exhibit random, unregulated, cross-linking and reduced ability to cross-link to ExbD. Strain W3110 expressed chromosomal TonB and ExbD, while plasmid-encoded wild-type TonB, pTonB(pKP325), was expressed in strain KP1344. Double Ala substitutions Y163A F180A (pKP1083), Y163A F202A (pKP1106), Y163A W213A (pKP1115), and Y163A F230A (pKP1130) were also expressed in strain KP1344. Arabinose was used to induce expression of the plasmid-encoded TonB as follows: 0.00075% for pKP325, 0.016% for pKP1083, 0.003% for pKP1106, 0.004% for pKP1115, and 0.2% for pKP1130. Cross-linking was performed with formaldehyde as described in Materials and Methods, and complexes were detected on immunoblots of 13% polyacrylamide gels using the monoclonal anti-TonB antibody 4F1 and ExbD polyclonal antibodies. The intermediate exposure on the left is a lighter exposure of the same samples in the middle panel. The asterisk denotes TonB degradation products. The molecular mass standards (kDa) are shown in the middle.
Table A-1: Lack of correlation between α-carbon distances in the crystal structures and ability of TonB Cys substitutions to form disulfide-linked dimers \textit{in vivo}

<table>
<thead>
<tr>
<th>Residue</th>
<th>Distance\textsuperscript{\textit{a}}</th>
<th>Dimer formation</th>
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<td>51.4 Å</td>
<td>59.2 Å</td>
</tr>
<tr>
<td>F202</td>
<td>6.5 Å</td>
<td>31.4 Å</td>
</tr>
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<td>W213</td>
<td>35.6 Å</td>
<td>45.1 Å</td>
</tr>
<tr>
<td>Y215</td>
<td>47.0 Å</td>
<td>51.4 Å</td>
</tr>
<tr>
<td>L229</td>
<td>20.7 Å</td>
<td>25.1 Å</td>
</tr>
<tr>
<td>F230</td>
<td>12.6 (8.0) Å</td>
<td>19.4 (18.4) Å</td>
</tr>
<tr>
<td>K231</td>
<td>8.6 (7.2) Å</td>
<td>13.6 (12.4) Å</td>
</tr>
<tr>
<td>I232</td>
<td>4.4 (3.7) Å</td>
<td>6.5 (2.7) Å</td>
</tr>
<tr>
<td>N233</td>
<td>8.1 (5.2) Å</td>
<td>5.7 (3.7) Å</td>
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</tbody>
</table>

\textsuperscript{a} Predicted distance between α-carbon atoms was calculated using the Swiss-PDBViewer, DeepView v4.0 (http://spdbv.vital-it.ch/). The distance between sulfur groups of optimized Cys rotamers is shown in parentheses. 1IHR and 1U07 are Protein Data Bank (PDB) identifiers for solved structures.

\textsuperscript{b} Relative levels of triplet dimer formation are shown in Fig. A-1 and 2.

A hyphen indicates that the dimers were relatively undetectable. The maximum distance between α-carbons of Cys residues that can form disulfide-linked dimers is \~7 Å (27).

For a summary of similarities between the 1IHR and 1U07 structures, see reference 24.
Table A-2: Idiosyncratic phenotypic profiles of the 7 functionally important TonB Cys substitutions

<table>
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<tr>
<th>Mutant(^b)</th>
<th>Col B</th>
<th>Col Ia</th>
<th>Col M</th>
<th>% Fe</th>
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<tr>
<td>pKP568(^d) (wt)</td>
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<td>Y163C</td>
<td>T,T,T</td>
<td>8,8,8</td>
<td>T,T,T</td>
<td>58 ± 2</td>
</tr>
<tr>
<td>F180C</td>
<td>7,7,7</td>
<td>4,4,4</td>
<td>4,4,4</td>
<td>48 ± 2</td>
</tr>
<tr>
<td>G186C</td>
<td>7,7,7</td>
<td>5,5,5</td>
<td>T,T,T</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>F202C</td>
<td>4,4,4</td>
<td>8,8,8</td>
<td>T,T,T</td>
<td>38 ± 1(^e)</td>
</tr>
<tr>
<td>W213C</td>
<td>6,6,6</td>
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<td>Y215C</td>
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<td>35 ± 2</td>
</tr>
<tr>
<td>F230C</td>
<td>7,7,7</td>
<td>8,8,8</td>
<td>2,2,2</td>
<td>38 ± 1</td>
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</tbody>
</table>

\(^a\) Spot titer assays were conducted in strain KP1406 (tonB aroB).
Numbers indicate the last 5-fold dilution at which sensitivity to the agent (colicin [Col] B, Ia, or M) was apparent in triplicate assays. T indicates tolerance (no sensitivity) to the particular agent tested. The results for three assays are shown.

Initial rates of $[^{55}\text{Fe}]$ferrichrome transport were determined in strain KP1344 ($\text{tonB}$).

pKP568 carries a gene encoding TonBC18G, which lacks the sole Cys residue in native TonB. wt, wild type.

TonB F202A exhibits 95% transport (26).
### Table A-S1: Double Mutant Cycle Analysis of TonB F125A, TonB Y163A, and TonB G186A

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<th>Col M</th>
<th>Col Ia</th>
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*Sensitivitya*
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a Spot titer assays were conducted in KP1406 (*tonB, aroB*). Numbers indicate the last 5-fold dilution at which sensitivity to the agent was apparent.

b TonB proteins were expressed at chromosomal levels

c T denotes tolerance (no sensitivity) to the particular agent tested
Table A-S2: Plasmids used in this study

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<th>Plasmid</th>
<th>Characteristics</th>
<th>Reference</th>
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<td>pKP325</td>
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<td>(23)</td>
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<tr>
<td>pKP477</td>
<td>pKP325 with entire <em>tonB</em> gene deleted from plasmid</td>
<td>(13)</td>
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<tr>
<td>pKP442</td>
<td>pKP325 with an <em>XhoI</em> site created with a silent point mutation at TonB A166 at the 830 bp of the <em>tonB</em> gene</td>
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<td>pKP325 (TonB S157A)</td>
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<td>pKP877</td>
<td>pKP325 (TonB Y163A)</td>
<td>Present study</td>
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<td>pKP509</td>
<td>pKP442 (TonB F180A)</td>
<td>(13)</td>
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Present study

Supplementary references:


For each mutant, the entire *tonB* gene was sequenced to identify the correct mutation and rule out the presence of unintended base changes.
Fig. A-1, Postle et al.
Fig. A-2. Postle et al.
Fig. A-3, Postle et al.
Fig. A-S1, Postle et al.
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**Fig. A-S2, Postle et al.**
Fig. A-S3, Postle et al.
VITA

Michael George Gresock

EDUCATION

Ph.D. in Biochemistry, Microbiology and Molecular Biology (expected August 2014)
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B.S. in Biological Sciences, summa cum laude (May 2007)
Saint Vincent College, Latrobe, Pa.

PUBLICATIONS


Postle K, Kastead KA, Gresock MG, Ghosh J, Swayne CD (2010) The TonB dimeric crystal structures do not exist in vivo. mBio (open access). PMCID: PMC3005593


AWARDS AND FELLOWSHIPS


INVITED TALKS AND POSTER PRESENTATIONS

2012 Pittsburgh Bacterial Meeting, Duquesne University: oral presentation

2012 Allegheny Branch of the American Society of Microbiology Symposium, Penn State University: first place graduate poster

2008 and 2009 Pittsburgh Bacterial Meeting, Duquesne University: poster presentations