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THE POSITIONAL REQUIREMENT FOR THE ASPARTATE RESIDUE IN THE EXBD TRANSMEMBRANE DOMAIN

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Abstract

The TonB system, found in *E. coli* and other Gram-negative bacteria, is responsible for harvesting the energy of proton motive force (pmf) at the inner membrane and transducing it to outer membrane transporters for the passage of iron-siderophore complexes and vitamin B12 into the cell. Known essential components of the TonB system are the inner membrane proteins TonB, ExbD, and ExbB. TonB is known to directly contact outer membrane transporters, and may enter an energized conformation in response to pmf from which the energy transduction event can take place. ExbB and ExbD are thought to harness pmf but their exact roles in this process are still unknown. ExbD has been proposed to have a signal transduction function, and it may also act as a chaperone to assist TonB in achieving the energized conformational state. ExbD contains a single, essential charged amino acid in its transmembrane domain, aspartate 25. This residue is highly conserved across species and in homologues of ExbD. Several possible functions for this residue have been suggested, including a function as a proton donor or acceptor that assists in the response of the system to pmf. In the work presented here, the spatial requirement for the aspartate residue in the transmembrane domain is tested. Plasmids encoding ExbD double substitution mutants, in which aspartate 25 is replaced with alanine and another transmembrane residue is replaced with aspartate, were constructed, and transformed into a strain lacking the *exbD* gene (as well as the *tolQR* operon of the homologous Tol system, because there is crosstalk between the systems). The strains were assayed for TonB system activity using spot titers of B group colicins and bacteriophage ϕ80. No TonB system activity was detected for any of the stable mutants constructed. Both ExbD(D25A, I33D) and ExbD(D25A, M35D) appeared to be highly unstable, and the mutant strain containing the pExbD(D25A, V43D) plasmid exhibited a severely growth-inhibited phenotype in the presence of ampicillin despite carrying an ampicillin resistance cassette on the plasmid.

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1. Introduction

1.1 Iron Transport & The Outer Membrane

Gram-negative bacteria are distinguished from their Gram-positive counterparts in the composition of their protective membrane structures. Gram-positive bacteria have a single plasma membrane surrounded by a thick peptidoglycan layer, which affords rigidity and protection from osmotic lysis to the bacteria. Gram-negative bacteria, on the other hand, possess both an inner and an outer membrane with just a thin layer of peptidoglycan in the aqueous space between the membranes, called the periplasm. The outer membrane primarily functions as a permeability barrier and is able to protect the peptidoglycan from peptidoglycan-lytic enzymes. Additionally, it possesses lipopolysaccharide on its surface, which protects bacteria from lipophilic molecules such as detergents and lipophilic antibiotics. While this protection is advantageous to the bacteria, the acquisition of nutrients across the outer membrane now becomes a challenge. Small, abundant, nutrients, under 600 Da in size, may passively diffuse through nonspecific porin channels. Larger or less abundant molecules (large malodextrins or nucleosides, for example) may cross the membrane through specific channels that contain a binding site for the target nutrient (Nikaido, 1994).

The transport of iron, however, requires particular attention. Iron is an essential nutrient, but is scarce in aerobic environments because it takes the form of insoluble ferric hydroxides. In addition, the successful scavenging of iron from the host plays an essential role in the pathogenesis of bacterial infections (Crosa, 1984). To obtain iron, bacteria secrete siderophores, high-affinity iron chelating compounds which are able to extract iron from its insoluble form. They can then be transported back into the bacterium as iron-siderophore complexes (Neilands, 1995). Additionally, in pathogenic bacteria, special outer membrane receptors exist that can take in iron directly from host iron-binding proteins (Torres and Payne, 1997). In accordance with the importance and scarcity of iron, and the large size of iron-siderophore complexes, high-affinity active transporters are used for the passage of iron-siderophores, as well as vitamin B12, into the periplasmic space (Postle, 1993). Active transport is a challenge in Gram-negative bacteria because the outer membrane is essentially unenergized; the large porins do not allow the establishment of an electrochemical gradient and the periplasm is devoid of high-energy phosphates. The TonB system allows Gram-negative bacteria to overcome this barrier by harnessing the energy of proton motive force at the inner membrane and transducing this energy to these high-affinity outer membrane receptors so that the bound ligand may pass into the periplasmic space.

1.2 The TonB System & Active Transport

The TonB protein was first identified as a mutation conferring resistance to the T1 bacteriophage; hence, the name "Ton" for "T-one," "B" because it was the second of two mutations identified (Luria and Delbrück, 1943). TonA would be recognized as the outer membrane receptor for T1 and renamed FhuA, however, the role of TonB would remain mysterious for many years (Kadner, *et al*., 1980). Other phenotypes for the *tonB* mutants, such as impaired iron-siderophore and vitamin B12 uptake at the outer membrane and resistance to B group colicins and filamentous bacteriophage ϕ80, were later revealed (Matsushiro, 1963; Frost

and Rosenberg, 1975; Davies and Reeves, 1975; Bassford, *et al*., 1976). The source of energy for these transport processes was found to be proton motive force (pmf) across the inner membrane, and the connection between the requirement for pmf and the need for the TonB protein led to the proposal that TonB might function in transducing the energy of pmf at the inner membrane to outer membrane transporters (Reynolds, *et al*., 1980; Hancock and Braun, 1976). Indeed, the topology of TonB as an inner membrane protein with a single transmembrane domain, a short N-terminal tail in the cytoplasm and a large C-terminal domain in the periplasmic space is appropriate for an energy transduction role (Roof, *et al*., 1991; Karlsson, *et al*., 1993a). Subsequent findings, such as the direct interactions between TonB and outer membrane transporters and the ability of TonB to conformationally respond to pmf, are also consistent with an energy transduction function, and this role for TonB is widely accepted (Weiner, 2005; Braun, 2006; Postle and Larsen, 2007). Seven TonB-dependent outer membrane receptors in *E. coli* K-12, each with specificity for a particular set of ligands, have been identified (Postle, 1993). Additionally, pathogenic *E. coli* O157:H7 also include an outer membrane receptor for host haem and heamoglobin proteins (Torres and Payne, 1997).

Mutants at the *exb* locus, originally isolated based on their resistance to B group colicins, were found to share similar phenotypes with *tonB* mutants; however, where *tonB* mutants could confer complete resistance to group B colicins, *exb* mutants had only a reduced sensitivity to these agents, and were only partially deficient in iron-siderophore and vitamin B12 transport (Guterman, 1973; Hantke and Zimmerman, 1981). It was later revealed that this more mild phenotype was actually the result of crosstalk with homologous Tol proteins, which are primarily associated with the transport of A group colicins and outer membrane stability (Braun, 1989).

The *exb* operon consists of two genes, *exbB* and *exbD* (Eick-Helmerich and Braun, 1989). Although the polarity of mutations in *exbB* on *exbD* initially complicated data about mutations in this operon, plasmid complementation studies show that both ExbB and ExbD are essential for TonB function in the absence of TolQ and TolR (Ahmer, *et al*., 1995). Like TonB, ExbB and ExbD are inner membrane proteins; ExbD shares a similar topology with TonB, while ExbB is quite different with its N-terminus in the cytoplasm, three transmembrane domains, and a large cytoplasmic loop between helices I and II (Kampfenkel and Braun, 1992; Kampfenkel and Braun, 1993). **Figure 1** shows the topologies and the locations of the predicted transmembrane domains of TonB, ExbB, and ExbD. Cross-linking data suggest that the three proteins interact via their transmembrane domains to form a complex of unknown size at the inner membrane (Skare, *et al*., 1993; Jaskula, *et al*., 1994; Ollis, *et al*., 2009). Interestingly, the functional half-life of TonB when protein synthesis is stopped is only about 15-30 minutes, but TonB, ExbB, and ExbD are proteolytically stable for far longer, at least 90 minutes for TonB (Skare and Postle, 1991; Kathleen Postle, unpublished data). This suggests that other, unidentified proteins might also contribute to TonB function.

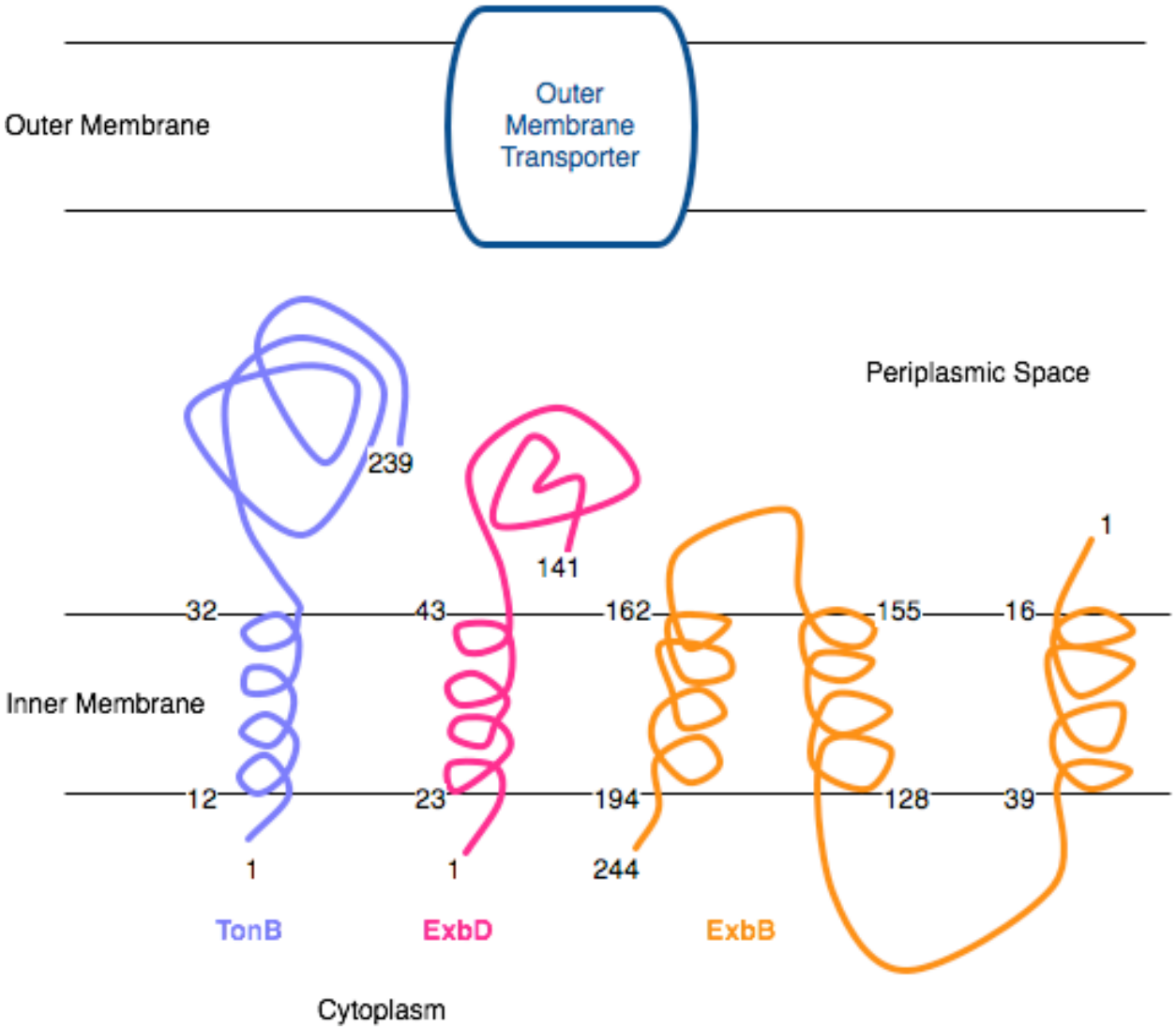

Figure 1: The Predicted Membrane Topologies of the TonB System Proteins TonB and ExbD have similar topologies, both with their N-termini in the cytoplasm, a single transmembrane domain, and a large domain in the periplasmic space. ExbB is quite different with three transmembrane domains, its N-terminus in the periplasmic space, and a large cytoplasmic loop between transmembrane domains I and II. Positions at which transmembrane domains are predicted to begin and end are given in the diagram. Figure adapted from Postle and Larsen, 2004.

1.3 Models of TonB Function

Although the role of TonB in delivering energy to OM transporters is widely accepted, the mechanism of this energy transduction event is still under debate. Studies of proteinase K susceptibility provide evidence that TonB undergoes a conformational change in response to proton motive force: under normal conditions, TonB is completely degraded by proteinase K in intact spheroplasts (but not in whole cells, because proteinase K is not able to cross membranes). However, when the proton motive force of intact spheroplasts is collapsed by treatment with carbonylcyanide *m*-chlorophenylhydrazone (CCCP), a proteinase K-resistant TonB fragment can

be detected on Western blots. A correlation between the appearance of the fragment and the ability of TonB to respond to pmf is drawn based on the finding that when non-functional mutations at the TonB transmembrane domain are partially suppressed by mutations in ExbB, the proteinase K-resistant fragment can be detected, but only faintly (Larsen, *et al*., 1999). The ability to form the proteinase K-resistant fragment, and thus the pmf-responsiveness of TonB, is dependent on the presence of ExbB and ExbD (Larsen, *et al*., 1999; Held and Postle, 2002). The C-terminal 65 residues, while essential for TonB function, are not necessary for the formation of the fragment, suggesting the C-terminal domain is not important for TonB pmf-responsiveness (Larsen, *et al*., 1999).

The ability of TonB to fractionate both with the inner membrane and outer membrane in sucrose density gradient fractionations, and the susceptibility of the TonB N-terminus (predicted to lie in the cytoplasm) to labeling with an inner membrane-impermeable compound when TonB is functional, has led to a model in which TonB forms an energized conformation and completely leaves the inner membrane, "shuttling" across the periplasm to interact with outer membrane transporters. There, TonB is proposed to transduce the conformationally-stored energy and return to its un-energized state, which can be recycled back to the inner membrane (Letain and Postle, 1997; Larsen, *et al*., 2003). Alternatives to this model include a mechanical model, in which TonB remains anchored in the membrane and stabilizes a conformation of ligand-bound outer membrane transporters that allows the ligand to be released into the periplasmic space. In this model any step, even the dissociation of TonB from the transporters, may be the energy-requiring step (Chang, *et al*., 2001; Kaserer, *et al*., 2008).

1.4 The C-terminal Domain and the Outer Membrane

A proline-rich region in the periplasm immediately follows the transmembrane domain of TonB, but this is region unimportant for TonB function except in high salt concentrations, where the periplasmic space is widened, suggesting a purely physical role (Larsen, *et al*., 1993). The C-terminal domain, in contrast, plays a significant role in TonB function. Cross-linking, sucrose density gradient fractionation, and immunoprecipitation data suggest that the TonB C-terminus makes contact with both ligand-bound outer membrane transporters and other outer membrane proteins not important for TonB function, such as OmpA and Lpp (Higgs, *et al*., 2002b; Cadieux and Kadner, 1999). However, TonB has a greater affinity for ligand-bound transporters than unbound transporters both *in vivo* and *in vitro* (Higgs, *et al*., 2002b; Khursigara, 2004). Together, these results have introduced the possibility that TonB "scans" the outer membrane proteins through less-specific interactions in search of a ligand-bound transporter, to which it is able to bind with a greater specificity (Vakharia-Rao, *et al*., 2007; Kaserer, *et al*., 2008). This idea could be incorporated into both shuttling and mechanical models. Another explanation is that TonB is able to "dock" at proteins like OmpA and Lpp until it is able to find a ligand-bound transporter (Higgs, *et al*., 2002b). Fractionation of TonB with the outer membrane and cross-linking of TonB to outer membrane proteins occurs independently of proton motive force and the TonB N-terminus, but requires the TonB C-terminal domain; thus, energization of TonB and the interaction with the outer membrane are thought to be two independent events (Jaskula, *et al*., 1994; Letain and Postle, 1997).

In an attempt to better understand the complex interactions at the outer membrane, crystal and

NMR structures of the TonB C-terminus have been solved. Two crystal structures depict the TonB C-terminus as a rigid, beta-strand exchanged dimer, but the *in vivo* relevance of these structures remains to be established (Chang, *et al*., 2001; Koedding, *et al*., 2004b). The structures must represent an unenergized state, as the purified fragments lack the TonB transmembrane domain and the ability to interact with ExbB or ExbD. Furthermore, research comparing 77-, 86-, 96-amino acid and longer C-terminal fragments in solution found that while the 77- and 86-amino acid fragments were dimeric, 96-amino acid and longer fragments were monomeric and were still able to inhibit ferrichrome transport *in vivo* by competing with functional, full-length TonB. The 77-amino acid fragment was, in contrast, not able to inhibit TonB function (Koedding, *et al*., 2004a). This is significant because the first two crystal structures were solved from purified 85- and 77-amino acid fragments, respectively (Chang, *et al*., 2001; Koedding, *et al*., 2004b). A later study showed that a 92-amino acid fragment yielded a much less rigid, but still dimeric crystal structure, and the NMR structure of a 137-amino acid C-terminal fragment was a monomer, highly ordered at its C-terminus with a 49-amino acid disordered region at the N-terminus of the fragment (Koedding, *et al*., 2005; Peacock, *et al*., 2005).

TonB dimerization can also be observed *in vivo*, however. ToxR fusions with TonB were created with ToxR in the cytoplasm, TonB in the periplasm, and either the ToxR or TonB transmembrane domain. ToxR dimerization, normally mediated by the ToxR periplasmic domain, begins a signal cascade that ends with transcription from the *ctx* promoter, so transcription controlled by *ctx* is a measure of TonB dimerization in this assay. This study showed that both the N-terminal and C-terminal domains, as well as ExbB and ExbD, contribute to the ability of TonB to dimerize at the cytoplasmic membrane (Sauter, *et al*., 2003). A further exploration of the relevance of the crystal structures examined the five aromatic amino acids in the C-terminal domain. That alanine substitutions for any two aromatic residues had a synergistic, not additive, affect on colicin transport suggested that these residues formed a single cluster, while in the crystal and NMR structures, distinct clusters of the aromatic residues are predicted (Chang, *et al*., 2001; Ghosh and Postle, 2004; Peacock, *et al*., 2005). Cysteine substitutions for all but one of these aromatic residues formed disulfide-linked homodimers *in vivo*, even though only one of these aromatic amino acids is predicted by the crystal structure to be close enough to support disulfide crosslinking, providing more evidence for a single aromatic cluster and a dynamic C-terminal region that might be able to move in and out of the conformation seen in the crystal structures (Chang, *et al*., 2001; Ghosh and Postle, 2005).

All TonB-dependent outer membrane transporters and colicins have a conserved "TonB box" necessary for functional interaction with TonB. In outer membrane transporters, the TonB box exists near the N-terminus, at the periplasmic side of the protein (Postle and Larsen, 2007). Initially, the region around Q160 in TonB was thought to be important for interaction between the TonB and the TonB box because TonB box mutations could be suppressed with mutations in this region, and because TonB Q160C could form disulfide-linked dimers with cysteine substitutions in the BtuB TonB box (Heller, *et al*., 1988; Bell, *et al*., 1990; Cadieux and Kadner, 1999). However, subsequent studies showed that a five amino acid region centered on Q160 could be deleted without affecting TonB function. A revised hypothesis is that this region may be important in mediating transitions of the C-terminus between a "disordered" state and an "ordered" state induced by the recognition of a ligand-bound transporter (Vakharia-Rao, *et al*., 2007).

Interactions with FhuA, FepA, and BtuB TonB-box peptides all shifted the NMR spectrum in a similar way, indicating specific interaction with the TonB C-terminus at the same location for all three transporters (Peacock, *et al.,* 2005). However, it is also worth noting that the aromatic amino acid alanine substitutions had idiosyncratic colicin import profiles, suggesting that there are differences in the way TonB interacts with different TonB boxes (Ghosh and Postle, 2005). Crystal structures of TonB fragments bound to outer membrane transporters have also been solved, but much remains mysterious about the way TonB acts on outer membrane transporters to allow ligands to pass into the periplasm (see Weiner, 2005 and Postle and Larsen, 2007 for reviews covering this topic).

1.5 The TonB N-terminal Domain, ExbB and ExbD

The precise role of ExbB and ExbD in TonB function has so far been elusive. ExbB and ExbD are important for TonB stability *in vivo*, though the effect of an *exbD* mutation is less severe than an *exbB* mutation, and overexpressed ExbB alone is able to stabilize TonB overexpressed from a plasmid (Fischer, *et al.,* 1989; Skare and Postle, 1991). ExbB also stabilizes ExbD at the inner membrane (Fischer, *et al*., 1989). Additionally, ExbB is able to stabilize a cytoplasmic TonB mutant lacking its transmembrane domain, leading to a hypothesis that ExbB acts as a chaperone to stabilize TonB in the cytoplasm and facilitate its insertion into the membrane (Karlsson, *et al*., 1993b). However, ExbB and ExbD are also functionally important; even if TonB is stabilized by the deletion of protease OmpT, it still requires the *exbBD* operon for function (Skare, *et al*., 1993). Furthermore, without ExbB, ExbD, or its N-terminal domain, TonB is unable to respond to pmf. This has led to the characterization of ExbB and ExbD as the "energy-harvesting complex" for TonB (Larsen, *et al*., 1999; Held and Postle, 2002; Postle and Larsen, 2007).

TonB, ExbB, and ExbD all cross-link to one another, and the transmembrane domains of these proteins are known to be important for at least some of these interactions, suggesting these proteins form a complex at the inner membrane (Skare, *et al*., 1993; Jaskula, *et al*., 1994; Ollis, *et al*., 2009). Additionally, it has been shown that TonB and ExbD co-elute with His-tagged ExbB on a nickel affinity column (Braun, *et al*., 1996). However, the stoichiometry of this proposed complex is unknown. The ratio of TonB:ExbB:ExbD in cells is known to be 1:7:2 in both iron-starved and iron-rich conditions, and the ability of the TonB N-terminus to dimerize suggests a potential 2:14:4 complex stoichiometry, but there is no direct evidence for a complex this large (Higgs, *et al*., 2002a). In the Mot system, the complex formed by ExbB and ExbD homologues MotA and MotB has a 4:2 stoichiometry, raising the possibility that the members of the TonB system may form a similar complex, with some ExbB existing outside of the complex as well (Kojima and Blair, 2004). It is important to note that TonB and ExbD also interact via their periplasmic domains, but observable crosslinking is dependent on functional transmembrane domains for both proteins (Ollis, *et al*., 2009).

The TonB transmembrane domain mutations Δ17, S16L, and H20Y, which disrupt TonB function, TonB-ExbB crosslinking, and the pmf-responsiveness of TonB (though these mutants can still fractionate with the outer membrane), can be partially suppressed by ExbB mutations A39E, V36D, and V35E. All of these mutants are also less stable than wild-type TonB, but not unstable enough to account for the loss of function (Larsen, *et al*., 1994; Larsen, *et al*., 1999).

These suppressors are intriguing but mysterious; the reason these suppressors are able to recover some of the lost function is unknown. However, they do provide further evidence that the TonB-ExbB TM domain interactions are crucial for the pmf-responsiveness of TonB, and suggest that TonB interacts with ExbB transmembrane domain I. Further exploration of the TonB transmembrane domain revealed that the only truly essential residue in this domain is the H20 residue - the rest, including S16, can be individually changed to alanine without significantly affecting function. However, it is important to note that the S16 residue does have some significance; when the entire transmembrane domain except S16 and H20 has been replaced with alanine residues, the TonB protein is nearly fully functional, but when S16 is also replaced with alanine in that context, the protein is almost completely inactive (Larsen, *et al*., 2007)

Two mutations have been identified in ExbD that completely disrupt TonB function: D25N, in the single ExbD transmembrane domain, and L132Q, located in the periplasmic space. While both have no detectable activity, ExbD(D25N) is notable in that wild-type ExbD is not able to support TonB system function when the two are coexpressed (Braun, *et al.,* 1996). Both the D25 residue and pmf are important for crosslinking of ExbD to TonB but not ExbD to ExbB (Ollis, *et al*., 2009). In ExbB, E176 (in transmembrane domain III) was identified as important, but in contrast to D25 in ExbD, replacement of the acidic residue with its uncharged analog yielded near-complete TonB functionality; E176D and E176Q both have iron transport rates about 80% of wild type, while E176A is completely deficient in iron transport and colicin import. Thus, it is the size and not the charge of this residue that appears to be important. Additionally, an ExbB (T148A, T181A) double mutant is completely inactive, although alone each of these mutations retains near wild type functionality (Braun and Herrmann, 2004). These residues are found in transmembrane helices II and III, respectively, and though they are not predicted to lie very close to each other in the membrane, these data suggest they have redundant functions.

While ExbB and ExbD are clearly important, hypotheses for their function in the TonB system are varied. One hypothesis is that ExbB and ExbD are transmitting a proton motive force "signal" to TonB to induce or disrupt a particular conformation (Ghosh and Postle, 2005). The combined topologies of the ExbB and ExbD, with large domains in both the cytoplasm and the periplasmic space, suggest a signaling role for these proteins is a possibility. Based on the shared topology between ExbD and TonB, the idea that ExbD acts as a chaperone to assist in disorder-to-order transitions at the TonB C-terminus has also been proposed (Vakharia-Rao, *et al*., 2007). Observed pmf-dependent interactions between the periplasmic domains of TonB and ExbD support this idea (Ollis, *et al*., 2009). ExbD might serve both of these functions; perhaps it is through its function as a chaperone that ExbD is able to transmit the proton motive force signal.

As part of a signal transduction function, it has been suggested ExbB and ExbD might act as liaisons relaying a signal initiated at a proton channel (Larsen, *et al*., 1999). However, homology between the ExbB and ExbD proteins and MotA and MotB suggests that ExbB and ExbD could actually form an ion channel themselves (Cascales, *et al*., 2001). The MotA and MotB proteins are thought to form a proton channel with which they generate torque for the bacterial flagellum. As is the case for ExbD, conserved D32 in MotB transmembrane domain is also known to be crucial for that system's function (Zhou, *et al*., 1998). Zhai, *et al*. propose that the aspartate residue might provide the specificity of the channel, act as a proton donor to a cytoplasmic residue, or confer conformational changes to the complex as a result of its own protonation and

deprotonation. Molecular modeling based on conserved residues in ExbB and ExbD and what is known about the MotA MotB complex has allowed two potential proton pathways in a hypothetical ExbB ExbD ion channel to be identified, but the models assume a 1:1 or 1:1:1 complex of ExbB:ExbD(:TonB), in sharp contrast to the cellular and proposed complex stoichiometry of these proteins (Zhai, *et al*., 2003). Further exploration of this model shows that some residues along the proposed pathways can be mutated without effect on TonB-dependent transport, so these models likely need revision (Braun and Herrmann, 2004).

It has also been suggested that the conformational changes of TonB could involve a proton transfer between TonB H20 to ExbD D25 (Braun and Braun, 2002). This could be incorporated into either the chaperone or the signal transduction functions proposed. However, recent data that TonB H20N shows no detectable decrease in activity contradict that idea (Cheryl Swayne and Kathleen Postle, unpublished data).

In the absence of ExbB, ExbD, TolQ and TolR, TonB fractionates entirely with the outer membrane. Thus, in addition to any of the previously mentioned potential roles for ExbB and ExbD, these proteins might also facilitate the return of TonB from the outer membrane (Letain and Postle, 1997). Similarly, it's been noted that TonB could be protected from direct contact with the inner membrane by existing in complex with ExbB and ExbD, making it thermodynamically more favorable for TonB to leave the inner membrane (Postle and Larsen, 2007).

1.6 The Tol System

The Tol system is of interest in studies of the TonB system because ExbB and ExbD are homologous to TolQ and TolR; 26.3% and 25% of amino acids are identical and 79.1% and 70% have similar chemical properties, respectively (Eick-Helmerich and Braun, 1989). Topology of TolQ resembles that of ExbB, with its N-terminus in the periplasmic space, three transmemebrane domains, and a large cytoplasmic loop between transmembrane domains I and II (Vianney, *et al*., 1994). TolR topology is likewise similar to that of ExbD, with a single transmembrane domain and a large C-terminal domain in the periplasmic space (Muller, *et al*., 1993). Tol system mutants exhibit a pleiotropic phenotype: they are hypersensitive to deleterious agents such as bile salts and antibiotics, form outer membrane vesicles, and "leak" proteins from the periplasmic space (Bernadac, *et al*., 1998; Lazzaroni, *et al*., 1999). Mutations in the Tol system may also affect cell division (Gerding, *et al*., 2007). The complete Tol system includes the TolA, TolQ and TolR proteins, thought to interact with each other via their transmembrane domains at the inner membrane, and the TolB-Pal complex at the outer membrane (Bouveret, *et al*., 1995; Derouiche, *et al*., 1995; Lazzaroni, *et al*., 1995; Germon, *et al*., 1998). In the absence of ExbB and ExbD, TolQ and TolR can partially support TonB function, and vice versa. However, the crosstalk between these systems is low enough that it is not thought to be relevant to the function of either system under normal conditions (Braun, 1989; Braun and Herrmann, 1993; Brinkman and Larsen, 2008).

A set of colicins distinct from the colicins that have parasitized TonB, called A group colicins, require Tol system proteins for import, but in contrast to B group colicins, import of A group colicins does not seem to require energy or a functional energy harvesting complex for passage

into the cell (Cascales, *et al*., 2007; Goemaere, *et al*., 2007a). It has been suggested that A group colicins enter by a Brownian ratchet mechanism, where random motion causes the colicins to first enter transporters, but binding to the Tol proteins to prevents their backwards motion out of the cell (Journet, *et al*., 2001).

TolA is the counterpart to TonB that utilizes the energy harvested, presumably by TolQ and TolR, at the inner membrane (Lloubès, *et al*., 2001). While TolA and TonB are not homologous, they do share a topology and a conserved $SX_3HX_6LX_3S$ motif at the inner membrane, and fusions of the TolA N-terminal domain with the TonB periplasmic domain are still able to support TonB-dependent transport (Braun and Herrmann, 1993; Koebnik, 1993). Similar to mutational studies on TonB, the S18L substitution and mutations at H22 in TolA prevented crosslinking to TolQ and TolR, and impaired function as assayed by both by outer membrane permeability and colicin sensitivity. In an additional commonality between the systems, suppressors for the TolA transmembrane domain mutations have been isolated in transmembrane domain I of TolQ (Germon, *et al*., 1998).

Like TonB, TolA undergoes a conformational change in response to proton motive force. However, when a functional transmembrane domain or the energy-harvesting complex is absent in the Tol system, the proteinase K-resistant fragment that normally appears only in spheroplasts where pmf has been dissipated now appears in untreated spheroplasts as well (Germon, *et al*., 2001). This is different from the pattern observed for the TonB system, where proteinase K-resistant fragments do not appear at all, even in the treated spheroplasts, if a functional transmembrane domain or any component of the energy-harvesting complex is missing (Larsen, *et al*., 1999; Held and Postle, 2002).

The characteristics of TonB and TolA go on to further diverge. TolA has never been implicated in nutrient acquisition; instead, co-immunoprecipitation experiments reveal that the conformational change in response to pmf might allow TolA to interact with the protein Pal at the outer membrane (Cascales, *et al*., 2000). This interaction also requires both TolQ and TolR (Cascales, *et al*., 2001). This is quite different from TonB, which is able to interact with the outer membrane independently of pmf (Jaskula, *et al*., 1994; Letain and Postle, 1997). While a direct link between TolA-Pal interaction and outer membrane stability has not been demonstrated, it has been shown that transmembrane mutants completely or partially deficient in outer membrane stability are accordingly completely or partially unable to co-immunoprecipitate Pal (Cascales, *et al*., 2001; Goemaere, *et al*., 2007a).

Both similarities and differences can also be found in the details of the interactions between members of the energy-harvesting complexes. Like ExbD, TolR is able to dimerize through both its transmembrane and periplasmic domains (Journet, *et al*., 1999; Goemaere, *et al*., 2007b; Zhang, *et al*., 2009). As is the case their TonB homologues, TolR is stabilized by TolQ, but in contrast to the TonB system, TolQ has not shown a detectable stabilizing effect on TolA (Journet, *et al*., 1999). Importantly for the present study, the D23 residue in TolR (which is analogous to D25 in ExbD) is important for energy-requiring functions of the Tol system (Cascales, *et al*., 2001; Goemaere, *et al*., 2007a).

That the Tol system is necessary for both energy-dependent and energy-independent functions of

adds a level of interpretation to studies of the Tol system transmembrane domains not found in the TonB system. While some mutations are deficient in both categories of function, others are only deficient in the energy-requiring functions; for example, sensitivity to colicins is reduced for S18 and H22 mutations in TolA, but is unaffected in mutations of the TolR D23 residue or other polarized residues in TolQ that are nonetheless necessary for outer membrane stability (Germon, *et al*., 1998; Goemaere, *et al*., 2007a). This has been interpreted as a way to differentiate between residues important for responding to pmf and residues with other functions, but this interpretation is imperfect. For a few residues in TolQ, substitutions of a given residue with certain amino acids result in both outer membrane stability and colicin import defects, while substitutions of with other amino acids at the same position are only deficient in outer membrane stability. As an example, T178M is less sensitive to colicins, while T178A is fully sensitive, but both show a destabilized outer membrane phenotype. For at least one set of mutants that fall into this category, it appears that the mutation that prohibits colicin import also does not allow the TolQRA complex to form, a viable explanation for these discrepancies (Goemaere, *et al*., 2007a). Still, these examples highlight the careful way that these data must be interpreted.

There is some indication that, despite their homology, significant differences in the mechanisms of the Tol- and TonB-system energy harvesting complexes exist. One example is the differences between phenotypes of mutations at the glutamate 173 residue in TolQ and the analogous glutamate 176 in ExbB. For E173 in TolQ, both glutamine and leucine substitutions exhibit both the outer membrane destabilized and colicin tolerant phenotypes, raising the possibility that the role for this residue is unrelated to proton motive force (Goemaere, *et al*., 2007a). However, although TolQ(E173Q) is completely deficient in observable Tol-dependent functions, it is still able to support some TonB function in the absence of ExbB, as is the ExbB(E176Q) mutant (Braun and Herrmann, 2004). This could indicate that the charge on this residue in TolQ is required for the protein to specifically interact with TolA, but not TonB, or it could reflect a difference in sensitivities of the Tol and TonB systems to mutations. However, it might also suggest that the TolQ and TolR proteins function quite differently from ExbB and ExbD.

The ExbB T148 and T181 residues and their analogs T145 and T178 in TolQ provide another example of differences between the function of conserved transmembrane residues. Individual alanine substitutions at ExbB T148 or T181 are fully active, though a double mutant with mutations at these two points has no detectable activity, suggesting a redundant function (Braun and Herrmann, 2004). In the Tol system, though, an alanine substitution at just one of these positions completely abolishes outer membrane stability but not colicin import, indicating that these residues are both important for responding to pmf (Goemaere, *et al*., 2007a). Note, however, that protein levels were determined in the former experiment using an indirect and sometimes unreliable method, which may account for this discrepancy.

Particularly important for this work, early studies on the D23 residue in TolR showed that while D23R is completely unable to support TolA-Pal immunoprecipitation and outer membrane stability, D23A supported some TolA-Pal interaction and exhibited a less severe outer membrane defect (Cascales, *et al*., 2001). In contrast, D25A in ExbD shows no detectable activity (Ollis, *et al*., 2009). This result is particularly intriguing because alanine should not support any of the functions proposed for the aspartate residue. It is possible that another residue in the Tol system can partially function for D23 when the system is not disrupted by the insertion of a positively

charged residue at the D23 position. That TolR(D23A) is later characterized as completely inactive might reflect different sensitivities of assays, or indicate that this was an anomalous result perhaps related to protein expression levels, but this is not clear (Goemaere, *et al*., 2007a).

The many differences between the systems should be kept in mind when considering how findings in the Tol system might relate to the TonB system. However, research that has been done in the Tol system does have interesting implications for explorations of ExbD and ExbB. In particular, the suppression of D23A by an A185D substitution in TolQ is intriguing. A185 is at a similar height in the membrane as D23 (Goemaere, *et al*., 2007a). This suggests that the two proteins might work together to the same end, as would be the case with an ion channel, or that they interact with one another such that swapping residues is able to restore a tight, lock and key fit that is lost when just one is mutated. It's also interesting to note that within TolQ, E173 mutations in the TolQ helix III can be suppressed by H151E, which falls at position of similar height in the helix II (Goemaere, *et al*., 2007a). Additionally, a recent study of disulfide crosslinking in the TolR transmembrane domain has introduced the possibility that the protein is able to rotate within a complex to interact with different TolQ components of the complex at different times (Zhang, *et al*., 2009).

In the present study, the spatial requirement of the charged residue in the ExbD transmembrane domain was explored. Twenty ExbD double substitution mutants where the essential aspartate was replaced by alanine and another residue of the transmembrane domain was replaced with aspartate were constructed, one for each residue other than aspartate 25 of the putative transmembrane domain. These are referred to as “aspartate relocation” mutants. Each was assayed for TonB system activity by determining the sensitivity of these mutants to colicins and bacteriophage ϕ80, which have parasitized the TonB system. TonB-dependent activity could not be detected for any of these mutants, indicating either a strict spatial requirement for the charged residue in the ExbD transmembrane domain or a requirement for the correct positioning of other residues in the transmembrane domain relative to aspartate. It should also be noted that other residues in the ExbD transmembrane domain might independently be important for TonB system activity, thus hypothetically preventing an aspartate substitution at that position from being active even if the role of the aspartate residue itself could be fulfilled from that location. This seems unlikely, though, because recent cysteine scanning of the transmembrane domain showed that cysteine substitutions at all positions except D25 were active (Mingchao Xie and Kathleen Postle, unpublished data). In addition, the introduction of a charged residue at a different location in the transmembrane domain may have unintended effects on the insertion, stability, or protein-protein interactions of ExbD. Indeed, two of the ExbD variants generated for this study could not be detected by immunoblotting with a polyclonal antibody, and another shows growth impairment in the presence of ampicillin.

2. Methods

2.1 Plasmid Construction

Plasmids were constructed using QuickChange site-directed mutagenesis to generate a single amino acid substitution in template plasmid. All plasmids were constructed using pKP1191, a pPro24-derivative plasmid encoding ExbD(D25A), as a template, with complementary primers encoding base pair changes for the appropriate aspartate substitution (Lee and Keasling, 2005; Ollis, et al., 2009). See **Appendix** for primer sequences and Polymerase Chain Reaction (PCR) reaction conditions. PCR product was digested with 2 μL DpnI 4-6 hours to remove template DNA and concentrated by ethanol precipitation, after which the pelleted DNA was resuspended in 10 μL ddH_20. Concentrated PCR product (1-5 μL) was electroporated into electrocompetent DH5α with a 1.8 kV shock, expanded in SOC broth, plated onto Luria-Bertani (LB) plates supplemented with 100 μg mL^{-1} ampicillin, and incubated overnight at 37°C. Colonies from this plate were streaked onto LB plates supplemented with 100 μg mL^{-1} ampicillin. Plasmids from these clones were isolated from 10 mL overnight LB cultures supplemented with 100 μg mL^{-1} ampicillin via alkaline lysis using the QIAPrep Spin Miniprep kit (Qiagen, Inc., Santa Clara, CA). The *exbD* gene of the isolated plasmids was sequenced using the primer oKP1032 (5' CAAATTGAAATTAAACATT 3') by The Pennsylvania State University Nucleic Acid Facility, University Park, PA, to screen for the targeted mutation and verify that no mutations other than those targeted exist in the *exbD* gene.

Plasmids were transformed into RA1045 by TSS transformation, incubated 1 hour in LB at 37°C with shaking, plated onto LB plates supplemented with 100 μg mL^{-1} ampicillin, and incubated overnight at 37°C (Chung, *et al.*, 1989). **Table 1** provides a complete list of all strains and plasmids used.

Strain	Relevant Phenotype	Reference
W3110	wild type	Hill and Harnish, 1981
RA1045	Δ*exbD* Δ*tolQR*	Brinkman and Larsen, 2008
RA1045/pKP999	pExbD	Ollis, *et al*., 2009
RA1045/pKP1191	pExbD(D25A)	Ollis, *et al*., 2009
RA1045/pKP1197	pExbD(D25A, V43D)	this study
RA1045/pKP1199	pExbD(D25A, T42D)	this study
RA1045/pKP1201	pExbD(D25A, V26D)	this study
RA1045/pKP1202	pExbD(D25A, V29D)	this study
RA1045/pKP1213	pExbD(D25A, M27D)	this study
RA1045/pKP1214	pExbD(D25A, L40D)	this study
RA1045/pKP1215	pExbD(D25A, A41D)	this study
RA1045/pKP1229	pExbD(D25A, L28D)	this study
RA1045/pKP1272	pExbD(D25A, L31D)	this study
RA1045/pKP1273	pExbD(D25A, I24D)	this study
RA1045/pKP1276	pExbD(D25A, F23D)	this study
RA1045/pKP1277	pExbD(D25A, I32D)	this study
RA1045/pKP1287	pExbD(D25A, I33D)	this study
RA1045/pKP1288	pExbD(D25A, F34D)	this study
RA1045/pKP1322	pExbD(D25A, M35D)	this study
RA1045/pKP1349	pExbD(D25A, A38D)	this study
RA1045/pKP1351	pExbD(D25A, V36D)	this study
RA1045/pKP1359	pExbD(D25A, A37D)	this study
RA1045/pKP1360	pExbD(D25A, P39D)	this study
RA1045/pKP1413	pExbD(D25A, L30D)	this study

Table 1: *Escherichia coli* Strains and Plasmids Used

2.2 Inductions

To determine levels of inducer needed to express the ExbD mutants at wild type levels, overnight LB cultures with 100 μg mL^{-1} ampicillin were subcultured 1:100 into T-broth supplemented with 100 μg mL^{-1} ampicillin and various concentrations of sodium propionate, pH8 (NaProp) or glucose to induce or repress, respectively, expression from the pPro24-derivative plasmids (Lee and Keasling, 2005). Subcultures were grown to exponential growth phase (about 0.5 OD_{550} in Spectronic 20 spectrophotometer with a 1.5 cm path length) and 0.2 ODmL was harvested. Harvested culture was combined with an equal volume of 20% (w/v) trichloroacetic acid (TCA) and TCA precipitated as previously described (Postle, 2007).

TCA precipitated protein samples were electrophoresed on 15% SDS polyacrylamide gels prepared as described previously (Laemmli, 1970). Electrophoresed proteins were transferred to polyvinylidene fluoride (PVDF) membranes 7 hours at 20V, 2 hours at 50V, or 1 hour at 100V. Membranes were incubated in blocking buffer (5% powdered milk, 0.1% Tween-20 in 1X PBS) 30 minutes, incubated in blocking buffer with 1:5000, 1:10000, 1:15000 anti-ExbD polyclonal antibody 1 hour, washed with blocking buffer for three ten minute washes, incubated with blocking buffer and 1:10000 horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Sigma-Aldrich, St. Louis, MO) 1 hour, and washed with 0.1% Tween-20 in 1X PBS for three ten minute washes. Polyclonal anti-ExbD antibody was created by Penelope Higgs (Higgs, *et al*., 2002a). Signal was detected using chemiluminescent Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Rockford, IL) and exposed to x-ray film with exposures ranging from less than 5 seconds to overnight. Membranes were stained with Coomassie blue to

ensure that equal amounts of protein were loaded in each lane.

2.3 Spot Titer Assays

Spot titers assays were performed as described previously (Postle, 2007). To summarize, 1:100 T-broth subcultures were grown to exponential growth phase, harvested in the volume of 0.08 ODmL per plate, combined with 3 mL melted T-top agar per plate, and plated in 3 mL aliquots on T-plates. T-broth subcultures, T-top agar and T-plates were supplemented with 100 μg mL^{-1} ampicillin for all strains carrying a plasmid, and appropriate amounts of NaProp or glucose were added to achieve approximately wild type levels of ExbD expression from plasmids. Five-fold serial dilutions of colicins B, Ia, and M and ten-fold serial dilutions of ϕ80 in λ-Ca^{++} buffer were prepared and for each agent, 10 μL of each dilution was spotted onto three plates per strain. For each strain expressing mutant ExbD, an undiluted spot of each agent was also spotted to assay for very low levels of activity. Plates were incubated upright for 18 hours at 37°C and scored by the highest dilution at which clearing of the bacterial lawn could be seen.

To check levels of expression of ExbD, T-broth subcultures used for plating were harvested, TCA precipitated, electrophoresed, and immunoblotted as described for inductions.

2.4 *In vivo* Formaldehyde Cross-Linking

Formaldehyde cross-linking was performed essentially as described previously, using 1:100 T-broth subcultures supplemented with 100 μg mL^{-1} ampicillin and the appropriate amount of glucose or NaProp as determined by inductions (Higgs, *et al.*, 1998). Samples were solubilized in 1X Laemmli sample buffer and electrophoresed on a 13% SDS-polyacrylamide gel, transferred to PVDF membrane 20V, 7 hours, and immunoblotted as described for inductions using 1:5000 anti-ExbD polyclonal antibody.

2.5 Media and Culture Conditions

LB, T-broth, T-plates and T-top agar were prepared as previously described (Miller, 1972).

To maintain plasmids, strains with plasmids were cultured, subcultured and plated with 100 μg mL^{-1} ampicillin, except where otherwise noted. RA1045/pKP1197, which is hypersensitive to ampicillin, was maintained on LB plates with 100 μg mL^{-1} ampicillin but could not grow in overnight LB culture with 100 μg mL^{-1} ampicillin. For this reason, all RA1045/pKP1197 overnight cultures were grown without ampicillin, but in subsequent subculturing and plating for spot titers 100 μg mL^{-1} ampicillin was used.

Growth curves were generated by taking OD_{550} measurements of 1:100 T-broth subcultures every half hour.

3. Results

3.1 Some "Aspartate Relocation" Mutants Have Notably Higher or Lower Steady State ExbD Levels Compared to Wild Type ExbD

In order to achieve conditions in which the activity of wild type ExbD and the mutated ExbD proteins could be directly compared, inductions at a range of inducer concentrations were performed to determine the level of inducer needed to achieve detection of the mutant proteins at approximately chromosomal levels. Expression of the mutant ExbD proteins was induced from the pPro24-derivative plasmids with sodium propionate, pH8 (Lee and Keasling, 2005). For some ExbD mutants, baseline protein levels were higher than those of chromosomally encoded ExbD, so expression was repressed with glucose. Achieving levels close to those of chromosomally-encoded ExbD is important for the interpretation of the spot titer assays.

Expression of all but three ExbD mutants at near wild type levels can be achieved with 1mM or less NaProp, or with 0.01% or less glucose. For comparison, wild type ExbD requires 0.5 mM NaProp for expression at approximately chromosomal levels. **Table 2** shows levels used to achieve expression closest to wild type for each mutant based on inductions. **Figure 2** shows levels of expression of all mutants at the concentration of inducer or repressor given in **Table 2**.

Also apparent in **Figure 2** is a shift in the migration of some ExbD variants on the SDS-PAGE gel. This is not surprising, as single amino acid substitutions can often affect the apparent molecular mass of a protein (Noel, *et al*., 1979).

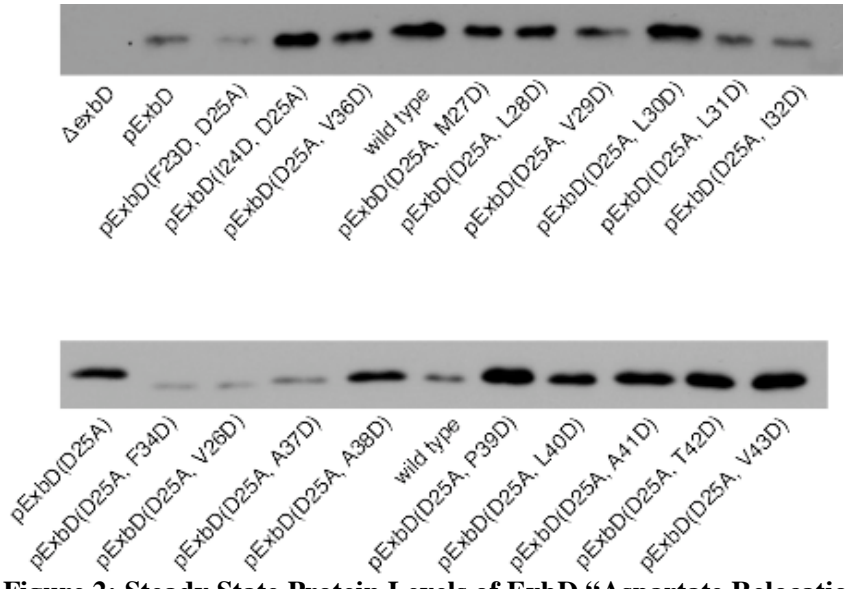

Figure 2: Steady State Protein Levels of ExbD "Aspartate Relocation" Mutants At Inducer or Repressor Levels Determined to be Closest to Wild Type. T-broth 1:100 subcultures were grown to exponential growth phase, harvested, TCA precipitated, electrophoresed on a 15% SDS-polyacrylamide gel, and immunoblotted with polyclonal anti-ExbD antibody to verify expression levels with the levels of inducer or repressor given in Table 2. pExbD(D25A, I33D) and pExbD(D25A, M35D), for which expression cannot be detected, were not included.

	Relevant Phenotype	[NaProp] or % glucose
RA1045/pKP999	pExbD	0.5 mM NaProp
RA1045/pKP1191	pExbD(D25A)	0.5% glucose
RA1045/pKP1276	pExbD(D25A, F23D)	-
RA1045/pKP1273	pExbD(D25A, I24D)	0.5 mM NaProp
RA1045/pKP1201	pExbD(D25A, V26D)	1 mM NaProp
RA1045/pKP1213	pExbD(D25A, M27D)	0.01% glucose
RA1045/pKP1229	pExbD(D25A, L28D)	1 mM NaProp
RA1045/pKP1202	pExbD(D25A, V29D)	0.01% glucose
RA1045/pKP1413	pExbD(D25A, L30D)	1 mM NaProp
RA1045/pKP1272	pExbD(D25A, L31D)	-
RA1045/pKP1277	pExbD(D25A, I32D)	-
RA1045/pKP1287	pExbD(D25A, I33D)	50 mM – NO EXPRESSION
RA1045/pKP1288	pExbD(D25A, F34D)	1 mM NaProp
RA1045/pKP1322	pExbD(D25A, M35D)	50 mM – NO EXPRESSION
RA1045/pKP1351	pExbD(D25A, V36D)	-
RA1045/pKP1359	pExbD(D25A, A37D)	-
RA1045/pKP1349	pExbD(D25A, A38D)	-
RA1045/pKP1360	pExbD(D25A, P39D)	0.5% glucose
RA1045/pKP1214	pExbD(D25A, L40D)	0.0005% glucose
RA1045/pKP1215	pExbD(D25A, A41D)	1 mM NaProp
RA1045/pKP1199	pExbD(D25A, T42D)	0.005% glucose
RA1045/pKP1197	pExbD(D25A, V43D)	-

Table 2: Levels of Inducer (NaProp) or Repressor (Glucose) Needed for Expression of Mutant ExbD at Approximately Chromosomal Levels T-broth 1:100 subcultures in T-broth with different levels of inducer or repressor were grown to exponential growth phase, TCA precipitated, electrophoresed on a 15% SDS-polyacrylamide gel, transferred to PVDF membrane and immunoblotted with polyclonal anti-ExbD antibody to determine levels of inducer or repressor necessary for chromosomal levels of expression. The values given represent the best approximation of appropriate levels for each mutant.

Two proteins, ExbD(D25A, I33D) and ExbD(D25A, M35D) could not be detected on Western blots even with 50 mM NaProp, the maximum amount of inducer tested. For both strains, the plasmid preparation was retransformed into RA1045 by TSS transformation and the experiment was repeated to ensure that a spontaneous mutation unique to the clone used was not causing this result. The same result was observed. **Figure 3** shows inductions for these mutants.

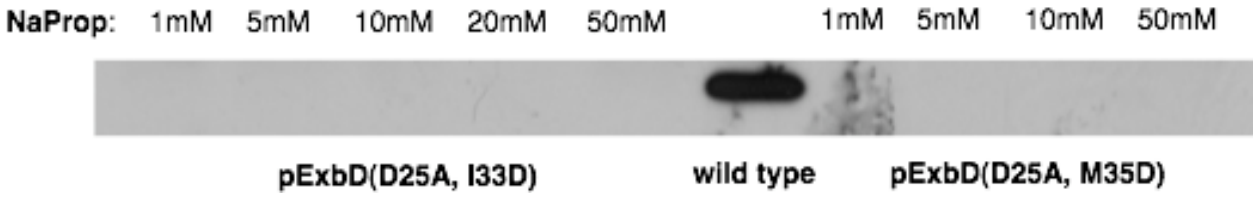

Figure 3: Detectable Expression of ExbD(D25A, I33D) and ExbD(D25A, M35D) Cannot Be Induced from a pPro24-Derivative Plasmid TCA precipitated protein from 1:100 T-broth subcultures were harvested in exponential growth phase and run on 15% SDS polyacrylamide gel, transferred to PVDF membrane, immunoblotted with polyclonal anti-ExbD antibody, and detected with chemiluminescence. High levels of inducer (NaProp) were used to try to achieve detectable levels of these proteins. For comparison, 0.5mM NaProp is sufficient to achieve chromosomal levels of pExbD expression. Overnight exposure to film is shown. While a strong signal for wild type, chromosomal ExbD is observed, no ExbD can be detected for pExbD(D25A, I33D) or pExbD(D25A, M35D).

In contrast, ExbD(D25A) and ExbD(D25A, P39D) are detected at much higher levels than the other ExbD mutants generated. Both RA1045/pKP1191 and RA1045/pKP1360 showed ExbD levels significantly higher than the native levels of ExbD even with 0.5% glucose used to repress transcription from the plasmid. **Figure 4** shows inductions for these mutants, and the levels of both proteins with 0.5% glucose can also be seen in **Figure 2**.

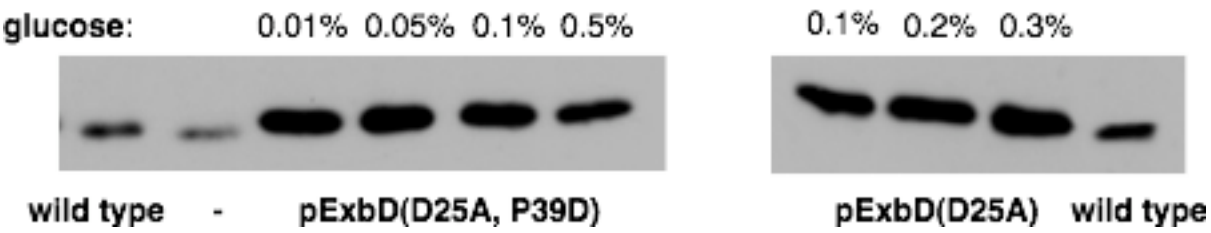

Figure 4: ExbD(D25A) and ExbD(D25A, P39D) Are Detected At High Levels Even With Large Amounts of Repressor TCA precipitated protein from 1:100 T-broth subcultures were harvested in exponential growth phase and run on 15% SDS polyacrylamide gel, transferred to PVDF membrane, immunoblotted with polyclonal anti-ExbD antibody, and detected with chemiluminescence. The signal for pExbD(D25A) and pExbD(D25A, P39D) is significantly stronger than the signal for wild type, chromosomal ExbD even when 0.3% or 0.5% glucose is used to repress expression from the plasmid. (Note: The level of pExbD(D25A) observed with 0.1% glucose, which should be higher than the levels observed with 0.2% and 0.3%, appears to be affected by its location on the edge of the gel, which sometimes diminishes signal. Nonetheless, the high levels of pExbD(D25A) relative to wild type even with large amounts of glucose can still be clearly observed.)

3.2 All ExbD "Aspartate Relocation" Mutants are Completely Tolerant to B Group Colicins and ϕ80

ExbD aspartate 25 is essential for TonB system function (Braun, *et al*., 1996; Ollis, *et al*., 2009). To determine if an aspartate residue at another position in the transmembrane domain could support TonB system function in the absence of aspartate 25, the activity of the TonB system with the "aspartate relocation" ExbD mutants was assessed using spot titer assays. The assay analyzed the sensitivity of these strains to colicins B, Ia, M and bacteriophage ϕ80, which parasitize the TonB system for entry into the cell. This assay was chosen because it is sensitive to low levels of TonB system activity (Larsen, *et al*., 2003). All mutants assayed were completely tolerant to all agents tested. **Table 4** provides a summary of all assays performed, and **Figure 5** shows ExbD levels for each spot titer assay.

Some of these assays were performed before the best levels of inducer or repressor to achieve chromosomal ExbD levels had been determined, and for other plasmids chromosomal levels of the mutant ExbD proteins cannot be achieved. It is unlikely, but possible, that for the mutant ExbD proteins that are underexpressed, higher levels of expression would support a very small amount of colicin or ϕ80 transport. Similarly, ExbD overexpression may have a dominant negative effect on TonB system activity (Anne Ollis and Kathleen Postle, unpublished data). Therefore, it is possible that lower levels of expression in strains in which ExbD is overexpressed would support low levels of activity as well. Had activity been observed for these mutants that

are underexpressed or overexpressed, the differing levels of expression might have affected quantification of the functionality of the mutant proteins. However, particularly because all of the mutant ExbD proteins generated here were completely tolerant to B group colicins and ϕ80, varied protein levels do not prevent reasonable interpretation of the data.

ST #	*Strain*	*ExbD*	*[NaProp], %gluc*	*ColB*	*ColIa*	*ColM*	*ϕ80*
1	W3110	wild type		777	666	457	788
1	RA1045	--		T	T	T	T
1	RA1045/pKP999	pExbD	1mM NaProp	666	667	555	777
1	RA1045/pKP1191	pExbD(D25A)	0.01% glucose	T	T	T	T
1	RA1045/pKP1202	pExbD(D25A, V29D)	0.01% glucose	T	T	T	T
2	W3110	wild type		677	666	444	788
2	RA1045	--		T	T	T	T
2	RA1045/pKP1213	pExbD(D25A, M27D)	0.01% glucose	T	T	T	T
3	W3110	wild type		667	666	44	778
3	RA1045	--		T	T	T	T
3	RA1045/pKP1201	pExbD(D25A, V26D)	-	T	T	T	T
3	RA1045/pKP1214	pExbD(D25A, L40D)	0.005% glucose	T	T	T	T
3	RA1045/pKP1229	pExbD(D25A, L28D)	1mM NaProp	T	T	T	T
4	W3110	wild type		777	777	444	888
4	RA1045	--		T	T	T	T
4	RA1045/pKP1191	pExbD(D25A)	0.05% glucose	T	T	T	T
4	RA1045/pKP1202	pExbD(D25A, V29D)	0.01% glucose	T	T	T	T
4	RA1045/pKP1213	pExbD(D25A, M27D)	0.01% glucose	T	T	T	T
4	RA1045/pKP1214	pExbD(D25A, L40D)	0.005% glucose	T	T	T	T
5	W3110	wild type		778	666	444	888
5	RA1045	--		T	T	T	T
5	RA1045/pKP1272	pExbD(D25A, L31D)	-	T	T	T	T
5	RA1045/pKP1276	pExbD(D25A, F23D)	-	T	T	T	T
5	RA1045/pKP1277	pExbD(D25A, I32D)	-	T	T	T	T
6	W3110	wild type		888	777	555	889
6	RA1045	--		T	T	T	T
6	RA1045/pKP1215	pExbD(D25A, A41D)	1mM	T	T	T	T
6	RA1045/pKP1288	pExbD(D25A, F34D)	0.5mM	T	T	T	T
7	W3110	wild type		777	555	444	888
7	RA1045	--		T	T	T	T
7	RA1045/pKP1273	pExbD(D25A, I24D)	-	T	T	T	T
7	RA1045/pKP1349	pExbD(D25A, A38D)	0.005% glucose	T	T	T	T
7	RA1045/pKP1351	pExbD(D25A, V36D)	-	T	T	T	T
8	W3110	wild type		777	667	555	899
8	RA1045	--		T	T	T	T
8	RA1045/pKP1197	pExbD(D25A, V43D)	-	T	T	T	T
8	RA1045/pKP1359	pExbD(D25A, A37D)	-	T	T	T	T
8	RA1045/pKP1360	pExbD(D25A, P39D)	0.01% glucose	T	T	T	T
9	W3110	wild type		888	666	666	10 10 10
9	RA1045	--		T	T	T	T
9	RA1045/pKP1197	pExbD(D25A, V43D)	-	T	T	T	T
9	RA1045/pKP1202	pExbD(D25A, V29D)	0.01% glucose	T	T	T	T
9	RA1045/pKP1213	pExbD(D25A, M27D)	0.01% glucose	T	T	T	T
9	RA1045/pKP1272	pExbD(D25A, L31D)	-	T	T	T	T
9	RA1045/pKP1273	pExbD(D25A, I24D)	0.5 mM NaProp	T	T	T	T
9	RA1045/pKP1276	pExbD(D25A, F23D)	-	T	T	T	T
10	W3110	wild type		777	666	665	999
10	RA1045	--		T	T	T	T
10	RA1045/pKP999	pExbD	-	665	666	655	888
10	RA1045/pKP1199	pExbD(D25A, T42D)	0.005% glucose	T	T	T	T
10	RA1045/pKP1349	pExbD(D25A, A38D)	-	T	T	T	T
10	RA1045/pKP1413	pExbD(D25A, L30D)	0.5 mM NaProp	T	T	T	T

Table 4: **Spot Titer Assays for "Moved Aspartate" Mutants** T-broth 1:100 subcultures for each mutant were grown to exponential phase, combined with melted T-top agar and appropriate supplements, and plated on T-plates with appropriate supplements. Five fold dilutions of colicins B, Ia, and M and ten fold dilutions of bacteriophage ϕ80 were spotted onto solidified T-top agar. Plates were incubated upright 18 hours at 37°C and given

scores based on the highest dilution at which clearing of the bacterial lawn was seen for each agent; for example, a score of 7 means for a colicin means that clearing was seen at a dilution of 5^{-7} but not 5^{-8}. Assays for ExbD(D25A), ExbD(D25A, F23D), ExbD(D25A, L31D), ExbD(D25A, A38D), ExbD(D25A, L40D), and ExbD(D25A, V43D) were performed twice. Assays for ExbD(D25A, M27D) and ExbD(D25A, V29D) were performed three times. All others were performed once. Activity of ExbD(D25A, I33D) and ExbD(D25A, M35D), for which no ExbD could be detected by immunobloting, was not assayed.

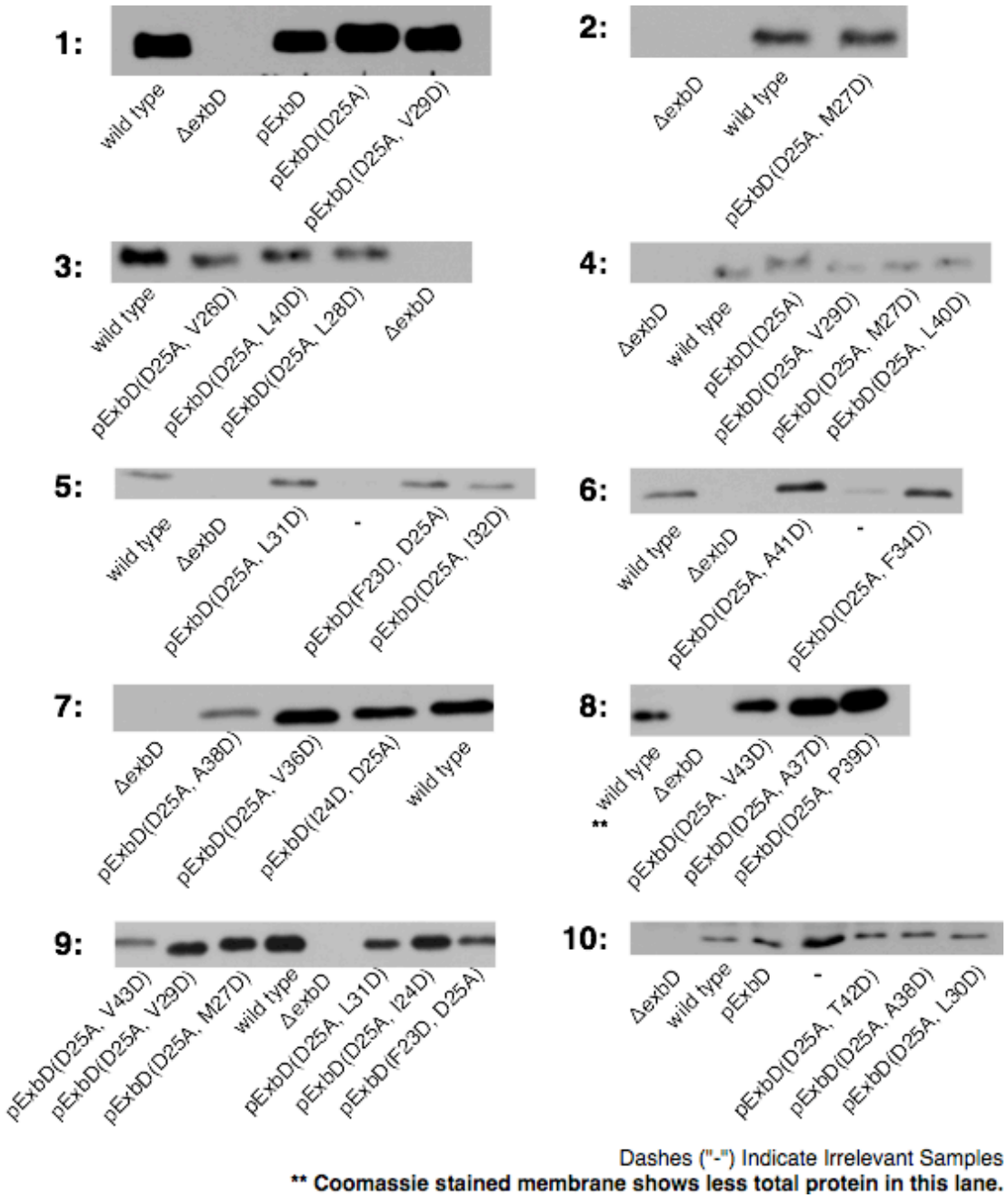

Figure 5: Expression of ExbD in Spot Titer Assays T-broth 1:100 subcultures were grown to exponential growth phase, harvested, TCA precipitated, electrophoresed on a 15% SDS-polyacrylamide gel, and immunoblotted with polyclonal anti-ExbD antibody to verify expression levels during each spot titer (see **Table 4** for amounts of glucose and NaProp used)

3.3 “Aspartate Relocation” Mutants Cross-Link *In vivo* to ExbB

That TonB system activity for the ExbD "aspartate relocation" variants was not detected could be explained by two potential interpretations: the relocated aspartate residue was not able to support TonB function, or the movement of the aspartate residue disrupted the signal sequence of ExbD, preventing insertion of the protein into the membrane, a known effect of aspartate insertions in other *E. coli* proteins (Gierasch, 1989). To distinguish between these two possibilities, *in vivo* formaldehyde crosslinking experiments were performed to determine if the detectable “aspartate relocation” variants could crosslink to ExbB or TonB, both interactions that would be indicative of a cytoplasmic membrane location and assembly of the energy-transduction complex. Wild-type ExbD crosslinks *in vivo* to both ExbB and TonB, while in contrast, ExbD(D25A) crosslinks with ExbB but not with TonB (Ollis, *et al.*, 2009). Because none of the mutants in which aspartate was reintroduced into the transmembrane domain of ExbD(D25A) were able to recover function, these variants were expected to exhibit the same crosslinking profile as ExbD(D25A). The TonB-ExbD complex could not be detected with wild type ExbD on this immunoblot due to the weak signal, so the formation of this complex could not be assessed. However, ExbB-ExbD complex at 41kDa could be detected for wild type ExbD and for all of the stable mutant ExbD proteins. Thus, it can be concluded that insertion of these proteins into the membrane does still occur. The ExbD monomer appears to have migrated off the gel in the first immunoblot pictured. The prominent band at around 15 kDa in the second immunoblot is likely ExbD monomer, but this cannot be verified because it cannot be compared to the strain in which the *exbD* gene is deleted.

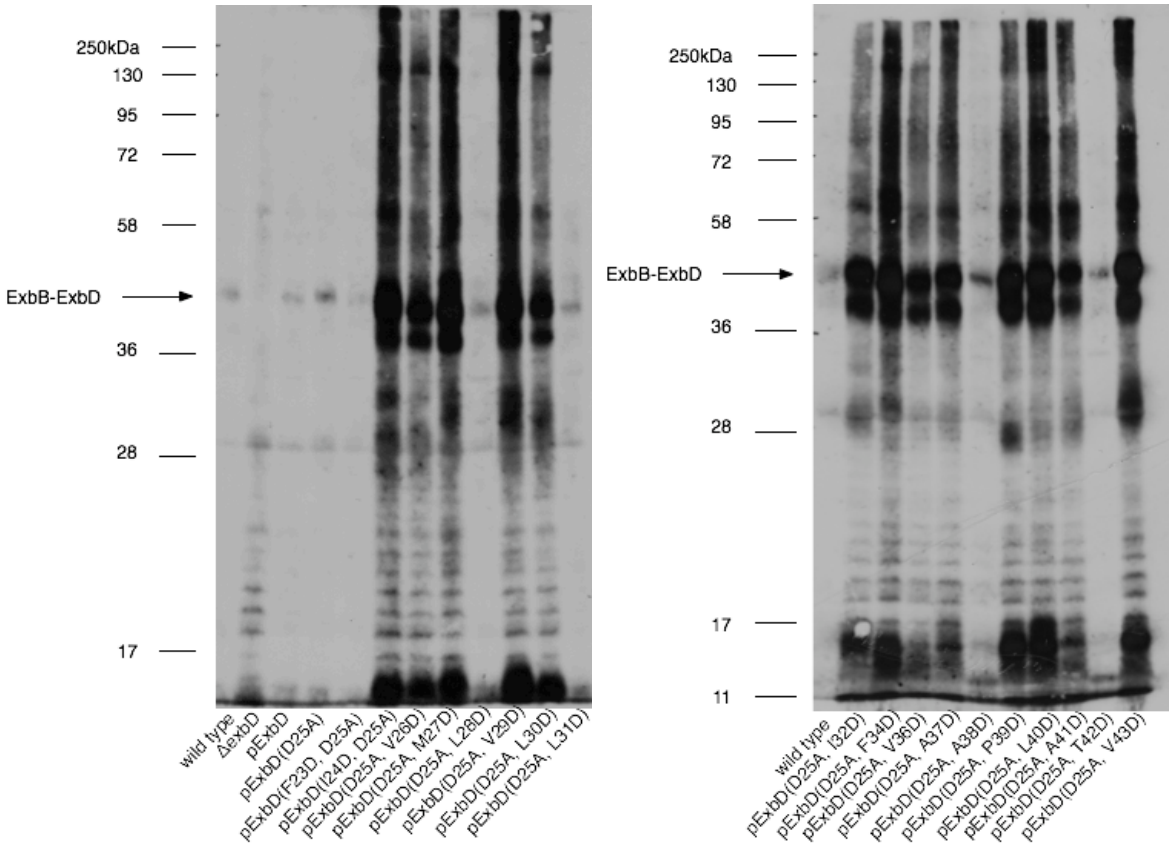

Figure 6: Anti-ExbD immunoblot of *In Vivo* Formaldehyde Cross-Linking of "Aspartate Relocation" Mutants T-broth 1:100 subcultures supplemented according to Table 2 were grown to exponential growth phase and harvested, and proteins were cross-linked with 1% formaldehyde. Samples were then pelleted and combined with 1X Laemmli sample buffer, then heated to 60°C for 5 minutes. Samples were run on a large 13% SDS-polyacrylamide gel and immunoblotted with 1:5000 polyclonal anti-ExbD antibody.

3.4 RA1045/pExbD(D25A, V43D) May Be Hypersensitive to Ampicillin, or ExbD(D25A, V43D) May Be Growth Inhibitory

During the course of these experiments, it was noticed that one strain exhibited impaired growth in the presence of ampicillin, which was used to maintain the plasmids constructed here. All plasmids in this experiment were derived from pPro24, which contains an ampicillin resistance cassette, and were maintained on plates and in culture with 100 μg mL^{-1} ampicillin. However, the strain RA1045/pKP1197, which encodes pExbD(D25A, V43D) in a Δ*exbD*Δ*tolQR* background, was observed to form very small colonies on LB plates with 100 μg mL^{-1} ampicillin and would not grow in overnight cultures with 100 or 50 μg mL^{-1} ampicillin, although it could grow in overnight cultures with 10 μg mL^{-1} ampicillin. In order to further explore this phenotype, growth curves for RA1045/pKP1197, RA1045, and two "aspartate relocation" strains that were known to grow normally were generated with 100, 50, 25 or 0 μg mL^{-1} ampicillin. All overnight cultures for this experiment were grown without ampicillin so that the use of ampicillin to maintain the

plasmid for the normally growing strains in the overnight cultures would not in and of itself give an advantage to these strains in the subculture with ampicillin. The growth curves in **Figure 7** show that RA1045/pKP1197 grows normally without ampicillin and that growth in the presence of ampicillin is slowed. RA1045/pKP1197 is not as sensitive to ampicillin as RA1045, which does not have the ampicillin resistance cassette and is ultimately unable to grow at all ampicillin concentrations tested (only 100 μg mL^{-1} shown). The effect of ampicillin is not dose-dependent, however; the growth curves for RA1045/pKP1197 with 100, 50, and 25μg mL^{-1} are essentially identical (**Figure 8**). Assuming that there is no mutation in the β-lactamase gene on the plasmid, two possible explanations for these data exist. In one explanation, ExbD(D25A, V43D) is severely growth inhibitory to the cell, creating a selective advantage for the cells that lose the plasmid during cell division, so that when RA1045/pKP1197 is grown overnight without ampicillin, most of the population does not contain the plasmid and is not able to survive in subculture. Alternatively, the protein encoded by RA1045/pKP1197 might create a hypersensitivity to ampicillin, possibly through interactions with the β-lactamase protein or through interactions with the Tol system, as defects in this system are known to cause cells to be hypersensitive to antibiotics and to leak β-lactamase and other periplasmic proteins across the outer membrane (Lazzaroni, *et al*., 1999).

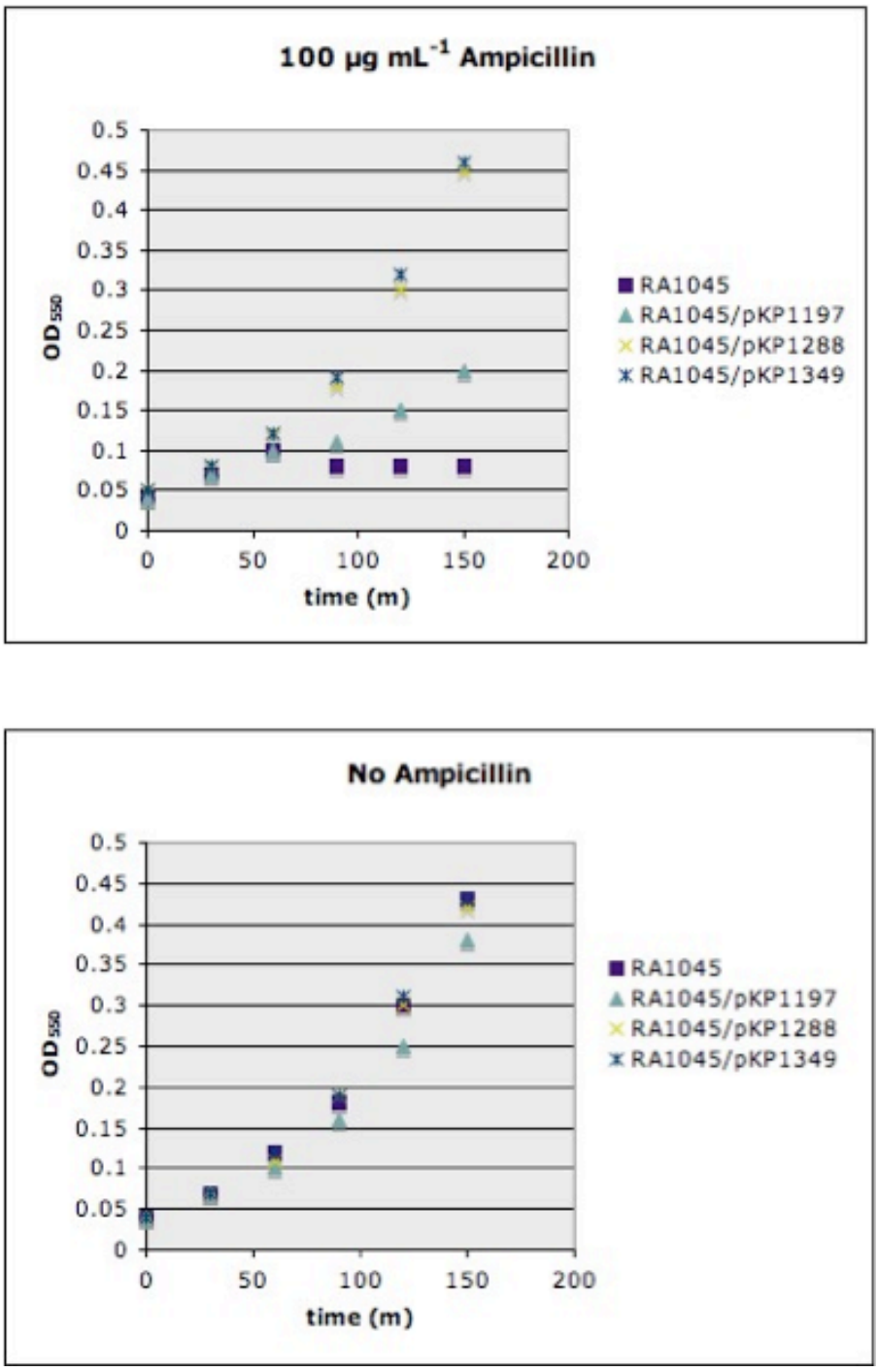

Figure 7: Growth Curves for RA1045, RA1045/pKP1197, RA1045/pKP1288 and RA1045/pKP1349 with and without Ampicillin OD_{550} for T-broth 1:100 subcultures (all subcultured from overnight cultures without ampicillin) was measured every 30 minutes to generate a growth curve for RA1045, RA1045/pKP1197, RA1045/pKP1288 and RA1045/pKP1349. RA1045 is completely unable to grow in ampicillin, RA1045/pKP1197 is able to grow in ampicilin, but its growth is slowed relative to its growth without ampicillin. In contrast, RA1045/pKP1288 and RA1045/pKP1349 are able to grow equally well with and without ampicillin.

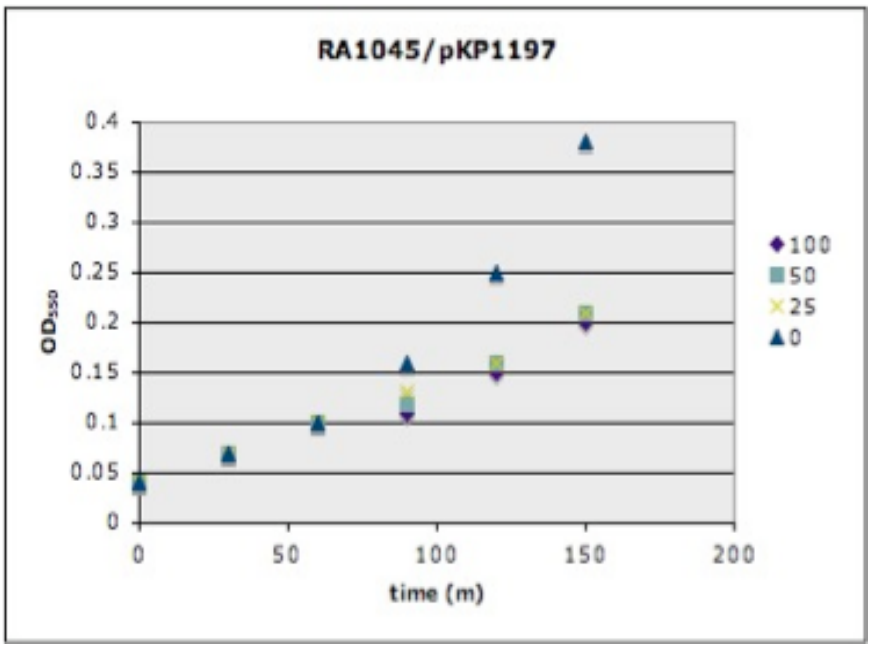

Figure 8: Growth Curves for RA1045/pKP1197 with 0, 25, 50, and 100 μg mL^{-1} Ampicillin OD_{550} for T-broth 1:100 subcultures was measured every 30 minutes to generate a growth curve for RA1045/pKP1197. RA1045/pKP1197 has slowed growth with all concentrations of ampicillin, but the growth is similar for all conditions where ampicillin is present, so the effect is not dose-dependent.

4. Discussion

The TonB protein is responsible for harnessing the energy of proton motive force at the inner membrane and physically contacting outer membrane transporters to transduce this energy, allowing the passage of iron-siderophore complexes and vitamin B12 into the periplasmic space. ExbD and ExbB are inner membrane proteins necessary for TonB-mediated energy transduction to outer membrane transporters in the absence of homologous proteins TolQ and TolR (Ahmer, *et al*., 1995; Held and Postle, 2002). ExbD has been proposed to serve a signal transduction function, and based on its topology might also serve as a chaperone, mediating conformational changes in TonB that could be used to store the energy of pmf (Ghosh and Postle, 2005; Vakharia-Rao, *et al*., 2007). An essential residue, aspartate 25, has been identified in the transmembrane domain of ExbD (Braun, *et al*., 1996). In this work, the positional requirement of the aspartate residue was explored.

4.1 Precise Positioning of Aspartate in the ExbD Transmembrane Domain is Required for TonB-Dependent Function

The results presented in this work indicate that the aspartate residue in the ExbD transmembrane domain is completely nonfunctional if it is moved to any other position in the transmembrane domain. While these data do not overtly support any particular role for ExbD, the strict requirement for aspartate at position 25 does provide some insight into the function of this residue and the ExbD transmembrane domain.

One possible function of the aspartate residue is to mediate protein-protein interactions. The presence of a single negatively charged residue, aspartate, in the ExbD transmembrane domain and a single positively charged residue, histidine, in the TonB transmembrane domain lent itself well to a theory that the two proteins contact each other through ionic interaction at these residues. In support of this idea, both aspartate 25 in ExbD and histidine 20 in TonB are required for the crosslinking of these proteins, while neither is necessary for cross-linking of ExbD or TonB with ExbB (Ollis, *et al*., 2009). It has also been suggested that aspartate may accept or donate a proton from or to the TonB histidine 20 residue to mediate a conformational change in TonB (Braun and Braun, 2002). In contrast to these ideas, recent data showed that the substitution of uncharged asparagine for histidine 20 has complete TonB system activity, making both of these possibilities unlikely (Cheryl Swayne and Kathleen Postle, unpublished data).

The aspartate residue may still physically contact the TonB transmembrane domain in a way that does not involve an ionic interaction, but this seems unlikely. Alternatively, aspartate may be important for interaction with ExbB; although crosslinking of ExbB and ExbD is still observed when aspartate is replaced with an uncharged residue, suggesting the physical interaction between the two is still possible, there nonetheless might be a functional interaction that requires aspartate. Furthermore, as discussed in the introduction, there is evidence for participation of another, as of yet unidentified, protein in TonB-dependent energy transduction. The aspartate residue may be important for interactions with this protein. This is perhaps the most likely of these three possibilities, as it would allow the aspartate to make an electrostatic interaction.

One proposed outcome of this experiment was that aspartate relocations to positions sequentially close to 25, such as 24 or 26, would support TonB system function. In the context of potential protein-protein interactions, this outcome would have provided evidence that ExbD undergoes a rotational motion, or that an interacting protein rotates or otherwise shifts around ExbD in the membrane. It would have also strengthened the hypothesis that TolR undergoes rotational motion (Zhang, *et al*., 2009). However, this was not observed. Another possible outcome was that aspartate substitutions on the same face of the helix as aspartate 25 would be able to support TonB function, possibly suggesting vertical movement in ExbD or an interacting protein relative to the membrane, but this was also not seen. Instead, the exact position of the aspartate residue appears to be crucial. If aspartate does directly participate in protein-protein interactions, this result would imply that the proteins are stationary relative to each other, that only precise movements between proteins allow the interaction to occur, or that the positions of other residues relative to the aspartate residue are crucial.

The possibility that correct positioning of other residues relative to aspartate in the transmembrane domain might be necessary might suggest a second, distinct site of interaction involving the ExbD transmembrane domain, or it could imply a lock and key fit, where the areas of interaction in ExbD and a protein it interacts with must fit quite precisely with each other for a functional interaction to occur. In the latter case, the immediate environment of the aspartate residue would be important. This possibility is supported by the result that a glutamate substitution for aspartate 25 has very low but still detectable TonB system activity, indicating that the shape of the residue has some importance (Anne Ollis and Kathleen Postle, unpublished data). The need for a certain environment in the space surrounding a functionally important charged residue has also been demonstrated in TonB: although S16 can be substituted with alanine without affecting TonB activity, and substituting the entire transmembrane domain except S16 and H20 with alanine still yields a nearly fully functional protein, when a final alanine substitution for S16 is added, the protein is essentially non-functional (Larsen, *et al*., 2007). This might represent a threshold of disturbance to the environment surrounding H20 that TonB could no longer tolerate, or alternatively, S16 might itself carry out an important, but redundant function, but either scenario re-enforces the idea that the charged residue in ExbD might likewise not function independently of its surrounding transmembrane amino acids. The lock and key fit proposed to occur between transmembrane helices II and III of TolQ would also suggest that precise interactions between helices could be important for the function of the Tol and TonB energy-harvesting complexes, but this has not been experimentally demonstrated (Goemaere, *et al*., 2007a).

Another proposed function for the aspartate residue, broadly, is to accept and release protons as part of the TonB system response to pmf. More specifically, the homology between the TonB energy harvesting complex ExbB/ExbD and the MotA/MotB proteins, which are thought to function as an proton channel, has led to the hypothesis that ExbB, ExbD, and perhaps TonB might too come together to form a proton channel, with aspartate 25 serving as a proton acceptor (Zhai, *et al*., 2003). The findings presented here would fit well with this scenario, where the passage of a proton from one site to the next would be likely to require exact positioning of the residues involved. Indeed, an experiment similar to the one presented here was done examining a conserved aspartate residue in the transmembrane domain of an ATPase subunit, where the aspartate was "moved" to one position above, one position below, and three positions below its

original location. As was observed in the present experiment, none of these mutants were active, although a suppressor mutation that substituted an aspartate residue at a similar height in the membrane on a adjacent helix was isolated (Miller, *et al*., 1990). On the other hand, studies on multidrug/proton antiporter MdfA showed that drug resistance activity for a completely inactive double substitution mutant, with neutral amino acids substituted for negatively charged residues in the proton pathway, can be partially restored by a single glutamate substitution at either of two locations in the register between the two original charged residues (Sigal, *et al*., 2009). The authors suggest that the apparent flexibility of the location of the charged residues is related to the "spatially promiscuous," multisubstrate nature of the system, but this result does still indicate that exact positioning of a key charged residue is not necessarily required for translocation of charged substrates.

Results in more closely related systems, however, support the idea that only aspartate substitutions at the exact height of the original aspartate in the membrane can recover the loss of function observed with D25A or an analogous mutation. PomA and PomB, which form a sodium channel, are orthologs of MotA and MotB, and are also necessary for flagellar motion (Sudo, *et al*., 2009). They would therefore exhibit similarity with ExbB and ExbD, respectively. An aspartate scanning study of *Vibrio alginolyticus* PomA transmembrane domains II and III found that PomA(N194D) was able to partially restore the loss of function generated by a PomB(D24N) mutation; these two residues are proposed to lie at very similar vertical positions in the membrane (Terashima, *et al*., 2010).

Similarly, the suppressor TolQ(A185D) in the highly homologous Tol system is able to restore the loss of function generated by the TolR(D23A) mutation. It was suggested that A185 is also at a similar height in the membrane as D23 (Goemaere, *et al*., 2007a). The two potential interpretations of these data (either suggesting ion channel function or close packing of the helices) and the many differences between the transmembrane mutation phenotypes in the Tol and TonB systems should be kept in mind when considering these results. Nonetheless, it would also be interesting to know if any aspartate substitutions in the ExbB transmembrane domains could likewise recover some TonB system function in the presence of ExbD(D25A).

In conclusion, the data presented here do not provide any evidence to support models in which ExbD or the proteins it may be interacting with move, either rotationally or vertically, in order to make a functional interaction in the membrane. Instead, they fit better with models in which ExbD is stationary, or precise movements or interactions are required for the aspartate residue to serve its purpose.

4.2 Undetectable Expression of Some Aspartate Relocation Mutants Suggests Signal Peptide Defects, Disrupted Protein-Protein Interactions, or Promoter Mutations

Other phenotypes unrelated to TonB system function were also observed for some of the "aspartate relocation" mutants. Two of the twenty mutants constructed were undetectable in this experiment. ExbD(D25A, I33D) and ExbD(D25A, M35D) both could not be detected by Western blotting even when a maximum amount of inducer, one hundred times the concentration necessary to induce expression of the wild type protein, was used to attempt to induce

expression. This suggests that these proteins are highly proteolytically unstable, although it is also possible that mutations in the promoter region, perhaps selected for by a toxic effect of these proteins, are the cause of this phenotype.

Proteolytic instability of ExbD(D25A, I33D) and ExbD(D25A M35D) could be caused by a defect in the signal sequence of these proteins such that they are localized to the cytoplasm. Interruption of the hydrophobic region of an *E. coli* signal sequence with a negatively charged residue has been noted previously as a pattern in non-functional signal sequence mutations (Gierasch, 1989). All other ExbD "aspartate relocation" proteins were able to crosslink *in vivo* with ExbB, indicating proper insertion into the inner membrane. If the ExbD(D25A, I33D) and ExbD(D25A M35D) proteins are, in fact, insertion defects, this would mean that only aspartate substitutions at particular positions in the transmembrane domain could interrupt insertion. That an insertion defect might only be seen at particular positions would not be unprecedented, as charged amino acid insertions in the hydrophobic portion of the LamB signal sequence at positions 12-15 that severely impair insertion have been isolated, while an aspartate substitution at position 17 has only a minor effect on insertion; the hydrophobic core of LamB is considered to extend from positions 8 to 25 (Stader, *et al*., 1986).

Signal sequence mutations have been observed in TonB-system proteins before. An aspartate substitution at position 26 in TonB and a TrpC-TonB fusion causes the protein to be localized in the cytosol according to spheroplast susceptibility studies, and it has been shown in the TrpC-TonB fusion that the insertion defect can be suppressed by mutations of the *prlA* gene known to suppress signal sequence mutations (Skare, *et al*., 1989; Jaskula, *et al*., 1994). In these experiments, TonB(G26D) can be still detected as a cytoplasmic protein (Jaskula, *et al*., 1994).

There are several reasons why a signal sequence mutation might cause ExbD to be rapidly degraded. Uninserted ExbD could become misfolded or aggregate, be recognized as a protein foreign to the cytosol, and be degraded. Also, there is some evidence that translation of some *E. coli* proteins is coupled to secretion, and so a signal peptide defect that prevents membrane insertion of ExbD might also prevent ExbD from being synthesized (Hall, *et al*., 1983). However, an ExbD variant with the transmembrane domain completely deleted can be expressed, (Anne Ollis and Kathleen Postle, unpublished data), so it is unlikely that the degradation of the ExbD(D25A, I33D) and ExbD(D25A M35D) proteins occurs in the cytoplasm.

Another explanation for the lack of detectable ExbD(D24A, I33D) and ExbD(D25A, M35D) proteins is that they are being inserted into the inner membrane properly, but are highly unstable because they are unable to make a key stabilizing interaction with another protein in the inner membrane. ExbB is known to stabilize both ExbD and TonB, and several TonB transmembrane domain mutants that lose their ability to cross-link strongly or specifically to ExbB are less stable than wild type TonB (Fischer, *et al*., 1989; Larsen, *et al*., 1999, Larsen, *et al*., 2007). Despite instability in the absence of ExbB, though, wild-type ExbD has been expressed from a pPro24-derivative plasmid in the absence of ExbB before, so it is unlikely that a lack of ExbD-ExbB interaction is the cause of the extreme instability of these ExbD mutants (Ollis, *et al*., 2009). However, these aspartic acid substitutions in the ExbD transmembrane domains might disrupt interactions with another, unidentified protein necessary for ExbD stability. Additionally, I33 and M35 are positioned on a helical wheel projection next to residues I24 and L40, which form

disulfide crosslinked homodimers when substituted with cysteine (Mingchao Xie and Kathleen Postle, unpublished data). This raises the possibility that aspartate substitutions at I33 and M35 might be disrupting key ExbD self-interaction.

Sequencing the promoter regions of these plasmids would be a good first step in exploring these possible explanations. If no mutations in the promoter region are found, the plasmid could be introduced into strains with known suppressors of signal sequence mutations or with periplasmic protease deletions. If one of these strains allows expression of the protein, it would provide strong evidence for either a defect in insertion or a disruption of stabilizing interactions at the inner membrane, respectively. Additionally, arabinose-inducible expression systems have been shown to achieve stronger overexpression of ExbD, suggesting that these undetectable proteins might be observable in this system with high levels of inducer (Anne Ollis and Kathleen Postle, unpublished data). If observable protein levels can be obtained this way, spheroplast susceptibility studies could be used to determine the intracellular location of these variants.

4.3 The Higher Detectable Levels of ExbD(D25A) and Some "Aspartate Relocation" Mutants Could Be Attributed to Increased Translation or More Favorable Protein Folding or Insertion

In contrast to the ExbD variants discussed in the previous section, two mutant ExbD proteins generated were detected at much higher levels than plasmid-encoded wild type ExbD, and even when transcription was repressed with high amounts of glucose, these proteins were present at levels higher than those of chromosomal ExbD. ExbD(D25A) and ExbD(D25A, P39D) could not be brought to chromosomal levels with 0.5% glucose. Many of the other "aspartate relocation" mutants at positions throughout the transmembrane domain also required repression with glucose, or were detectable at about wild type levels with no inducer or repressor, in contrast to plasmid-encoded wild type ExbD, which requires sodium propionate as an inducer for detection at chromosomal levels. However, unlike the two ExbD variants mentioned above, all the other "aspartate relocation" proteins could be repressed to at or below wild type levels with 0.01% glucose.

The higher levels of detectable D25A are consistent with similar results with ExbD(D25N) and ExbD(D25C), indicating that the aspartate codon is somehow able to affect cellular levels of the ExbD protein (Ollis, *et al*., 2009; Mingchao Xie and Kathleen Postle, unpublished data). Interestingly, the D25 substitution mutations do not have a longer steady state proteolytic half-life, so possibilities other than proteolytic instability must be considered (Mingchao Xie and Kathleen Postle, unpublished data). These results are particularly interesting in light of the observation that, although ExbB and ExbD are transcribed from the same operon, translationally coupled, and have similar steady state proteolytic half-lives, the ratio of cellular ExbB to ExbD is 3.5 to 1 (Higgs, *et al*., 2002a). One explanation for such a discrepancy would be a translational downshift mediated by an important RNA secondary structure or a site for binding of an unidentified repressor protein. Alternatively, if the folding or insertion of ExbD into the membrane is less favorable than it is for ExbB, this could also account for the differences in cellular levels of the proteins. Because the vast majority of ExbD in the cell is localized at the inner membrane and not the cytosol, degradation of newly synthesized ExbD in the cytosol would probably not have an observable effect on the steady state half-life of the protein.

The differences in detectable levels of the ExbD aspartate substitution and "aspartate relocation" variants compared to wild type ExbD could be explained in either context, but because there is no clear pattern in the levels of detection of these mutant ExbD proteins, it is possible that several factors are at work. The aspartate substitutions might be detected at higher levels because the replacement of the aspartate codon disrupts a sequence necessary for a translational downshift. The diverse stabilities of the "aspartate relocation" variants, however, are more difficult to explain in this context. If translational repression is the only factor affecting steady state ExbD levels, it would imply that the introduction of an aspartate codon (GAT or GAC) as far as fifty base pairs away and surrounded by an entirely different sequence could partially recover a dramatic loss of transcriptional repression caused by the D25A mutation, GAC→GCC. It is more likely that all of the "aspartate relocation" variants experience the same relief from RNA-mediated repression that D25A does, but this effect is masked by varying degrees of proteolytic instability that may result from the movement of the aspartate residue.

It is also possible that, in a situation converse to the signal sequence disruptions previously discussed, replacement of aspartate for an uncharged residue might allow more efficient insertion of ExbD into the inner membrane. This explanation could account for the increased detection of the ExbD aspartate substitution variants, but not ExbD(D25A, P39D). On the other hand, the increased detection of ExbD(D25A) and ExbD(D25A, P39D) could be explained by more thermodynamically favorable helix formation; neither aspartate nor proline support helix formation, but aspartate is significantly less disruptive than proline, so both the replacement of aspartate with alanine and the replacement of proline with aspartate would probably make helix formation for ExbD more favorable (Pace and Scholtz, 1998). However, ExbD(D25N) and ExbD(D25C), which substitute residues with similar helix propensities as aspartate, could probably not be explained this way. In either case, the diverse ExbD levels of some other "aspartate relocation" mutants would indicate differing tolerances for aspartate at various locations in the transmembrane domain. Both situations would also imply that the aspartate residue drastically impairs the ability of nascent, wild type ExbD to enter the membrane in a properly folded state before it is proteolytically degraded in the cytoplasm; we are unaware, however, of another case where such dramatic effects are caused by a charged residue that occurs in the wild type sequence of a transmembrane domain, and a system where much more wild type ExbD is synthesized than is able to become a functional protein would be inefficient for the cell.

Differentiating increased translation and better folding or insertion of the newly synthesized protein experimentally may be difficult. If the hypothetical misfolded or uninserted ExbD proteins could still be efficiently immunoprecipitated, then a pulse-chase experiment might reveal an increased half-life for the newly synthesized mutants compared to newly synthesized wild-type ExbD. *In vitro* analysis of ExbD half-life might also reveal differences between wild-type ExbD and the highly detectable variants, but the absence of potentially necessary chaperones would complicate interpretation of these results. Quantifying the differences between levels of wild-type ExbD and the ExbD variants with the same amount of inducer would be illuminating; if the ratio of the levels any ExbD variant to wild-type ExbD was more than 3.5:1, it would suggest than something other than disruption of a translational downshift is causing the variant to be detected at higher levels. Analysis of P39D and other transmembrane aspartate substitutions without the D25A substitution might also be interesting, but the potential effects of

adding a second charged residue to the transmembrane domain would need to be taken into account.

4.4 The Growth Inhibitory Phenotype of ExbD(D25A, V43D) Might Be Due to Crosstalk with Other Cellular Systems

A particularly unusual phenotype was observed in RA1045/pKP1197, which encodes ExbD(D25A, V43D), and like all the other mutants generated for this study, expresses the mutant ExbD protein from a pPro24-derivative plasmid in a Δ*tolQR*Δ*exbD* background. This strain exhibited impaired growth in the presence of ampicillin despite containing the pPro24-encoded β-lactamase gene. There is uncertainty whether the slowed growth is a result of a growth-inhibitory trait of the mutant ExbD protein that favors the survival of cells that lose the plasmid in cell division, or a true hypersensitivity to ampicillin. This could be resolved by comparing the percentage of cells of this strain that retain the plasmid in the absence of ampicillin to the percentage of cells in a normally-growing strain that retain the plasmid in the absence of ampicillin; if there is a selective pressure, RA1045/pKP1197 should lose the plasmid more rapidly. A similar phenotype was also observed for plasmid-encoded ExbD(Y112C, L132Q) in the same background strain (Anne Ollis and Kathleen Postle, unpublished data).

It is important to note that the exact boundaries of the transmembrane domain have not been experimentally determined, and that V43 is the last position in the predicted transmembrane domain, and so it might, in reality, lie in the periplasm. Additionally, the V43D substitution creates a series of hydrophilic residues from positions 42 to 44 of ExbD (threonine aspartate aspartate). Thus, it is possible that the transmembrane region would shift or would form incorrectly in the presence of the V43D mutation. Crosslinking between the mutant ExbD protein and ExbB suggests that the protein can be inserted into the membrane, and the protein appears to have a normal proteolytic stability relative to the other aspartate substitution mutants generated, but the structure of ExbD near the interface of the inner membrane and the periplasmic space might still be drastically altered. An altered protein structure might initiate a stress response in the cells, responsible for the growth-inhibitory phenotype and the selective pressure for the cells to lose the plasmid; however, because the ExbD(D25A, V43D) protein seems to be properly inserted at the inner membrane and proteolytically stable relative to the other mutants generated here, it is unlikely that this protein aggregates in the cytosol or is rapidly degraded to initiate such a response. Alternatively, the mutant protein might impair growth by interfering with some essential function of the cell that occurs at the inner membrane or in the periplasmic space, such as transport of outer membrane proteins or ATP synthesis, through an atypical interaction this mutation allows.

If this strain truly does exhibit hypersensitivity to ampicillin, several intriguing possibilities are raised. A hallmark of mutations in the Tol system is instability of the outer membrane, which causes periplasmic proteins, such as β-lactamase, to leak out of the cell, and renders the cells more sensitive to some antibiotics (Lazzaroni, *et al.*, 1999). ExbD(D25A, V43D) could be interacting with some component of the Tol system to further destabilize the outer membrane. However, given that the background strain is a Δ*tolQR* strain which already lacks the Tol energy-harvesting complex, it is difficult to speculate how ExbD(D25A, V43D) might further exacerbate an already "leaky" outer membrane phenotype despite homology with Tol system component

TolR. Perhaps this mutant is able to interact with TolA and prevent some energy-independent function, or causes TolA to make deleterious interactions with its normal outer membrane contacts. The mutant ExbD might also interact directly with β-lactamase to inhibit its function. Again, the possibility that the V43 residue might lie in the periplasmic space, which would allow a direct interaction between this residue and a periplasmic protein like β-lactamase to occur, should be considered. Additionally, it should be noted that position 44 in ExbD is also an aspartate residue. The possibility that two aspartate residues side-by-side might mimic the structure of ampicillin, allowing this mutant ExbD to competitively inhibit the function of β-lactamase was considered, but ultimately discarded. Although the positioning of the amine and carboxyl groups in the aspartate-aspartate peptide bond shows some similarity to ampicillin, as shown in **Figure 9**, that known β-lactamase inhibitors and substrates contain rings (while no amino acids with rings exist near the V43D mutation) and that the ExbD(Y112C, L132Q), which exhibits a similar phenotype, does not show any notable structural similarity to β-lactam antibiotics or inhibitors counters this idea (Matagne, *et al*., 1998). It is also possible that the mutant ExbD, in a variation of its potential role as a chaperone for TonB, stabilizes a conformation of β-lactamase that is recognized by proteases, forcing β-lactamase to be rapidly degraded.

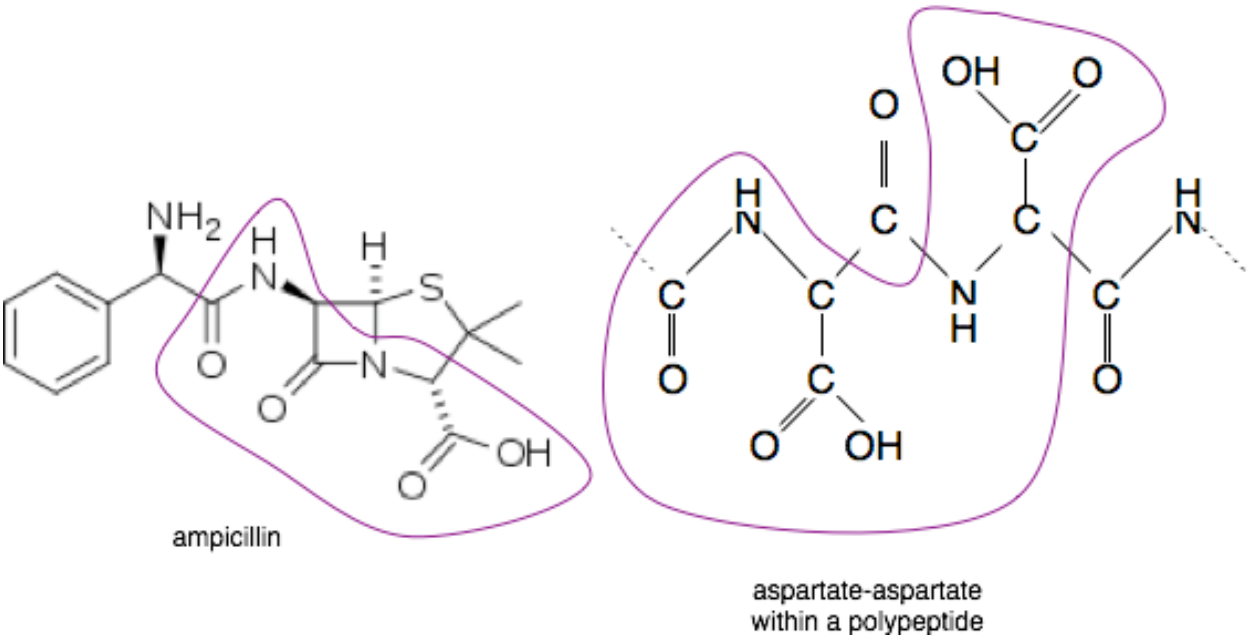

Figure 9: The Structures of Ampicillin and an Aspartate-Aspartate Peptide Bond within a Polypeptide The chemical structures of ampicillin and the aspartate-aspartate sequence are compared. The circled regions highlight structural similarity. However, for other reasons, it seems unlikely that the ExbD(D25A, V43D) protein inhibits β-lactamase by competing with ampicillin.

In summary, these findings reinforce the importance of the conserved aspartate residue in the ExbD transmembrane domain. Aspartate substitutions at no other position in the transmembrane domain can recover the loss of function generated by the D25A mutation. The ExbD aspartate presumably must exist at a particular position or chemical environment in order to function. This result could support a number of current ideas about the role of the ExbD aspartate as part of the TonB system. Additionally, growth and stability phenotypes of aspartate substitutions in the

ExbD transmembrane domain provide foundation for speculation about the structure and function of the transmembrane domain as a whole.

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Appendix

All plasmids used in this study were constructed using QuickChange site-directed mutagenesis to introduce an aspartate substitution using pKP1191, a pPro24-derivative plasmid encoding ExbD(D25A), as a template. Polymerase Chain Reaction (PCR) was used with complementary primers encoding the appropriate nucleotide changes necessary to introduce a given aspartate substitution. **Table 5** shows the primers used for construction of each plasmid. All primers were synthesized by Invitrogen (Carlsbad, CA).

Plasmid Name	Introduced Mutation	Primer A (Coding, 5' →3')	Primer B (Noncoding, 5' → 3')
pKP1197	V43D	CCGTTAGCGACG**GAT**GATGTGAAGGTG	CACCTTCACATC**ATC**CGTCGCTAACGG
pKP1199	T42D	GCACCGTTAGCG**GAT**GTAGATGTGAAG	CTTCACATCTAC**ATC**CGCTAACGGTGC
pKP1201	V26D	CCGTTTATCGCC**GAT**ATGTTGGTTCTG	CAGAACCAACAT**ATC**GGCGATAAACGG
pKP1202	V29D	GCCGTGATGTTG**GAT**CTGCTGATTATC	GATAATCAGCAG**ATC**CAACATCACGGC
pKP1213	M27D	TTTATCGCCGTG**GAT**TTGGTTCTGCTG	CAGCAGAACCAA**ATC**CACGGCGATAAA
pKP1214	L40D	GTGGCGGCACCG**GAT**GCGACGGTAGATGTG	CACATCTACCGTCGC**ATC**CGGTGCCGCCAC
pKP1215	A41D	GCGGCACCGTTA**GAT**ACGGTAGATGTG	CACATCCTACCGT**ATC**TAACGGTGCCGC
pKP1229	L28D	ATCGCCGTGATG**GAT**GTTCTGCTGATT	AATCAGCAGAAC**ATC**CATCACGGCGAT
pKP1272	L31D	GATGTTGGTTCTG**GAC**ATTATCTTTATGGTG	CACCATAAAGATAAT**GTC**CAGAACCAACATC
pKP1273	I24D	AACGTGACGCCGTTT**GAT**GCCGTGATGTTGGTT	AACCAACATCACGGC**ATC**AAACGGCGTCACGTT
pKP1276	F23D	ATCAACGTGACGCCG**GAT**ATCGCCGTGATGTTG	CAACATCACGGCGAT**ATC**CGGCGTCACGTTGAT
pKP1277	I32D	TTGGTTCTGCTG**GAC**ATCTTTATGGTGGCG	CGCCACCATAAAGAT**GTC**CAGCAGAACCAA
pKP1287	I33D	GTTCTGCTGATT**GAC**TTTATGGTGGCG	CGCCACCATAAA**GTC**AATCAGCAGAAC
pKP1288	F34D	CTGCTGATTATC**GAT**ATGGTGGCGGCA	TGCCGCCACCAT**ATC**GATAATCAGCAG
pKP1322	M35D	CTGATTATCTTT**GAT**GTGGCGGCACCG	CGGTGCCGCCAC**ATC**AAAGATAATCAG
pKP1349	A38D	ATCTTTATGGTGGCG**GAT**CCGTTAGCGACGGTA	TACCGTCGCTAACGG**ATC**CGCCACCATAAAGAT
pKP1351	V36D	CTGATTATCTTTATG**GAC**GCCGGCACCGTTAGCG	CGCTAACGGTGCCGC**GTC**CATAAAGATAATCAG
pKP1359	A37D	ATTATCTTTATGGTG**GAC**GCACCGTTAGCGACG	CGTCGCTAACGGTGC**GTC**CACCATAAAGATAAT
pKP1360	P39D	TTTATGGTGGCGGCA**GAT**TTAGCGACGGTAGAT	ATCTACCGTCGCTAA**ATC**TGCCGCCACCATAAA
pKP1413	L30D	GCCGTGATGTTGGTT**GAC**CTGATTATCTTT	AAAGATAATCAG**GTC**AACCAACATCACGGC

Table 5: Primers Used for Construction of "Aspartate Relocation" Mutants Complementary primers encoding the necessary base pair changes to introduce an aspartate substitution were used. The location of the aspartate codon is highlighted in the primer sequences.

Throughout the course of the construction of these plasmids, sequences of plasmid potentially carrying the targeted mutation were often found to contain extra, inserted regions matching the primer sequences immediately following the targeted mutation. In order to attempt to minimize the occurrence of these incorrect sequences, a variety of reaction conditions were tested. Variables altered include annealing temperature, $MgCl_2$ concentration, and number of cycles. However, no consistent relationship between the success of the PCR reaction and any of these variables was observed.

PCR reaction conditions for each plasmid constructed follow.

pKP1197 & pKP1199
composition
27.5 µL ddH_20
10 µL 5X Phusion HF buffer (New England BioLabs, Ipswich, MA)
4 µL dNTPs (2.5 mM each)
1 µL pKP1191 (5ng/µL)
1.5 µL Primer A (125 ng/µL)
1.5 µL Primer B (125 ng/µL)
4 µL 50mM $MgCl_2$ (New England BioLabs, Ipswich, MA)
0.5 µL Phusion Hot Start II DNA Polymerase (2U/µL) (New England BioLabs, Ipswich, MA)

cycle
initial denaturation: 98°C, 30 seconds

denaturation: 98°C, 10 seconds
annealing: 60.6°C, 30 seconds
extension: 72°C, 7 minutes
[30 cycles]

final extension - 72°C, 10 minutes
hold - 4°C

pKP1201, pKP1202, & pKP1215
composition
29.5 µL ddH_20
10 µL 5X Phusion HF buffer
4 µL dNTPs (2.5 mM each)
1 µL pKP1191 (5ng/µL)
1.5 µL Primer A (125 ng/µL)
1.5 µL Primer B (125 ng/µL)
2 µL 50mM $MgCl_2$
0.5 µL Phusion Hot Start II DNA Polymerase (2U/µL)

cycle
initial denaturation: 98°C, 30 seconds

denaturation: 98°C, 10 seconds
annealing: 60.6°C, 20 seconds
extension: 72°C, 7 minutes
[30 cycles]

final extension - 72°C, 10 minutes
hold - 4°C

pKP1213 & pKP1214
composition
31.5 µL ddH_20
10 µL 5X Phusion HF buffer
2 µL dNTPs (5.0 mM each)
1 µL pKP1191 (5ng/µL)
1.5 µL Primer A (125 ng/µL)
1.5 µL Primer B (125 ng/µL)
2 µL 50mM $MgCl_2$
0.5 µL Phusion Hot Start II DNA Polymerase (2U/µL)

cycle
initial denaturation: 98°C, 30 seconds

denaturation: 98°C, 10 seconds
annealing: 90.2°C, 30 seconds
extension: 72°C, 7 minutes
[25 cycles]

final extension - 72°C, 10 minutes
hold - 4°C

pKP1229
composition
31.5 µL ddH_20
10 µL 5X Phusion HF buffer
2 µL dNTPs (5.0 mM each)
1 µL pKP1191 (5ng/µL)
1.5 µL Primer A (125 ng/µL)
1.5 µL Primer B (125 ng/µL)
2 µL 50mM $MgCl_2$
0.5 µL Phusion Hot Start II DNA Polymerase (2U/µL)

cycle
initial denaturation: 98°C, 30 seconds

denaturation: 98°C, 10 seconds
annealing: 78.3°C, 30 seconds
extension: 72°C, 7 minutes
[25 cycles]

final extension - 72°C, 10 minutes
hold - 4°C

pKP1272, pKP1273, pKP1276, pKP1277, pKP1287, pKP1288, pKP1322, & pKP1349
composition
31.5 µL ddH_20
10 µL 5X Phusion HF buffer
2 µL dNTPs (5.0 mM each)
1 µL pKP1191 (5ng/µL)
1.5 µL Primer A (125 ng/µL)
1.5 µL Primer B (125 ng/µL)
2 µL 50mM $MgCl_2$
0.5 µL Phusion Hot Start II DNA Polymerase (2U/µL)

cycle
initial denaturation: 98°C, 30 seconds

denaturation: 98°C, 10 seconds
annealing: 80.3°C, 30 seconds
extension: 72°C, 7 minutes
[25 cycles]

final extension - 72°C, 10 minutes
hold - 4°C

pKP1351, pKP1359, & pKP1360
composition
33.5 µL ddH_20
10 µL 5X Phusion HF buffer
2 µL dNTPs (5.0 mM each)
1 µL pKP1191 (5ng/µL)
1.5 µL Primer A (125 ng/µL)
1.5 µL Primer B (125 ng/µL)
0.5 µL Phusion Hot Start II DNA Polymerase (2U/µL)

cycle
initial denaturation: 98°C, 30 seconds

denaturation: 98°C, 10 seconds
annealing: 60.6°C, 30 seconds
extension: 72°C, 7 minutes
[25 cycles]

final extension: 72°C, 10 minutes
hold: 4°C

pKP1413
composition
38 μL ddH_20
5 μL 10X PfuUltra HF buffer (Stratagene, LaJolla, CA)
2 μL dNTPs (5.0 mM each)
1 μL pKP1191 (5ng/μL)
1.5 μL Primer A (125 ng/μL)
1.5 μL Primer B (125 ng/μL)
1 μL PfuUltra High-Fidelity DNA Polymerase (2.5U/μL) (Stratagene, LaJolla, CA)

cycle
initial denaturation: 98°C, 2 minutes

denaturation: 98°C, 30 seconds
annealing: 65°C, 30 seconds
extension: 72°C, 6 minutes
[30 cycles]

final extension: 72°C, 10 minutes
hold: 4°C

Academic Vita of Christine Dubowy

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Education

The Pennsylvania State University, University Park, PA
B.S. in Biochemistry and Molecular Biology with Honors - May 2010
Thesis: *The Positional Requirement for the Aspartate Residue in the ExbD Transmembrane Domain*
Dean's List eight of eight semesters

Cocalico High School, Denver, PA
High School Diploma - January 2006

Research Experience

Undergraduate Research in Molecular Microbiology - August 2008 to April 2010
Penn State University, University Park, PA
* Conducted research in the laboratory of Kathleen Postle, PhD
* Used site-directed mutagenesis to examine the role of the protein ExbD in the transport of iron-siderophore complexes and vitamin B12 across the outer membrane of *E. coli*.

Nemours Summer Undergraduate Research - Summer 2009
Nemours Foundation/Alfred I. duPont Hospital for Children, Wilmington, DE
* Research supervised by Katherine King, MD and Zhaoping He, PhD
* Investigated the pig as a potential animal model for research on the role of pepsinogen C in the lungs and its potential as a marker of human disease.

Presentations

"Pepsinogen C in Lung Injury" - August 2009
* Presented at the Nemours Summer Student Research Seminar, and to the otolaryngology and pulmonology departments at A.I. duPont Hospital for Children

Honors and Awards

Penn State President's Fund for Undergraduate Research - Fall 2008 to Spring 2010
Nemours Student Research Scholarship - Summer 2009
Schreyer Honors College Academic Excellence Scholarship - Spring 2006 to Fall 2009
National Merit Scholar - Spring 2006 to Fall 2009

Community Service

Kitchen Volunteer, "New Faces of An Ancient People" Traditional Powwow **-** April 2007 & 2008
"Operations" Volunteer, Penn State IFC/Penhellenic Dance Marathon **-** February 2006 & 2007

Recent Work Experience

Server, Outback Steakhouse, State College, PA and Glen Mills, PA - October 2007 to May 2010
Commons Desk Clerk, PSU Housing & Food Services, University Park, PA - February 2007 to May 2010
Science Camp Instructor, Science Explorers, Blue Ball, PA - Summer 2007 & Summer 2008