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CHARACTERIZATION OF EXBB TRANSMEMBRANE DOMAINS FOR TONB-DEPENDENT ENERGY TRANSDUCTION IN *ESCHERICHIA COLI*

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ABSTRACT

In Gram negative bacteria, the TonB system serves as an energy conduit, connecting cytoplasmic membrane (CM) proton motive force (pmf) to active transport of ligands at the outer membrane (OM) through TonB-gated transporters. In *Escherichia coli*, CM proteins TonB, ExbB, and ExbD form a complex which links pmf energy to transport of important nutrients vitamin B12 and iron-siderophore complexes into the cell. TonB and ExbD have a similar topology with a short N-terminal cytoplasmic domain, one transmembrane domain (TMD) and a large C-terminal periplasmic domain. In contrast, ExbB has three TMDs and large cytoplasmic loop and tail domains. Because the majority of TonB/ExbD and ExbB occupy different cellular compartments, important interactions are thought to occur through the TMDs.

Characterization of functionally important residues in the TMDs and periplasmic domains of TonB and ExbD have provided mechanistic insight for TonB system function. However, the functional role of ExbB is undefined. Currently, ExbB has three speculative functions, as a scaffolding protein, as a signal transducer and as a proton translocator. Mutagenic studies in the large cytoplasmic loop and tail domains have concluded that these regions are essential for ExbB function. In addition, Cys substitutions in the C-terminal tail identified residues important for signal transduction from the cytoplasm to periplasm. The ExbB TMDs have not been well-studied and it is unclear if all TMDs are essential for ExbB function.

This study addressed the functional importance of the ExbB TMDs for TonB-dependent energy transduction. New predictions of ExbB TMD boundaries replaced three

prior incongruent ExbB TMD predictions. Substitution of each half ExbB TMD with alanine determined that all TMDs were essential for activity. Additional substitution of individual ExbB TMD residues identified ExbB scaffolding and signal transduction functions. Substitution of all protonatable residues in the TMD ruled out the hypothesis that ExbB residues function in proton translocation. This knowledge was then applied to characterize ExbB Δ120-129-mediated proton leakage via addition of TMD substitutions. The proton leakage rate was reduced by substitution of important ExbB TMD residues, suggesting proton conductance was occurring through ExbB. However, substitution of non-essential ExbB residues had the most profound effect on the ExbB Δ120-129-mediated proton leakage. This implied that the proton leakage was occurring through a non-native ExbB mechanism. The mutagenic studies presented here, reinforce the proposed ExbB scaffolding and signal transducer functions and refute a proton pathway formed through the ExbB TMDs. This work clarified our understanding of ExbB functionand what role(s) the TMDs play in TonB-dependent energy transduction.

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CHAPTER 1

INTRODUCTION TO TONB-DEPENDENT TRANSPORT

Transport across the Gram negative bacterial cell envelope

The ability to acquire nutrients from the environment is required for all living organisms. In pathogenic bacteria, highly efficient nutrient acquisition is an important factor for pathogenesis [Mahan et al., 2000]. Iron is a key nutrient as it is a vital co-factor for a number of enzymes involved in multiple biological processes such as the electron transport chain, peroxide reduction, and DNA replication [Bullen and Griffiths, 1999]. While iron is plentiful in the environment, the amount of bioavailable iron is scarce because iron forms insoluble ferric hydroxides at physiological pH in aerobic environments and in mammalian hosts it is tightly bound in hemoglobin, lactoferrin, transferrin and other iron-binding proteins [Bullen and Griffiths, 1999]. To acquire iron from the environment, bacteria produce a diverse group of iron scavenging compounds, termed siderophores, which can bind iron at high affinities ($K_d = 10^{-30}$M) [Chipperfield and Ratledge, 2000; Wandersman and Delepelaire, 2004].

However, the architecture of the Gram negative bacterial cell envelope impairs entry of iron-siderophore complexes into cells. Gram negative bacteria contain two concentric membranes: an inner, cytoplasmic membrane (CM) and an outer membrane (OM). The periplasmic space, an aqueous compartment that lies between these two membranes, contains a thin layer of peptidoglycan to maintain cellular structure. The CM is composed of a phospholipid bilayer and contains integral membrane proteins which generate and maintain an electrochemical gradient known as proton motive force (pmf). Pmf is harnessed for ATP generation and drives diverse cellular functions. In contact with the external environment, the asymmetric OM comprises an inner leaflet of phospholipids and an outer leaflet of lipopolysaccharides which forms a barrier against

diffusion of hydrophobic agents [Nikaido, 2003]. Size limitations imposed by OM porin proteins exclude harmful environmental agents but also prevent the passive diffusion of essential iron-siderophore compounds (Figure 1-1). Because the OM lacks direct access to energy for active transport, energy must be supplied from other sources.

The TonB system in the CM transduces pmf energy, by an uncharacterized mechanism, to OM TonB-gated transporters (TGTs) proteins allowing transport of important nutrients into the cell. The TonB transport system in *Escherichia coli* K12 can transport iron-siderophore compounds and vitamin B12. In other species of Gram negative bacteria, the TonB system also transports other nutrients including, sucrose, nickel, maltodextrin, and heme [Krewulak and Vogel, 2011]. Each ligand has a specific OM TGT with at least 7 different TGTs in *E. coli*, while *Bacteroides Thetaiotaomicron* has up to 120 different TGTs [Noinaj et al., 2010; Schauer et al., 2008]. TGTs are 22 stranded β-barrel proteins containing an N-terminal plug domain. TonB is known to interact with the TGTs through an N-terminal ~7 amino acid TonB-recognition sequence called the "TonB box" and is also thought to interact with other parts of the receptor [Devanathan and Postle, 2007]. In addition to the transport of nutrients, bacteriophages and colicins hijack the TGTs to gain entry into the cell [Cascales et al., 2007].

The CM components of the TonB system in *E. coli* consist of 3 integral membrane proteins: TonB, ExbB and ExbD. ExbB and ExbD harness CM pmf energy and TonB physically connects this energy to active transport of OM ligands through TGTs [Cadieux and Kadner, 1999; Gresock et al., 2011; Ogierman and Braun, 2003; Skare et al., 1993]. In other Gram negative species there are multiple sets of TonB system proteins thus increasing the system complexity [Kuehl and Crosa, 2010]. Because *E. coli*

expresses only one set of TonB system genes, this makes *E. coli* K12 the most ideal system to study the mechanisms of TonB-dependent transport.

Identification of TonB system components: An historical view

The *tonB* locus was initially identified through studies of resistance to the bacteriophage T1 (T one) [Anderson, 1946; Luria and Delbruck, 1943]. Early studies attributed inactive TonB mutations to seemingly diverse phenotypes, such as tolerance to group B colicins and phage T1 and ϕ80, iron starvation, hyper-excretion of enterochelin, deficient in the uptake of an array of ferrous compounds, and vitamin B12 [Wookey, 1982]. The deletions in the *exbB* locus, which includes both *exbB* and *exbD*, were originally associated with insensitivity to colicin B through hyperexcretion of enterochelin [Gutermann and Dann, 1973]. Later it became clear that hyperexcretion of enterochelin caused by iron starvation in *exbB* strains was increasing colicin B tolerance through competitive binding to their common transporter FepA [Pugsley and Reeves, 1976, 1977]. The *exbB* locus was unlike a *tonB* phenotype since uptake of ferrous compounds still occurred albeit at a lower rate. The reason for an intermediate phenotype was later attributed to paralogous protein TolQ which could partially replace ExbB function via cross-talk. Deletion of both TolQ and ExbB eliminated TonB-dependent activities [Braun, 1989; Braun and Herrmann, 1993].

Because *exbB* and *exbD* are transcribed as an operon, early studies using Tn10 gene insertions also had polar effects on *exbD* expression [Ahmer et al., 1995]. Individual mutations in ExbD identified its importance for TonB system activities, and

expression of ExbB and ExbD individually confirmed that ExbB/D functions in TonB-dependent transport are codependent [Braun et al., 1996; Held and Postle, 2002].

TonB and ExbD in TonB-dependent transport

TonB and ExbD share a similar topology, containing a single N-terminal transmembrane domain (TMD) with the majority of their residues localized to the periplasm (Fig 1-2) [Hannavy et al., 1990; Kampfenkel and Braun, 1992; Postle and Skare, 1988]. TonB can be divided into three main structural domains: the N-terminal transmembrane domain, containing an important His residue, a central domain containing a non-essential Pro rich motif, and the C-terminal domain, which directly interacts with OM TGTs [Larsen et al., 1997; Larsen et al., 1993; Peacock et al., 2005; Skare et al., 1993]. ExbD contains two characterized functionally important residues, D25 in the TMD and L132 in the periplasmic domain [Braun et al., 1996]. While crystal structures for both TonB and ExbD carboxy terminal domains have been published [Chang et al., 2001; Kodding et al., 2005; Garcia-Herrero et al., 2007], the structures have been shown to lack *in vivo* relevance [Ghosh and Postle, 2005; Ollis and Postle, 2011, 2012a, 2012b; Postle et al., 2010].

The TonB TMD has been extensively studied demonstrating that TonB H20 is the sole important residue. Simultaneous alanine substitution of all TonB TMD residues except for conserved S16 and H20 retained full function. S16 is on the same face as H20, yet a S16A substitution on a wild-type TMD scaffold maintained near wild-type activity whereas H20A was inactive [Larsen et al., 2007]. Additional studies which substituted

TonB H20 to all 19 residues found that a structurally similar Asn supported full function. The positional requirement of TonB H20 was also tested by moving His to different TMD locations in a H20A TonB. Only TonB H20A V24H, located on the same face as H20, one turn towards the periplasm, restored significant activity [Swayne and Postle, 2011]. Although the ExbD TMD has been less extensively studied, an essential residue located near the cytoplasmic TMD boundary, D25, is essential for TonB response to pmf [Ollis et al., 2009; Ollis and Postle, 2012a, 2012b].

ExbB in TonB-dependent transport

ExbB adopts a N-out, C-in orientation and consists of three TMDs, a large cytoplasmic loop (residues ~40-129) and a C-terminal tail (starting at residues ~197 -224) (Fig 1-2)[Kampfenkel and Braun, 1993a; Karlsson et al., 1993b]. Three different predictions of ExbB TMD boundaries were determined through ExbB fusions/hydropathy plot analysis and resulted in the following predictions: TMD1 (16-39), (25-42) and (23-42); TMD2: (128-155), (131-160) and (141-159); TMD3 (162-194), (177-199), and (172-194) [Kampfenkel and Braun, 1993a; Karlsson et al., 1993a; Zhai et al., 2003].

E*xbB stabilizes TonB and ExbD in vivo*

While ExbB is stable independently of TonB and ExbD, TonB and ExbD stability is dependent upon the presence of ExbB. Earlier studies observed that the presence of ExbB enhanced TonB stability [Fischer et al., 1989; Skare and Postle, 1991]. While the exact domain through which ExbB stabilizes TonB is unknown, one mode of TonB stabilization could occur through the cytoplasmic domains of ExbB, as cytoplasmically

localized TonB without its signal sequence is rapidly degraded in the absence of ExbB [Karlsson et al., 1993b]. Nonetheless, multiple subsequent studies have confirmed a direct protein-protein interaction both *in vitro* and *in vivo* [Ahmer et al., 1995; Braun et al., 1996; Skare et al., 1993]. ExbD stability is also dependent upon the presence of ExbB although the mechanism of stability is unknown. [Ahmer et al., 1995; Braun et al., 1996, unpublished data]. Consecutive ten amino acid deletions within ExbD residues 62-141 were each proteolytically unstable, requiring overexpression of ExbB to achieve chromosomal levels [Ollis and Postle, 2012a].

ExbB transmembrane domains

ExbB and ExbD are thought to couple pmf to TonB energization through an uncharacterized mechanism. Furthermore, a prediction for a putative proton translocation pathway including the TMDs of ExbB, ExbD and/or TonB was made based on sequence homology to the proton translocating flagellar motor proteins, MotA/B [Zhai et al., 2003]. This pathway included ExbB TMDs 2 and 3, while TMD1 residues were not included and hypothesized to serve a structural role. The composition of the putative pathway is as follows: Periplasm ->T181 (TMD3) -> H_2O (near G184 in TMD3) ->H20 (TonB) -> S16 (TonB)-> H_2O (near A188 in TMD3) -> T148 (TMD2) -> H_2O (near G144 in TMD2) -> D25 (ExbD) or T181 (TMD3) -> S155 (TMD2)-> H_2O (near G151 in TMD2) -> T148 (TMD2)-> H_2O (near G144 in TMD2) -> D25 (ExbD) -> cytoplasm [Zhai et al., 2003]. The validity these proton pathway predictions were tested through Ala substitution of ExbB TMD residues S155, T148 and T181 [Braun and Herrmann, 2004]. While no residue was individually important, double substitution of T148 and T181

eliminated ExbB function. Additional Ala substitution of the well conserved ExbB E176 in TMD3, which was not included in the pathway prediction, eliminated function. However, Gln/Asp substitution at 176 was functional suggesting an important structural role. Due to limited mutational analysis, the existence of a proton pathway composed of ExbB TMD residues has not been rigorously investigated.

While mutagenic studies of ExbB TMD1 have not been performed, suppressors of inactive TonB TMD mutants were identified at ExbB residues V34D, V35D and A39E [Larsen et al., 1999; Larsen et al., 1994]. The ExbB suppressors were not allele specific as they suppressed the TonB mutants indiscriminately, while ExbB A39E suppressed TonB TMD mutations more strongly [Larsen et al., 1999; Swayne and Postle, 2011]. While mode of ExbB suppression is unknown, all three ExbB suppressors are located on one face of the ExbB TMD1 helix and substitute a non-polar residue to one with a negative charge.

Studies of ExbB cytoplasmic domains

Both ExbB cytoplasmic loop and tail regions have been analyzed through mutagenic studies. Deletion analysis of the ExbB cytoplasmic loop and the ExbB C-terminal tail confirmed the functional importance of these domains [Bulathsinghala et al., manuscript in preparation; Jana et al., 2011]. Additional cysteine scanning in the predicted ExbB cytoplasmic C-terminal tail domain identified important residues Y195, N196, D211, A288 and G244 [Jana et al., 2011]. Inactive N196A and A288C substitutions demonstrated the first instance of signal transduction from the cytoplasm in the TonB system. TMD3 cytoplasmic boundary predictions placed residues Y195 and

N196 within TMD3 near the cytoplasmic edge suggesting signal transduction from ExbB TMDs as well. Additionally, Cys substitutions in the ExbB tail formed disulfide-linked complexes despite their predicted localization in the reducing cytoplasmic environment suggesting the ExbB tail could be in an environment sequestered from cytoplasmic reductases [Jana et al., 2011].

Deletion of 10 consecutive residues in the ExbB cytoplasmic loop (residues 40-129) eliminated activity except for Δ100-109 which retained partial activity. Inactive ExbB loop deletion mutants were also unstable and growth inhibitory [Bulathsinghala et al., manuscript in preparation]. While the reason for growth inhibition is unknown, this phenotype was unaffected by deletion of ExbD and TonB indicating the deleterious effect of ExbB mutant expression was uninfluenced by known TonB system proteins. Further characterization of the ExbB mutants determined that deletions Δ50-59 and Δ120-129 partially dissipated pmf. However, the degree of pmf-dissipation was not substantial enough to account for the observed growth inhibition. Furthermore, all other ExbB loop deletions did not reduce the pmf; therefore, proton leakage was not the sole reason for growth inhibition [Bulathsinghala et al., manuscript in preparation]. The determinants of ExbB Δ120-129-mediated proton leakage were investigated in this dissertation (Chapter 3).

Homologous proteins: Flagellar motor proteins MotA/B and OM maintenance proteins TolQ/R

Inner membrane energy harvesting complexes MotA/B and TolQ/R share homology with ExbB/D proteins [Zhai et al., 2003]. Flagellar motor proteins, MotA/B translocate protons, which through FliG interaction with the cytoplasmic MotA loop, couples pmf energy to flagellar rotation [Blair, 2003]. TolQ/R with TolA use pmf energy to maintain OM membrane integrity [Cascales et al., 2001; Fig 1-4]. These two protein complexes are most homologous to ExbB/D in the TMD portions, with the soluble portions likely specialized for each systems specific function [Zhai et al., 2003]. MotB, TolR and ExbD share similar topology, each containing a critical Asp residue in their N-terminal TMDs [Cascales et al., 2001; Zhou et al., 1998]. TolQ and ExbB TMDs are also highly similar sharing identical topology and similar TMD boundary predictions [Kampfenkel and Braun, 1993b; Vianney et al., 1994]. While MotA shares a similar topology to ExbB/TolQ, MotA is more distantly related and features four instead of three TMDs. MotA TMD3 and TMD4 are most related to ExbB/TolQ TMD2 and TMD3 [Zhai et al., 2003].

Due to their similar TMD sequences ExbB/D, MotA/B and TolQ/R are all thought to harness pmf by a similar mechanism [Braun and Blair, 2001; Cascales et al., 2001; Zhai et al., 2003]. MotAB proton conduction was presumed through the observation that the co-overexpression of MotA and a MotB fusion containing the first 60 residues fused to 50 residues from its pBR322 vector caused growth inhibition [Blair et al., 1990]. Addition of inactive MotA TMD substitutions and the MotB D32 substitution reduced/eliminated growth inhibition of the overexpressed MotA-MotB truncation

indicating that that the MotAB TMD residues were instrumental for the growth inhibitory phenotype [Blair and Berg, 1990; Hosking et al., 2006; Morimoto et al., 2010a; Wilson and Macnab, 1990; Zhou et al., 1998]. While studies with MotA and a truncated MotB investigated the possibility of MotAB function in proton translocation, congruent studies in ExbBD and TolQR have not been performed.

The TMDs of TolQ and MotA have been more extensively studied, through site-specific mutation of most protonatable TMD residues [Braun et al., 2004; Goemaere et al., 2007; Kim et al., 2008; Sharp et al., 1995a; Zhang et al., 2011]. The 4 TMDs of MotA contain 7 protonatable residues which could participate in a proton channel according to the predicted TMD arrangement [Sharp et al., 1995a]. Based on the limited number of protonatable residues, the MotA/B proton channel is thought to contain mainly water molecules and the critical Asp in the MotB TMD [Sharp et al., 1995b]. Protonation of Asp is believed to trigger conformational changes which apply force to the FliG rotor protein to drive flagellar rotation [Blair, 2003]. While the mechanism of pmf utilization is less defined in the Tol system, important TolQ protonatable residues S28, T145, and T178 were identified through inactive residue substitution suggesting they may have functional importance in a proton pathway [Goemaere et al., 2007; Zhang et al., 2011]. Additional TolQ substitutions of residues potentially important for structure, Gly and Pro, were also important for activity. The glycine residues in TolQ TMD2 are also highly conserved in ExbB. Conserved proline residues in the TMDs of MotA and TolQ were also important for function with inactive TolQ P138C and P187C/V substitutions, while P138V retained partial activity [Goemaere et al., 2007; Zhang et al., 2011]. Similarly

substitution of prolines in MotA TMD3 (P173) and TMD4 (P222), to all other amino acids did not restore significant flagellar rotation [Blair and Berg, 1991; Braun et al., 1999]. Effects of the MotA proline substitutions on proton leakage of the MotA-MotB truncation mutant were found to reduce the severity of proton leakage (as assayed by growth inhibition), suggesting that the proline residues may also be important in a MotA proton translocation mechanism [Braun et al., 1999].

Cysteine scanning in MotA and TolQ TMDs determined the probable TMD arrangement through the formation of disulfide-linked dimers. MotA TMD3 and TMD4 and the MotB TMD are predicted to comprise the main portions of the putative proton channel while MotA TMD1 and TMD2 are located on the periphery of the complex [Braun et al., 2004; Braun and Blair, 2001; Kim et al., 2008]. Similarly, cysteine scanning in the TolQ TMDs also identified a similar arrangement based on disulfide-linked dimer formation and suppressor studies, placing TolQ TMD2, TMD3 and the TolR TMD on a putative proton channel, while TMD1 is predict to be on the periphery [Zhang et al., 2011]. The current model of TolQ/R TMD arrangement does not include TolA, which is homologous to TonB in TMD region. Earlier models placed TolA at the periphery of the TolQ/R complex based on potential TolA-TolQ interaction from suppressor studies [Cascales et al., 2001].

TonB, ExbD and ExbB form a complex in the CM

In Vivo formaldehyde crosslinking studies have identified homo- and heteromultimeric interactions between TonB, ExbB and ExbD [Higgs et al., 2002b; Higgs et al., 1998; Ollis et al., 2009; Skare et al., 1993]. Because formaldehyde is

membrane permeable and specific for close protein-protein interactions, via formation of a methylene bridge, it is good indicator for membrane protein interactions [Means and Feeney, 1971]. However, formaldehyde crosslinking might not detect all interactions, since only lysine, the N-terminus and to a lesser extent arginine, tryptophan and cysteine residues are reactive with formaldehyde at short incubation times [Toews et al., 2008; Toews et al., 2010]. *In vivo* TonB forms formaldehyde-crosslinked complexes with ExbB, ExbD and the OM TGT, FepA [Ollis et al., 2009; Skare et al., 1993]. Additional interactions with non-TonB system proteins Lpp and OmpA have been identified indicating that TonB interactions at the OM are not limited to the TGTs, however their functional significance is not known [Higgs et al., 2002a; Skare et al., 1993]. Only the TonB-ExbD crosslink requires pmf and functional TonB system proteins [Ollis et al., 2009]. A TonB-ExbD complex can also be detected with anti-ExbD antibodies and additional ExbD-ExbB heterodimer, and ExbD homodimer complexes are detected [Ollis et al., 2009]. However, TonB-ExbB and ExbB-ExbD complexes are not identified with anti-ExbB antibodies; instead, an ExbB homodimer and an ExbB tetramer complex containing up to 85kDa unidentified protein (ExbB tetramer +X) are detected [Higgs et al., 1998; Jana et al., 2011]. It is unclear why interactions with ExbD and TonB are not detected with the ExbB-antibodies. However one possibility is that ExbB formaldehyde detected interactions with TonB and ExbD may be less abundant with respect to the detected ExbB homomultimeric complexes [Higgs et al., 1998]. Consistent with this conclusion, ExbB is more abundant than TonB and ExbD proteins. While the exact composition of the energy transduction complex is unknown, TonB, ExbD and ExbB are present in at cellular ratio of 1:2:7 respectively [Higgs et al., 2002a].

Detection of the TonB-ExbD formaldehyde-crosslinked complex requires pmf, ExbB, TonB H20 and ExbD D25 TMD residues [Ollis et al., 2009]. TonB and ExbD periplasmic domain interactions can also be detected through disulfide crosslinking in Cys substituted TonB and ExbD residues. Since ExbD D25 is important for pmf-harnessing functions, the significant reduction of TonB-ExbD disulfide-linked dimer formation with ExbD D25N identified specific, pmf-promoted interactions between the ExbD and TonB periplasmic C-terminal domains [Ollis and Postle, 2012b].

Current Model of TonB-dependent Energy transduction

A number of mechanical models and a shuttle model have been proposed to describe the TonB energy transduction process [Krewulak and Vogel, 2011]. Recently, the shuttle model hypothesis was tested and disproved since TonB was shown to retain full activity even when anchored to the CM via fusion to the ToxR cytoplasmic domain, indicating that TonB does not shuttle *in vivo* [Gresock et al., 2011]. Because the TonB TMD lacks any absolutely essential protonatable residues to respond to pmf directly, ExbB and ExbD likely harness pmf energy for TonB energization, through an unknown mechanism. Current data suggest that, protonation of the conserved Asp in the ExbD TMD, triggers conformational changes in the ExbD TMD which are then transmitted to TonB for energization [Ollis et al., 2009; Ollis and Postle, 2012a, 2012b]. Data support an essential role for ExbB in TonB energization since inactivating ExbB substitutions in the loop and C-terminal tail domains do not support formaldehyde-detectable conformational changes in the TonB and ExbD periplasmic domains indicative of TonB energization [Bulathsinghala et al., manuscript in preparation; Jana et al., 2011]. Through

direct contact with the TGTs, TonB with ExbBD transduce pmf energy for active transport of ligands at the OM [Cadieux and Kadner, 1999; Gresock et al., 2011; Ogierman and Braun, 2003; Skare et al., 1993]. It is not known how TonB re-establishes an energized conformation after energy discharge to a TGT (i.e. TonB recycling); however, the ExbB suppressor A39E of TonB ΔV17 is proposed to be defective in TonB recycling since ExbB suppression caused instability of TonB only in the presence of ligand [Larsen et al., 1999].

Recently, the initial stages of TonB energization have been characterized (Fig 1-3). Based on TonB and ExbD periplasmic interactions, initial TonB energization has been divided into three stages [Ollis and Postle, 2012a]. Stage I was characterized in inactive ExbD and TonB mutants where the periplasmic portions of TonB and ExbD are unable to detectably interact and suggest a state where nascent TonB and ExbD have not yet assembled. Initial, pmf-independent interaction between the periplasmic domains of TonB and ExbD occurs in stage 2, which requires ExbB [Held and Postle, 2002]. In the presence of wild type TMDs, ExbB, and pmf, TonB and ExbD periplasmic conformations can progress to stage 3, where TonB and ExbD interactions are detectable through formaldehyde crosslinking and in disulfide dimer formation between periplasmic C-terminal domain residues [Ollis et al., 2009; Ollis and Postle, 2012b]. Under wild-type conditions, TonB and ExbD conformations toggle between stages 2 and 3 representative of TonB cycling through energized and de-energized conformations [Ollis and Postle, 2012a].

Role of ExbB TMDs in TonB-dependent energy transduction

While ExbB is essential for TonB-dependent transport, its role in this process is undefined. Currently, ExbB has three hypothesized roles, as a scaffolding protein, as a signal transducer between the cytoplasm and the periplasm and as a proton translocator [Ahmer et al., 1995; Fischer et al., 1989; Karlsson et al., 1993b; Larsen et al., 1994; Pramanik et al., 2010; Zhai et al., 2003]. In any of these roles, the ExbB TMDs would likely be central for ExbB function in TonB-dependent transport.

This work analyzed the functional importance of the ExbB TMDs in TonB-dependent energy transduction. In Chapter 2, characterization of the ExbB TMDs was initiated through redefinition of the TMD boundaries, since these boundaries are poorly defined. New TMD predictions were made using advanced TMD prediction software and sequence conservation analysis. Consistent with the expected importance of the ExbB TMDs, substitution of each half TMD to Ala residues demonstrated that no ExbB TMD was indispensable for function. Additional characterization of the TMDs was accomplished through mutagenic analysis of individual ExbB TMD residues. Through this approach, functional regions within the TMDs important for TonB-ExbB assembly and ExbB multimerization were identified. In addition, individual residues involved in signal transduction from the TMDs to the periplasmic domains of TonB and ExbD were also identified in this study.

While this study supported ExbB function as a scaffolding and signal transducer, an ExbB function as a proton translocator was ruled out. Individual substitution of all protonatable residues within the TMDs did not eliminate function, indicating that ExbB residues do not participate in a proton translocation pathway. This conclusion was

seemingly in conflict with a previous finding where partial pmf-dissipation was caused by deletion of ExbB cytoplasmic loop residues 120-129. In Chapter 3, important ExbB TMD residues were substituted in the ExbB Δ120-129 mutant, to illuminate the reason for this discrepancy. Substitution of ExbB TMD residues in the Δ120-129 background did affect the rate Δ120-129-mediated proton leakage, confirming that the proton leakage was influenced by the ExbB TMDs. However, substitution non-essential residues in the ExbB TMDs disproportionately decreased the proton leakage rate compared to essential residues. This suggests that ExbB Δ120-129-mediated proton leakage may be occurring through a non-native ExbB conformation.

Analysis of the ExbB TMDs supported two of the three hypothesized roles for ExbB. First ExbB function as scaffolding protein was supported through several residue substitutions which altered ExbB homomultimer and heteromultimer assembly. Furthermore, this study identified ExbB facilitation in the initial assembly of the TonB-ExbD periplasmic domains. In addition to a scaffolding-like role, inactive ExbB TMD mutants blocked signal transduction to the periplasmic domains of TonB and ExbD which was not attributable to assembly defects. This implicates ExbB function in both initial assembly of the TonB-energization complex and in transduction of the pmf signal for TonB-dependent transport. Importantly, this study ruled out a role for ExbB as a proton translocator and raises important questions pertaining to how the TonB system harnesses and transduces pmf-energy for nutrient uptake at the OM.

Chapter 4 discusses implications of this work and outlines future directions to further define the mechanistic role of ExbB in TonB-dependent energy transduction.

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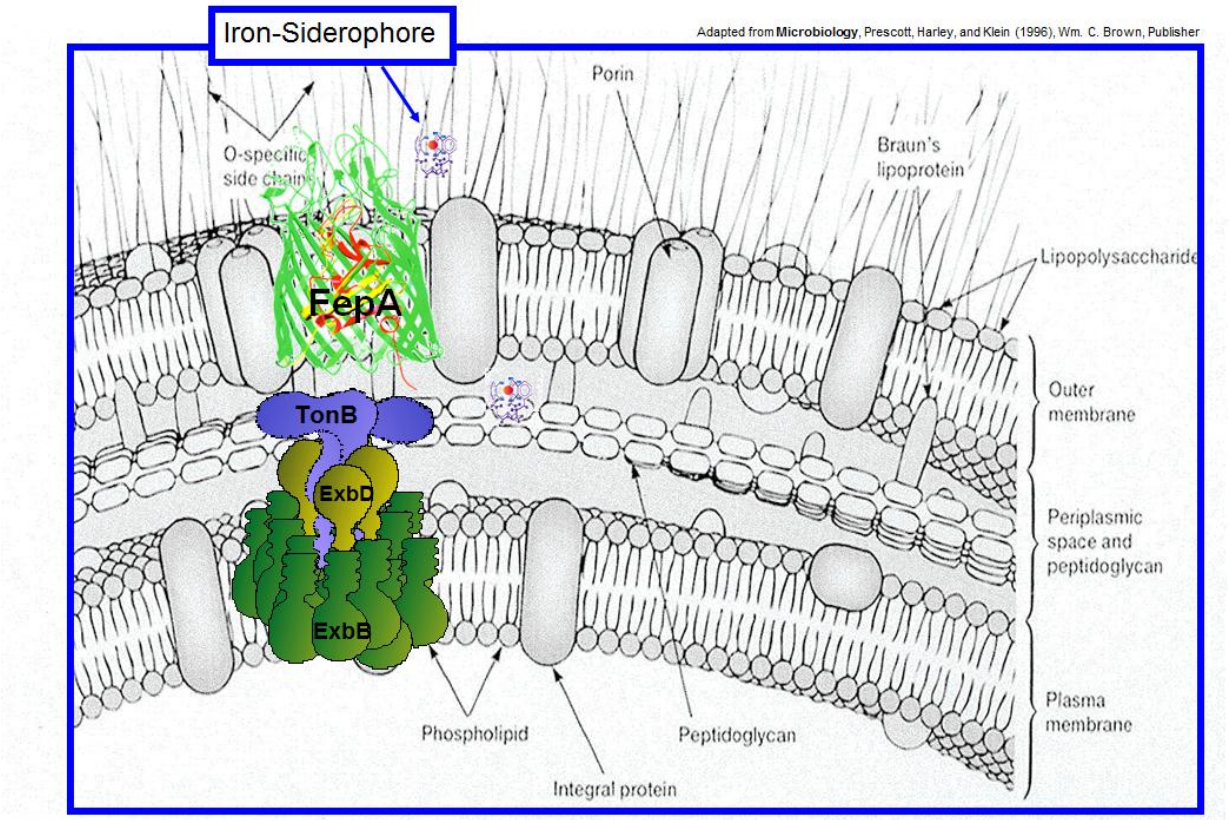

Figure 1-1 *The Gram negative bacterial cell envelope*. This schematic depicts the organization of the Gram negative bacterial envelope. The inner, cytoplasmic (plasma) membrane is the site where generation of proton motive force occurs. The asymmetric outer membrane is composed of an inner leaflet of phospholipids and an outer, protective leaflet of lipopolysaccharides. The periplasmic space (periplasm) is located between the two membranes and contains a thin layer of peptidoglycan. TonB system proteins: TonB, ExbB and ExbD form a complex in the cytoplasmic membrane. Since the composition of the TonB energization complex *in vivo* is unknown, this diagram illustrates a hypothetical TonB energization complex. The iron-siderophore, enterochelin, is depicted outside the outer membrane next to its specific TonB-gated transporter FepA and in the periplasm to portray TonB-dependent active transport. This illustration is not drawn to scale. The Gram negative cell envelope was adapted from [Prescott et al., 1996]. The FepA crystal structure is from [Buchanan et al., 1999].

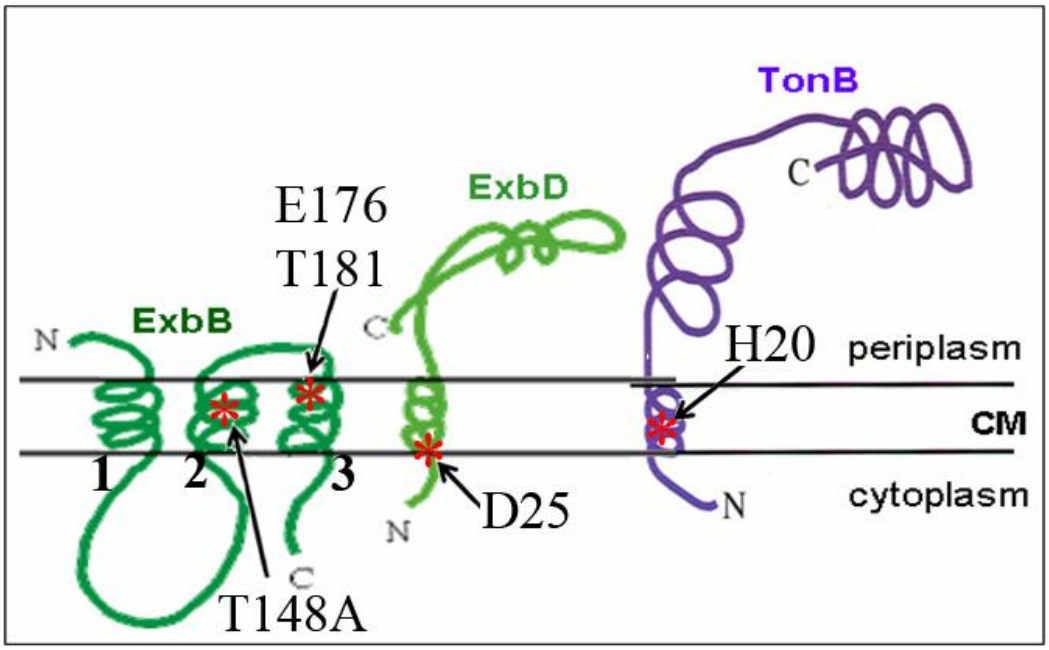

Figure 1-2 *Topology of TonB system proteins.* TonB, ExbB and ExbD are localized in the cytoplasmic membrane (CM). The periplasm and cytoplasm are indicated. TonB and ExbD share a similar topology with short, cytoplasmic N-terminal domains, a single TMD and large periplasmic domains. ExbB contains three TMDs, a small N-terminal periplasmic tail and large cytoplasmic loop and C-terminal tail domains. The ExbB TMDs are labeled 1 through 3. The general location of important TMD residues D25 (ExbD) and H20 (TonB) are indicated by an asterisk. The asterisks in ExbB indicate the general location of previously studied TMDs residues T148, E176 and T181, which are not essential for function. This illustration is not drawn to scale.

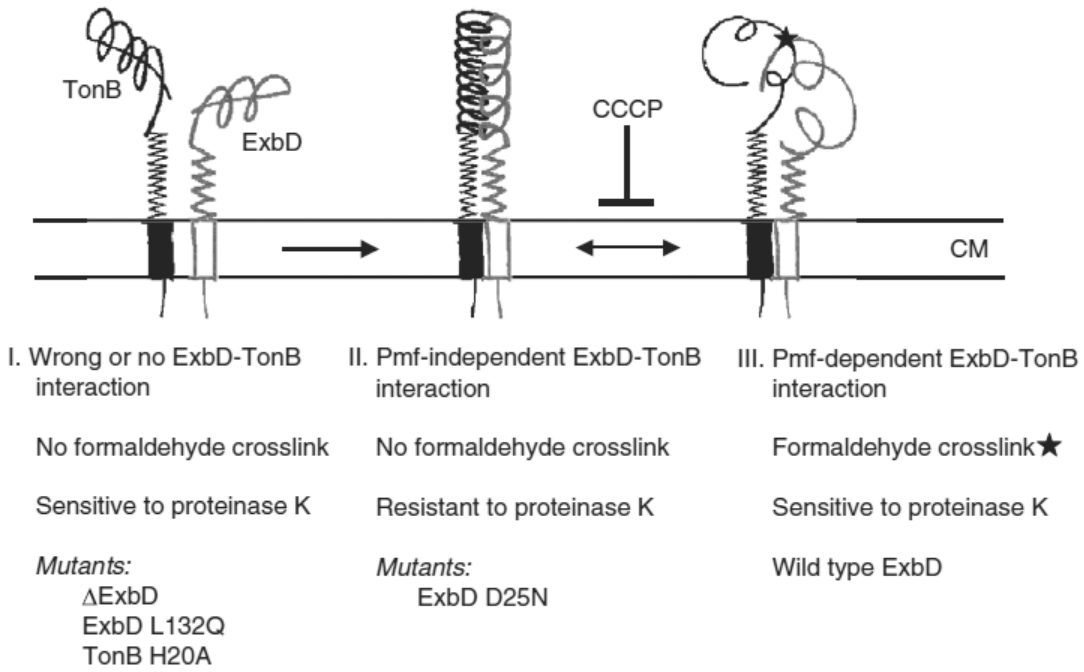

Figure 1-3 *Model of initial TonB energization stages.* Three stages of TonB energization are shown, listed left to right. Localized in the cytoplasmic domain (CM), TonB is shown in black with a filled in TMD and ExbD is shown in gray with an unfilled TMD. The boxed regions within the CM represent the TMDs, unstructured periplasmic regions are in shown as zig zag lines, while potential C-terminal domain structures are shown. This figure in not drawn to scale. ***While ExbB is not shown, it is assumed to be present for all stages.*** Characteristic TonB proteinase K sensitivity and formaldehyde detectable interactions and mutants which stall TonB-ExbD interactions (or prevent interactions) are also listed below each stage. In stage 1, TonB and ExbD periplasmic domains are not assembled. TonB is proteinase K sensitive and does not form a TonB-ExbD formaldehyde crosslinked complex. Assembly mutants TonB H20A and L132Q mutants prevent assembly of the TonB-ExbD periplasmic domains. In stage 2 pmf - independent TonB and ExbD periplasmic domain interactions are resistant to proteinase K treatment and do not formaldehyde crosslink. Collapse of pmf (via addition of protonophore CCCP) or the ExbD D25N TMD substitution stalls TonB conformations in this stage. In the presence of pmf, ExbB, and wild type TonB and ExbD TMDs, TonB

and ExbD conformations progress to stage 3 where TonB is sensitive to proteinase K and TonB-ExbD interactions can be detected via formaldehyde crosslinking (indicated with a star). *In vivo*, TonB and ExbD interactions cycle between stages 2 and 3. Reprinted with permissions from [Ollis and Postle, 2012a]

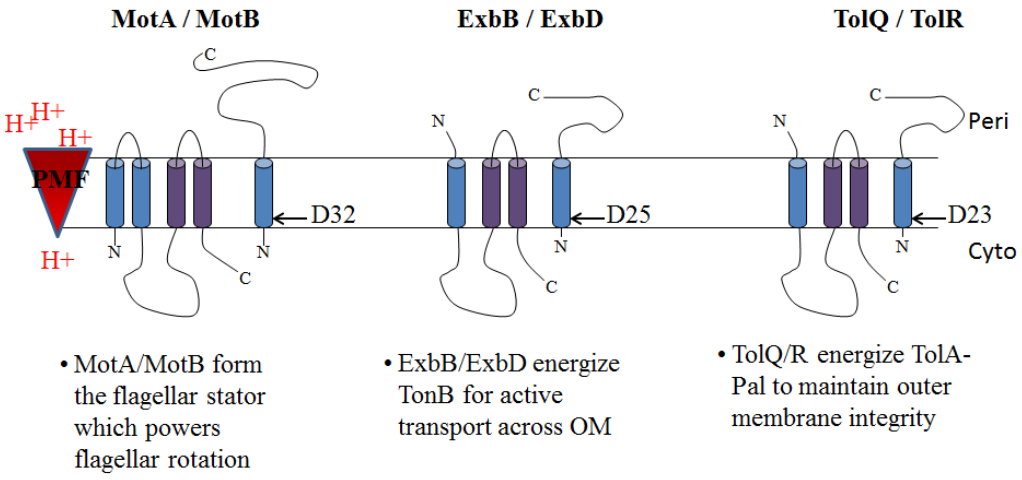

Figure 1-4 *Illustration of pmf-harnessing systems: TonB, Tol and MotA/B flagellar motor*. The Tol-Pal system is depicted on the right, TonB system in the middle and MotA/B flagellar motor is on the left. ExbB, TolQ and MotA share a similar topology with large cytoplasmic domains and three or four TMDs. TMDs are arranged in ascending numbers from left to right, TMD1, TMD2 and TMD3 (and TMD4 for MotA).The last two TMDs of ExbB/TolQ/MotA which share the greatest homology are shown in purple. ExbD, TolR and MotB also have similar topologies with a single TMD containing the critical Asp residue and large periplasmic domains. The red triangle depicts proton motive force (pmf).

CHAPTER 2

MUTATIONS IN *ESCHERICHIA COLI* EXBB TRANSMEMBRANE DOMAINS IDENTIFY SCAFFOLDING AND SIGNAL TRANSDUCTION FUNCTIONS AND EXCLUDE PARTICIPATION IN A PROTON PATHWAY

This chapter is in preparation for submission to *Journal of Bacteriology* and is formatted slightly different than the preceding chapter to accommodate publication guidelines. KRB is the first author of the work and has performed all the experiments.

Chapter 2

Mutations in *Escherichia coli* ExbB Transmembrane Domains Identify Scaffolding and Signal Transduction Functions and Exclude Participation in a Proton Pathway

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Abstract

The TonB system functions as an energy conduit, connecting proton motive force (pmf) at the cytoplasmic membrane to nutrient uptake through high affinity transporters at the outer membrane. Current data suggest that cytoplasmic membrane proteins ExbB and ExbD harness pmf energy through an unknown mechanism and connect it to TonB. Remaining anchored at the cytoplasmic membrane, TonB transmits this energy to high affinity TonB-gated transporters through direct interaction. In *Escherichia coli,* recent studies have clarified the functional roles for TonB and ExbD in this process. However, the functional role of ExbB is not well understood. ExbB adopts an unusual N-out, C-in topology, contains three transmembrane domains (TMDs) and large cytoplasmic loop and tail domains. Here, the functional role of the ExbB TMDs was examined. New ExbB TMD predictions replaced three prior, incongruent TMD predictions. Substitution of each half TMD with alanine eliminated TonB-dependent transport indicating essential functions for all three ExbB TMDs. Individual TMD residue substitutions identified ExbB scaffolding-like functions in TonB-ExbB and ExbB multimer formation. Furthermore, inactive TMD mutants prevented TonB-ExbD pmf-dependent periplasmic interaction constituting functions in signal transduction from the ExbB TMD to the periplasm. Importantly, individual substitution of all protonatable residues in the ExbB TMDs excluded the hypothesis that ExbB residues function in proton translocation. With both structural and functional roles identified, these results indicate a central role for the ExbB TMDs in TonB-dependent energy transduction.

Introduction

In Gram negative bacteria, the TonB system couples cytoplasmic membrane (CM) proton motive force (pmf) to active transport of ligands at the outer membrane (OM) through TonB-gated transporters (TGTs) (for reviews see (1-5). The TonB system is composed of three CM proteins TonB, ExbB and ExbD and several different TGTs in the OM that recognize diverse ligands. In *Escherichia coli* K-12, the TonB system transports iron-siderophore compounds and vitamin B12, while in other Gram negative bacteria additional nutrients including nickel, sucrose, heme, and maltodextrin are transported (6-9).

TonB and ExbD have the similar topology of one N-terminal transmembrane domain (TMD) and a large periplasmic domain (10, 11). In contrast, ExbB contains three TMDs with a large soluble cytoplasmic loop between TMDs 1 and 2, and a soluble cytoplasmic C-terminal tail (12, 13). ExbB appears to be the scaffold upon which TonB and ExbD assemble since TonB and ExbD are proteolytically unstable in the absence of ExbB, while ExbB stability is independent of TonB and ExbD (13-16, unpublished results).

TonB physically interacts with OM TGTs, connecting CM pmf energy to OM ligand transport (17-20). A proton pathway through ExbB, ExbD and/or TonB TMD residues has been proposed based on the similar sequence homology to the flagellar pmf harnessing motor proteins, MotA and MotB (21). Through sequence alignment two different pathways mediated primarily by ExbB TMD residues were proposed. The first includes residues in TonB: T181 -> H_2O (near G184) ->H20 (TonB) -> S16 (TonB)->

H_2O(near A188) -> T148 -> H_2O(near G144) -> D25 (ExbD). The second does not: T181 -> S155-> H_2O(near G151) -> T148 -> H_2O(near G144) -> D25 (ExbD) (21).

We demonstrated that all the residues except H20 in the TonB TMD can be substituted with Ala without significantly decreasing TonB activity (22). Recently, we also showed that H20 can only be substituted with the non-protonatable residue Asn and still retain full activity (23). These studies removed H20, and thus the TonB TMD, from consideration as a participant in a proton translocation pathway. However, both ExbB and ExbD are required for TonB conformational response to pmf (24-27).

The predicted proton pathways through ExbB from Zhai and Saier were evaluated by Braun and Herrmann in 2004. They found that individual alanine substitution of predicted ExbB proton pathway residues S155, T148 in TMD2 and T181 in TMD3 were functional, while double T148A/T181A substitution was inactive for unknown reasons. ExbB E176 in TMD3, which is not within the originally proposed proton pathway prediction, was inactive when substituted to Ala but fully active with Asp and, more importantly, Gln substitution suggesting that E176 was not on a proton translocation pathway (28). In that study, however, other potential TMD residues that could affect ExbB activity, including those within ExbB TMD1, were not evaluated.

The first task for identifying residues on a proton pathway is to define TMD boundaries. In the past, widely different predictions have been made for the location of ExbB TMD boundaries: TMD1 (16-39), (25-42) and (23-42); TMD2: (128-155), (131-160) and (141-159); TMD3 (162-194), (177-199), (172-194) (12, 13, 21). We recently reassessed the likely cytoplasmic boundary of ExbB TMD3 using the MPEX TMD prediction algorithm combined with an analysis of conserved regions to likely end at

residue 196 (29). In this study, we extended that analysis to the rest of ExbB TMD boundaries using the TOPCON program that takes a similar approach and uses a consensus of multiple prediction programs (30). This resulted in significant redefinition of these regions, predicting TMD1 from 22-42, TMD2 from 132-152 and TMD3 from 178-198. Here we show that block alanine substitution of each half ExbB TMD inactivated ExbB, indicating that all 3 TMDs were essential for function. Subsequent substitution of individual TMD residues identified residues critical for ExbB function. Several TMD residues were important for signal transduction or assembly of the energy transduction complex. No residues were important for proton translocation. Thus, like the TonB TMD, ExbB TMDs do not appear to be part of a proton translocation pathway.

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Methods and Materials

Strains and Plasmids

The bacterial stains and plasmids used in this study are listed in Table 2-1. Plasmid pKP660 contains ExbB and ExbD which are expressed from the arabinose promoter of pBAD24 (25). ExbB substitutions were constructed by site-directed mutagenesis PCR using a pKP660 template as previously described (31). ExbB TMD half-Ala substitutions were made using 30 cycle extra-long PCR with half of the replacing alanine sequence on each forward and reverse primer. Correct sequences of both *exbB* and *exbD* genes were confirmed in all mutant plasmids by the Penn State Genomics Core Facility (University Park, PA).

Media and Culture Conditions

Lubia-Bertani (LB), Tryptone (T), and M9 minimal salts were prepared as described previously (32, 33). Agar plates, T-top agar and liquid cultures were supplemented with 100μg/ml of ampicillin and plasmid specific amount of L-arabinose to equal chromosomal protein levels. M9 salts were supplemented with 1.0% glycerol (wt/vol), 0.4 mg of thiamine/ml, 1 mM $MgSO_4$, 0.5 mM $CaCl_2$, 0.2% Casamino Acids (wt/vol), 40 mg of tryptophan/ml, and 1.85 μM$FeCl_3$. Cultures were grown with aeration at 37 °C.

Spot titer Assays:

To determine ExbB activity, strains expressing mutant ExbB proteins at chromosomal levels were grown to mid-exponential phase and plated in T-top agar on T-plates containing identical arabinose concentrations and 100 μg/ml ampicillin. Chromosomal level of expression was confirmed by immunobloting. 5-fold dilutions of colicins and 10-fold dilutions of phage were spotted on the bacterial lawns in triplicate. Plates were incubated ~18 hours at 37 °C. Scoring was reported as the reciprocal of the highest dilution that produced clearing (34). For the individual and double cys substitutions, an insignificant amount ExbB homodimer was detected only after long exposure of non-reducing anti-ExbB immunoblots, indicating that spontaneous disulfide-linked dimer formation was not responsible for any reduced activity *in vivo* (data not shown). Additionally, no evidence of intramolecular crosslinks occurring through the native ExbB C25 residue was observed (data not shown).

[^{55}Fe] ferrichrome Transport

Initial rate of iron transport was performed as described previously (34, 35). Strains containing plasmids were grown in M9 minimal media supplemented with 100 μg/ml ampicillin and L-arabinose to maintain chromosomal expression levels. Cells were grown to mid-exponential-phase, harvested and suspended in assay buffer. Initial rates of [^{55}Fe] ferrichrome transport were determined in triplicate. Chromosomal level of expression was confirmed through trichloroacetic acid (TCA) precipitation of assayed samples and ExbB levels were visualized by immunoblot with anti-ExbB (36).

In Vivo Formaldehyde Crosslinking

Saturated overnight LB cultures were subcultured to M9 media containing 100ug/ml ampicillin and arabinose for chromosomal levels of ExbB expression. Cells were grown to mid-exponential phase and 0.5 $ODml_{550}$ cells were harvested and pelleted at room temperature and suspended in 938μl 100mM Na^{2+} phosphate buffer, pH 6.8. Cells were then treated with 1% paraformaldehyde for 15 minutes at room temperature then pelleted at room temperature and solubilized at 60 °C for 10 minutes in Laemmli sample buffer (LSB) (37, 38). Rapid formaldehyde crosslinking used for ExbB half-Ala substitutions was performed similarly with the following exceptions. Cells were grown and harvested as above and suspended in 938μl 100mM Na^{2+} phosphate buffer, pH 8.0. Cells were treated with 1% paraformaldehyde for 3 minutes at room temperature. The crosslinking reaction was then quenched with 1% acetic acid. Crosslinked samples were

resolved on 13% or 11% SDS- polyacrylamide gels and detected by immunoblotting with anti-ExbB, anti-ExbD or anti-TonB antibodies (36, 39).

Proteinase K sensitivity

TonB sensitivity to proteinase K was performed as previously described (26, 40, 41). Spheroplasts were generated as previously described (40), and treated with 60μM of protonophore CCCP (carbonyl cyanide m-chlorophenylhydrazone) or solvent only, DMSO, 5 minutes prior to proteinase K treatment. Proteinase K at 25 μg ml^{-1} was added for 2, 5, 10, or 15 minutes followed by the addition of protease inhibitor, 1.0 mM PMSF (phenylmethylsulfonyl fluoride). After limited proteolysis, samples were then precipitated with trichloroacetic acid (TCA) and resolved on 13% SDS-polyacrylamide gels. Proteins visualized on immunoblots probed with TonB-specific antibodies (39). Non-proteinase K treated whole cell samples were also immunobloted with ExbB-specific antibodies to confirm chromosomal expression levels (36).

Results

Defining ExbB transmembrane domain boundaries

To address the role of the three ExbB TMDs in TonB-dependent energy transduction, we began by reassessing their predicted boundaries. The recently described TOPCONS program uses a consensus of multiple different topology prediction algorithms including evolutionary conservation based programs and the "biological" hydrophobicity scale from experimentally generated measurements involving Sec61-

mediated integration of TMD helices (Bernsel et. al. 2009). TOPCON predictions for TMD boundaries were as follows: TMD1: 22-42, TMD2: 132-152 and TMD3: 178-198 (Fig. 2-1) (http://topcons.cbr.su.se).

Sequence alignments of ExbB sequences with expect scores from e^{-28} to e^{-7} showed that the TMD regions are more highly conserved than the predicted soluble domains, as had been noted previously for more closely related species (28) (Fig. 2-2). Sequence conservation for TMD1, the least conserved TMD, did not indicate clear boundaries but was consistent with the TOPCONS prediction. TMD2 contained conserved Leu (133), Pro (141) Gly (144-147-151), and Thr (148) residues (Fig. 2-2). For TMD3, the TOPCONS-predicted periplasmic boundary occurred at residue 178, which would omit a highly conserved Glu 176. We extended the analysis to include Glu 176, even though E176Q was fully active in the 2004 study of Braun and Herrmann because it is so highly conserved (28). TMD3 sequence similarity drops considerably after residue 196, as noted previously (Fig.2-2) (29). TMD3 contained conserved Leu (178), Thr (181), Pro (190) and Asn (196) residues (Fig 2-2).

All ExbB TMDs are essential for function

Large segments of the TonB TMD can be simultaneously substituted with Ala residues without affecting its activity (22). To determine rapidly if any portions of the three ExbB TMDs were dispensable, half of each predicted TMD (~11 consecutive amino acids) was substituted as a block with Ala on pKP660, which encodes the *exbB, exbD* operon under arabinose control. The six resulting plasmids were designated as a group to encode "half-Ala TMD" ExbBs. Individually they were designated according to

the location of the Ala block substitution, e.g. “TMD1 peri” for substitution of alanines on the periplasmic half of TMD1. Function of each half-Ala TMD was assessed by spot titer assay of sensitivity to colicins B, D, Ia, M and phage ϕ80, each of which requires the TonB system to enter and subsequently kill *E. coli*. All of the mutants could be expressed at near chromosomal levels and were completely tolerant (insensitive) to all agents tested, indicating that each half TMD was essential for ExbB function (Table 2-2).

To determine if the half-Ala TMD mutants were dominant, each was expressed at least 100 X greater than the chromosomal level. Overexpressed half-Ala TMDs from TMDs 2 and 3 significantly reduced the ability of wild type ExbB to support [^{55}Fe] transport to ~5% or less (Fig. 2-3). TMD1 peri and cyto were the least impaired only reducing activity to ~20% and ~50% of wild type respectively. These results suggested that each of the half-Ala TMD for TMD 2 and 3 retained significant ability to assemble into a multimeric complex, and suggested that TMD1 might play a more direct role in assembly.

Half-Ala TMDs do not support formation of the pmf-dependent TonB-ExbD complex

In vivo interactions in the TonB system can be identified by crosslinking with monomeric formaldehyde (20, 25, 29, 36, 37). Wild-type ExbB crosslinks to form homodimers and a homotetrameric complex that includes ~85kDa of unknown protein known as “ExbB tetramer + X” (29, 37). When expressed in the absence of wild-type ExbB, the least dominant half-Ala TMD1 cyto reduced formation of the ExbB tetramer + X, consistent with the results from the dominance study (Fig. 2-4). The half-Ala TMD2 cyto also reduced formation of this complex, but likely for other reasons than a defect in

assembly since this mutant was dominant. The remaining half-Ala TMD mutants did not reduce tetramer + X formation. Additionally, TMD1 peri and TMD2 cyto contained two complexes in the ExbB tetramer + X region with the lower complex migrating near 130 kDa (Fig. 2-4). While the composition of these two complexes was not clear, it is likely that neither complex is a pure ExbB homotetramer considering that such a complex would migrate at ~100kDa (29). Instead, half-Ala substitution in TMD1 and TMD2 may have altered ExbB conformations to the extent that alternative crosslinkable conformations of ExbB were favored and migrated differently in SDS-PAGE.

The uniform inactivity of the half-Ala TMD mutants was almost certainly due to their uniform inability to support formation of the TonB-ExbD formaldehyde crosslink that typifies an active TonB system (25). This pmf-dependent complex was absent from ExbD crosslinking profiles of all the variants (Fig.2-5). ExbD co-expressed with TMD2 cyto was present at lower levels, suggesting that its proteolytic stability was reduced. Despite the reduced ExbD levels, an ExbD-ExbB complex was still apparent suggesting that ExbB-ExbD assembly was not disrupted. Consistent with their decreased ability to form the ExbB tetramer + X complex, ExbB TMD1 cyto and TMD2 cyto mutants significantly eliminated or reduced TonB-ExbB complex detection by anti-TonB antibodies (Fig. 2-6).

Identification of important residues in ExbB TMDs

Three roles for ExbB have been proposed: as a scaffold that stabilizes and mediates assembly of TonB and ExbD, as transducer of signals between cytoplasm and periplasm, and as a proton translocator (14-16, 21, 27, 29). To identify residues

important for these roles, we created substitutions throughout the three ExbB TMDs based on conservation and theoretical importance for TMD helix packing and/or for pmf-coupling. Selected residues were substituted to Ala on pKP660 using site-directed mutagenesis. The ExbB mutants were initially assayed for TonB-dependent [^{55}Fe] transport activity. This discriminative assay can resolve the activity level of partial-function mutants relative to wild type (35). Mutants identified as inactive by that criterion could still have low levels of activity. These were additionally tested in spot titers of colicins B, D, Ia, M and phage ϕ80, which is a more sensitive, but less discriminative, assay for TonB function (35) (Fig. 2-7, 2-8, 2-9 and Table 2-3).

Effects of substitutions at protonatable residues in ExbB TMDs

Polar residues within TMDs play roles in proton translocation and in driving helix assembly (42, 43). We analyzed Ala substitutions at S34 and T37 (TMD1), Y132, T135, S140, and T148 (TMD2), and T181 (TMD3). Previously, ExbB T148A and T181A were 99% active in iron transport assays, however steady state levels of the mutants were not determined (28). Here, Ala substitution at all ExbB hydroxyls within the predicted TMDs were expressed at chromosomally encoded levels.

In these studies, T148A reduced [^{55}Fe] transport activity to ~22%, while ExbB T181A supported intermediate activity (61%) (Fig. 2-7). Braun and Herrmann also evaluated double Ala substitutions T148A/T181A and found them completely inactive (28). The inactivity of T148A/T181A double substitution was confirmed here with spot titers (Table 2-3). The difference in our results and those of Braun and Herrmann could be due to protein overexpression in their study.

Of the remaining substitutions at hydroxyl residues, only ExbB S34A and T135A eliminated [^{55}Fe] transport activity entirely (Fig. 2-7), while T135A retained slight activity in the more sensitive spot titers assays (Table 2-3). The remaining hydroxyl residue substitutions T37A, Y132A and S140A, were fully active in [^{55}Fe] transport and not considered further (Fig. 2-7).

Charged residues play critical roles in proton binding for proton translocating protein complexes such as H^+ F_0-F_1 ATPase, M_2 viral protein, flagellar motor protein MotB, and bacteriorhodopsin [reviewed in (42)]. Of the two charged residues in the ExbB TMDs, K24A in TMD1 was fully functional; ExbB E176A in TMD3 supported ~25% activity, ruling out an essential role in proton translocation (Fig. 2-8). Our observation that additional substitutions of E176 to Asp or Gln supported 70% and 25% activity respectively confirmed this conclusion (Fig. 2-8). In the Braun and Herrmann study, ExbB E176Q supported 80% iron transport activity (28). Taken together these results indicated that E176 does not play a key role in proton translocation.

Effect of substitutions at unique function glycine, proline and tryptophan residues

Common TMD packing motifs containing Gly residues have been implicated in driving helix assembly (44, 45). ExbB and closely related homolog, TolQ, both contain a $G_{144(141)}xxG_{147(144)}$ $xxxG_{151(148)}$ packing motif in TMD2 (TolQ positions in parenthesis). For TolQ TMD2, G141A, G144A, and G148A are completely inactive in pmf-dependent OM stability, TolA-Pal co-immunoprecipitation and pmf-independent colicin uptake assays, suggesting that corresponding ExbB residues may also be functionally important (46). However, none of the Ala substitutions of corresponding ExbB TMD2 Gly residues

144, 147 and 151 eliminated activity, supporting ~90%, 67%, and ~45% [^{55}Fe] transport activity respectively (Fig. 2-9). In addition, Ala substitution of less conserved Gly residues G29 (TMD1), G131 and G137 (TMD2) and highly conserved G184 (TMD3) were fully functional (Fig. 2-9).

Considering that ExbB and TolQ TMD sequences are highly conserved and that TolQR can partially substitute for ExbBD functions in TonB-dependent energy transduction, it is almost certain that ExbB and TolQ TMD functions are mechanistically similar (47-49). Therefore, it is likely that activity differences between TolQ and ExbB Ala substituted glycines reflected a disparity in the different assay sensitivities rather than actual mechanistic differences. Additionally, the steady state levels of the TolQ substitutions in that study are unknown, further complicating activity comparisons to corresponding ExbB substitutions. Given the 100% Gly conservation for distantly related ExbB alignments, glycines at these positions almost certainly comprise an important helix packing motif as Goemaere et al. postulated (46) (Fig 2-2). Ala substitution of the remaining individual Gly residues might be too conservative to highlight their full functional importance.

Proline-kinked α-helices influence TMD helix-helix interactions and can be important for propagating conformational changes during signal transduction (44, 50, 51). In both MotA and TolQ systems, the proline residues are proposed to influence proton translocation functions via regulation of proton flow through helix movements mediated by Pro-kinked helices (46, 52, 53). Substitutions of conserved proline residues P173 and P222 in the TMD domains of the homologous flagellar motor protein MotA eliminated torque generation (52, 54). Similar to results with MotA P222A, TolQ P187V

(ExbB P190A) eliminated Tol-dependent activities (46). In contrast, TolQ P138V (ExbB P141, MotA P173) was partially active.

In these studies we obtained an intermediate result for Ala substitution at ExbB P141 (TMD2), which supported ~45% activity in [^{55}Fe] transport. While P190A in TMD3 had a more detrimental effect, it still supported ~15% transport activity (Fig. 2-8). The presence of low but detectable activity for ExbB P190A but not the corresponding prolines in MotA and TolQ probably reflected variances in the sensitivities of the different activity assays for the three paralogues. These reduced but intermediate ExbB activities of substitutions at Pro residues appeared to support a role in TMD helix assembly, but did not argue strongly for an absolute role in proton gating.

Tryptophan can play a structural role in TMD anchoring and positioning within the membrane by stabilizing interactions at membrane-water interfaces (43, 55). Tryptophan located within the TMD interior has demonstrated roles in oligomer assembly and in an ion gating mechanism (56, 57). According to our TMD predictions, ExbB W150 in TMD2 was located near the membrane-water interface and ExbB W38 in TMD1 was located within the TMD boundaries. While ExbB W150A was fully active in [^{55}Fe] transport, W38A supported only 18% activity (Fig. 2-8). W38 is located on the same helical face as functionally important S34 residue in TMD1, suggesting that this face was somehow important for ExbB function.

ExbB facilitates, but is not essential for, formation of an initial pmf-independent TonB-ExbD interaction

To assess effects of ExbB mutants in more mechanistically informative assays, we first need to more fully understand the role of wild-type ExbB. Three stages in the initial energization of TonB at the CM have been identified using the pmf-sensitive assays of TonB proteinase K resistance and formation of TonB-ExbD formaldehyde crosslinks (25, 26). In Stage I, TonB and ExbD are not detectably associated; TonB is sensitive to proteinase K and does not form a formaldehyde crosslink with ExbD in vivo (26). In Stage II, which does not require pmf, TonB and ExbD periplasmic domains assemble such that their interaction prevents proteinase K degradation of TonB residues 1-155 (~23 kDa). When pmf is collapsed, TonB-ExbD interactions are arrested at stage II, demonstrated by essentially complete conversion of all TonBs to the ~23 kDa proteinase K resistant fragment, apparently due to protection by ExbD. Only when both pmf and ExbB are present does TonB energization progress from Stage II to Stage III. In that Stage, TonB and ExbD undergo pmf-dependent rearrangement of their conformational relationship such that formaldehyde crosslinkable residues in both proteins are in proximity to crosslink. This shift in the conformational relationship renders the entire length of TonB proteinase K sensitive again.

To determine the role of ExbB in progression from Stage I to Stage II of TonB energization, the proteinase K sensitivity of TonB in the absence of ExbB was assessed. In strain RA1017 (Δ*exbBD* Δ*tolQRA*), a small but detectable proportion of TonB could form the characteristic proteinase K resistant fragment after 2 min of treatment with proteinase K, indicating that TonB and ExbD had a capacity to interact in the absence of

ExbB (Fig. 2-10, middle panel). Because the proportion of TonB in this form did not increase upon the addition of protonophore CCCP, which stalls TonB at Stage II, ExbB was still clearly required for the initial efficient TonB-ExbD Stage II assembly (Fig 2-10, compare top and middle panel, +CCCP). These results indicated that, while not absolutely essential for initial Stage II TonB-ExbD interactions, ExbB greatly facilitated them. The small proportion of the TonB proteinase K resistant fragment that formed in the absence of ExbB was still dependent upon ExbD, since in absence of both ExbB and ExbD, the characteristic ~23 kDa fragment did not form (Fig 2-10, bottom panel). Instead, a small population of TonB was degraded to a ~28kDa fragment as noted previously (26).

Identification of ExbB TMD residues that mimic effects of ExbD D25N on TonB energization

ExbD, and homologous proteins TolR and MotB TMDs all contain a highly conserved protonatable Asp residue which is considered a key component of their respective putative proton pathways (21, 27, 58, 59). The ExbD D25N mutant stalls the TonB-ExbD interaction at the pmf-independent Stage II regardless of whether or not pmf is present, suggesting protonation of D25 is essential for conformational response to pmf (26). Thus behavior of the inactive ExbD D25N substitution in the proteinase K sensitivity assay provides an example where proton translocation is prevented. It is important for the interpretation of our data here to also recognize that ExbD D25N also decreases the stability of the TonB-ExbD Stage II interaction in the presence of CCCP for unknown reasons. The degradation of TonB stalled at Stage II by the ExbD D25N

mutation may reflect dissociation of TonB and ExbD D25N, or increased accessibility of ExbD D25N and subsequently TonB, to proteinase K. Any ExbB TMD substitutions that mimicked the behavior of ExbD D25N in this assay would be candidates to be on a proton translocation pathway.

All ExbB substitution mutants supported the Stage II initial assembly of the pmf-independent TonB-ExbD interaction to a much greater extent than seen in the absence of ExbB (compare levels of the proteinase K resistant fragment in Fig. 2-10, 2 min time point from middle panel +CCCP with Fig. 2-11, 2 min time points +CCCP). There was mutant-specific variability in the stability of proteinase K-resistant form in the presence of CCCP over time. For those mutants that had the least deleterious effects in the phenotypic assays (P141A, G147A, G151A, E176A, T181A) the TonB proteinase K-resistant fragment remained stable over the 15 min time course, similar to results in the presence of wild-type ExbB. For ExbB S34A, W38A, T135A, P190A, Y195A and the T148A/T181A double mutant, the TonB proteinase K resistant fragment was unstable over the 15 min time course, mimicking the behavior of TonB in the presence of ExbD D25N [Fig. 2-11; (26)]. To confirm that S34A, W38A, T135A, T148A/T181A and P190A were unable to support a TonB transition from Stage II to Stage III, their ability to support formation of the pmf-dependent TonB-ExbD formaldehyde crosslinked complex was determined. Consistent with the proteinase K resistance assay results, none of them was able to (Fig. 2-12, 2-13, 2-14, 2-15, 2-16, 2-17, 2-18).

The behavior of ExbB W38A, P190A and Y195A indicated that other factors in addition to proton translocation could affect conversion of TonB from Stage II to Stage III. Tryptophan and proline are not protonatable residues and were likely to play a

structural role. Although Y195 is potentially a protonatable residue, Y195F is fully functional, indicating that aromaticity is the only important aspect of the tyrosine residue at that position (29). Thus this assay could rule out residues as participants in proton translocation by their ability to support a wild-type conformational response of TonB to collapse of pmf. To test participation of S34A, T135A, or T148A/T181A in proton translocation would require additional substitutions and assays described below.

ExbB S34A and W38A are assembly mutants that define a TMD1 cyto face important for TonB-ExbB interactions.

Residues whose only role was in proton translocation would not be expected to impair ExbB assembly functions, and would thus be dominant. Assessment of dominance of ExbB S34A, W38A, T135A, T148A, Y195A, P190A and the double mutant T148A/T181A was carried out as for the half-Ala TMD mutants above.

ExbB S34A did not reduce [^{55}Fe] transport lower than ~70% of chromosomally encoded ExbB activity, compared to over-expressed wild-type ExbB, which reduced [^{55}Fe] transport to ~85% of chromosomally encoded activity (Fig. 2-19). Thus ExbB S34A was non-dominant, suggesting that it could not assemble into a complex with wild-type ExbB. Likewise, ExbB W38A exhibited only moderate dominance. The ability of assembly mutants S34A and W38A to form the ExbB tetramer + X complex was surprisingly not impaired, suggesting that there were subsequent essential configurations of wild-type ExbB tetramer in which they could not participate during energy transduction (Fig. 2-20). Where they appeared to be impaired was in their ability to

crosslink to TonB in vivo. S34A eliminated the TonB-ExbB complex while W38A reduced it (Fig. 2-21). Even though ExbB S34A did not stabilize TonB in a half-life study (data not shown), it clearly still maintained some sort of interaction because it supported greater formation of the TonB-ExbD Stage II complex than the absence of ExbB did (Fig 2-11). This implied S34A altered but did not abolish TonB-ExbB interaction through the TMDs. ExbB W38A consistently gave rise to high levels of the tetramer + X complex as well as a high level of background complex formation, possibly suggesting that it was getting delayed in moving out of the tetramer + X conformation (Fig. 2-20).

These data are consistent with the iron transport results where S34A was completely inactive and W38A had about 20% iron transport activity. ExbB S34 and W38, which share a face on the cytoplasmic half of TMD1, appeared to identify a face that was important for assembly with TonB. Consistent with that, the half-Ala TMD1 cyto in which they reside was the least dominant of the half-Ala TMDs, reducing activity to ~45% (Fig. 2-3).

ExbB P190 and P141 mediate efficient transition from ExbB homodimers to ExbB tetramers

ExbB appears to be a dimer of dimers, which subsequently interacts with an unknown protein to form the ExbB tetramer + X (29). ExbB P141A and P190A exhibited dominant negative phenotypes with P190A being more dominant of the two, reducing wild-type activity to ~10%. Both ExbB P141A and P190A exhibited an increase in ExbB homodimer formation suggesting that they could not easily transition from dimer to

tetramer. For P190A, the ExbB tetramer + X complex was essentially absent (Fig. 2-22 and 2-23). It is important to note that failure to form tetramer + X complex is not proof that it no longer exists, only that the relationships between crosslinkable residues in that complex has changed. In this case however, since a correspondingly high level of dimer complex was found instead, the data suggested that ExbB P190A existed primarily as a dimer, with dominance due to preventing normal tetramerization of wild-type ExbB. These results removed ExbB P141 and P190 from playing a role in proton translocation and were consistent with the iron transport results where activity was reduced but not absent in the Ala substitutions.

Substitution analysis of ExbB T135 and the T148/T181 combination

Potential roles of important polar ExbB TMD residues in proton translocation were further investigated by determining which other near-cognate residues could functionally substitute. Both Ser and Thr have been found on proton translocation pathways, whereas Cys, which has a similar overall structure, has not (42). Cysteine can form hydrogen bonds (60). Valine substitutes a methyl group for the hydroxyl of threonine and thus has a somewhat similar shape while being non-polar. Substitution of ExbB T135 with cysteine and serine supported 62% and 86% activity in [^{55}Fe] transport, respectively. Even substitution with valine retained 8% activity (Fig. 2-7). Because all the T135 C/S/V substitutions increased activity relative to T135A (0%), it indicated that hydrophobicity, size and shape, or ability to form a hydrogen bond were important at this position, rather than the ability to translocate protons.

Additional single substitutions at ExbB T148 and T181 further supported the contention, based on the Ala substitutions that neither was on a proton translocation pathway. Because the T148S substitution, which could conceivably contribute to a proton pathway, did not restore significant activity over and above the T148A substitution (28% vs 23% in the [^{55}Fe] transport assay, Fig. 2-7), there appeared to be a preference for Thr at that position that was not due to a requirement for proton translocation. ExbB T148C and T148V substitutions resulted in ~3% and 0% activity respectively (Fig 2-7 and Table 2-3). In contrast to T148, all T181 substitutions were active. ExbB T181 Ser, Cys, and Val substitutions supported 81%, 33% and 42% [^{55}Fe] transport respectively, demonstrating that T181A was not part of a proton translocation pathway (Fig 2-7).

The double mutant T148A/T181A was completely inactive. This was not due to a requirement for T181 as shown above. Double substitutions of T148/T181 to Cys, Ser, or Val residues were assayed for [^{55}Fe] transport and/or in spot titers (Fig. 2-7, Table 2-3). The double Ser substitution retained ~21% [^{55}Fe] transport activity. Since ExbB T181S supported 80% activity, this was not an additive effect but instead reflected the activity of the least active substitution of the pair, T148S. This result suggested that the two residues were not acting synergistically. The ExbB T148C/T181C substitution supported nearly full sensitivity to colicin Ia in spot titers, where the requirement of the TonB system for pmf is absolute (Table 2-3). Taken together, these results argue against the idea that T148 or T181 play any role in proton translocation. Consistent with the effect of the single T148V substitution, the ExbB T148V/T181V mutant was completely inactive (Table 2-3).

The role of ExbB T148

The inactive or reduced activity of individual T148 substitutions described above suggested that T148 was more important than T181 for ExbB activity. To define that role, T148C/V substitutions were analyzed using the more mechanistically informative assays applied to the single substitutions. T148V concomitantly reduced ExbB tetramer + X and homodimer complex detection in formaldehyde crosslinking. T148V/T181V reduced ExbB tetramer +X detection, with what appeared to be a compensatory increase in ExbB homodimers (Fig. 2-24). Double T148/T181 Ala and Cys substitutions did not affect ExbB multimer formation, suggesting that the phenotype of the Val substitutions reflected structural interference. The structural interference did not prevent ExbB T148V from being highly dominant (Fig. 2-19). ExbB T148V also had a unique phenotype in the proteinase K sensitivity assays that assess TonB-ExbD interactions (Fig 2-11). While the rest of the inactive ExbB TMD mutants had a TonB proteinase K profile similar to ExbD D25N, T148V produced a TonB proteinase K profile where the TonB proteinase K resistant fragment was present at nearly 100% in the cell population, even in the presence of pmf (spheroplasts without added CCCP). ExbB T148V also did not reduce the stability of the TonB proteinase K resistant fragment under any circumstance. Thus ExbB T148V appeared to keep TonB permanently stalled in Stage II such that it remained in contact with ExbD.

ExbB S34A is an assembly mutant

The ExbB S34A, in the first TMD, although entirely inactive, was unlikely to be on a proton pathway. It was not dominant in the [^{55}Fe] transport assay and prevented formation of the TonB-ExbB formaldehyde crosslink that is sensitive to mutations in their TMDs (Fig 2-21) (41). These data suggested that S34 played a structural role in allowing correct assembly. Additional S34 substitutions supported this conclusion. S34C/T restored very low but detectable activity in spot titers, suggesting that both size and polarity are important at S34 (Table 2-3). Confirming the role for ExbB S34A in assembly with TonB, both ExbB S34C and S34V restored a low but detectable level of the TonB-ExbB formaldehyde crosslinked complex (Fig. 2-25).

Discussion

New TMD predictions for the ExbB/TolQ/MotA/PomA family

The ExbB/TolQ/MotA/PomA family are integral cytoplasmic membrane proteins. ExbB and TolQ have three TMDs with the N terminus in the periplasm. MotA and PomA have four TMDs, with the "extra" TMD occurring at the N terminus in the cytoplasm. There is a high degree of homology among the C-terminal two TMDs of all four proteins (Fig. 2-26). That, and the fact that they all are in multi-protein complexes that require pmf as the energy source, unites them as a family. However, the *E. coli* ExbB, TolQ, and MotA proteins and the MotA orthologue, PomA from *Vibrio alginolyticus* have dissimilar published TMD predictions [ExbB: (12, 13, 21) TolQ: (61, 62) MotA: (63-65) PomA: (66)]. Because all four proteins are likely to have similar

mechanisms of action, we subjected TolQ, MotA and PomA to analysis with TOPCONS. The results significantly altered the previous predictions for the last two TMD of each protein, and brought them into greater congruency with the ExbB TMD predictions (Fig. 2-27). Based on that information, we aligned the last two TMDs of each protein based on 6 shared highly conserved residues: those corresponding to ExbB G137, G144, G151, and P141 in the second to last TMD, and those corresponding to ExbB T181 and G184 in the last TMD (Fig. 2-26). For MotA, residues corresponding to ExbB P141, G144, T181, and G184 are absolutely conserved among over 50 comparisons in many different species (67).

Glycine residues in TMDs drive helix assembly and are thus important markers by which conserved TMDs can be aligned. ExbB, TolQ and PomA all have a $G_{144}xxG_{147}$ $xxxG_{151}$ motif in the second to last TMD (ExbB residues 132-152). Such glycine zipper motifs are highly conserved and play a role in helix packing (45). MotA lacks the central Gly residue (G147 in ExbB) and in its place carries the most conservative replacement, Ala, least likely to interfere in packing. All 4 second-to-last TMDs have an additional Gly residue (Gly137 in ExbB). We suggest that this redefinition of the boundaries of the last two TMDs is an accurate one, reflecting high degree of residue conservation and current best identifications of transmembrane domain helices. It is important to note that while our predictions are for 20-residue TMDs, longer TMDs are not ruled out. But wherever the TMD boundaries lie, they will be based upon the high degree of residue conservation.

By significantly redefining the TMD boundaries, the TOPCON predictions for MotA and TolQ may alter interpretations of previous studies. Previously defined TMD

boundaries place MotA P173 (corresponding to P141 in ExbB) at the cytoplasmic edge of the second to last TMD, where it was proposed to interact with the essential pmf-responsive MotB residue Asp32, also at the cytoplasmic edge of the TMD (67). The redefined TMD boundaries for MotA place the conserved P173 in the center of the TMD, where it may be sufficiently distant from MotB D32 that the proposed interaction does not occur. Unless the ExbD/TolR/MotB/PomB TMD boundaries are redefined in the future, this suggests that proline corresponding to MotA P173 serves a structural role and is not directly involved in proton translocation as previous studies have suggested (52, 53). A non-essential structural role for the P173 in MotA is consistent with our findings where the corresponding ExbB P141A retained 45% activity. In addition, ExbB P141A supported formation of both Stage II and Stage III TonB-ExbD interactions.

In studies of ExbB paralogues, the PomA T186I and MotA T209W, which correspond to ExbB T181, were both inactive (64, 68). Our results suggested that the observed inactivity was likely due to the non-conservative nature of the substitutions; the corresponding ExbB T181 was highly tolerant to substitutions with Ala, Ser, Val, and Cys residues. To the extent that the four proteins have similar mechanisms, the conserved Thr in the second-to-last PomA and MotA TMDs does not appear to be important for anything other than optimal helix packing (44).

ExbB TMDs are not on a proton translocation pathway

We mutated all the potentially interesting residues in the three ExbB TMDs with the goal of identifying the role of ExbB in TonB-dependent energy transduction (Fig. 2-28). The results suggest that its role is primarily in signal transduction from the

cytoplasm to the periplasm and in correct assembly of the TonB-ExbD complex. We could find no evidence that any ExbB TMD residues were on a proton translocation pathway. Since the majority of amino acid sequences of ExbB and TonB-ExbD are positioned on opposite sides of the cytoplasmic membrane, the interaction of the three ExbB TMDs with the two TMDS of TonB and ExbD respectively probably provides the means of communication between cytoplasm and periplasm.

The role of ExbB TMD1

ExbB TMD1 is the least conserved among the corresponding TMDs of the ExbB/TolQ/MotA/PomA family. The properties of the half-Ala cyto substitution and the two inactive substitutions, S34 and W38 within that region indicated that ExbB interacts with TonB through this TMD. The primary sequences of the corresponding TMDs among the family therefore likely reflect different additional TMDs with which each family member interacts in energy transduction, outer membrane integrity and flagellar rotation (Fig. 2-26).

The inactive ΔVal17 deletion in the single TonB TMD is suppressed by ExbB alleles V35E, V36D, and A39E (40, 41). Based on previous definitions of ExbB TMD1 boundaries, these residues were thought to be near the cytoplasmic boundary of TMD1. However, the redefinition of the boundaries in this study places them well within the cytoplasmic half, but on an opposite face relative to S34 and W38 (Fig. 2.29). The suppressors somehow reestablish ExbB TMD1 interactions with the TonB TMD. It is notable that the suppressors all exchange the negatively charged Asp or Glu for the native Ala or Val residues (40, 41). Similar suppressors exist between TonB/ExbB paralogs

TolA/TolQ, where a TolQ TMD1 face corresponding to ExbB S34 supports similar conserved functional interactions (69, 70).

The role of ExbB TMD2

All individual ExbB TMD2 Ala substitutions formaldehyde crosslinked with TonB, with ExbD and could form the ExbB homo-dimer and tetramer + X complex, thus eliminating key roles in assembly. Most exhibited little to moderate decreases in ExbB activity. This TMD appeared to play a role in signal transduction because ExbB T135A, the only inactive Ala substitution in TMD2, did not support the Stage III interaction between TonB and ExbD. In addition, the T148V substitution caused TonB-ExbD to stably stall at Stage II. Because the half-Ala TMD2 cyto reduced ExbD stability, there appeared to be some sort of role in interaction as well.

The role of ExbB TMD3

With one exception, all individual ExbB TMD3 substitutions formaldehyde crosslinked with TonB, with ExbD and could form the ExbB homo-dimer and homotetramer + X. The exception was ExbB P190A which had ~ 15% activity and appeared to be stalled at the homo-dimer state. Thus the kink induced by P190 appeared to be important for the transition from homodimer to homotetramer + X. Consistent with that idea, ExbB P141A in TMD2 led to a more modest decrease in activity, but like ExbB P190A, accumulated higher levels of formaldehyde crosslinked homodimers. In the re-evaluated TMD boundaries, P141 and P190 were located at similar depths (Fig. 2-28).

Prolines are known to play important roles in signaling mediated by transmembrane domains (50).

Idiosyncratic profile of reduced activity ExbB TMD mutants in colicin uptake.

The conformation of the disordered TonB C-terminus is hypothesized to be managed by ExbD protein such that it recognizes ligand-bound transporters by induced fit. Different Cys substitutions in the TonB C-terminus have different phenotypes in a variety of assays that measure transporter-specific sensitivity to colicins, phage, and ability to transport Fe-ferrichrome (71, 72). The C-terminus of TolA has similar. It is logical that the carboxy terminus of TonB would interact differentially with different transporters and colicin ligands, all of which have different amino acid sequences. It is not logical that mutations in ExbB would have idiosyncratic phenotypes because ExbB supposedly only interacts with TonB and ExbD in the energy transduction complex and not with the transporter proteins to which energy is transduced. Nonetheless, idiosyncratic phenotypes were observed for some ExbB substitutions in colicin sensitivity assays. For example, ExbB T148C/T181C was nearly insensitive to colicin D and completely insensitive to colicin M while retaining significant sensitivity to colicin B and nearly wild-type sensitivity to colicin Ia. Colicins B and D both use the outer membrane transporter FepA as a receptor, however, colicin D exerts its effects as a tRNAse in the cytoplasm whereas colicin B kills cells by creating a pore in the cytoplasmic membrane. Colicin Ia, which uses the transporter Cir as a receptor, also kills by creation of a cytoplasmic membrane protein pore. It seems unlikely that ExbB T148C/T181C differentially alters TonB conformations and transporter recognition. Alternatively, after

periplasmic entry of Tol-dependent colicin A, TolQ was required for colicin A killing activity suggesting additional colicin interactions with TolQ at the CM (73). This possibility has not been explored for ExbB substitutions.

How does the TonB system harness pmf?

While the use of cytoplasmic membrane pmf energy for TonB-dependent transport across the outer membrane has been well established, the mechanism by which pmf is harnessed remains unknown (1, 40, 74-76).

Our studies here ruled out participation of ExbB TMD residues in proton translocation and instead supported a role in signal transduction and direct interaction with the TonB TMD. Since no ExbB residues play a direct role in proton binding, it is theoretically possible that protons could traverse the membrane through an aqueous channel formed, a least in part, by the ExbB TMDs. In helical wheel projections of ExbB TMD2 and TMD3, conserved small residues line one face of each TMD from residues in G147 to A134 in TMD2 and T181 to A187 in TMD3 which could hypothetically form part of an aqueous channel (Fig. 2.29). Although an aqueous channel model has been proposed for proton translocation in the Mot system, it has not been experimentally explored (67). Repositioning of the TMD boundaries in this study also raised questions about that model.

The TonB TMD has been eliminated from any direct role in harnessing pmf. It is instead important to retain TonB in the cytoplasmic membrane and allow direct interaction of its periplasmic domain with the ExbD periplasmic domain (23, 77-79). ExbD emerges as the only protein with a TMD residue—an essential conserved Asp—

that somehow responds to pmf and mediates initial stages of TonB energization as ExbD transits from a homo-dimer to two different conformations of TonB-ExbD heterodimer (25, 26, 78). It is not clear how a single Asp would, by itself, harness pmf and use it to transmit conformational changes to the TonB C-terminus. The cellular ratio of ExbB:ExbD:TonB is 7:2:1, with TonB known to function as a dimer; which suggests that there is excess ExbB unassociated with ExbD or TonB. In the Mot and Tol systems, the complex consists of 4 MotA/TolQ: 2MotB/TolR (67, 70). In both Mot and Tol systems there is similar excess of ExbB paralogues relative to the ExbD paralogues (80, 81), suggesting that pools of uncomplexed ExbB/MotA/TolQ exist, which complicates any analysis.

It has been known for years that unidentified proteins exist which are essential for TonB activity (82). At least one of these proteins is characterized by a short half-life and, because they have not turned up in genetic selections, they are likely to be either redundant or essential (83, 84). Both ExbD and ExbB formaldehyde crosslink to unidentified proteins *in vivo* (25, 29, 37). The most abundant ExbB *in vivo* formaldehyde-crosslinked complex contains an ExbB tetramer linked to an unknown protein or proteins of ~ 85 kDa. In this study, the two substitutions P190A and T148V that disrupted formation of the tetramer + X also rendered ExbB non-functional. The ExbB tetramer + X complex does not require TonB or ExbD to form, suggesting that it is a stable and abundant complex (37). It is not known through which domain of ExbB this complex forms. Likewise, ExbD crosslinks in vivo to a protein of ~ 18kDa (25). This crosslink grows more abundant with inactive ExbD.

What might be the function of the unknown proteins? It is possible that an unidentified protein could contribute TMDs and form a more complete proton translocator. Alternatively, for the ExbD paralogue, TolR, it has been shown that rotation of the helices is important for function (85). If the ExbD helices also need to rotate, perhaps this is accomplished by the interaction of ExbB cytoplasmic domains with unidentified cytoplasmic proteins. It has been recently shown that the thrombopoietin receptor has multiple dimeric conformations achieved through rotation of helices (86). It is likely that the identification of unknown proteins will be important for future progress in understanding the mechanism.

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Table 2-1. Strains and Plasmids

Strains	**Genotype**	**Source**
W3110	*F-IN(rrnD-rrE) 1*	Hill and Harrnish (1981)
RA1017	W3110 *exbBD::kan* Δ*tolQRA*	Larsen 2007

Plasmids	**Genotype**	**Source**	**[Arabinose][a]**
pBAD24	Empty vector control	(Guzman *et al.*, 1995)	NA
pKP660	pBAD24 expressing *exbBD* from the pBAD promoter	This Study	0.0003
pKP1461	*exbB*(K24A),*exbD*	Present study	0.0003
pKP878	*exbB*(C25A),*exbD*	(Jana et. al. 2011)	0.0003
pKP1474	*exbB*(G29A),*exbD*	Present study	0.0007
pKP1462	*exbB*(S34A),*exbD*	Present study	0.0003
pKP1498	*exbB*(S34C),*exbD*	Present study	0.0003
pKP1499	*exbB*(S34T),*exbD*	Present study	0.0003
pKP1463	*exbB*(T37A),*exbD*	Present study	0.0005
pKP1471	*exbB*(W38A),*exbD*	Present study	0.0004
pKP1475	*exbB*(G131A),*exbD*	Present study	0.0003
pKP663	*exbB*(Y132A),*exbD*	Present study	0.0003
pKP1470	*exbB*(T135A),*exbD*	Present study	0.0003
pKP1612	*exbB*(T135C),*exbD*	Present study	0.0003
pKP1521	*exbB*(T135S),*exbD*	Present study	0.0003
pKP1522	*exbB*(T135V),*exbD*	Present study	0.0005
pKP1476	*exbB*(G137A),*exbD*	Present study	0.0003
pKP1465	*exbB*(S140A),*exbD*	Present study	0.0003
pKP1466	*exbB*(P141A),*exbD*	Present study	0.0003
pKP1477	*exbB*(G144A),*exbD*	Present study	0.0003

Table 2-1. ***continued***

Plasmids	Genotype	Source	[Arabinose][a]
pKP1478	*exbB*(G147A),*exbD*	Present study	0.0003
pKP1419	*exbB*(T148A),*exbD*	Present study	0.0003
pKP1422	*exbB*(T148C),*exbD*	Present study	0.001
pKP1421	*exbB*(T148S),*exbD*	Present study	0.0005
pKP1437	*exbB*(T148V),*exbD*	Present study	0.0005
pKP1464	*exbB*(W150A),*exbD*	Present study	0.0003
pKP1479	*exbB*(G151A),*exbD*	Present study	0.0003
pKP1327	*exbB*(E176A),*exbD*	Present study	0.0005
pKP1523	*exbB*(E176D),*exbD*	Present study	0.0003
pKP1472	*exbB*(E176Q),*exbD*	Present study	0.0003
pKP1420	*exbB*(T181A),*exbD*	Present study	0.0003
pKP1422	*exbB*(T181S),*exbD*	Present study	0.0003
pKP1437	*exbB*(T181V),*exbD*	Present study	0.0005
pKP1480	*exbB*(G184A),*exbD*	Present study	0.0003
pKP1473	*exbB*(P190A),*exbD*	Present study	0.0003
pKP664	*exbB*(Y195A),*exbD*	(Jana et. al., 2011)	0.0003
pKP1081	*exbB*(N196A),*exbD*	(Jana et. al., 2011)	0.0006
pKP1520	*exbB*(N196D),*exbD*	Present study	0.0003
pKP1519	*exbB*(N196H),*exbD*	Present study	0.0003
pKP1423	*exbB*(T148A, T181A),*exbD*	Present study	0.0008
pkP1623	*exbB*(T148C, T181C),*exbD*	Present study	0.0003
pKP1424	*exbB*(T148S, T181S),*exbD*	Present study	0.0003
pKP1437	*exbB*(T148V, T181V),*exbD*	Present study	0.0008
pKP1194	pBAD24 expressing *exbD* from the pBAD promoter	(Ollis, Kumar and Postle 2012)	0.05

Table 2-2. ExbB half alanine substitutions are non-functional in Spot titer assays.

		Sensitivity[a]				
Mutant[b]	Ara (%)[c]	Col B	Col D	Col Ia	Col M	ϕ80
Δ*exbBD* Δ*tolQRA*	NA	T,T,T	T,T,T	T,T,T	T,T,T	T,T,T
pExbBD	0.00005%	S,S,S	S,S,S	S,S,S	S,S,S	S,S,S
TMD1, peri	0.0001%	T,T,T	T,T,T	T,T,T	T,T,T	T,T,T
TMD1, cyto	0.0001%	T,T,T	T,T,T	T,T,T	T,T,T	T,T,T
TMD2, peri	0.0001%	T,T,T	T,T,T	T,T,T	T,T,T	T,T,T
TMD2, cyto	0.0001%	T,T,T	T,T,T	T,T,T	T,T,T	T,T,T
TMD3, peri	0.0001%	T,T,T	T,T,T	T,T,T	T,T,T	T,T,T
TMD3, cyto	0.0001%	T,T,T	T,T,T	T,T,T	T,T,T	T,T,T

[a] Colicins and phage ϕ80 were spotted in 5-fold and 10-fold dilutions respectively. Clearing was indicated as "S" which denotes sensitivity and "T" denotes tolerance (insensitivity) to the agent tested. Results of triplicate experiments are shown.

[b] All mutants were expressed near chromosomal levels in strain RA1017 (Δ*exbBD* Δ*tolRQA*). RA1017 contained the empty vector plasmid pBAD24. The following are the corresponding plasmid names for the mutants listed: pExbBD (pKP660); TMD1 peri (pKP1459);TMD1 cyto (pKP1460); TMD2 peri (pKP1481); TMD2 cyto (pKP1482); TMD3 peri (pKP1483); TMD3 cyto (pKP1484).

[c] Percentage of L- arabinose added to growth media and plates to achieve near chromosomal expression levels. "NA" not applicable.

Table 2-3. Sensitivity of low activity ExbB substitutions to TonB-dependent colicins and phage ϕ80

		Sensitivity[a]				
Substitution	Ara (%)[c]	Col B	Col D	Col Ia	Col M	ϕ80
WT	NA	8,8,8	6,6,6	7,7,7	6,6,6	8,8,8
exbBD tolQRA	NA	T,T,T	T,T,T	T,T,T	T,T,T	T,T,T
pExbBD	0.00005	7,7,7	4,4,4	7,7,7	6,6,6	7,7,7
S34A	0	T,T,T	T,T,T	T,T,T	T,T,T	T,T,T
S34C	0.00005	T,T,T	T,T,T	2,2,2	3,3,3	3,3,3
S34T	0	T,T,T	T,T,T	U,U,U	T,T,T	1,1,1
T135A	0.00005	3,3,3	T,T,T	4,4,4	3,3,3	3,3,3
T148A	0.00005	7,7,7	2,2,2	7,7,7	4,4,5	3,3,3
T148C	0.0001	3,3,3	T,T,T	4,4,4	T,T,T	T,T,T
T148V	0.00005	T,T,T	T,T,T	T,T,T	T,T,T	T,T,T
T181A	0.00005	7,7,7	3,3,3	7,7,7	6,6,6	8,8,8
T181C	0.0001	7,7,7	3,3,3	7,7,7	6,6,6	8,8,8
T181V	0.00005	7,7,7	3,3,3	7,7,7	3,3,3	7,7,7
T148A, T181A	0.00005	T,T,T	T,T,T	T,T,T	T,T,T	T,T,T
T148C, T181C	0.0001	3,3,3	T,T,T	4,4,4	T,T,T	4,4,4
T148V, T181V	0.00005	T,T,T	T,T,T	T,T,T	T,T,T	T,T,T

[a] Colicins and phage ϕ80 were spotted in 5-fold and 10-fold dilutions respectively. Results are presented as the highest dilution that provided clearing. U, undiluted and T, tolerant (insensitive). Results of triplicate experiments are shown.

[b] WT indicates strain W3110 and Δ*exbBD* Δ*tolRQA* indicates strain RA1017. Both contained the empty vector control from which all plasmids were expressed, pBAD24. All mutants were expressed near chromosomal levels in RA1017. Corresponding plasmid names are listed in table 1.

[c] Percentage of L- arabinose added to growth media and plates to achieve near chromosomal expression levels. “NA” not applicable

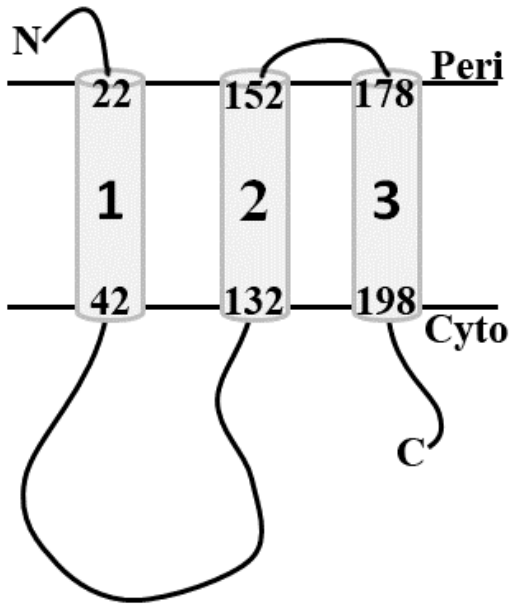

FIG. 2-1. *Revision of ExbB TMD boundaries: ExbB topology*. ExbB topology within the cytoplasmic membrane (parallel black lines), and the TOPCON-predicted boundaries for TMDs 1, 2, and 3 are shown. The majority of ExbB is localized in the cytoplasm (cyto), containing a large cytoplasmic loop and the C-terminal tail domain. The N terminus of ExbB is in the periplasm (peri).

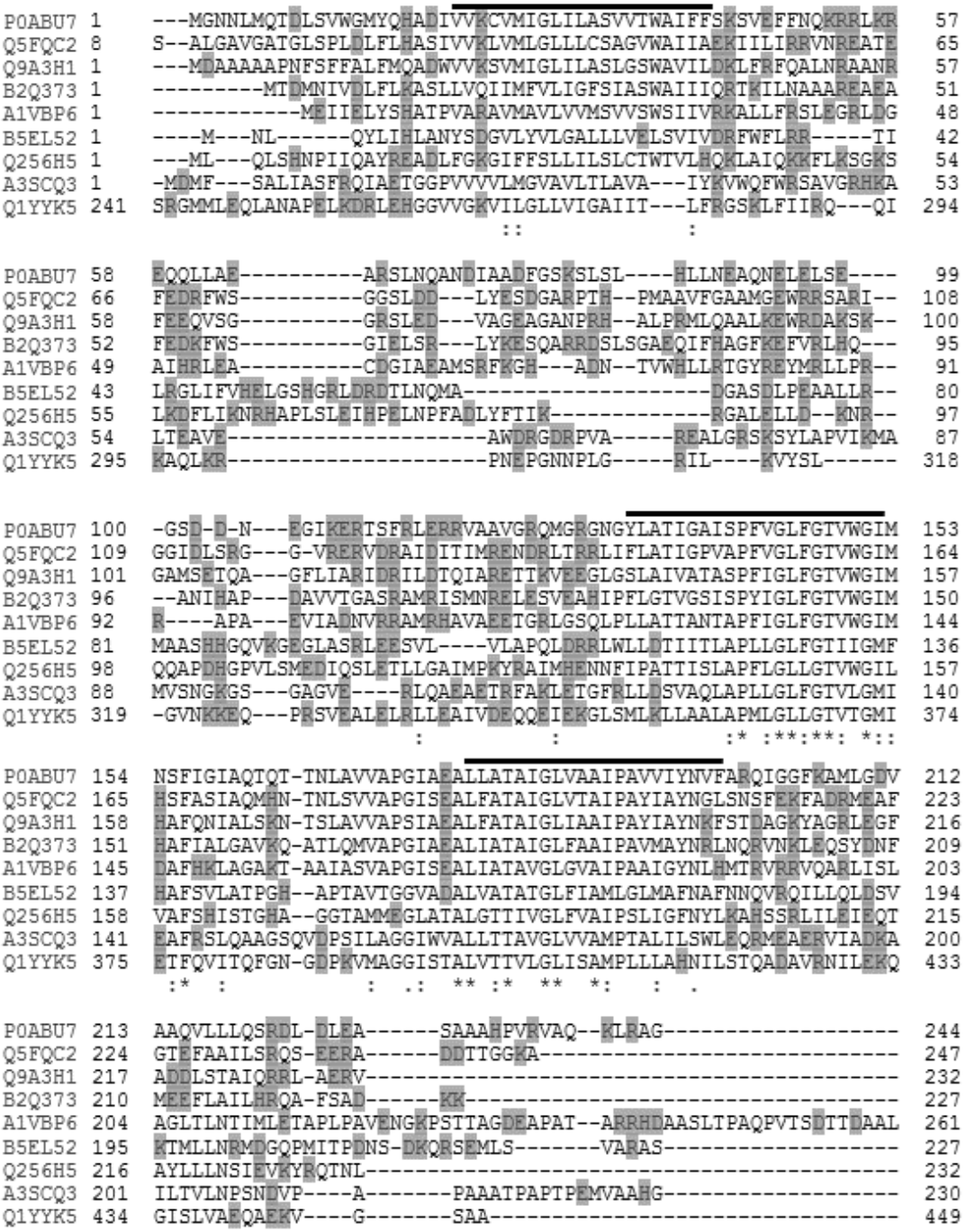

FIG. 2-2. *Revision of ExbB TMD boundaries: ExbB sequence alignment*. Distantly related ExbB sequences were aligned using clustal omega multiple sequence alignment program (http://www.ebi.ac.uk/Tools/msa/clustalo/). Charged residues are highlighted in grey.

TOPCONS-predicted TMDs are indicated by a black bar above the sequence: TMD1 (22-42), TMD2 (132-152), TMD3 (178-198). Names of distantly related species, their corresponding accession numbers, and expect scores relative to ExbB from *Escherichia coli* K12 are: *Escherichia coli* (P0ABU7, e $^{-130}$), *Gluconobacter oxydans* (Q5FQC2, 2e $^{-28}$), *Caulobacter crescentus* (Q9A3H1, 9e $^{-29}$), *Providencia stuartii* (B2Q373, 9e $^{-21}$), *Desulfovibrio vulgaris* (A1VBP6, 1e $^{-20}$), *Acidithiobacillus ferrooxidans* (B5EL52, 4e $^{-7}$), *Chlamydophila felis* (Q256H5, 4e $^{-7}$), *Sulfitobacter sp. EE-36* (A3SCQ3, 3e $^{-7}$), *Photobacterium profundum* (Q1YYK5, 4e $^{-7}$).

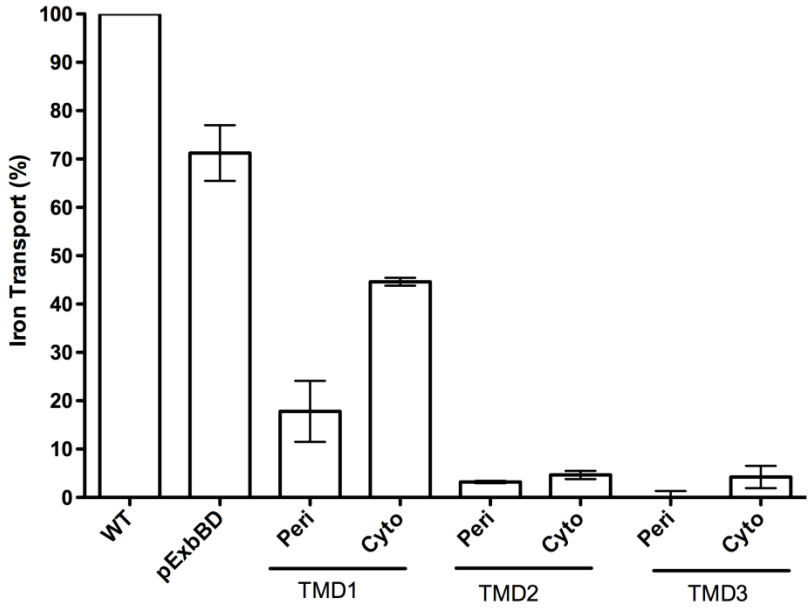

FIG. 2-3. *All ExbB half-Ala substitutions are mostly dominant.* ExbB half-Ala substitution mutants co-expressed with ExbD were induced at subculture with 0.01% L-arabinose in wild type (WT) strain W3110. All mutants were overexpressed greater than 100-fold. Initial rates of [^{55}Fe]-ferrichrome transport was determined from multiple triplicate experiments and normalized to W3110 (100%). Substituted portions and their corresponding plasmid numbers were: pExbBD (pKP660); TMD1 peri (pKP1459); TMD1 cyto (pKP1460); TMD2 peri (pKP1481); TMD2 cyto (pKP1482); TMD3 peri (pKP1483) ; TMD3 cyto (pKP1484).

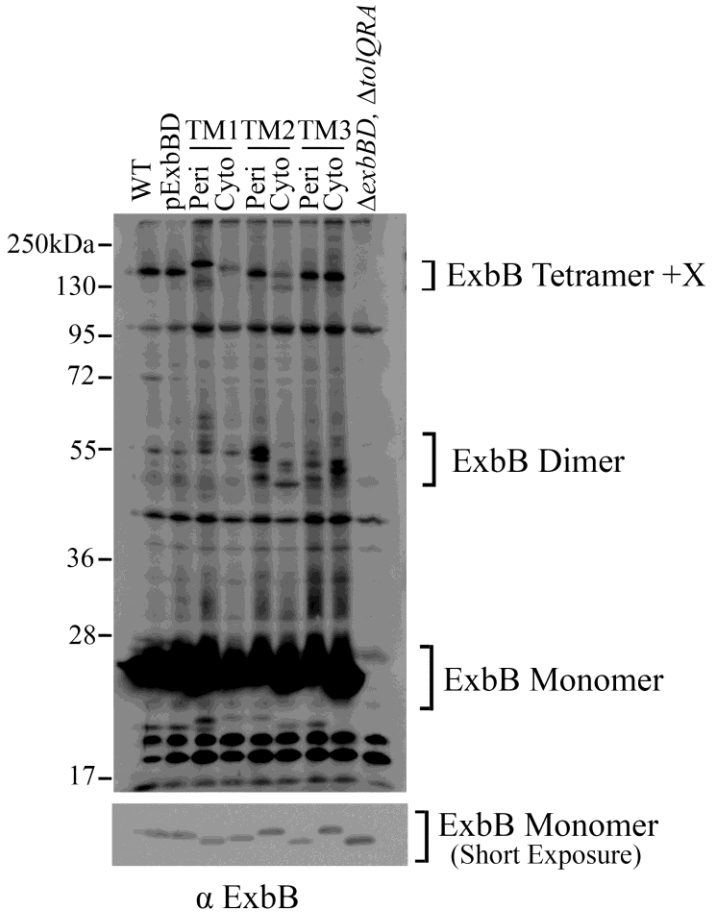

FIG 2-4. Formaldehyde crosslinking of *ExbB half-Ala TMDs using anti-ExbB antibody.* ExbB half-Ala mutants and the plasmid encoded (pExbBD) control were expressed to near chromosomal levels in strain RA1017 (Δ*exbBD* Δ*tolQRA*) and parent strain W3110 served as the wild type (WT) chromosomal control. The corresponding locations of the half-Ala substitutions are indicated above the each lane as TMD1, TMD2 or TMD3 and Peri or Cyto. Cultures grown to mid-exponential phase were cross-linked with monomeric formaldehyde and solubilized in LSB at 60 °C. Samples were resolved on 11% SDS-polyacrylamide gels and immunoblotted with specific anti-ExbB antibody.

Positions and composition of complexes are indicated on the right. Molecular mass standards are indicated on the left. A shorter exposure of the same immunoblot is shown in the lower panel for monomer levels. Corresponding plasmid names and percentages of L-arabinose used to induce chromosomal level expression are as follows: pExbBD (pKP660) 0.0004%; TMD1-peri (pKP1459) 0.0005%; TMD1-cyto (pKP1460) 0.0005%; TMD2-peri (pKP1481) 0.0005%; TMD2-cyto (pKP1482) 0.0009%; TMD3-peri (pKP1483) 0.0005%; TMD3-cyto (pKP1484) 0.0008%.

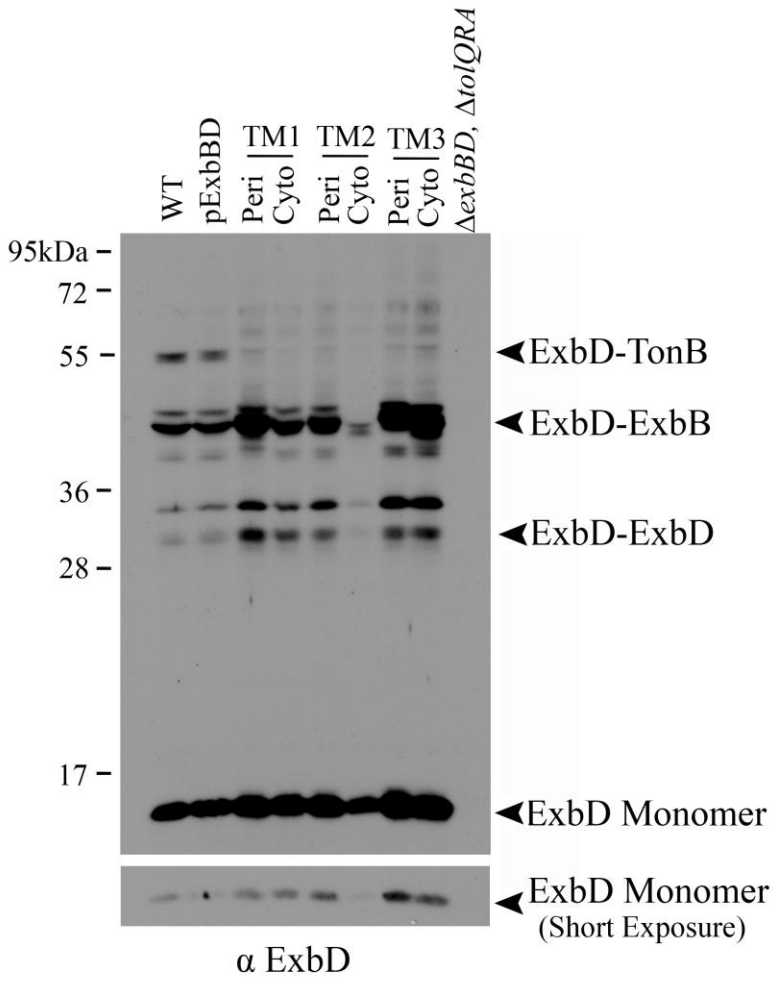

FIG 2-5. *ExbB half-Ala TMDs do not form the TonB-ExbD complex using anti-ExbD antibody.* ExbB half-Ala mutants and the plasmid encoded (pExbBD) control were expressed to near chromosomal levels in strain RA1017 (Δ*exbBD* Δ*tolQRA*) and parent strain W3110 served as the wild type (WT) chromosomal control. The corresponding locations of the half-Ala substitutions are indicated above the each lane as TMD1, TMD2

or TMD3 and Peri or Cyto. Cultures grown to mid-exponential phase were cross-linked with monomeric formaldehyde and solubilized in LSB at 60°C. Samples were resolved on 13% SDS-polyacrylamide gels and immunoblotted with specific anti-ExbD antibody. Positions and composition of complexes are indicated on the right. Molecular mass standards are indicated on the left. A shorter exposure of the same immunoblot is shown in the lower panel for monomer levels. Corresponding plasmid names and percentages of L-arabinose used to induce chromosomal level expression are as follows: pExbBD (pKP660) 0.0004%; TMD1-peri (pKP1459) 0.0005%; TMD1-cyto (pKP1460) 0.0005%; TMD2-peri (pKP1481) 0.0005%; TMD2-cyto (pKP1482) 0.0009%; TMD3-peri (pKP1483) 0.0005%; TMD3-cyto (pKP1484) 0.0008%.

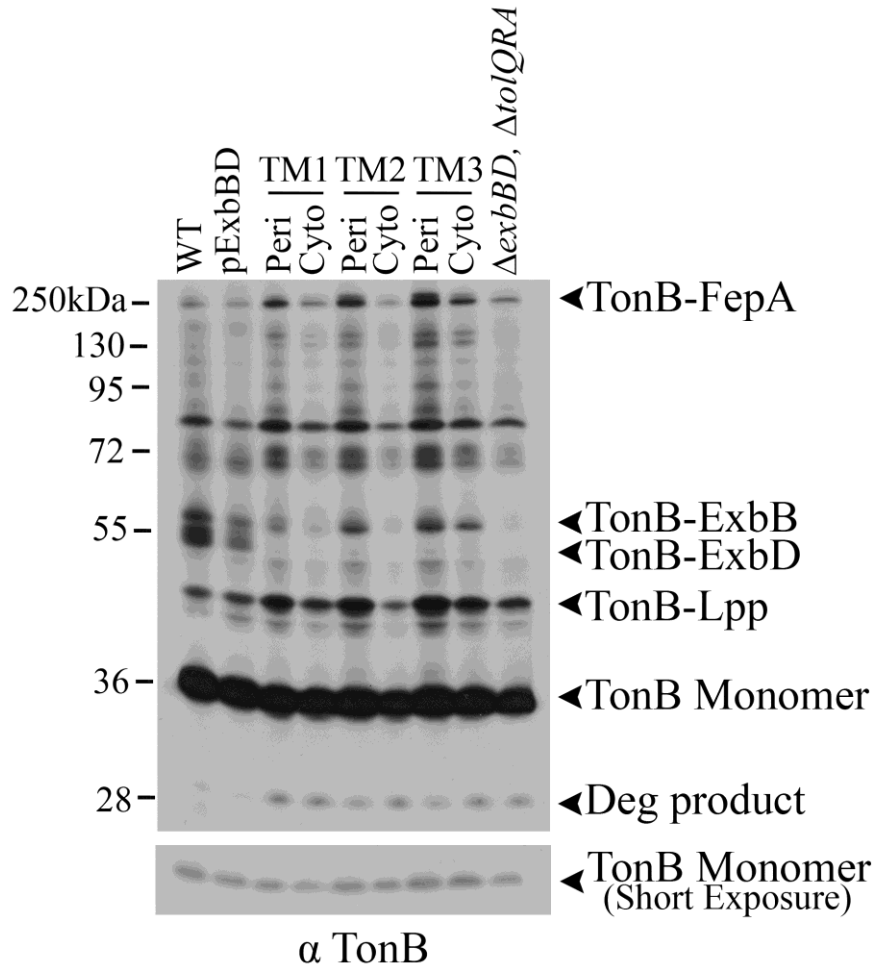

FIG. 2-6. *ExbB half-Ala TMDs do not form the TonB-ExbD complex using anti-TonB antibody.* ExbB half-Ala mutants and the plasmid encoded (pExbBD) control were expressed to near chromosomal levels in strain RA1017 (Δ*exbBD* Δ*tolQRA*) and parent strain W3110 served as the wild type (WT) chromosomal control. The corresponding locations of the half-Ala substitutions are indicated above the each lane as TMD1, TMD2 or TMD3 and Peri or Cyto. Cultures grown to mid-exponential phase were cross-linked with monomeric formaldehyde and solubilized in LSB at 60°C. Samples were resolved

on 11% SDS-polyacrylamide gels and immunoblotted with specific anti-TonB antibody. Positions and composition of complexes are indicated on the right. Molecular mass standards are indicated on the left. A shorter exposure of the same immunoblot is shown in the lower panel for monomer levels. Corresponding plasmid names and percentages of L-arabinose used to induce chromosomal level expression are as follows: pExbBD (pKP660) 0.0004%; TMD1-peri (pKP1459) 0.0005%; TMD1-cyto (pKP1460) 0.0005%; TMD2-peri (pKP1481) 0.0005%; TMD2-cyto (pKP1482) 0.0009%; TMD3-peri (pKP1483) 0.0005%; TMD3-cyto (pKP1484) 0.0008%.

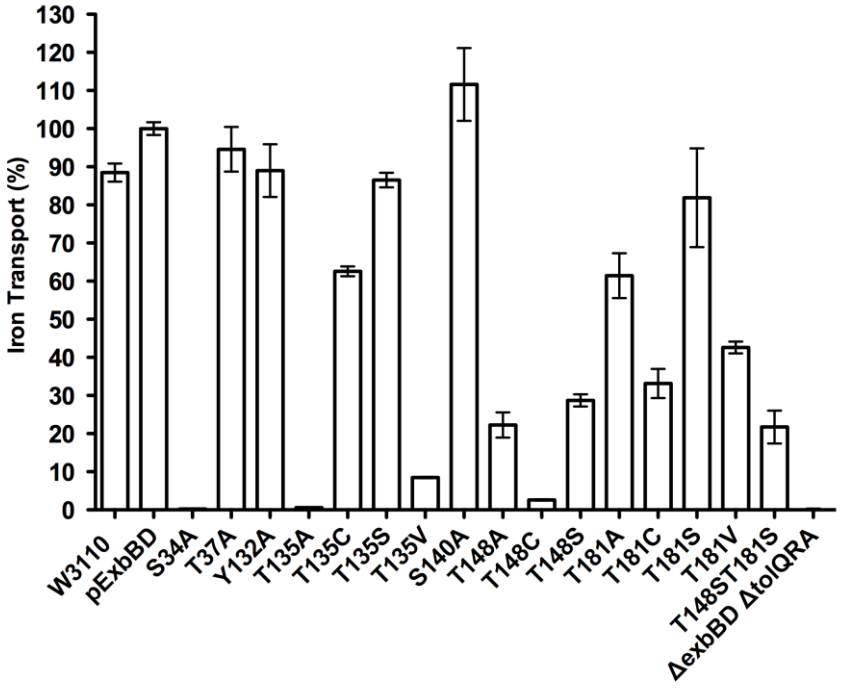

FIG. 2-7. *Activity of ExbB TMD substitutions: substitution of hydroxyl residues.* ExbB TMD substituted mutants were expressed at chromosomal levels in strain RA1017 (Δ*exbBD* Δ*tolQRA*) and initial rates of [^{55}Fe]-ferrichrome transport were measured as described in the Materials and Methods. Triplicate samples were taken at all time points and the initial transport rates were calculated by linear regression. Linear regression slopes were normalized to plasmid encoded wild type ExbBD (pExbBD) (100%). Normalized % activities of at least 2 independent triplet experiments were averaged and deviations are shown.

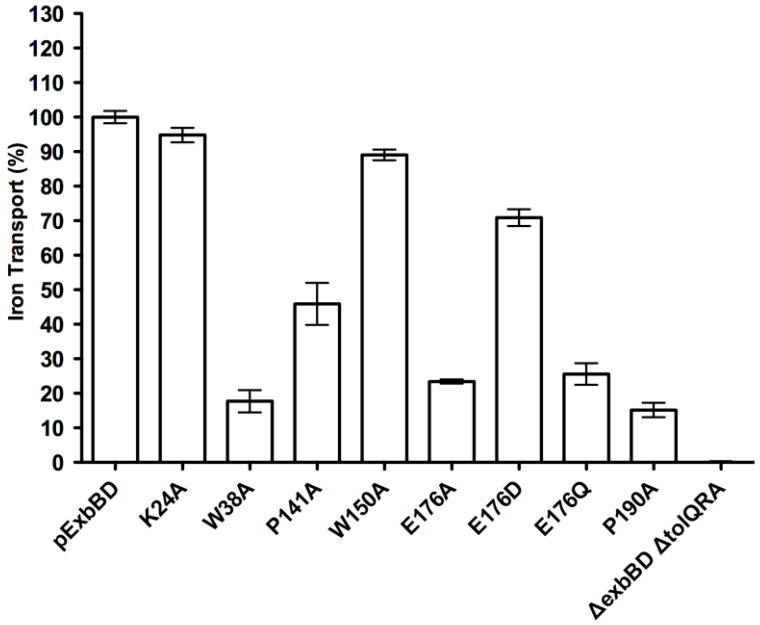

FIG. 2-8. *Activity of ExbB TMD substitutions: substitution of proline, charged, and the remaining polar residues.* ExbB TMD substituted mutants were expressed at chromosomal levels in strain RA1017 (Δ*exbBD* Δ*tolQRA*) and initial rates of [^{55}Fe]-ferrichrome transport were measured as described in the Materials and Methods. Triplicate samples were taken at all time points and the initial transport rates were calculated by linear regression. Linear regression slopes were normalized to plasmid encoded wild type ExbBD (pExbBD) (100%). Normalized % activities of at least 2 independent triplet experiments were averaged and deviations are shown.

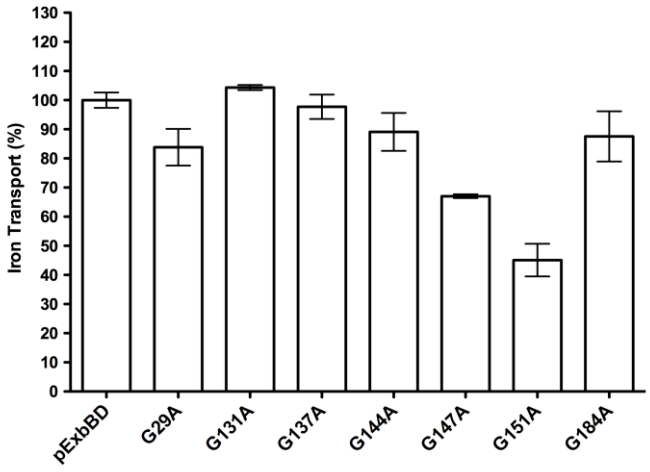

FIG. 2-9. *Activity of ExbB TMD substitutions: substitution of glycine residues.* ExbB TMD substituted mutants were expressed at chromosomal levels in strain RA1017 (Δ*exbBD* Δ*tolQRA*) and initial rates of [^{55}Fe]-ferrichrome transport were measured as described in the Materials and Methods. Triplicate samples were taken at all time points and the initial transport rates were calculated by linear regression. Linear regression slopes were normalized to plasmid encoded wild type ExbBD (pExbBD) (100%). Normalized % activities of at least 2 independent triplet experiments were averaged and deviations are shown.

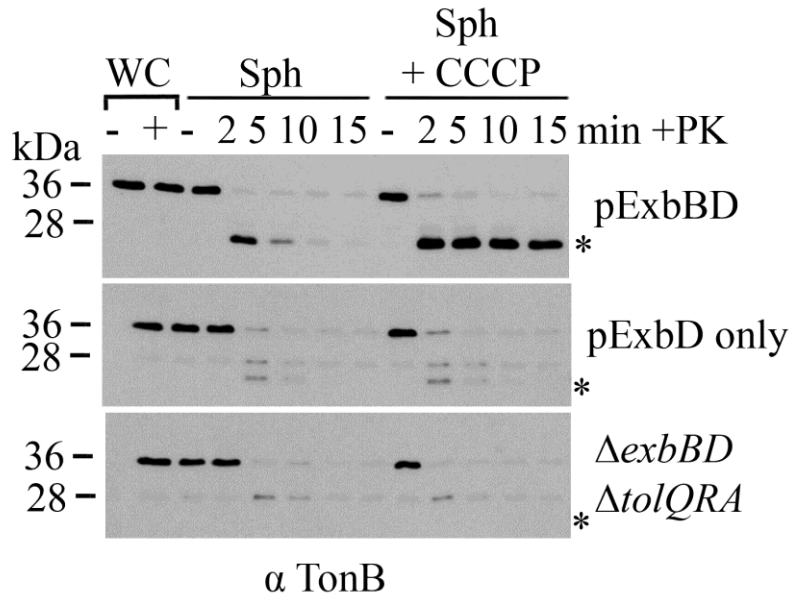

FIG. 2-10. *ExbB is required for efficient TonB-ExbD assembly*. Spheroplasts were treated with DMSO (sph) or CCCP (sph + CCCP), and subsequently treated without (-) or with (+) proteinase K for a time course of 2, 5, 10 and 15 min followed by the addition of protease inhibitor, PMSF and immediate precipitation with trichloro-acetic acid (TCA). TCA-precipitated samples were resolved on 13% SDS-polyacrylamide gels and immunoblotted with anti-TonB antibodies. Molecular mass markers are indicated on the left. Plasmid expressed proteins or the deletion strain are indicated on the right of each panel. The TonB-proteinase K- resistant ~23kDa fragment is indicated as "*". Spheroplasts were generated from Δ*exbBD* Δ*tolQRA* strain (RA1017), expressing pExbBD (pKP660) or pExbD (pKP1194) at chromosomal levels. RA1017 containing the empty vector, pBAD24, is shown in the bottom panel.

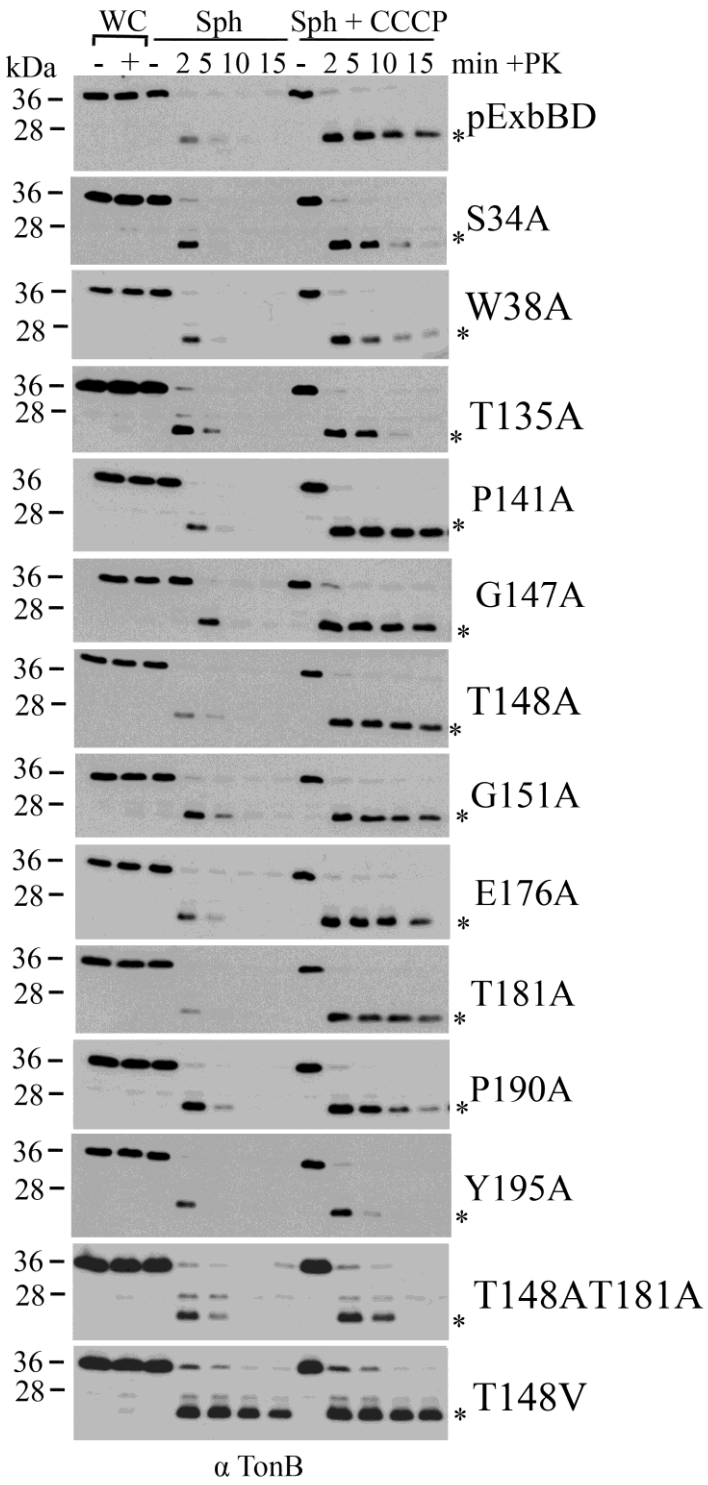

FIG. 2-11. *ExbB is required for conformational response of TonB to pmf*. Spheroplasts were treated with DMSO (sph) or CCCP (sph + CCCP), and subsequently treated without

(-) or with (+) proteinase K for a time course of 2, 5, 10 and 15 min followed by the addition of protease inhibitor, PMSF and immediate precipitation with trichloro-acetic acid (TCA). TCA-precipitated samples were resolved on 13% SDS-polyacrylamide gels and immunoblotted with anti-TonB antibodies. Molecular mass markers are indicated on the left. Plasmid expressed proteins or the deletion strain are indicated on the right of each panel. The TonB-proteinase K- resistant ~23kDa fragment is indicated as "*". Spheroplasts were generated in RA1017 (Δ*exbBD* Δ*tolQRA*) expressing plasmid encoded ExbB TMD mutants at chromosomal levels as well as ExbD. This figure is a composite of multiple representative immunoblots.

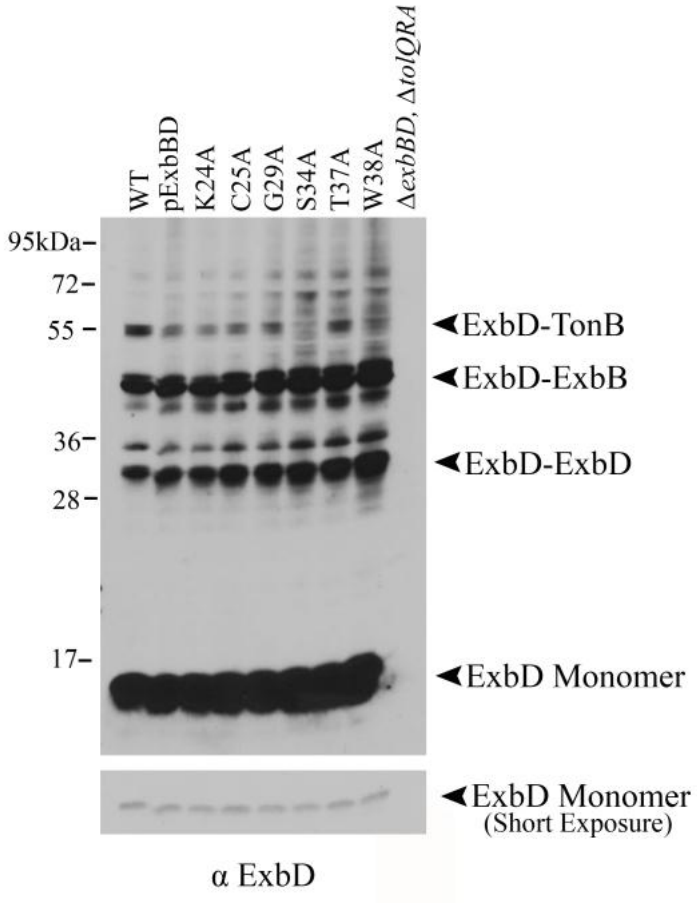

FIG. 2-12. *Formaldehyde crosslinking profile of ExbB TMD1 Ala substituted mutants using anti-ExbD antibody.* ExbB Ala substituted TMD mutants were expressed at chromosomal levels in strain RA1017 (Δ*exbBD* Δ*tolQRA*). W3110 served as the wild type (WT) chromosomal control. ExbB TMD mutants are indicated above each lane. Cultures were grown to mid-exponential phase, crosslinked with monomeric formaldehyde and solubilized in LSB at 60°C. Samples were resolved on 13% SDS-polyacrylamide gels and immunoblotted with anti-ExbD antibody. Positions and composition of complexes are indicated on the right. Molecular mass standards are indicated on the left. A shorter exposure of the same immunoblot is shown in the lower panel for monomer levels.

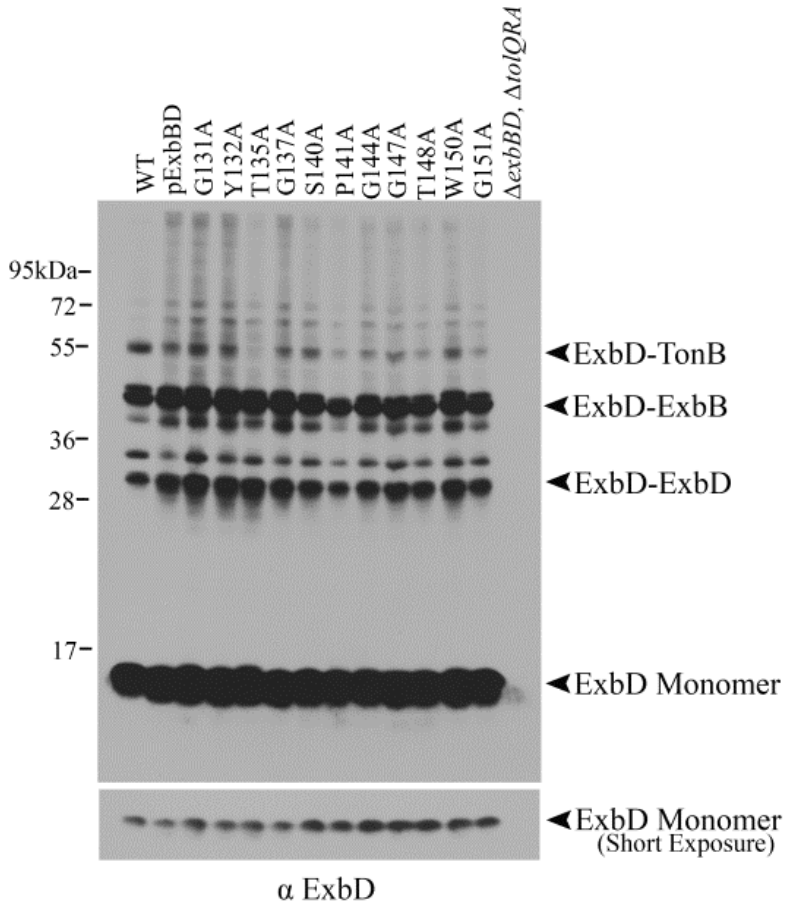

FIG. 2-13. *Formaldehyde crosslinking profile of ExbB TMD2 Ala substituted mutants using anti-ExbD antibody.* ExbB Ala substituted TMD mutants were expressed at chromosomal levels in strain RA1017 (Δ*exbBD* Δ*tolQRA*). W3110 served as the wild type (WT) chromosomal control. ExbB TMD mutants are indicated above each lane. Cultures were grown to mid-exponential phase, crosslinked with monomeric formaldehyde and solubilized in LSB at 60°C. Samples were resolved on 13% SDS-polyacrylamide gels and immunoblotted with anti-ExbD antibody. Positions and composition of complexes are indicated on the right. Molecular mass standards are indicated on the left. A shorter exposure of the same immunoblot is shown in the lower panel for monomer levels.

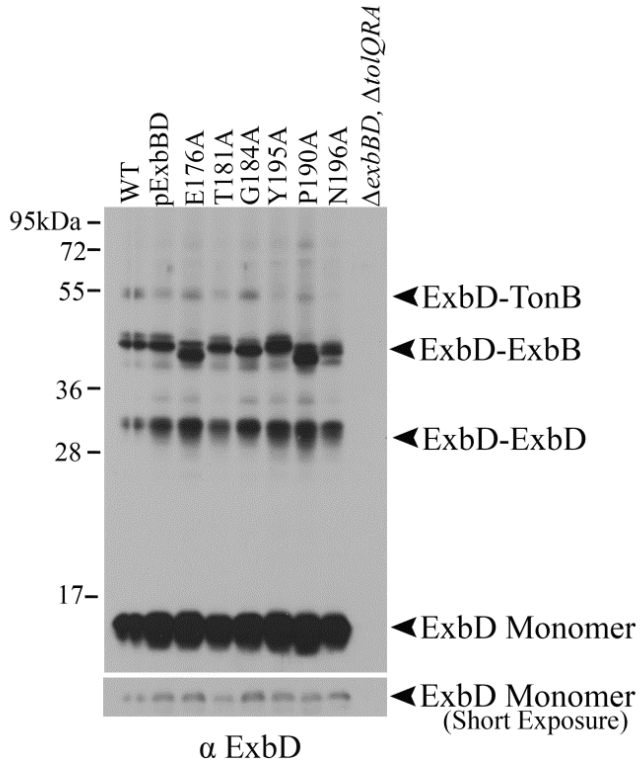

FIG. 2-14. *Formaldehyde crosslinking profile of ExbB TMD3 Ala substituted mutants using anti-ExbD antibody.* ExbB Ala substituted TMD mutants were expressed at chromosomal levels in strain RA1017 (Δ*exbBD* Δ*tolQRA*). W3110 served as the wild type (WT) chromosomal control. ExbB TMD mutants are indicated above each lane. Cultures were grown to mid-exponential phase, crosslinked with monomeric formaldehyde and solubilized in LSB at 60°C. Samples were resolved on 13% SDS-polyacrylamide gels and immunoblotted with anti-ExbD antibody. Positions and composition of complexes are indicated on the right. Molecular mass standards are indicated on the left. A shorter exposure of the same immunoblot is shown in the lower panel for monomer levels. Aberrant migration of the ExbD-ExbB complex of ExbB E176A and ExbB P190A corresponds to the faster migration of the ExbB monomer (refer to Fig. 2.22).

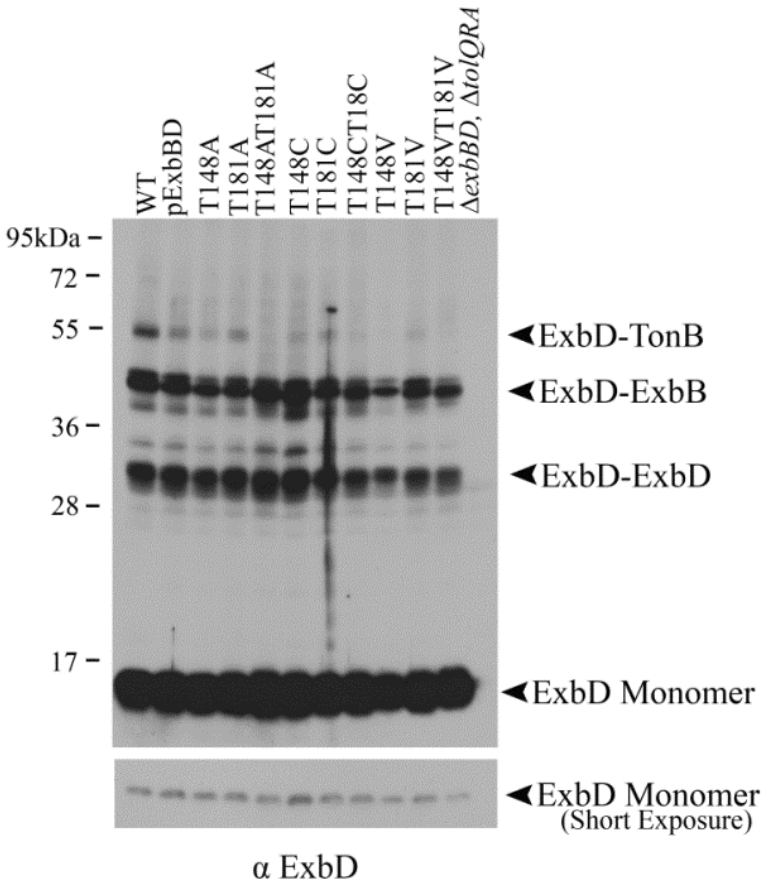

FIG. 2-15. *Formaldehyde crosslinking profile of ExbB T148 and T181 substitutions using anti-ExbD antibody.* Substituted TMD mutants were expressed at chromosomal levels in strain RA1017 (Δ*exbBD* Δ*tolQRA*) and parent strain W3110 (WT) served as the wild type chromosomal control. Plasmids expressing ExbB substitutions are indicated above each lane. Cultures were grown to mid-exponential phase and crosslinked with monomeric formaldehyde then solubilized in LSB at 60°C. Samples were resolved on 13% SDS-polyacrylamide gels then immunoblotted with anti-ExbD antibody. Positions and composition of complexes are indicated on the right. Molecular mass standards are indicated on the left. A shorter exposure of the same immunoblot is shown in the lower panel for monomer levels.

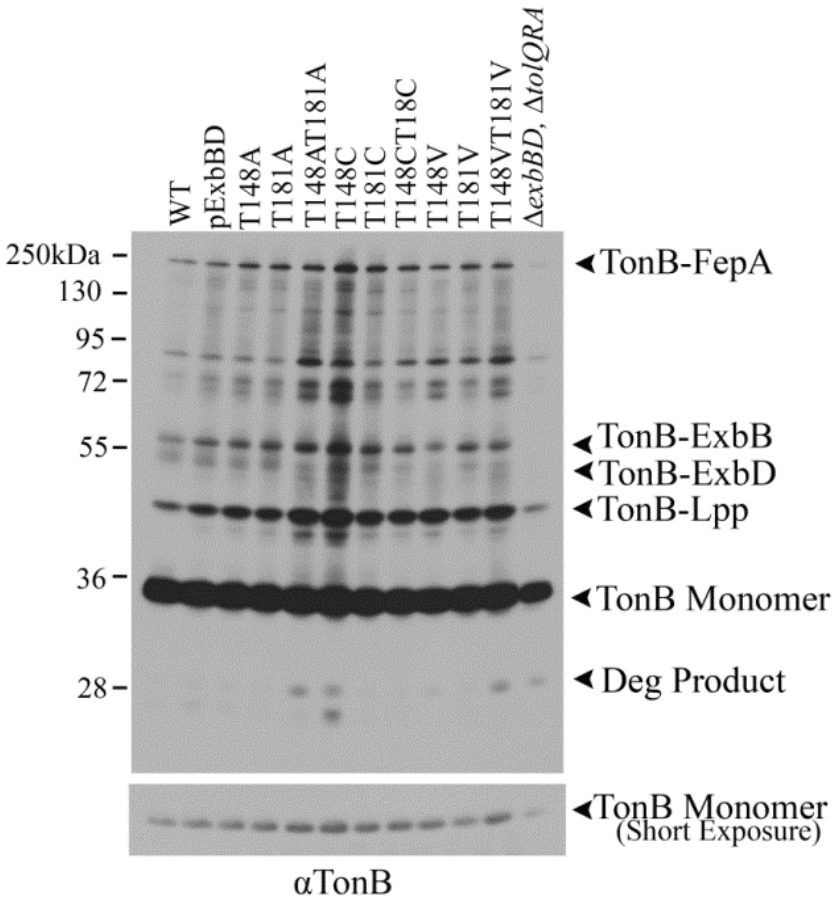

FIG. 2-16. *Formaldehyde crosslinking profile of ExbB T148 and T181 substitutions using anti-TonB antibody.* Substituted TMD mutants were expressed at chromosomal levels in strain RA1017 (Δ*exbBD* Δ*tolQRA*) and parent strain W3110 (WT) served as the wild type chromosomal control. Plasmids expressing ExbB substitutions are indicated above each lane. Cultures were grown to mid-exponential phase and crosslinked with monomeric formaldehyde then solubilized in LSB at 60°C. Samples were resolved on 11% SDS-polyacrylamide gels then immunoblotted with anti-TonB antibody. Positions and composition of complexes are indicated on the right. Molecular mass standards are indicated on the left. A shorter exposure of the same immunoblot is shown in the lower panel for monomer levels.

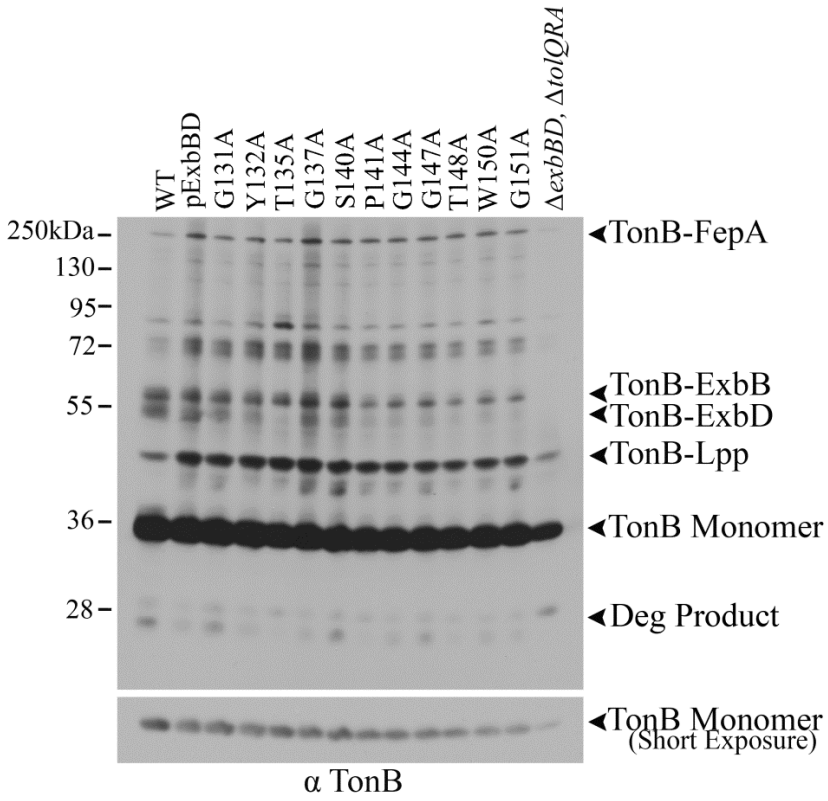

FIG. 2-17. *Formaldehyde crosslinking profile of ExbB TMD 2 Ala substituted mutants using anti-TonB antibody.* ExbB Ala substituted TMD mutants were expressed at chromosomal levels in strain RA1017 (Δ*exbBD* Δ*tolQRA*) and parent strain W3110 (WT) served as the wild type chromosomal control. ExbB TMD mutants are indicated above each lane. Cultures grown to mid-exponential phase were crosslinked with monomeric formaldehyde and solubilized in LSB at 60°C. Samples were resolved on 11% SDS-polyacrylamide gels and immunoblotted with anti-TonB antibody. Positions and composition of complexes are indicated on the right. Molecular mass standards are indicated on the left. A shorter exposure of the same immunoblot is shown in the lower panel for monomer levels.

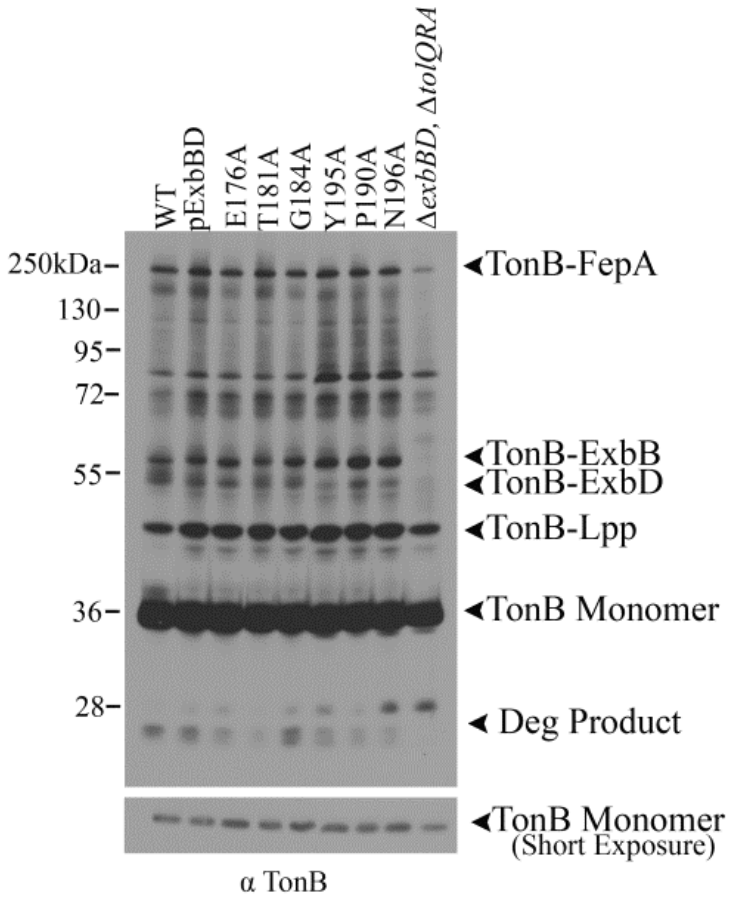

FIG. 2-18. *Formaldehyde crosslinking profile of ExbB TMD3 Ala substituted mutants using anti-TonB antibody.* ExbB Ala substituted TMD mutants were expressed at chromosomal levels in strain RA1017 (Δ*exbBD* Δ*tolQRA*) and parent strain W3110 (WT) served as the wild type chromosomal control. ExbB TMD mutants are indicated above each lane. Cultures grown to mid-exponential phase were crosslinked with monomeric formaldehyde and solubilized in LSB at 60°C. Samples were resolved on 11% SDS-polyacrylamide gels and immunoblotted with anti-TonB antibody. Positions and composition of complexes are indicated on the right. Molecular mass standards are indicated on the left. A shorter exposure of the same immunoblot is shown in the lower panel for monomer levels.

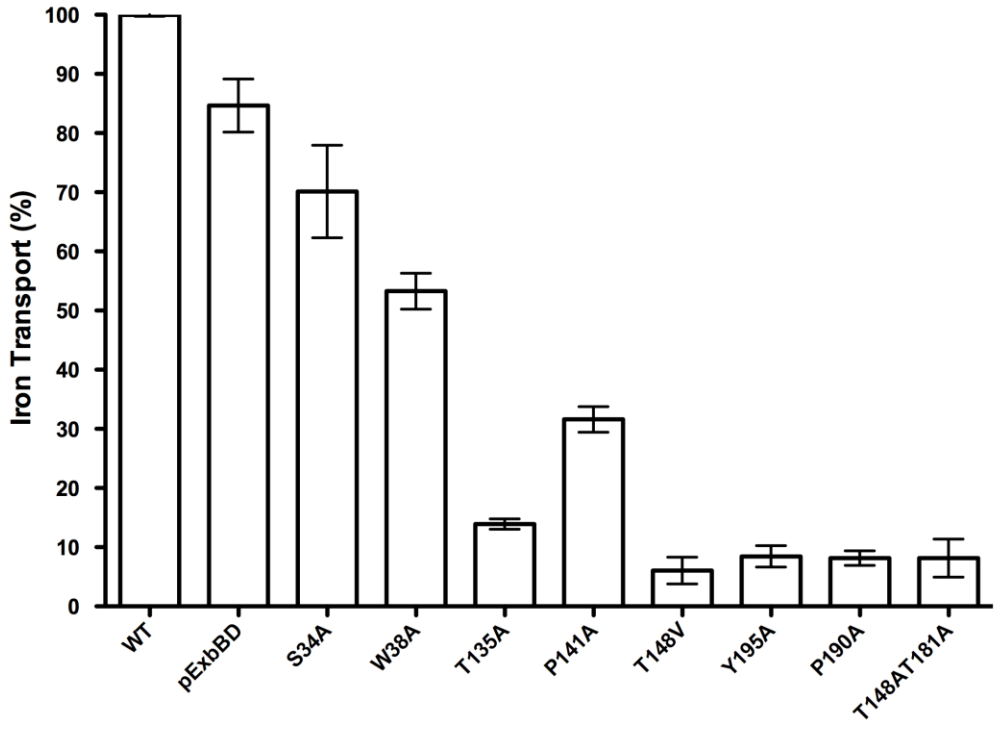

FIG. 2-19. *ExbB S34A and W38A TMD mutants are not dominant*. ExbB mutants were co-expressed with ExbD using 0.01% L- arabinose in wild type strain W3110. All mutant proteins were overexpressed greater than 100-fold compared to chromosomal levels, based on serial dilutions and immunoblot analysis (data not shown). Initial rates of [^{55}Fe]-ferrichrome transport were measured from triplicate samples and normalized to W3110 (100%). Averaged percentages of at least two triplicate experiments are shown.

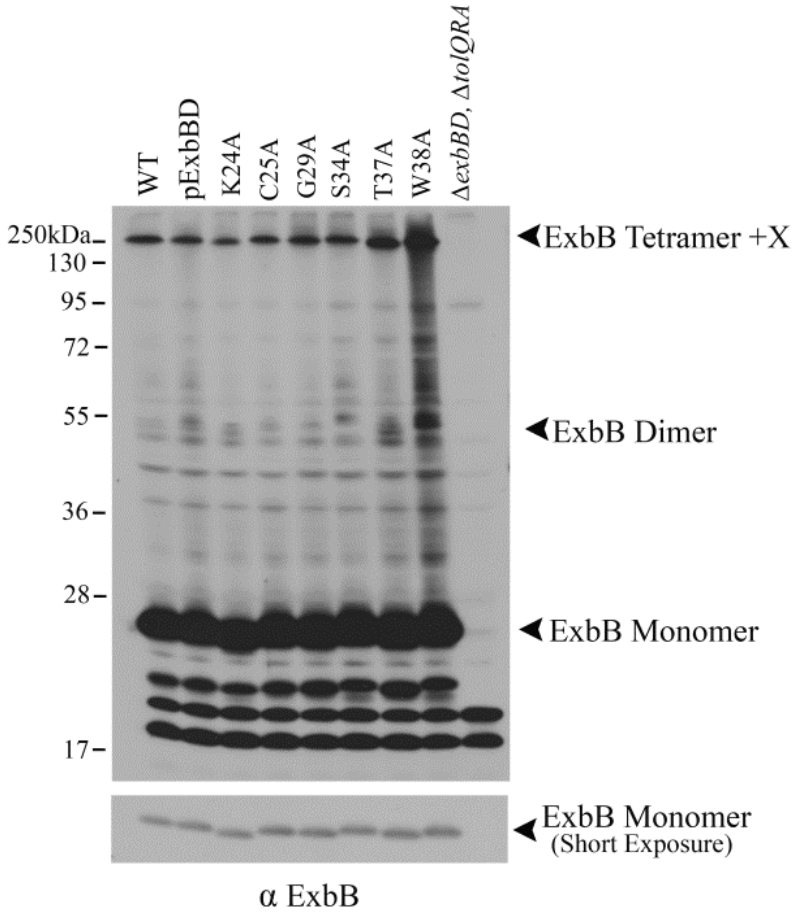

FIG. 2-20. *Formaldehyde crosslinking profile of ExbB TMD1 Ala substituted mutants using anti-ExbB antibody.* ExbB Ala substituted TMD mutants were expressed at chromosomal levels in strain RA1017 (Δ*exbBD* Δ*tolQRA*) and W3110 (WT) served as the wild type chromosomal control. ExbB TMD mutants are indicated above each lane. Cultures were grown to mid-exponential phase, crosslinked with monomeric formaldehyde and solubilized in LSB at 60°C. Samples were resolved on 13% SDS-polyacrylamide gels and immunoblotted with anti-ExbB antibody. Positions and composition of complexes are indicated on the right. Molecular mass standards are indicated on the left. A shorter exposure of the same immunoblot is shown in the lower panel for monomer levels.

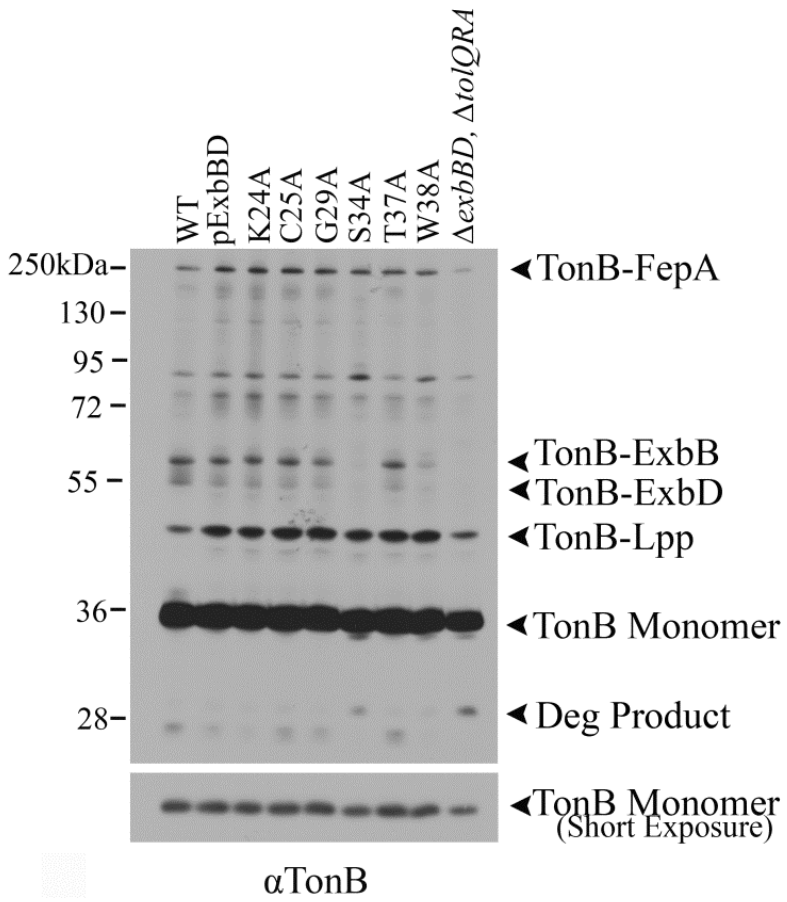

FIG. 2-21. *ExbB S34A and W38A reduce the TonB-ExbB formaldehyde-crosslinked complex.* ExbB TMD1 mutants were expressed at chromosomal levels in strain RA1017 (Δ*exbBD* Δ*tolQRA*), and parent strain W3110 (WT) served as the wild type chromosomal control. Identities of mutants are indicated above each lane. Cultures grown to mid-exponential phase were crosslinked with monomeric formaldehyde and solubilized in gel sample buffer at 60°C. Samples were resolved on 11% SDS-polyacrylamide gels and immunoblotted with anti-TonB antibody. Positions and composition of complexes are indicated on the right. Molecular mass standards are indicated on the left. A shorter exposure of the same immunoblot is shown in the lower panel for comparison of monomer levels.

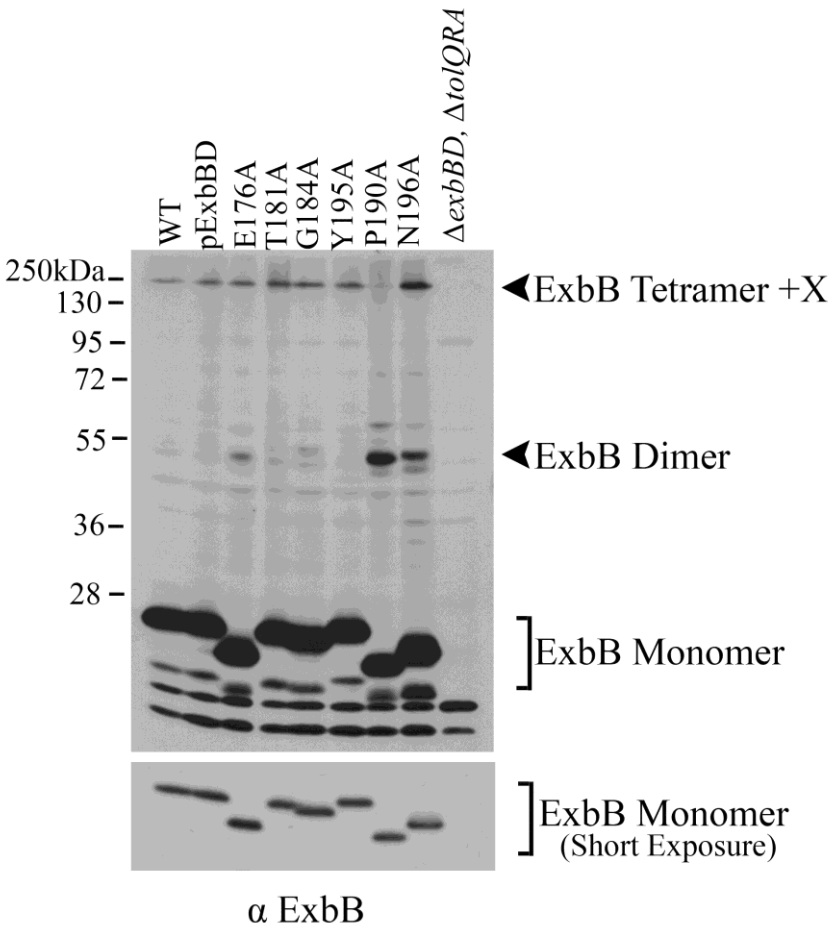

FIG. 2-22. *ExbB P190A reduces ExbB tetramer + X complex formation*. ExbB TMD3 mutants were expressed at chromosomal levels in strain RA1017 (Δ*exbBD* Δ*tolQRA*), and parent strain W3110 (WT) served as the wild type chromosomal control. Identities of mutants are indicated above each lane. Cultures grown to mid-exponential phase were crosslinked with monomeric formaldehyde and solubilized in gel sample buffer at 60°C. Samples were resolved on 13% SDS-polyacrylamide gels and immunoblotted with anti-ExbB antibody. Positions and composition of complexes are indicated on the right. Molecular mass standards are indicated on the left. A shorter exposure of the same immunoblot is shown in the lower panel for comparison of monomer levels.

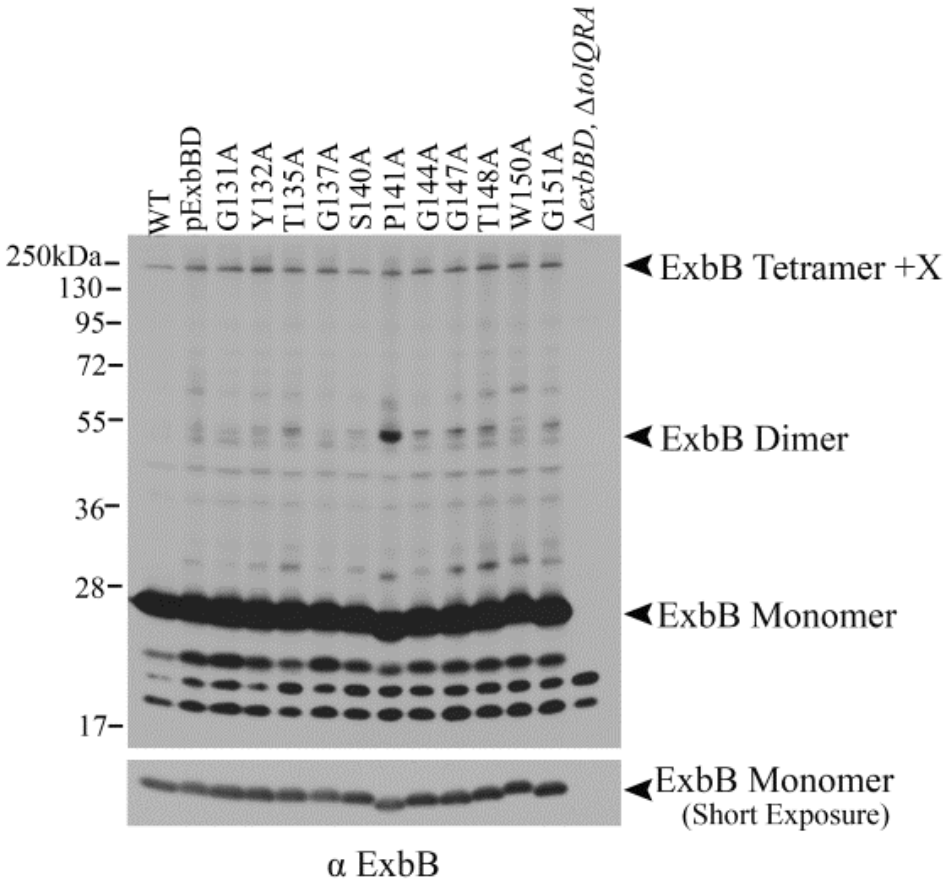

FIG. 2-23. *Formaldehyde crosslinking profile of ExbB TMD 2 Ala substituted mutants using anti-ExbB antibody.* ExbB Ala substituted TMD mutants were expressed at chromosomal levels in strain RA1017 (Δ*exbBD* Δ*tolQRA*) and W3110 (WT) served as the wild type chromosomal control. ExbB TMD mutants are indicated above each lane. Cultures were grown to mid-exponential phase, crosslinked with monomeric formaldehyde and solubilized in LSB at 60°C. Samples were resolved on 13% SDS-polyacrylamide gels and immunoblotted with anti-ExbB antibody. Positions and composition of complexes are indicated on the right. Molecular mass standards are indicated on the left. A shorter exposure of the same immunoblot is shown in the lower panel for monomer levels.

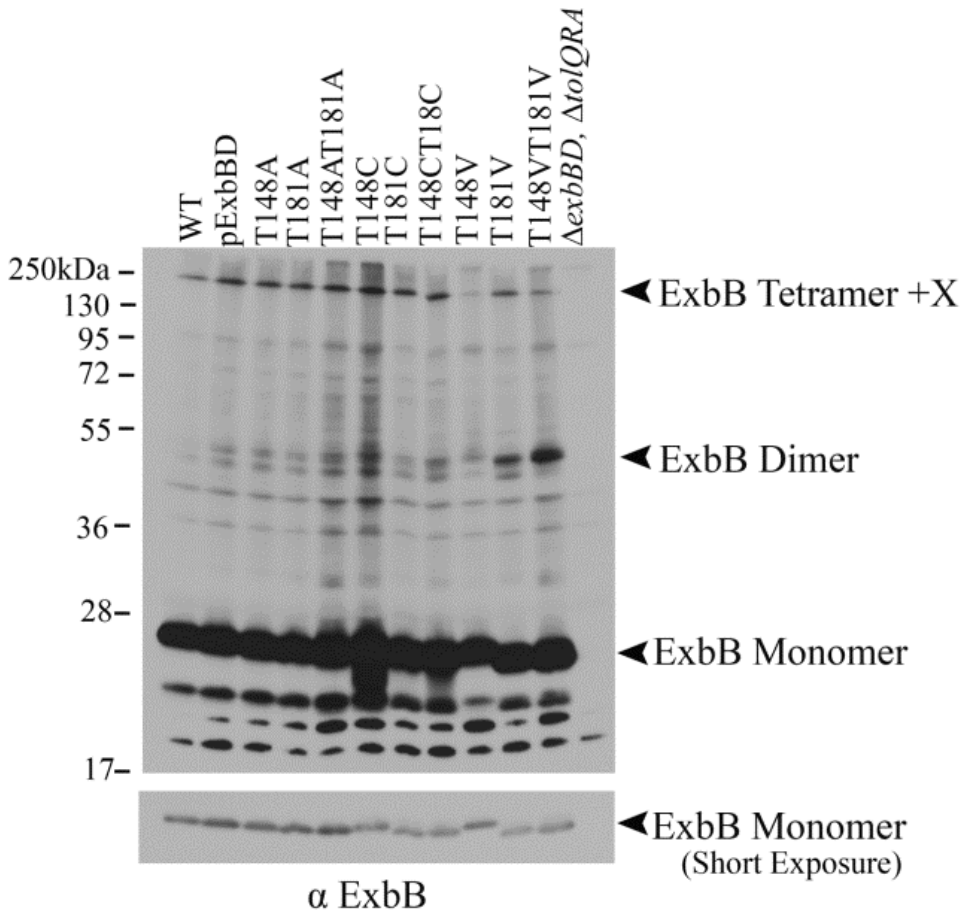

FIG. 2-24. *Formaldehyde crosslinking profile of ExbB T148 and T181 substitutions using anti-ExbB antibody.* Substituted TMD mutants were expressed at chromosomal levels in strain RA1017 (Δ*exbBD* Δ*tolQRA*) and parent strain W3110 (WT) served as the wild type chromosomal control. Plasmids expressing ExbB substitutions are indicated above each lane. Cultures were grown to mid-exponential phase and crosslinked with monomeric formaldehyde then solubilized in LSB at 60°C. Samples were resolved on 13% SDS-polyacrylamide gels then immunoblotted with anti-ExbB antibody. Positions and composition of complexes are indicated on the right. Molecular mass standards are indicated on the left. A shorter exposure of the same immunoblot is shown in the lower panel for monomer levels.

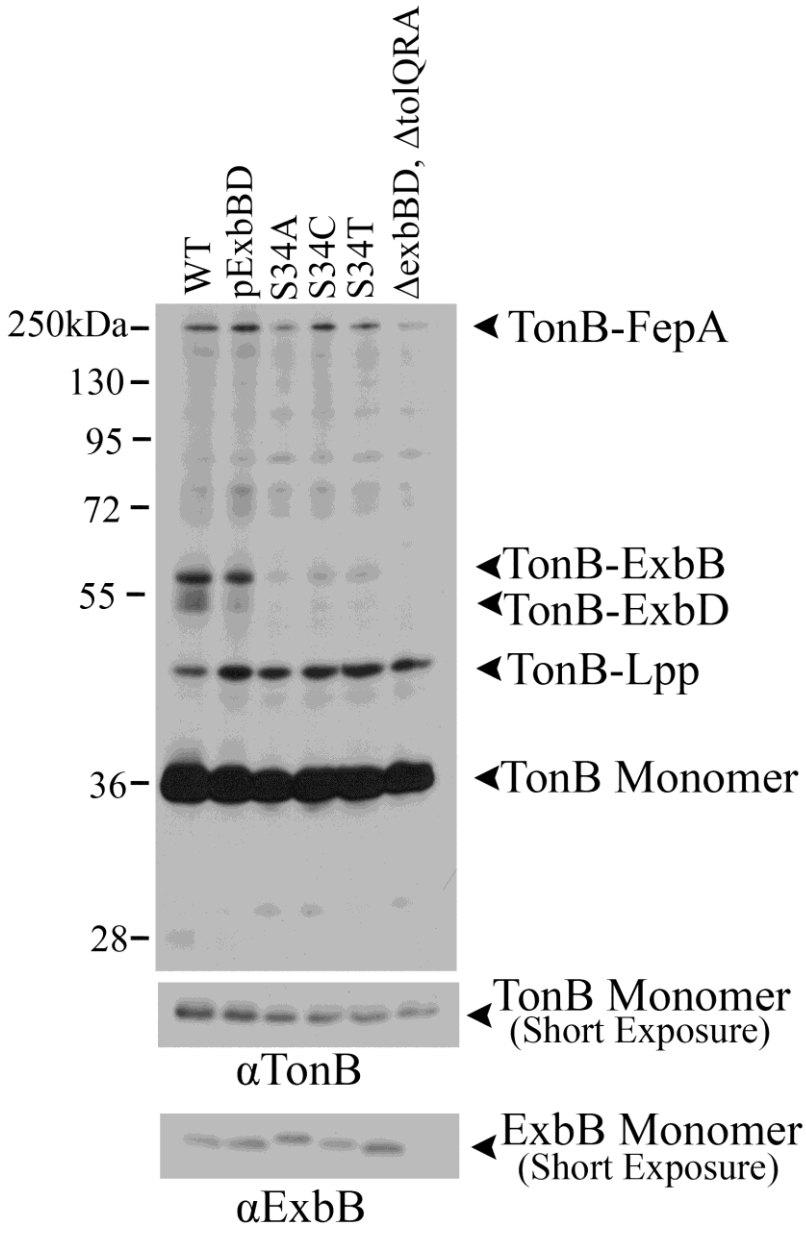

FIG. 2-25. *Conservative S34 substitutions restore low but detectable levels of the TonB-ExbB formaldehyde crosslinked complex.* ExbB S34 substitutions were expressed at chromosomal levels in strain RA1017 (Δ*exbBD* Δ*tolQRA*), and parent strain W3110 (WT) served as the wild type chromosomal control. Identities of mutants are indicated above each lane. Cultures grown to mid-exponential phase were crosslinked with

monomeric formaldehyde and solubilized in gel sample buffer at 60°C. Samples were resolved on 11% SDS-polyacrylamide gels and immunoblotted with anti-TonB antibody. Positions and composition of complexes are indicated on the right. Molecular mass standards are indicated on the left. A shorter exposure of the same immunoblot is shown in the lower panel for comparison of TonB monomer levels. For ExbB monomer levels, the same samples were resolved on a 13% SDS-polyacrylamide gel and immunoblotted with anti-ExbB antibody.

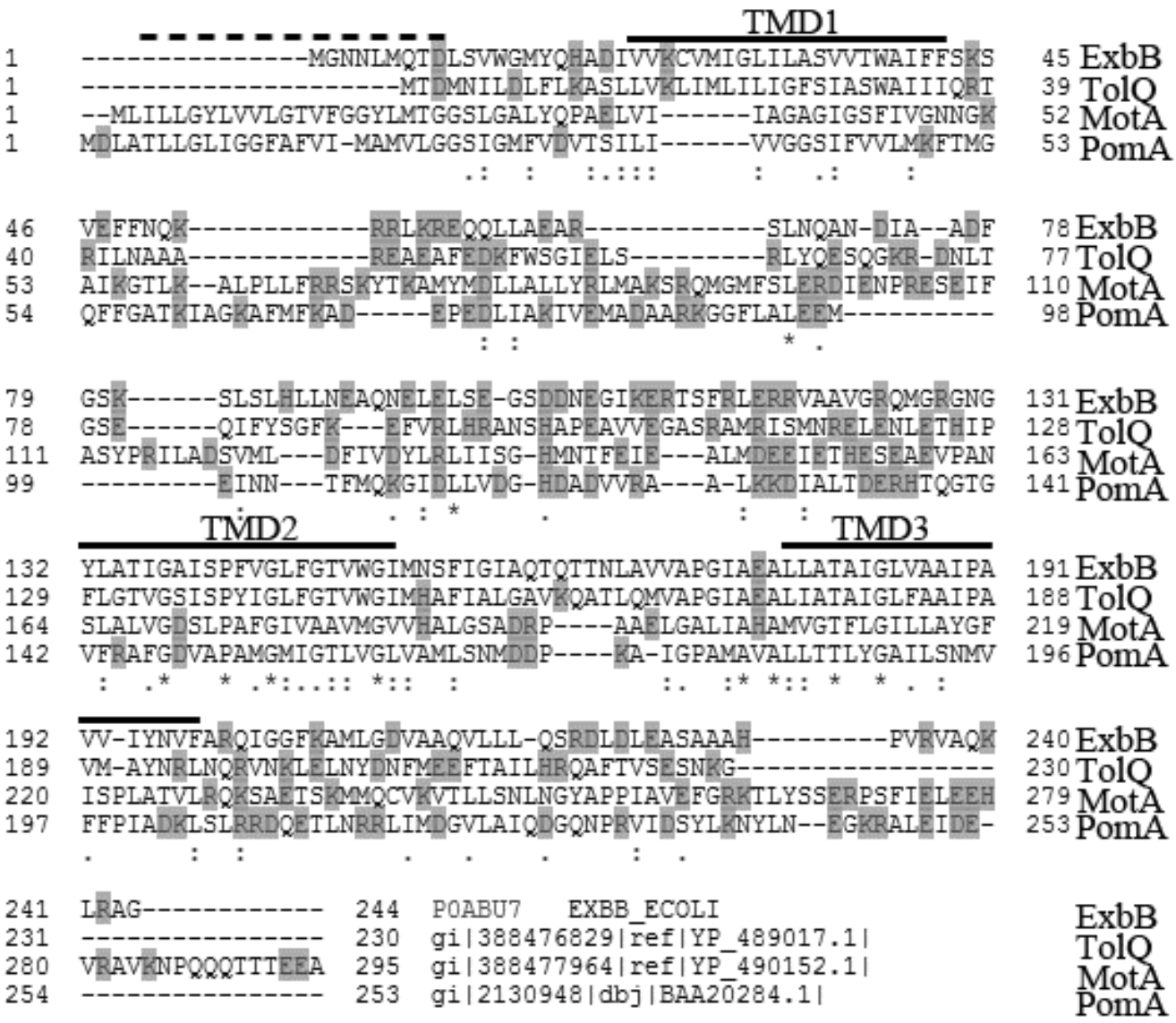

FIG. 2-26. S*equence alignment of ExbB, TolQ, MotA and PomA.* ExbB, TolQ, MotA, and PomA sequences were aligned using clustal omega multiple sequence alignment program (http://www.ebi.ac.uk/Tools/msa/clustalo/). Charged residues are highlighted in grey. Predicted ExbB TMDs from Fig. 2-1 are indicated by a black bar above the sequence.

The dashed bar indicates the predicted location of the MotA/PomA TMD1 (Dean et. al, 1984). ExbB (YP_491200.1), TolQ (YP_489017.1) and MotA (YP_490152.1) sequences are from *Escherichia coli K12* strain W3110. The PomA sequence is from *Vibrio alginolyticus* (BAA20284.1). Accession numbers are shown in parenthesis. The importance of glycines in alignment of transmembrane domains among these proteins has been recognized previously (Cascales et. al, 2001; Kojima et. al, 2001).

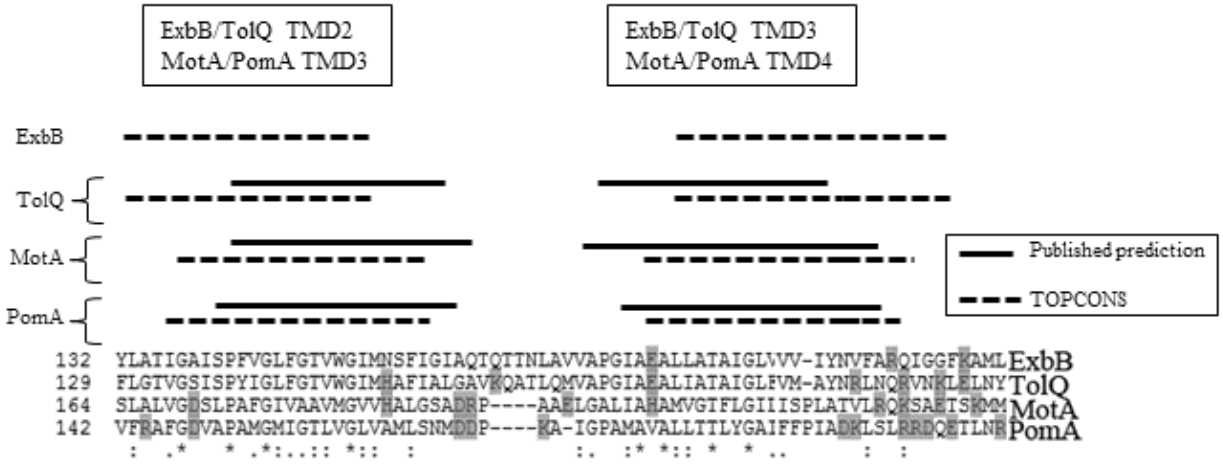

Fig 2-27. *TMD predictions of ExbB/TolQ TMD2/3 and MotA/PomA TMD3/4.* Sequence alignment of the last two TMD of ExbB, TolQ, MotA and PomA is shown. In sold lines above the alignment are the TMD predictions for ExbB presented in this study and the published TMD predictions for TolQ, MotA and PomA (Vianney et. al, 1994; Sharp et. al, 1995; Li et. al. 2011). The dashed line shows the TOPCON predicted TMDs. The sequences were aligned using clustal omega multiple sequence alignment program (http://www.ebi.ac.uk/Tools/msa/clustalo/). Charged residues are highlighted in grey. ExbB (YP_491200.1), TolQ (YP_489017.1) and MotA (YP_490152.1) sequences are from *Escherichia coli K12* strain W3110. The PomA sequence is from *Vibrio alginolyticus* (BAA20284.1). Accession numbers are shown in parenthesis.

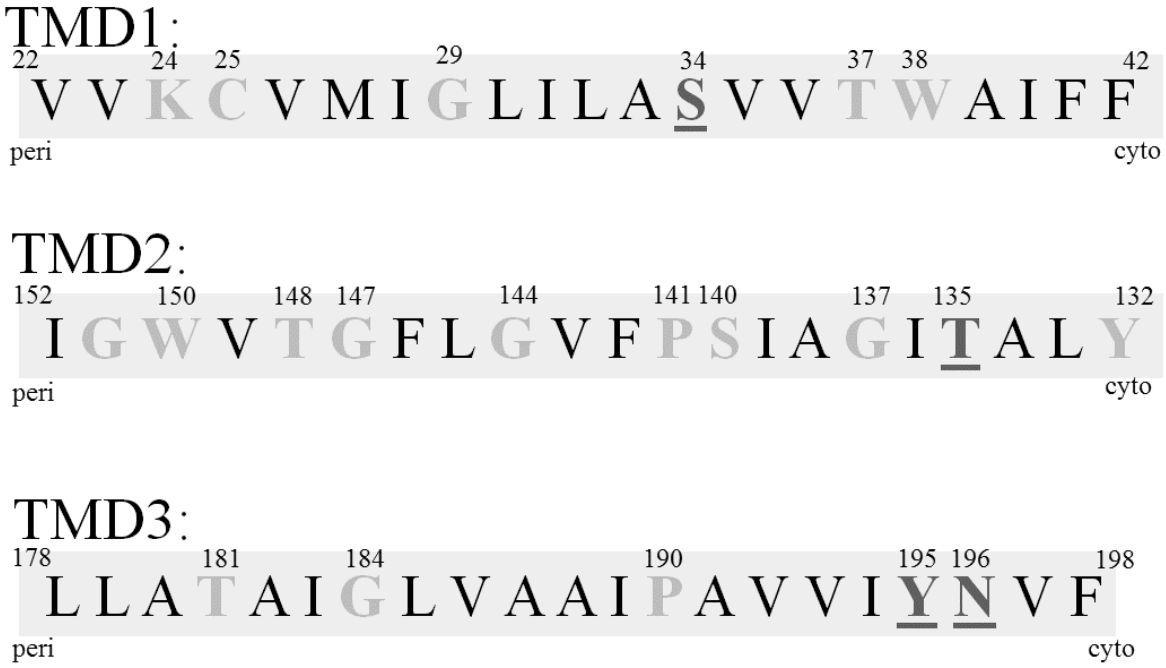

FIG. 2-28. *Summary of residue substitutions in ExbB transmembrane domains.* Predicted ExbB TMDs are depicted as boxes. Grey residues within each box indicate the position of Ala substitutions. All substituted resides are shown in grey: light grey were functional and dark grey/underlined were non-functional when substituted to alanine. The corresponding amino acid numbers are listed above the residues. ExbB Y195A and N196A substitutions were previously reported (Jana et. al, 2011).

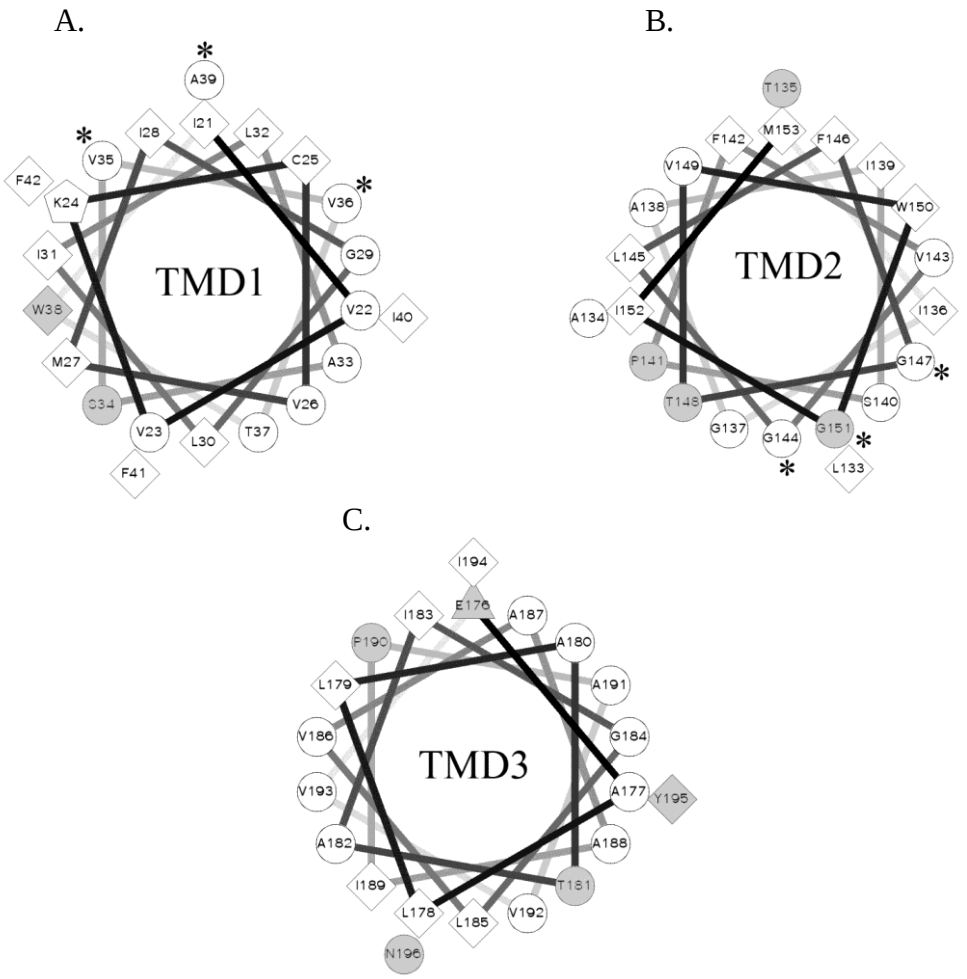

FIG. 2-29. *Helical Wheel projection of ExbB TMDs.* ExbB TMD α-helices are projected as helical wheels from periplasm to cytoplasm, using the helical wheel projection program: (http://rzlab.ucr.edu/scripts/wheel/wheel.cgi). Functionally important residues are marked in gray. Hydrophilic residues are presented as circles, hydrophobic as diamonds, negatively and positively charged residues as triangles and pentagons respectively. (A) TMD1. The location of the TonB TMD ExbB suppressors, V35E, V36D and A39E are indicated by asterisks (Larsen et. al. 1994; 1999). (B) TMD2. Conserved Gly residues are indicated by asterisks (C) TMD3.

CHAPTER 3
CHARACTERIZATION OF EXBB-MEDIATED PROTON LEAKAGE IN *ESCHERICHIA COLI*

Chapter 3

Characterization of ExbB Δ120-129-mediated proton leakage in *Escherichia coli*

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Summary

In the TonB system, cytoplasmic membrane pmf is harnessed by ExbB and ExbD for active transport of nutrients across the outer membrane. While the mechanism of pmf harnessing is uncharacterized, the absolute requirement for ExbD D25 for the TonB response to pmf has been established. In the previous chapter, results from the substitution of protonatable residues showed that no protonatable residues were essential for function, but instead ExbB TMD residues were implicated in signal transduction and a scaffolding-like function. Recent characterization of ExbB Δ120-129, which deletes 10 cytoplasmic loop residues adjacent to TMD2, was shown to be leaking protons. In this study, substitution of important ExbB TMD residues reduced ExbB Δ120-129-mediated proton leakage demonstrating that proton flow was influenced by the ExbB TMDs. However, the effect of the TMD substitutions on the proton leakage rate did not correlate with the residues' importance in a wild type ExbB, suggesting that proton leakage caused by the ExbB Δ120-129 mutant was occurring through an aberrant mechanism not indicative of normal ExbB functions.

Introduction

The asymmetric outer membrane (OM) of Gram negative bacteria provides a protection barrier against harsh external environments. However, passive diffusion across the OM is limited to ~600Da by OM porin proteins [Nikaido, 2003]. Nutrients too large or scarce such as iron-siderophore complexes and B12 in *Escherichia coli* are actively transported across the OM through ligand specific transporters known as TonB-gated transporters (TGT). Energy for active transport is derived from proton motive force (pmf) generated at the cytoplasmic membrane (CM) which is traduced by integral CM proteins, TonB, ExbB and ExbD [for reviews see Cornelis et al., 2009; Cornelissen and Hollander, 2011; Krewulak and Vogel, 2011; Kuehl and Crosa, 2010; Noinaj et al., 2010; Zimbler et al., 2009].

TonB physically contacts the TGT at the OM through a conserved N-terminal domain called the TonB-box as well as through less characterized domains [Cadieux and Kadner, 1999; Gresock et al., 2011; Ogierman and Braun, 2003; Skare et al., 1993]. Because the TonB TMD lacks essential protonatable residues to directly harness pmf, presumably ExbB/D connect TonB to pmf energy [Swayne and Postle, 2011; Held and Postle, 2002; Ollis et al., 2009; Ollis and Postle, 2012a].

TonB and ExbD have a similar topology containing a single N-terminal transmembrane domain (TMD) and large periplasmic C-terminal domains [Kampfenkel and Braun, 1992; Roof et al., 1991]. ExbB adopts an N-out, C-in topology, contains three transmembrane domain segments, and cytoplasmic loop and tail domains [Kampfenkel and Braun, 1993a; Karlsson et al., 1993b].

Proton translocating functions of ExbB have been suggested by homology with the flagellar motor protein MotA [Kojima and Blair, 2001; Zhai et al., 2003]. However, mutational analysis of protonatable residues in the ExbB TMDs refuted a proton pathway involving ExbB TMD residues [Chapter 2, Braun and Herrmann, 2004]. Analysis of ExbB TMD residues suggested a scaffolding role for correct TonB/ExbB/ExbD assembly. If a proton translocation channel exists, ExbB TMDs and the ExbD TMD containing an essential Asp residue would likely comprise the channel [Chapter 2, Braun et al., 1996]. Inactive substitutions of ExbD D25, and ExbB TMD substitutions demonstrate pmf-dependent signal transduction from the TMDs to the periplasmic domains of TonB and ExbD [Jana et al., 2011; Ollis et al., 2009; Ollis and Postle, 2012b, Chapter 2].

Recently, 9 consecutive ten amino acid deletions of the ExbB loop domain also demonstrated signal transduction from the cytoplasmic domain. In addition to eliminating pmf-dependent TonB-ExbD interaction, expression of the loop deletion mutants inhibited growth [Bulathsinghala et al., manuscript in preparation]. Growth inhibition cause by overexpression of homologous flagellar motor proteins MotA and the 60 N-terminal residues of MotB were presumably dissipating pmf through proton leakage [Blair and Berg, 1990]. However, proton leakage was not attributed to the growth inhibition of the ExbB deletion mutants with pmf dissipation caused by Δ50-59 and Δ120-129 being insufficient to inhibit growth. In this study, substitution of ExbB TMD residues confirmed that ExbB Δ120-129-mediated proton leakage was occurring through the ExbB TMDs. However, the importance of the TMD substitutions did not correlate

with the reduction of ExbB Δ120-129-mediated proton leakage suggesting a non-wild type mechanism of proton conductance.

Materials and methods:

Strains and Plasmids

The bacterial stains and plasmids used in this study are listed in Table 3-1. Plasmid pKP660, contains ExbB and ExbD which are expressed from the arabinose promoter of pBAD24 [Ollis et al., 2009]. pKP657 was constructed using 30 cycle extra-long PCR to delete ExbB residues 120-129 from a pKP660 template. ExbB Δ120-129 derivatives were constructed by site-directed mutagenesis PCR using a pKP657 template as previously described [Vakharia-Rao et al., 2007]. Correct sequences of both exbB and exbD genes were confirmed for all mutants by the Penn State Genomics Core Facility (University Park, PA).

Media and Culture Conditions

Lubia-Bertani (LB) and M9 minimal salts were prepared as described previously [Miller, 1972; Shedlovsky and Brenner, 1963]. Agar plates and liquid cultures were supplemented with 100μg/ml of ampicillin and plasmid specific amount of L-arabinose to equal chromosomal protein levels. M9 salts were supplemented with 1.0% glycerol (wt/vol), 0.4 mg of thiamine/ml, 1 mM $MgSO_4$, 0.5 mM $CaCl_2$, 0.2% Casamino Acids (wt/vol), 40 mg of tryptophan/ml, and 1.85 μM$FeCl_3$. Cultures were grown with aeration at 37°C.

EtBr Accumulation assays

EtBr accumulation assays were performed as previously described with the following modifications [Takatsuka and Nikaido, 2007]. Cultures were grown in M9 minimal media to 0.4-0.5 OD_{550}. Protein expression of plasmid encoded ExbB Δ120-129 mutants and the pExbBD controls in GM1 derivative strain, KP1445, were induced with L-arabinose, at concentrations listed below, for 15 minutes prior to EtBr addition. For the proton leakage control, 30μM CCCP was added to KP1445, containing empty vector pBAD24, 15 minutes prior to EtBr addition. After 15 minutes induction 2, 0.4 OD_{550} ml cells were transferred directly to a 1.5ml disposable cuvette. The final volume was adjusted to 2 ml using the M9 growth media containing identical arabinose concentrations. EtBr fluorescence was measured on a Perkin Elmer LS55 fluorescence spectrometer immediately after addition of EtBr (final concentration of 6.25μM) at the 520nm and 590nm EtBr excitation and emission wavelengths respectively. The widths of the excitation and emission slits were set at 15nm. Data was collected continuously for 180 seconds using the time drive application from the FLWINLAB software. The rate of EtBr accumulation was linear and the slopes were calculated using linear regression. Rate of EtBr accumulation from at least 2 independent triplicate assays were graphed as arbitrary fluorescence units (FU)/time. Immediately prior to EtBr addition, protein samples were harvested and trichloroacetic acid (TCA)-precipitated and resolved on 13% SDS-polyacrylamide gels and visualized on immunoblots probed with anti-ExbB antibodies [Higgs et al., 2002a]. Corresponding ExbB Δ120-129 plasmids used here are listed in Table 3-1. Arabinose percentages are as follows: 0.1% for pKP657 (ExbB

Δ120-129) and derivative plasmids additionally containing substitutions S34A, W38A, T135A, T181A, P190A, and Y195A; 0.002% ExbB Δ120-129 (E176A); 0.001% for ExbB Δ120-129 derivative plasmids containing P141A, G147A, T148A, T138C, T148V, G151A and N196A.

Results:

ExbB TMD mutations decrease ExbB Δ120-129-mediated proton leakage

ExbB Δ120-129 at the cytoplasmic loop between TMD 1 and 2 leaks protons, as measured by the loss of EtBr efflux capacity through AcrAB/TolC (Figure 3-1) [Bulathsinghala et al., manuscript in preparation; Takatsuka and Nikaido, 2007]. To determine if this proton leakage was attributable to a normal ExbB function, we attempted to correlate the function of TMD substitutions with their abilities to prevent proton leakage. Failure of AcrAB-TolC to efflux EtBr due to loss of pmf results in steadily increasing fluorescence as EtBr enters the cell and binds to nucleic acids [Paixão et al., 2009; Takatsuka and Nikaido, 2007]. EtBr accumulation directly correlates to increasing CCCP-mediated collapse of pmf [Bulathsinghala et al., manuscript in preparation]. To assay the effect of ExbB Δ120-129 + X mutants on the rate of EtBr accumulation, the mutants were expressed at a level similar to ExbB Δ120-129 and the rate of EtBr accumulation was measured as EtBr fluorescence/time. Because the proton translocation functions would be essential for TonB system activity, ExbB TMD

substitutions affecting the proton translocation rate of ExbB Δ120-129 should have similarly reduced function.

Substitution of important ExbB TMD residues did not correlate with the degree of ExbB Δ120-129-mediated proton leakage. Previous characterization of important ExbB TMD residues suggested roles in assembly and did not identify any residues specifically important for proton translocation functions (Chapter 2). Therefore, no reduction in proton leakage of Δ120-129 + TMD substitutions S34A, W38A, T135A, T181A, P190A and Y195A could be explained by the unimportance of these residues in proton channel formation (Fig 3-2).

However, if ExbB Δ120-129 proton leakage is mechanistically similar to wild type, substitution of residues which did reduce the rate of ExbB Δ120-129-mediated proton leakage should similarly reduce activity since pmf is essential for TonB system activity. ExbB P141A, G147A, G151A, and E176A substitutions had significant function (45%, 70%, 50%, 25% [^{55}Fe] transport activity respectively) yet reduced/eliminated ExbB Δ120-129-mediated proton leakage, suggesting aberrant proton translocation of ExbB Δ120-129 (Chapter 2, Fig 3-2). Furthermore, ExbB Δ120-129 + T148A/C/V and T148A/T181A all reduced ExbB Δ120-129-mediated proton leakage similarly, despite varying activity of T148A/C/V activities in [^{55}Fe] transport (Chapter 2, Fig 3-2). Inactive ExbB N196A was the only mutant which correlated with a reduction in the proton leakage rate (Fig 3-2)[Jana et al., 2011]. Since Asn is not a protonatable residue, the reduced proton leakage rate may affect some aspect of ExbB assembly. However, consistent with our previous results (Chapter 2), most residues affecting the

rate of EtBr uptake were not protonatable, suggesting that instead ExbB assembly functions were affecting the rate of EtBr uptake.

It is worth noting that while ExbB Δ120-129 was unstable and could not be overexpressed relative to chromosomal, all Δ120-129 + X mutants that reduced the proton leakage rate were also more stable and could be overexpressed (Fig. 3-4). Additionally, when ExbB Δ120-129 + substitutions: G147A, T148A/C/V, G151A, T148A/T181A, E176A, N196A were overexpressed, the rate of EtBr accumulation also increased (Fig. 3-3). Since overexpressed wild type ExbBD did not increase the EtBr accumulation rate, the pmf dissipation was attributable to the Δ120-129 deletion and not a wild type ExbB overexpression phenotype. This suggests that the TMD substitutions were reducing the rate of proton translocation and further correlates the rate of EtBr accumulation to the amount of mutant ExbB protein present. By contrast, overexpression of Δ120-129 + P141A did not increase EtBr accumulation meaning that this substitution fully eliminated that ability of ExbB Δ120-129 to leak protons (Fig 3-3).

Discussion:

Deletion studies in the cytoplasmic loop of ExbB identified ExbB Δ120-129-mediated proton leakage [Bulathsinghala et al., manuscript in preparation]. Because ExbB has proposed proton harnessing functions, it seemed reasonable that proton flow could be occurring directly through the ExbB TMDs. If this was true, then important residues within the TMDs would be required for ExbB Δ120-129-mediated proton

leakage. To test this assumption, inactive or reduced activity ExbB TMD substituted mutants were added to ExbB Δ120-129 and the rate of proton leakage was determined. Elimination or reduction ExbB Δ120-129-mediated proton leakage containing TMD substituted residues demonstrated that proton flow was occurring through ExbB TMDs potentially via a putative proton channel.

Similar to proposed ExbD functions, proton harnessing in the homologous TolQ/R and MotA/B systems are thought to involve protonation of the critical Asp residue in the ExbD/TolR/MotB TMDs [Cascales et al., 2001; Kojima and Blair, 2001; Zhou et al., 1998]. Since ExbB Δ120-129 mediated proton leakage is uninfluenced by deletion of ExbD containing the essential D25 [Bulathsinghala et al., manuscript in preparation], this suggested that ExbB Δ120-129-mediated proton leakage may have occurred through an aberrant mechanism not representative of a wild type ExbB. If the mode of ExbB Δ120-129 proton translocation is representative of a wild type proton pathway, the TMD substitutions which reduce the rate of ExbB Δ120-129-mediated proton leakage should be equally important for ExbB function. However, ExbB TMD substitutions did not reduce the proton leakage rate proportionately to their corresponding [^{55}Fe] transport activity (Fig 3-2, Chapter 2). This suggested that the mode of ExbB Δ120-129-medated proton leakage may be mechanistically different from pmf-harnessing functions of a wild type ExbB. In particular, ExbB P141A, G147A and G151A were ~45%, 67% and ~45% active in [^{55}Fe] transport yet abolished/reduced the rate of ExbB Δ120-129-medated proton leakage (Fig 3-2, 3-3).

Importance of ExbB multimer assembly for ExbB Δ120-129-mediated proton leakage

An altered ExbB conformation caused by Δ120-129 deletion could have distorted some aspect of pmf-harnessing since overexpressed wild type ExbB did not dissipate pmf (Fig 3-3). Because this deletion is near the predicted TMD2 boundary, the mutant sequence was analyzed by TOPCONS topology prediction program (http://topcons.cbr.su.se). The predicted TMD residues were identical to a wild type ExbB suggesting that the ExbB topology was not severely altered (data not shown). Thus, proton leakage due to distorted ExbB Δ120-129 conformations would potentially arise from altered ExbB multimer interactions.

Highlighting the importance of ExbB multimer assembly in proton leakage, the substitutions P141A, N196A and E176A reduced the Δ120-129-mediated proton leakage rate and increased the formaldehyde crosslinked ExbB homodimer suggesting altered ExbB assembly [Chapter 2, Jana et al., 2011]. Furthermore, ExbB E176A was non-dominant in [^{55}Fe] transport assay when overexpressed in a wild type strain indicating reduced ExbB multimer assembly abilities (Data not shown). Since P190A more severely impaired ExbB assembly, eliminating the ExbB tetramer +X formaldehyde-crosslinked complex, but did not eliminate Δ120-129-mediated proton leakage, this further suggests an altered mechanism of proton translocation through this mutant. Similarly, G147A and G151A substitutions in the conserved TMD2 $G_{141}xxxG_{147}xxxG_{151}$ packing motif reduced/eliminated the rate proton leakage but did not eliminate ExbB activity (Fig 3-4, 3-5, Chapter 2). In TolQ and MotA proton channel predictions, these residues correspond

to regions of helix-helix assembly consistent with important ExbB assembly functions [Goemaere et al., 2007; Kim et al., 2008].

Stability of ExbB Δ120-129 + X mutants

In addition to reducing the rate of ExbB Δ120-129-medated proton leakage, the Δ120-129 + X substitutions differentially affected ExbB stability. It is unlikely that the ExbB TMD substitutions are effecting localization since individually, the TMD mutants and the Δ120-129 still formaldehyde crosslink to ExbD (Chapter 2, Bulathsinghala et al., manuscript in preparation]. Instead, differential stability of individual ExbB Δ120-129 + X mutants could suggest varied protease susceptibility or cellular protease concentrations. One candidate protease, FtsH, is a well characterized membrane bound protease that degrades misfolded/unassembled CM proteins [Ito and Akiyama, 2005]. Additionally, FtsH degradation of growth inhibitory overexpressed translocon protein, SecY and subunit a from the F_0 ATP synthase suggest additional degradation functions of deleterious proteins [Akiyama et al., 1996]. Addition of ExbB TMD substitutions which alleviate Δ120-129-medated proton leakage may not be targeted for FtsH degradation and can be more stably expressed. Alternatively, instead of varying protease affinities, a cellular stress response triggered by Δ120-129-medated proton leakage could up-regulate membrane proteases leading to ExbB mutant instability [Bury-Mone et al., 2009]. Addition of ExbB TMD mutants that decreased the rate of proton leakage would not trigger a stress response and would therefore be more stable.

Through substitution of important ExbB TMD residues, this study demonstrated that while ExbB Δ120-129-mediated proton leakage was probably occurring through the

ExbB TMDs, that leakage did not support the idea that wild type ExbB is, by itself, a proton translocator. While ExbBD were thought to function as pmf-harnessers, the results presented here and in Chapter 2 suggest that alternative models for TonB system pmf-utilization should be considered.

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Table 3-1. Stains and plasmids

Strains	**Genotype**	**Source or reference**
GM1	*ara Δ(pro-lac) thi,* F*' pro lac*	[Sun and Webster, 1987]
KP1445	GM1 *exbB::Tn*10 *recA::cat*	(Bulthasinghala et. al. , submitted)
Plasmids		
pBAD24	Empty vector control	[Guzman et al., 1995]
pKP657	*exbBΔ120-129, exbD*	(Buthasinghala, et al. submitted)
pKP1535	*exbBΔ120-129* (S34A), *exbD*	Present study
pKP1616	*exbBΔ120-120* (W38A), *exbD*	Present study
pKP1543	*exbBΔ120-120* (T135A), *exbD*	Present study
pKP1618	*exbBΔ120-120* (P141A), *exbD*	Present study
pKP1629	*exbBΔ120-120* (G147A), *exbD*	Present study
pKP1616	*exbBΔ120-120* (T148A), *exbD*	Present study
pKP1617	*exbBΔ120-120* (T148C), *exbD*	Present study
pKP1536	*exbBΔ120-120* (T148V), *exbD*	Present study
pKP1630	*exbBΔ120-120* (G151A), *exbD*	Present study
pKP1620	*exbBΔ120-120* (E176A), *exbD*	Present study
pKP1619	*exbBΔ120-120* (T181A), *exbD*	Present study
pKP1573	*exbBΔ120-120* (P190A), *exbD*	Present study

Table 3-1 Strains and Plasmids (*cont.*)		
pKP1621	*exbBΔ120-120* (Y195A), *exbD*	Present study
pKP1622	*exbBΔ120-120* (N196A), *exbD*	Present study
pKP1544	*exbBΔ120-120* (T148A,T181A), *exbD*	Present study

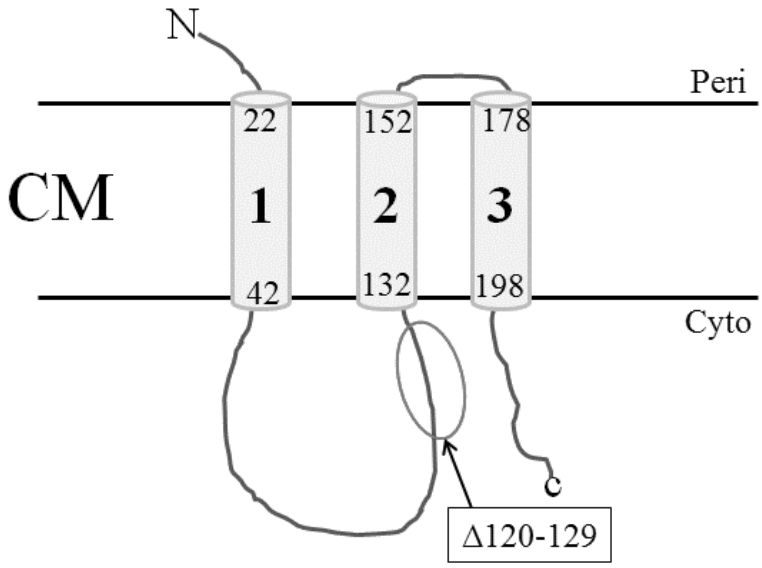

Figure 3-1 *ExbB topology and location of the loop deletion.* This cartoon depicts ExbB topology and the predicted TMD boundaries from chapter 2. The two parallel black lines represent the cytoplasmic membrane; both cytoplasm (cyto) and periplasm (peri) are indicated. The majority of ExbB is localized in the cytoplasm, containing large cytoplasmic loop and C-terminal tail domains. ExbB TMDs 1, 2, and 3 are indicated with the predicted amino acid boundary numbers presented within each predicted helix, respectively. The circle indicates the location of the Δ120-129 deletion characterized in this study.

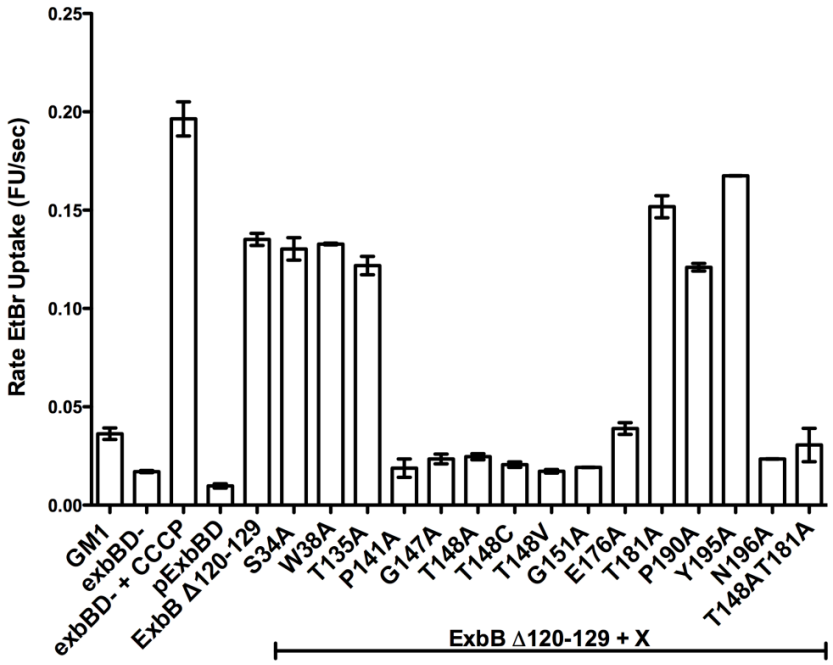

Figure 3-2. *ExbB TMD mutants decrease the rate of ExbB Δ120-129-mediated proton leakage*. Plasmid encoded ExbB Δ120-129 containing TMD substitutions and ExbD were expressed in KP1445 (GM1*exbB::Tn10*, *recA::cat*) at levels similar to ExbB Δ120-129, ExbD for 15 minutes prior to harvesting at mid-exponential phase. Arabinose induction levels are listed in Materials and Methods and protein levels are shown in Fig. 3-4. A pExbBD control was overexpressed to demonstrate no proton leakage by wild type ExbB. KP1445 was treated with 30μM of protonophore (CCCP) for 15 minutes prior to harvesting to demonstrate the effect of pmf dissipation on EtBr accumulation. The 30μM CCCP concentration is sufficient to inhibit cell growth as reported in [Bulathsinghala et al., manuscript in preparation]. Proton leakage was measured indirectly via the rate of EtBr accumulation. Cellular EtBr accumulation was indicated by the increment of EtBr fluorescence due to nucleic acid intercalation at excitation and emission wavelengths 520 and 590nm, respectively. Fluorescence was continuously measured for 180 seconds and the rate of EtBr accumulation was calculated by linear regression and presented as fluorescence units (FU/sec).

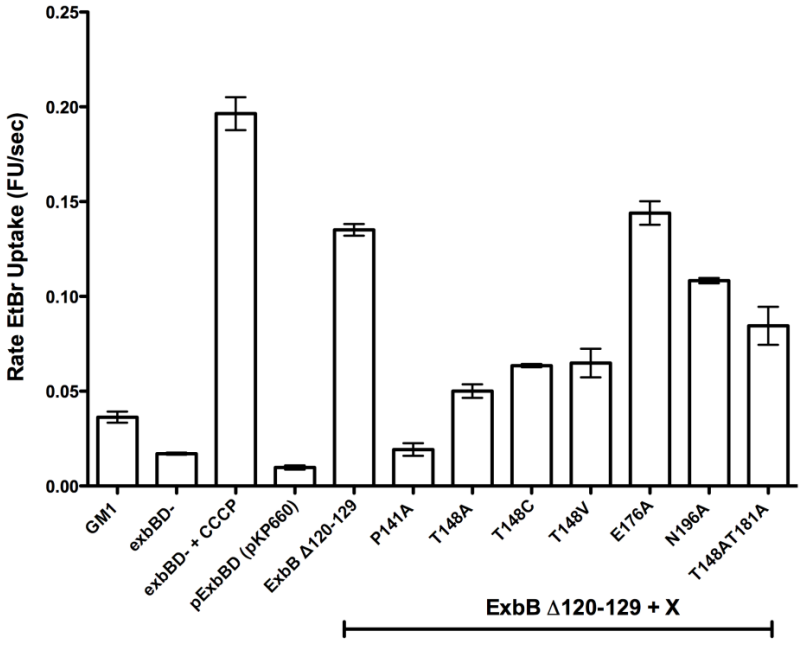

Figure 3-3. *Rate of ExbB Δ120-129+ X-mediated proton leakage is increased by overexpression.* Plasmid encoded ExbB Δ120-129 containing TMD substitutions and ExbD were maximally expressed in KP1445 (GM1*exbB::Tn10*, *recA:: cat*) with 0.1% arabinose for 15 minutes prior to harvesting at mid-exponential phase and protein levels are shown in Fig. 3-4. A pExbBD control was overexpressed to demonstrate no proton leakage by wild type ExbB. KP1445 was treated with 30μM of protonophore (CCCP) for 15 minutes prior to harvesting to demonstrate the effect of pmf dissipation on EtBr accumulation. The 30μM CCCP concentration is sufficient to inhibit cell growth as reported in [Bulathsinghala et al., manuscript in preparation]. Proton leakage was measured indirectly via the rate of EtBr accumulation. Cellular EtBr accumulation was indicated by the increment of EtBr fluorescence due to nucleic acid intercalation at excitation and emission wavelengths 520 and 590nm, respectively. Fluorescence was continuously measured for 180 seconds and the rate of EtBr accumulation was calculated by linear regression and presented as fluorescence units (FU/sec).

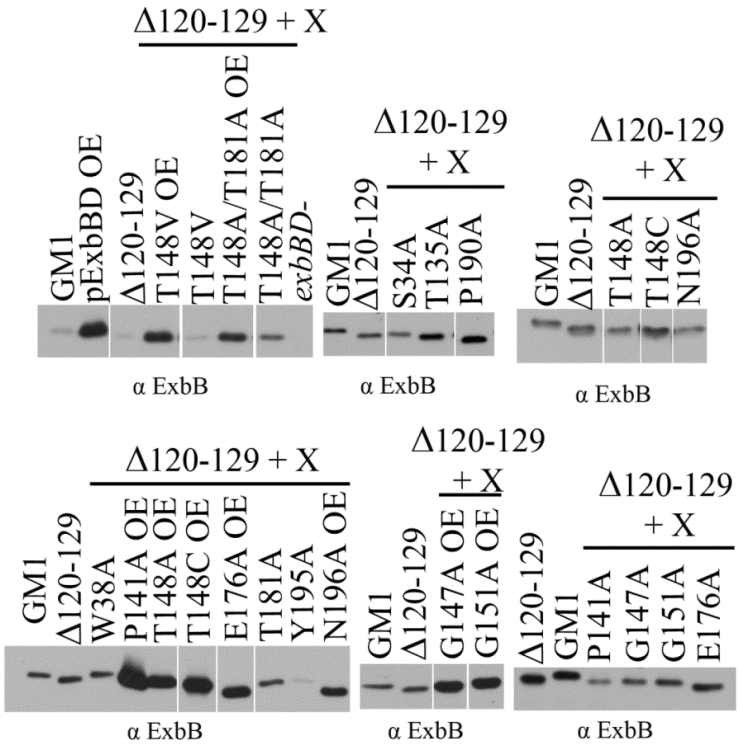

Fig 3-4. *Protein levels of ExbB Δ120-129+ X mutants from EtBr uptake experiments.* ExbB Δ120-129 containing ExbB TMD substitutions (ExbB Δ120-129 + X) were expressed in strain KP1445 (GM1*exbB::Tn10*, *recA::cat*) with varying levels of arabinose to equal an ExbB Δ120-129 expression level. ExbB Δ120-129 + X mutants which could be overexpressed relative to an ExbB Δ120-129 expression level with 0.1% arabinose are denoted "OE". Arabinose induction levels are listed in Materials and Methods. Cultures were grown to mid-exponential phase and protein expression was induced 15 minutes prior to harvesting for the EtBr Uptake assay. Samples were collected immediately prior to EtBr addition from cultures corresponding to the data shown in figures 3-2 and 3-3, TCA-precipitated and solubilized at 95°C. Samples were resolved on 13% SDS-polyacrylamide gels and immunoblotted with anti-ExbB antibody. Similar length immunoblot exposures from multiple experiments are shown. To evaluate relative protein levels between experiments, compare GM1 and Δ120-129 ExbB levels from each experiment to Δ120-129 + X mutants.

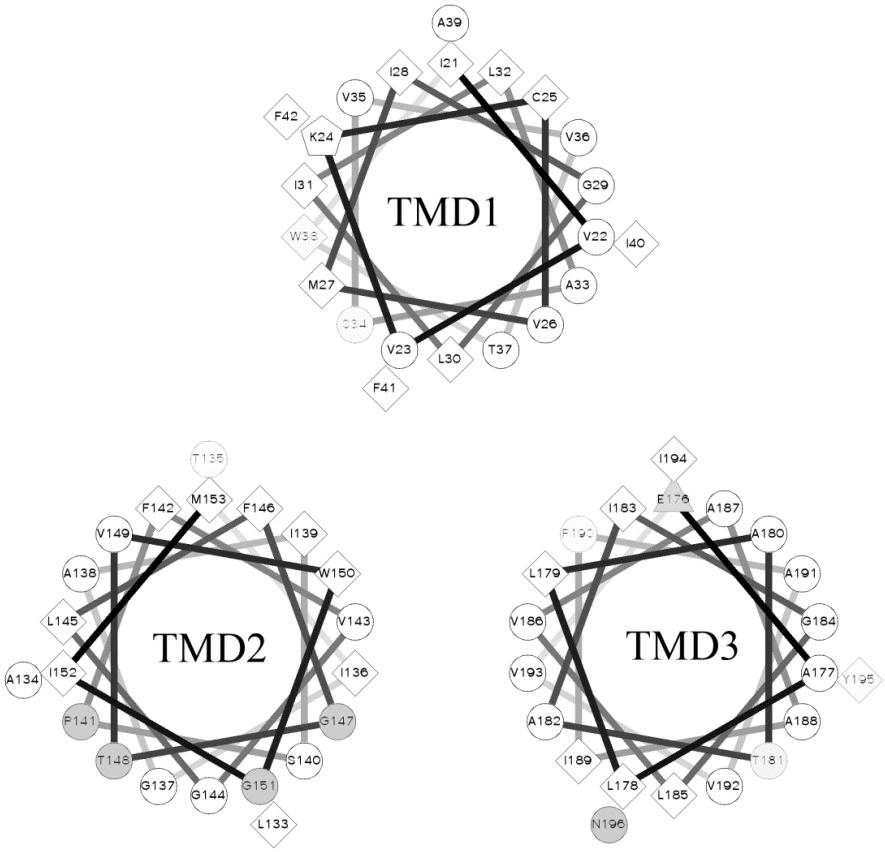

Figure 3-5. *Helical Wheel Projection of ExbB TMDs: Effect of Ala substitutions on the rate ExbB Δ120-129-mediated proton leakage.* ExbB TMD α-helices are projected as helical wheels from periplasm to cytoplasm, using the helical wheel projection program: (http://rzlab.ucr.edu/scripts/wheel/wheel.cgi). Ala substituted residues which reduced the rate of ExbB Δ120-129-mediated proton leakage are indicated in gray and are as follows: P141A, T148A, G147A, G151A (TMD2); E176A, N196A (TMD3). Tested residues which did not decrease the rate of proton leakage are marked in light gray and are as follows: S34A, W34A (TMD1); T135A (TMD2); T181A, P190A, Y195A (TMD3). Hydrophilic residues are presented as circles, hydrophobic as diamonds, negatively and positively charged residues as triangles and pentagons respectively.

CHAPTER 4

DISCUSSION

Discussion

ExbB is centrally important for TonB-dependent energy transduction serving both structural and functional roles, protecting TonB and ExbD from proteolytic degradation and supporting the TonB conformational response to pmf [Ahmer et al., 1995; Fischer et al., 1989; Held and Postle, 2002; Ollis et al., 2009]. While the functional domains of TonB and ExbD have been well characterized, mechanistic insight of ExbB functions have not been extensively explored [Larsen et al., 2007; Larsen et al., 1997; Larsen et al., 1993; Ollis et al., 2012; Ollis and Postle, 2012b; Postle et al., 2010; Swayne and Postle, 2011]. ExbB inactive mutations in the cytoplasmic loop and tail domains prevent pmf-dependent TonB-ExbD periplasmic interaction indicating ExbB-dependent signal transduction. Furthermore, expression of ExbB cytoplasmic loop deletions Δ50-59 and Δ120-129 partially dissipate pmf energy, raising the possibility that ExbB could be directly involved in harnessing pmf. [Bulathsinghala et al., manuscript in preparation; Jana et al., 2011]. A proton pathway involving ExbB, ExbD and/or TonB TMDs was predicted [Zhai et al., 2003]. Despite the likely importance of the ExbB TMDs for TonB system function, only two inactivating TMD mutants have been identified N196A and double substitution T148A/T181A [Braun and Herrmann, 2004; Jana et al., 2011; Zhai et al., 2003]. Limited mutational analysis of ExbB TMD residues has kept the precise functional role of ExbB in the realm of speculation.

This work examined the functional importance of the ExbB TMDs and began with new predictions of ExbB TMD boundaries. Ala substitutions of each half-TMD and individual residue substitutions demonstrated that all three ExbB TMDs are essential for function. Furthermore, specific residues were important for ExbB scaffolding functions

and for signal transduction. Finally, important ExbB TMD residues substituted in the ExbB Δ120-129 background affected the rate of proton leakage, raising the possibility that proton translocation occurs through the ExbB TMDs. However this possibility was excluded since substitution of unessential TMD residues most strongly reduced proton leakage. This instead suggests that proton leakage occurred through non-native conformations in the ExbB TMDs.

This work ruled out the hypothesis that protonatable ExbB TMD residues were functioning in a proton pathway. Instead, substitution analysis supports an ExbB role in signal transduction and in complex assembly through a scaffolding-like function. Non-dominance and absence of the TonB-ExbD crosslink implicated a role for ExbB TMD1 in an assembly function with TonB (Fig. 4-1). Such an interaction could be confirmed through Cys scanning in both TonB and ExbB TMDs. Substitution analysis in TMD 2 and 3 revealed a role in ExbB multimer formation (Fig. 4-1). While no individual substitution eliminated a formaldehyde-crosslinked ExbB-ExbD complex, Ala substitution of the cytoplasmic half of TMD2 reduced ExbD stability suggesting a role in assembly with ExbD.

All inactive ExbB TMD substitutions eliminated the functionally relevant TonB-ExbD formaldehyde-crosslinked complex. While this suggested a role in signal transduction, the absence of the TonB-ExbD complex for most mutants was attributable to defects in complex assembly. However, no assembly defects were detected for the ExbB T135A substitution, implicating a role for T135 in signal transduction mediated by the ExbB TMDs.

Pmf-dependent conformational changes in the TonB system

In pmf-harnessing complexes, proton translocation is coupled to cellular work via conformational changes. Examples of conformational change coupled to proton conduction include lac permease and the F_1F_0 ATP synthase. Lac permease co-transports lactose/protons across the CM through TMD rearrangement which allows alternate access of the lactose binding pocket the periplasm and cytoplasm for lactose release [Guan and Kaback, 2006]. The F_0F_1 ATP synthase is comprised of two subunits: the membrane embedded F_0 subunit which is adjoined to the soluble catalytic F_1 subunit through the F_1 protein, γ. Proton conduction through the F_0 subunit generates torque via rotation of the c-ring proteins relative to the a-b stator proteins in the F_0 complex. This torque is coupled to the F_1 subunit through rotation of the γ protein which triggers conformational changes in the F_1 catalytic subunits resulting ATP generation [Okuno et al., 2011]. Similarly, ExbD-mediated conformational changes in the TonB C-terminal domain are pmf-dependent and are functionally important for TonB-dependent ligand transport [Ghosh and Postle, 2005; Larsen et al., 1999; Ollis et al., 2009; Ollis and Postle, 2012b].

Future Directions: Identification of pmf-dependent conformational changes in ExbB

While ExbD D25-influenced conformational changes in the periplasmic domains of TonB and ExbD have already been demonstrated, additional TMD conformational changes promoting these TonB-ExbD interactions have not been investigated [Ollis et al., 2009; Ollis and Postle, 2012a, 2012b]. In support of changes in TMD conformations during the TonB energization cycle, disulfide crosslinking studies of Cys substituted homologous MotA/B and TolQ/R identified dynamic interactions between the TMDs

[Braun et al., 2004; Braun and Blair, 2001; Kim et al., 2008; Zhang et al., 2011; Zhang et al., 2009]. In particular, disulfide trapped TolQ interactions likely represented multiple different conformational stages of energy transduction which were not distinguished by this approach, making clear mechanistic insights difficult. While application of cysteine scanning to the ExbB TMDs would likely provide similarly complex results, addition of inactive ExbB TMD substitutions to this approach would sort potential disulfide-trapped interactions into different functionally relevant categories. Through comparative ExbB TMD substitution analysis of different disulfide-trapped interaction profiles, mechanistically informative interpretations can be made. Because ExbB T148V and T135A supported different TonB proteinase K profiles, these substitutions could identify different ExbB TMD conformational states (Chapter 2).

Comparisons between homologous/orthologous proteins TolQ, MotA and PomA can assist our understanding of ExbB functions. Since these proteins are most homologous through their last two TMDs which are implicated in pmf-harnessing functions, highly conserved structural glycines and 2 proline residues corresponding to ExbB TMD2/3 suggest similar helix-helix interactions and consequently a similar proton channel composition [Chapter 2, Cascales et al., 2001; Kojima and Blair, 2001; Zhai et al., 2003]. The unconserved domains likely serve specialized roles for the unique functions of each protein. MotA TMD1 and TMD2 have a proposed structural role in TMD assembly of the flagellar stator [Kim et al., 2008]. Likewise, ExbB TMD1 substitutions presented here also suggest a structural role in TonB-ExbB interaction.

While the loop domains of MotA and ExbB are not homologous, both domains are essential for activity indicating important functional roles [Bulathsinghala et al.,

manuscript in preparation; Morimoto et al., 2010b; Muramoto and Macnab, 1998]. In MotA, conformational changes in the loop domain propagate rotation through electrostatic interactions with the FliG rotor protein. Inactivating mutants in the MotA and MotB TMDs stalled MotA loop conformational changes, reminiscent to how ExbB and ExbD TMD mutations stalled TonB-ExbD periplasmic interactions in proteinase K sensitivity assays (Chapter 2). To determine if functionally important conformational changes occur in the ExbB cytoplasmic domain, a similar approach to the MotA loop can be initiated where conformational changes in the ExbB loop could be trapped with ExbB/ExbD/TonB TMD inactivating mutations and probed through treatment with different proteases [Kojima and Blair, 2001]. However due to the cytoplasmic localization of the ExbB loop domain, protease susceptibility studies would require preparation of inside-out membrane vesicles causing dissipation of pmf. Since pmf is required to detect TonB and ExbD conformational changes, this approach may not be able to capture dynamic conformational changes in the ExbB loop domain. However, it is possible that inactivating substitutions could stabilize/stall in the ExbB loop in a stable conformation such that it could be observed through this approach.

TonB-system mediated proton translocation

Pmf energy generated at the CM is harnessed through specialized protein complexes for a variety of cellular functions including motility, enzymatic catalysis, outer-membrane maintenance, ATP-generation, and substrate import/export [Cascales et al., 2001; Decoursey, 2003; Nikaido and Takatsuka, 2009; Stoffels et al., 2012]. The

basic biophysical mechanism of proton translocation through these complexes is thought to be similar with proton channels consisting mainly of water molecules and containing key protonatable side chain(s) [Decoursey, 2003]. Despite a similar biophysical mechanism, protein proton translocators are diverse ranging from a simple 15 residue homo-dimer in gramicidin to highly complex systems such as the 36 TMD AcrB homotrimeric complex [Kelkar and Chattopadhyay, 2007; Seeger et al., 2009]. Due to this structural diversity, it is difficult to identify proton translocator proteins based on the primary sequence or secondary structure alone. Instead, proton translocation could be suggested by pmf-dependent protein functions. However, this phenotypic classification is not sufficient to confirm a role in proton translocation. Additional spatial structural knowledge of the TMD arrangement and identification of key residues is required to conceive a proton translocation mechanism.

The pmf-dependent function of the TonB system suggests that the TonB-ExbB-ExbD complex may directly translocate protons. ExbB function as an independent proton conducting component of the TonB system was tested and refuted in this study. A proton conduction role of ExbB was postulated based on homology to the MotA flagellar motor protein [Zhai et al., 2003], which was initially suggested to be an independent proton conductor [Blair and Berg, 1990]. However, later studies demonstrated that MotA proton conduction (assayed via a growth inhibition phenotype) was dependent upon co-expression of a MotB fusion protein containing 60 MotB N-terminal residues indicating that MotA was not itself a proton translocator [Stolz and Berg, 1991]. Yet, the misconception that MotA alone constituted a proton translocation pathway remained. This fed subsequent TMD comparisons of ExbB to MotA proton translocation functions

and a proposed proton pathway through ExbB TMD residues [Zhai et al., 2003]. The proposed pathway was tested by Braun and Hermann through substitution of protonatable T148, T181 and S155 residues. Even though substitution analysis of these ExbB residues did not support the existence of a proton pathway, the authors' conclusions were not supported by their data and thus required additional clarification [Braun and Herrmann, 2004]. Furthermore, the ExbB mutants were expressed at unknown levels which could result in an artifactual phenotype. To unambiguously clarify ExbB function, analysis of all protonatable ExbB TMDs residues concluded that no protonatable residues were essential for ExbB function excluding the possibility that ExbB is itself, a proton translocator.

Prior to this study, ExbB was thought to play a key role in pmf-harnessing for TonB-dependent transport. Previous mutagenesis in the TonB and ExbD TMDs similarly eliminated all residues except for ExbD D25 from participation in a proton pathway [Larsen et al., 2007; Swayne et al., 2011; Braun et al., 1996; unpublished data]. The results of this study call into question the mechanism through which the TonB system harnesses pmf energy. Proton conduction in MotAB is thought to occur through an aqueous channel formed by the MotAB TMDs. The existence of an aqueous proton channel in the TonB system has not been experimentally explored. Since chapter 3 concluded that ExbB Δ120-129-mediated proton leakage was occurring through a non-native mechanism, additional studies are required to investigate a wild type TonB system pmf-harnessing mechanism.

Considering that the ExbB Δ120-129 mutant is currently our only tool to directly assay ExbB-mediated proton translocation, there is a presumption that identification of

the cause of proton leakage would provide mechanistic insight. Because overexpressed wild type ExbB does not dissipate pmf, ExbB Δ120-129 most likely altered some aspect of proton conduction causing unregulated proton flow. Physical means to regulate proton flow include conformational rearrangement of TMDs which alternate proton channel access or by physical blockage of the proton translocation channel via a 'plug domain' [Abramson et al., 2003; Hosking et al., 2006; Moore et al., 2008]. While the soluble ExbB loop, tail domains or the ExbD/TonB periplasmic domains could theoretically function as a proton channel 'plug,' mutational analysis of these domains suggests such a role is unlikely [Ollis et al., 2012a; Larsen et al., 1993; Gresock et al., 2011; Jana et al., 2011; Bulathsinghala et al, manuscript in preparation]. Most deletions in the ExbB cytoplasmic loop did not leak protons suggesting this domain does not act as a 'channel plug' since all or most loop deletions would be expected to cause a proton leak [Bulathsinghala et al., manuscript in preparation]. Likewise, deletion of the ExbB C-terminal tail domain was not growth inhibitory [Jana et al., 2011]. While ExbD 'plug' domain functions could be suggested through comparison to the MotA/B system where an amphipathic helix 'plug' in the periplasmic domain of MotB is thought to regulate proton flow [Hosking et al., 2006; Morimoto et al., 2010a], the ExbD periplasmic domain is not homologous to MotB and consecutive 10 amino acid ExbD deletions were not growth inhibitory. This suggests that the ExbD periplasmic domain is not important to 'plug' the channel [Ollis et al., 2012]. Similarly, the periplasmic domain of TonB is very tolerant to deletions which are not deleterious to growth [Larsen et al., 1999; Larsen et al., 1993; Postle et al., 2010]. Alternatively, altered interactions with unidentified proteins observed in formaldehyde crosslinking studies could be influencing ExbB Δ120-129-

mediated proton leakage [Higgs et al., 1998; Jana et al., 2011; Ollis et al., 2009]. Isolation of suppressors that abolish the proton leakage phenotype could potentially identify novel system proteins.

Future Directions: Characterization of a putative proton channel formed through ExbB-ExbD-TonB TMDs

While this work identified residues important for ExbB signal transduction and scaffolding functions, additional studies are needed to characterize the composition of a putative proton channel. By employing a cysteine accessibility method to Cys substituted ExbB, ExbD and TonB TMD residues, the composition of an aqueous channel can be investigated. This approach has been successful in probing membrane aqueous ion channels in multiple systems [Akabas et al., 1994; Akabas et al., 1992; Bragg and Hou, 2000; Fillingame et al., 2003]. Since the Cys sulfhydryl group is more reactive in an aqueous environment, reactivity of Cys substitutions to different sulfhydryl reactive compounds can differentiate between aqueous and lipid facing residues [Mordoch et al., 1999]. Binding of sulfhydryl reactive agents to Cys within a proton channel would be expected to prevent proton translocation and inhibit activity. Furthermore, utilizing Cys reactive agents of different diameters would probe the pore size of the proton channel. This type of analysis has been performed in the F_0 atp synthase subunit a using NEM and Ag+ [Angevine et al., 2007]. Effect NEM and Ag^+ binding to Cys substituted TMD residues in ExbB Δ120-129 would identify reactive residues blocking proton translocation via reduction/elimination of ExbB Δ120-129-mediated proton leakage. A similar approach was used to probe the proton pathway of the AcrB of the AcrAB-TolC

multidrug efflux system demonstrating its application in whole cells [Takatsuka and Nikaido, 2007]. Preliminary studies using overexpressed ExbB Δ120-129 + T148C, which can still leak protons albeit at a lower rate, is the ideal residue to test the validity of this assay. MotA/TolQ/PomA residues corresponding to ExbB T148 are also predicted to be in a proton channel and in this study T148 substitutions reduced the proton leakage rate [Goemaere et al., 2007; Kim et al., 2008; Li et al., 2011, Chapter 3]. Additionally, since T181C is functional and T181A did not reduce Δ120-129-mediated proton leakage, this method could be used to determine if T181 is similarly located to T148 within a putative proton channel as previously proposed [Braun and Herrmann, 2004; Zhai et al., 2003].

Since ExbB Δ120-129-mediated proton leakage is likely occurring through a non-native ExbB conformation, examination the putative proton channel in a wild type TonB-ExbB-ExbD complex is desirable. This cysteine accessibility approach could be applied to TonB proteinase K sensitivity assays where obstruction of proton translocation could be probed through changes in TonB conformational sensitivity to pmf. In this approach, cys reactive agents could be added to spheroplasts and then changes in TonB conformations would then be probed with proteinase K in the presence and absence of CCCP. Reactive Cys substitutions which block the proton channel would result in a TonB proteinase K sensitivity profile that is insensitive to pmf since proton translocation would be obstructed. This approach can be extended to the TonB and ExbD TMDs to probe proton channel composition in a wild type TonB-ExbB-ExbD proton translocation complex.

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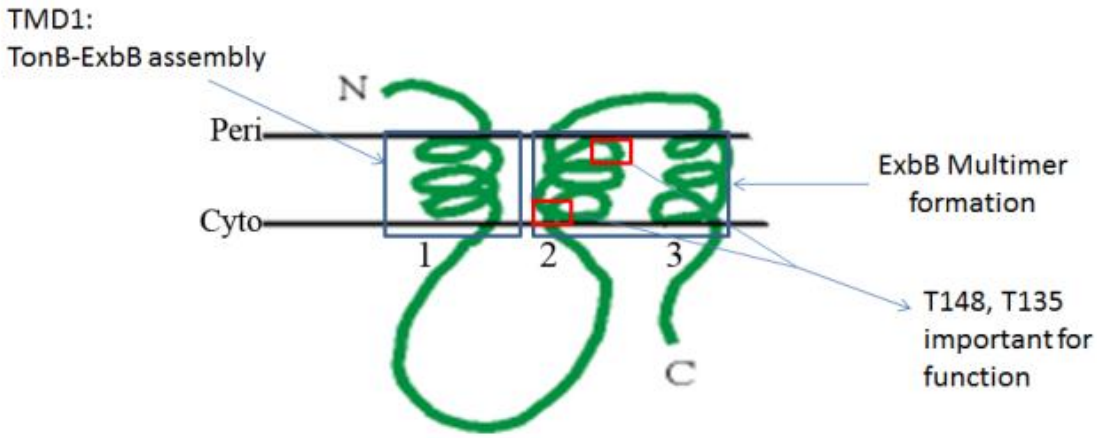

Fig. 4-1. *Functional role of the ExbB TMDs*. This cartoon summarizes ExbB functions identified through mutation analysis of the TMDs. The TMDs are numbered below their respective TMD. Peri and Cyto indicate periplasmic and cytoplasmic sides of the membrane.

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