The ExbD Periplasmic Domain Contains Distinct Functional Regions for Two Stages in TonB Energization

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Running title: Multiple functional regions in ExbD periplasmic domain
Abstract

The TonB system of Gram negative bacteria energizes active transport of diverse nutrients through high-affinity TonB-gated outer membrane transporters using energy derived from the cytoplasmic membrane protonmotive force. Cytoplasmic membrane proteins ExbB and ExbD harness the proton gradient to energize TonB, which directly contacts and transmits this energy to ligand-loaded transporters. In *Escherichia coli*, the periplasmic domain of ExbD appears to transition from protonmotive force-independent to protonmotive force-dependent interactions with TonB, catalyzing the conformational changes of TonB. A ten-residue deletion scanning analysis showed that while all regions except the extreme amino terminus of ExbD were indispensable for function, distinct roles for the amino and carboxy terminal regions of the ExbD periplasmic domain were evident. Like residue D25 in the ExbD transmembrane domain, periplasmic residues 42-61 facilitated the conformational response of ExbD to protonmotive force. This region appears important for transmitting signals between the ExbD transmembrane domain and carboxy terminus. The carboxy terminus, encompassing periplasmic residues 62-141, was required for initial assembly with the periplasmic domain of TonB, a stage of interaction required for ExbD to transmit its conformational response to protonmotive force to TonB. Residues 92-121 were important for all three interactions previously observed for formaldehyde-crosslinked ExbD: ExbD homodimers, TonB-ExbD heterodimers, and ExbD-ExbB heterodimers. The distinct requirement of this ExbD region for interaction with ExbB raised the possibility of direct interaction with the few residues of ExbB known to occupy the periplasm.
Introduction

In Gram negative bacteria, specific high-affinity TonB-gated transporters bind large, scarce, and essential nutrients for active transport across an unenergized outer membrane (OM). The TonB system, with a complex of TonB, ExbB, and ExbD in the cytoplasmic membrane (CM), couples energy derived from the CM protonmotive force (pmf) to TonB-gated transporters, energizing active transport of nutrients into the periplasm. TonB-dependent substrates include iron-siderophore complexes, vitamin B\textsubscript{12}, heme, maltodextrin, chitin oligosaccharides, sucrose, and nickel (1, 5, 6, 27, 39, 40).

The precise mechanism by which TonB transmits pmf energy to TonB-gated transporters is unknown. ExbB and ExbD are thought to harness pmf energy to TonB. TonB maintains its amino-terminal association with the CM, spans the periplasm to directly contact transporters through its periplasmic domain, transmits energy when ligand is bound, and is recycled to initiate the cycle again (3, 9, 17, 23, 30, 42). Conformational changes in the TonB periplasmic domain require ExbB, ExbD, pmf, and a functional TonB transmembrane domain (TMD) (23). Within the TonB TMD, all residues except His\textsubscript{20} can be replaced with alanine with no loss of function. His\textsubscript{20} serves a structural role and is not on a proton translocation pathway since this position tolerates a non-protonatable asn residue (20, 43). In contrast to the TMD, there are no essential residues in the TonB periplasmic domain (24, 36, 41).

ExbD (141 amino acids) has an identical topology to TonB (239 amino acids), with a carboxy-terminal periplasmic domain (more than 60% of the protein), a single transmembrane domain (TMD), and a short cytoplasmic amino terminus. ExbD directs the conformational changes of the TonB periplasmic domain, which can currently be divided into three stages (Fig. 1). In Stage I, the periplasmic domains of ExbD and TonB have yet to assemble together. In Stage II, ExbD and TonB can assemble such that TonB assumes a proteinase K resistant
conformation. This stage does not require the pmf. In Stage III, pmf allows rearrangement of the ExbD and TonB periplasmic domains such that they can be crosslinked by formaldehyde. Stages II and III require ExbB, which at a minimum appears to serve as a scaffold for the interactions between ExbD and TonB (31, 32).

The TMD is the most conserved of the three topological domains of ExbD, and conservation is shared with ExbD paralogues TolR and MotB (4). The TMD of each contains an essential conserved aspartate residue, with the potential for protonation, as has been suggested for the putative TolQ/R and MotA/B proton translocation pathways (2, 4, 44). ExbD D25 is required for the conformational response of both itself and TonB to pmf, which results in progression of TonB from Stage II to Stage III. D25 is also required for the apparent transition of ExbD from a homodimeric state to heterodimeric interaction with TonB through a 30-residue region of the ExbD periplasmic domain (92-121). The two dimeric states require nearly the same set of contact residues in the ExbD periplasmic domain (33).

Here we provide the first comprehensive study of the entirety of ExbD, providing detailed insights into functional domains of the *Escherichia coli* ExbD protein using a 10-residue deletion scanning analysis. This “global” mutagenesis approach identified two regions of the ExbD periplasmic domain with distinct functional roles. ExbD residues 42-61 were required for TonB to progress to its energized Stage III conformation but not for initial interaction with TonB that occurs in Stage II. No ExbD missense mutations have been found in this region of ExbD. Lastly, ExbD residues 62-141 (the carboxy terminus) proved important for proper assembly with TonB (Stage II). This region included a 30 residue subdomain that was important for all known ExbD protein-protein interactions.
Materials and Methods

Bacterial strains and Plasmids

Strains and plasmids used in this study are listed in Table 1. KP1522 was constructed by P1vir transduction of ΔexbD::cam from RA1021 into RA1016 (kind gifts from Ray Larsen), creating W3110, ΔexbD::cam, ΔtolQRA.

A set of ExbD 10 amino acid deletion mutants was constructed where the exbB, exbD operon was encoded on the plasmid. Plasmids pKP724 and pKP761 through pKP764 were constructed by in-frame deletion of ten exbD codons using extra-long PCR, as previously described (13). All were derivatives of pKP660 (31). Sequences of exbB and exbD were confirmed by DNA sequencing. A second set of ExbD deletion mutants, pKP1246 through pKP1259, was constructed where the exbB gene was not present on the plasmid. pKP1246 through pKP1259 were derivatives of the first set of plasmids that included exbB. The second set was constructed by extra-long PCR to create an in-frame deletion of exbB. Due to a possible requirement for translational coupling between exbB and exbD, deletion of exbB left intact the initiating ATG plus last 25 codons of exbB. Sequences of the exbB segment and exbD gene were confirmed by DNA sequencing.

pKP920, which expresses only ExbB, was also a derivative of pKP660. Extra-long PCR was used to delete exbD from its ATG start codon through 6 bases following the exbD TAA stop codon. The sequence of exbB was confirmed by DNA sequencing.

pKP1194, exbD in pBAD24, was constructed by digestion of pKP999 (exbD in pPro24) and pBAD24 with Ncol. Fragments were separated by gel electrophoresis. The 4542 bp fragment of pBAD24 and 506 bp fragment of pKP999, containing exbD, were purified by gel extraction and ligated together after treatment of the vector fragment with Antarctic Phosphatase. Proper
orientation of the insert was verified by FspI digestion. The exbD sequence in pBAD24 was confirmed by DNA sequencing.

Induction levels for ExbD deletion mutants

For assays in T broth (spot titers), the following percentages of arabinose were added at subculture to induce expression of ExbD mutants near native levels of ExbD:

- pKP660 = no inducer, pKP761 = .0001%, pKP760 = .0003%, pKP759 = .05% glucose (to repress basal levels of overexpression), pKP758 = .001%, pKP762 = .0003%, pKP757 = .001%, pKP756 = .0025%, pKP755 = .001%, pKP754 = .004%, pKP753 = .006%, pKP752 = .008%, pKP763 = .006%, pKP764 = .004%, pKP724 = .002%.

For assays in 1xM9, 37μM Fe (proteinase K accessibility, $[^{55}\text{Fe}]$-ferrichrome uptake, formaldehyde crosslinking, and sucrose density gradient fractionation), the following percentages of arabinose were added at subculture to induce expression of ExbD mutants near native levels of ExbD: pKP660 = no inducer, pKP761 = .0008%, pKP760 = .0008%, pKP759 = .3% glucose (to repress basal levels of overexpression), pKP758 = .002% glucose (to repress basal levels of overexpression), pKP762 = .0006%, pKP757 = .0007%, pKP756 = .006%, pKP755 = .008%, pKP754 = .15%, pKP753 = .18%, pKP752 = .2%, pKP763 = .18%, pKP764 = .2%, pKP724 = .006%.

Activity assays

Spot titer assays were performed as described previously (19, 35). Initial rates of $[^{55}\text{Fe}]$-ferrichrome uptake were determined as described previously (22).
In vivo formaldehyde crosslinking

Saturated overnight cultures were subcultured 1:100 into M9 minimal media (above) supplemented with L-arabinose. At mid-exponential phase, cells were treated with formaldehyde as previously described (13). Crosslinked complexes were detected by immunoblotting with ExbD-specific polyclonal antibodies (12) or TonB-specific monoclonal antibodies (21). To normalize levels of ExbD monomer after crosslinking, the following ODmL were loaded on the SDS-polyacrylamide gel:

- W3110 = 0.2
- RA1017/pKP660 = 0.25
- RA1017/pKP761 = 0.2
- RA1017/pKP760 = 0.4
- RA1017/pKP759 = 0.15
- RA1017/pKP758 = 0.2
- RA1017/pKP762 = 0.2
- RA1017/pKP757 = 0.2
- RA1017/pKP756 = 0.5
- RA1017/pKP755 = 0.35
- RA1017/pKP754 = 0.4
- RA1017/pKP753 = 0.5
- RA1017/pKP752 = 0.45
- RA1017/pKP763 = 0.45
- RA1017/pKP764 = 0.4
- RA1017/pKP724 = 0.42

Results

The ExbD periplasmic domain is important for ExbD stability and activity

To define functionally important regions of ExbD, a set of mutant proteins with consecutive 10-residue deletions was constructed.

Plasmid encoded exbD deletion mutants were expressed under control of the arabinose promoter in strain KP1522, which carries a chromosomal exbD deletion. ExbB, the first gene in the operon is present in KP1522 under control of its native promoter. For activity assays, attempts were made to express all ExbD deletion proteins at levels equal to native, chromosomally-encoded ExbD. Proteins with deletions in the amino terminus or TMD, appeared to be stably expressed (Fig. S1 A and B). However, 8 of the ExbD proteins with
periplasmic deletions covering the region from 62-141 could not be expressed at normal levels. We assume that these mutant proteins were synthesized, but rapidly degraded by proteases.

The same set of ExbD deletion mutants was then tested with plasmids encoding both exbB and exbD under control of the arabinose promoter. With a concomitant high level of ExbB expression, a subset of the ExbD proteins with periplasmic domain deletions (from residues 82-131) could be expressed at chromosomally encoded levels (Methods and Materials, Fig. S1 B). Accordingly, in subsequent experiments absolute levels of ExbB varied considerably because different induction conditions were used to achieve physiological levels of the various ExbD deletion proteins.

Spot titer s, which are capable of detecting very low levels of TonB activity, measure sensitivity to colicins and bacteriophage that enter and subsequently kill E. coli via the TonB system (19). 13 of the 14 exbD deletion mutants showed complete tolerance (insensitivity) to TonB-dependent colicins and bacteriophage (Table 2). Only ExbDΔ2-11 was active, supporting essentially full sensitivity to colicins (Table 2) and transporting 55Fe-ferrichrome at a rate near that of wild-type, plasmid-encoded ExbD (Table 3). Consistent with their insensitivity to phage and colicins, the other 13 deletion mutants supported no iron transport (data not shown).

Identification of an ExbD periplasmic domain required to energize TonB

ExbD is essential for the stages leading to TonB energy-dependent conformational changes (32). TonB and ExbD form an initial pmf-independent complex that renders both proteins resistant to exogenous proteinase K in spheroplasts. Protonmotive force subsequently promotes rearrangement of the initial TonB-ExbD periplasmic interactions that renders both proteins sensitive to proteinase K. The proteinase K resistant forms of TonB and ExbD can be
observed in two ways: either by treating cells with protonophores to stall the TonB-ExbD interaction at the pmf-independent stage or when the ExbD partner carries the D25N replacement.

The ExbD deletion mutant proteins were surveyed in spheroplasts for their ability to support the TonB proteinase K resistant conformation indicative of pmf-independent interaction of the ExbD and TonB periplasmic domains in Stage II (Fig. 1). Like wild-type ExbD, ExbDΔ2-11 fully supported formation of the TonB proteinase K resistant conformation after collapsing pmf by addition of the protonophore CCCP (Fig. 2A). ExbDΔ2-11, itself, exhibited sensitivity to proteinase K by 15 minutes of treatment, but stable resistance after pmf collapse, also like wild-type ExbD (Fig. 2B).

The majority of inactive ExbD deletion mutant proteins resulted in complete sensitivity of TonB to proteinase K, both in energized spheroplasts and CCCP-treated spheroplasts (Fig. 3). Sensitivity to proteinase K when pmf is collapsed is a characteristic of TonB and ExbD that have not yet assembled into the pmf-independent complex (Stage I, Fig. 1). This behavior can be due either to the absence of ExbB or ExbD or to the presence of structural changes in either protein that prevent proper assembly (32). Similar behavior was seen in the parent strain RA1017, which is deleted for exbB/D (Fig. 3).

The results with ExbDΔ42-51 and ExbDΔ52-61 were distinct from the other deletion mutants and resembled those obtained with the ExbD D25N mutant protein. Both deletion proteins supported the proteinase K resistant conformation of TonB at 2 minutes, irrespective of pmf [Fig. 2A; (32)]. In both cases the mutant protein was more susceptible to proteolysis, but not fully degraded, by 15 minutes in the presence of pmf and maintained more stable resistance to proteinase K after collapse of pmf. ExbDΔ42-51 and Δ52-61 themselves were resistant to
proteinase K in spheroplasts under all conditions tested, similar to wild-type ExbD in the presence of CCCP (Fig. 2B).

Deletion ExbDΔ12-21, which removes part of the cytoplasmic domain, supported a trace amount of the TonB proteinase K resistant conformation at only the earliest time point of 2 minutes (Fig. 3). Though at a much lower level, this signal was pmf-independent, like those for ExbDΔ42-51 and Δ52-61. Perhaps this initial assembly of TonB with the periplasmic domain of ExbDΔ12-21 was unstable or inefficiently formed. Overall, the regions of 12-21, 42-61, and 62-141 were apparently important in different ways for ExbD interactions with TonB.

Inactive ExbD deletion mutants exhibit changed protein-protein interactions

Because deletions within the various domains of ExbD differentially affected initial assembly with TonB, we also examined their effects on other interactions of ExbD. In vivo, formaldehyde crosslinks ExbD subunits to one another as well as to ExbB and TonB subunits (31). To determine which of those interactions were supported by the ExbD deletion proteins, their individual crosslinking profiles were analyzed. Formaldehyde is a conformation-sensitive crosslinking agent that forms a methylene bridge between specific reactive residues. This stringent requirement for close association means that, while the absence of a crosslink is evidence that the nature of the interaction has somehow changed, it is not proof that the proteins no longer interact. The proteins may still interact, but through conformations where the crosslinkable residues are no longer within crosslinking range. Cells expressing the deletion mutants were treated with monomeric paraformaldehyde, resolved on SDS-polyacrylamide gels, and immunoblotted using polyclonal ExbD-specific antibody. As previously observed, the only
active deletion protein, ExbDΔ2-11, formed all three known complexes at levels similar to wild-type ExbD [(31) and Fig. 4A].

None of the 13 inactive ExbD deletion mutants formed a detectable crosslink to TonB, an interaction that, to date, appears to occur only through an active conformation of ExbD (31). In cases where a potential TonB-ExbD heterodimer was present based on a complex of similar molecular mass (Δ72-81, for example), it was ruled out because the crosslinking profile in the absence of TonB remained the same (Fig. 4B). ExbDΔ42-51 and Δ52-61 profiles, which clearly lacked the TonB-ExbD heterodimer, were also identical with and without TonB (data not shown). ExbDΔ12-21 exhibited decreased levels of crosslinking to ExbB and of homodimer formation. As expected, ExbDΔ22-31 and Δ32-41, each missing half of the proposed ExbD TMD, formed no detectable complexes. The likelihood that absence of complexes for those deletions was due to their sequestration in the cytoplasm was investigated below.

The five ExbD proteins with deletions involving residues 42-91 formed some formaldehyde crosslinks with ExbB and formed ExbD homodimers (Fig. 4A). Increased formation of the ExbB-ExbD complex was likely due in part to increased levels of ExbB in the strains where high levels of arabinose were required for near-native expression of the ExbD mutants. However, the relative level of complex formed did not correlate with the level of inducer in all cases. ExbDΔ42-51 and Δ52-61 mediated increased association with ExbB, especially compared with the active ExbDΔ2-11, even though at least 10-fold less inducer was used compared to that needed for the ExbD deletions in the region from 62-141 (see Materials and Methods for induction levels). ExbDΔ62-71 exhibited low levels of complex formation with ExbB that were comparable to its homodimer propensity. ExbDΔ72-81 and Δ82-91 formed
homodimers with high efficiency, but also required high levels of inducer that subsequently gave rise to high levels of ExbB.

Three ExbD deletion mutants encompassing the region from residues 92-121 failed to form any of the expected complexes, including the crosslink to ExbB, a protein with only minor soluble periplasmic domains (Fig. 4A). Nonetheless, ExbB overexpression stabilized these deletion mutants to the point where they could be detected by immunoblot, suggesting that some interactions with ExbB remained (Fig. S1 B). Homodimer formation was weak for ExbDΔ92-101, and no detectable homodimers were trapped for ExbDΔ102-111 or ExbDΔ112-121. It seemed unlikely that this region affected ExbD export because these three proteins formed a formaldehyde crosslinked complex with an unknown protein (Fig. 4A), known to complex with wild-type ExbD (31). This unidentified complex was also observed for most of the other deletion proteins, each of which had been properly exported based on their ability to form complexes with ExbB and was not detected with either export-deficient (see below) ExbD TMD deletion mutant.

ExbD mutants with deletions in the extreme carboxy terminus, Δ122-131 and Δ132-141, each formed homodimers, and heterodimers with ExbB (Fig. 4A). The Δ122-131 protein showed increased homodimer formation and a strong complex with the unknown protein. Intensities of the ExbDΔ132-141 homodimer and complex with ExbB were comparable to those formed with wild-type ExbD.

Deletions within the ExbD TMD prevent CM insertion

The ExbD TMD is predicted to span residues 23-43. Therefore, two ExbD deletion mutants, Δ22-31 and Δ32-41, each had partial potential TMDs. No formaldehyde crosslinked
complexes were observed for either mutant, suggesting that these mutant proteins were not properly inserted in the CM. To directly test their localization, strains expressing the TMD deletion mutants ExbDΔ22-31 and Δ32-41 were fractionated on sucrose density gradients. Both mutant ExbD proteins fractionated with soluble proteins (Fig. 5A) and were sensitive to exogenous proteinase K only after lysis of spheroplasts (Fig. 5B), indicative of cytoplasmic localization. A faint band of the stable degradation product of ExbDΔ22-31 was still present after 15 minutes of proteinase K treatment in lysed spheroplasts. This may be a more proteolytically stable form of this deletion mutant.

**Discussion**

Although ExbD is an essential protein in the TonB system, it has not been subjected to a comprehensive mutagenesis study. Here we describe results of a 10-residue deletion scanning analysis that define functionally significant regions of ExbD. While all 10 periplasmic domain 10-residue ExbD deletions were inactive, two different functional regions within the ExbD periplasmic domain were identified. Residues from 62-141 were important for the pmf-independent contacts between TonB and ExbD periplasmic domains, and the region from 42-61 was important for the subsequent conformational response of assembled TonB-ExbD heterodimers to pmf (Fig. 6).

A functional unit consisting of the ExbD membrane-proximal periplasmic domain and TMD

Although ExbDΔ42-51 and ExbDΔ52-61 were fully capable of initial assembly with TonB, they were blocked in the transition to an energized TonB-ExbD interaction. These mutants exhibited similar phenotypes to those previously observed for ExbD-D25N in all assays, where TonB conformation remains stalled at Stage II [Fig. 1; (31, 32)]. Thus it appeared that the
TMD and the region immediately following it were directly involved in response of ExbD, and consequently TonB, to pmf. ExbD residues 44-66 are disordered in the NMR solution structure of the periplasmic domain (7), and the same region from residues 45-66 was also predicted to be disordered by PONDR™ analysis (Fig. S2). TonB residues 102-151 are disordered in the solution structure, with a region of residues ~35-170 predicted to be disordered by PONDR™ (20, 34). One possibility is that the disordered region of ExbD is important for the carboxy terminus of ExbD to achieve the conformation that allows it to energize TonB. ExbD residues 45-66 might, for example, propagate changes from the TMD to the structured carboxy terminus of ExbD, which is involved in direct interaction with the TonB periplasmic domain. Alternatively, the disordered regions of TonB and ExbD may need to find each other and collapse into a defined structure for TonB to be correctly energized.

The conformation of ExbD residues 62-141 is important for proper assembly with TonB. Protonmotive force-independent assembly of the ExbD and TonB periplasmic domains (Stage II, Fig. 1) is an essential stage prior to the action of the pmf (32). The entire ExbD carboxy terminus from residue 62-141 appeared to be important for that initial assembly with TonB. All deletions within this region stalled TonB at Stage I, indicating either complete absence of TonB-ExbD periplasmic domain interactions or lack of functionally important localized interactions. The 8 ExbD proteins with deletions involving residues 62-141 appeared to be proteolytically unstable, suggesting that deletions within this region had greater structural ramifications than those involving residues 42-61. Consistent with that idea, the carboxy terminal deletions encompassed the region of defined tertiary structure (residues 64-133) in the ExbD periplasmic domain NMR structure (Fig. 6). However, the conformation of at least 5
deletions within this region, from residues 62-91 and 122-141, was not so distorted as to prevent formation of an ExbD homodimer, a known biologically relevant interaction, previously detected in complex with ExbB \textit{in vivo}. The ExbD NMR structure requires significant conformational rearrangements to account for the \textit{in vivo} dimer contacts (33). None of these homodimer-competent deletion proteins, however, interacted with TonB. Thus it appears that ExbD homodimer formation has less stringent structural requirements than initial ExbD-TonB assembly does.

Inactivity due to deletion of the last 10 amino acids of ExbD, Δ132-141, may be an effect of removal of L132, previously shown to be important for ExbD activity and assembly of ExbD and TonB periplasmic domains (2, 32). A construct of ExbD fused at residue 134 to β-lactamase is active (18), which suggests that at least the last 7 residues of ExbD are dispensable, presumably with the important function of this region coming from L132.

Determinants of ExbD-ExbB formaldehyde crosslinks

Three deletions within the ExbD carboxy terminus, from residues 92-121 prevented formation of all three known ExbD formaldehyde-crosslinked complexes: ExbD homodimers, ExbD-TonB heterodimers, and ExbD-ExbB heterodimers. An inability to form structure-stabilizing interactions could contribute to the observation that these three deletion proteins were also the most proteolytically unstable. We showed previously with single cysteine replacements that residues within the 92-121 region are involved in direct homodimer and TonB-ExbD heterodimer formation (33). The requirement of residues 92-121 for formaldehyde-crosslinked interaction with ExbB was unexpected, because the majority of ExbB residues are localized to the cytoplasm. There is currently no evidence for direct interaction of the ExbD periplasmic
domain with ExbB. However, periplasmic domain interaction at the CM has been proposed between homologous proteins in the Mot and Tol protein systems (8, 15, 25). The potential for periplasmic domain interaction between ExbD and ExbB and a role for such interaction has yet to be explored. ExbDΔ42-51 and Δ52-61 exhibited increased formaldehyde crosslinking with ExbB, suggesting that this region might be important for subsequent conformational changes that release ExbD from ExbB contact.

Commonalities and differences with MotB
MotB, a parologue of ExbD, tolerates the deletion of 5 successive 10-residue segments immediately following its TMD (28). In contrast, 10-residue deletions at all sites in ExbD except the extreme amino terminus resulted in complete inactivity. This raises the question of how mechanistically similar ExbD and MotB are. The periplasmic domains of ExbD and MotB are entirely dissimilar in sequence, including the fact that ExbD lacks the peptidoglycan binding domain of MotB (37). The MotB periplasmic domain is also more than twice the size of the corresponding portion of ExbD. However, the general architecture of the proteins is similar in that each contains a highly conserved TMD with an essential aspartate, a periplasmic carboxy terminus that appears to define functional interactions of each protein, and a flexible region connecting these two domains. It may be that the periplasmic domains of MotB and ExbD have functionally diverged but common elements of the mechanism of harnessing energy derived from the pmf remain.

Pivotal roles for flexible regions of the periplasmic domain, adjacent to the TMD, in propagating conformational changes between the TMD and carboxy terminus may be a conserved mechanism between MotB and ExbD. In MotB, bi-directional signaling is proposed,
based primarily on crystal structures of the \textit{H. pylori} His\textsubscript{6}-MotB periplasmic domain containing various truncations of a linker region (residues 64-112) (29). It is currently unknown whether the ExbD region from 42-61 is important for TMD conformation, or vice versa, though it is clear the region is important for conformational response of ExbD to the pmf.

Identification of cytoplasmic residues important for ExbD function

Reasons for the complete inactivity of deletion proteins lacking the second half of the ExbD cytoplasmic domain, \(\Delta\)12-21, are unclear. This region may be important for proper or stable assembly of ExbD, because ExbD\(\Delta\)12-21 exhibited reduced protein-protein interactions. ExbD\(\Delta\)12-21 also failed to support the conformational response ofTonB to pmf. Recently, residues in the cytoplasmic carboxy terminus of ExbB, where contact with the cytoplasmic amino terminus of ExbD is possible, were also shown to be important for response of the ExbD and TonB periplasmic domains to pmf (16). Residues 12-17 do not appear to be essential for ExbD function because ExbD\(\Delta\)4-15, ExbD-H16A, and ExbD-D17A retain activity \([2], \text{Ollis and Postle, unpublished}\]. Therefore, residues 18-21 may make an important but currently unknown contribution to ExbD function.

In summary, this comprehensive deletion analysis identified two different functional regions within the ExbD periplasmic domain, with a clear division between the 20 membrane-proximal residues and the carboxy terminal 80 residues. Another region of the carboxy terminus, residues 92-121, previously determined to be important for interaction with TonB or another ExbD, was also found to be important in supporting ExbD-ExbB interaction. The importance of the overall conformation of the ExbD periplasmic domain, or potential to achieve multiple conformations, is in accordance with a role in regulating TonB conformation. It will be
important to further determine how these specific regions of the ExbD periplasmic domain function in the energization of TonB and the direction of apparent signal propagation between functional domains of ExbD.
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Figure legends

Fig. 1. Model for initial stages in TonB energization. Three sequential stages in TonB energization in the cytoplasmic membrane (CM) are shown from left to right. ExbB, assumed to be present for all stages, is not shown. Black constructs with filled transmembrane domains represent TonB; gray constructs with empty transmembrane domains represent ExbD. Jagged regions represent disordered domains. This model is not drawn to scale and represents a conceptual framework only. Mutants that stall TonB at each stage are listed below the stage.

Stage I reflects a lack of interaction between the TonB and ExbD periplasmic domains. ExbD L132Q and TonB H20A remain stalled at this Stage, which is characterized by proteinase K sensitivity and inability of TonB and ExbD to crosslink with formaldehyde.

In Stage II, the periplasmic domains of TonB and ExbD interact in a configuration that does not require the pmf. Collapse of the pmf by CCCP or the ExbD D25N mutation leave both TonB and ExbD stalled at this Stage, which is characterized by proteinase K resistance of the amino terminal 2/3 of TonB and of ExbD.

In Stage III, the conformational relationship between the TonB and ExbD periplasmic domains has changed such that formaldehyde crosslinkable residues in the periplasmic domains of both proteins move into close proximity (star). This new conformational relationship is also marked by complete TonB sensitivity to proteinase K. The transition between Stages II and III is reversible, with presence or absence of pmf acting as the toggle switch [adapted from (32)].


Fig. 2. ExbDΔ42-51 and Δ52-61 fully support the proteinase K resistant conformation of TonB that arises from pmf-independent interaction with ExbD but not pmf-dependent conformational changes. Spheroplasts were generated with a wild-type strain (W3110) or a ΔexbBD, ΔtolQRA
(RA1017) strain expressing plasmid-encoded ExbB with ExbD (RA1017/pKP660), ExbDΔ2-11 (RA1017/pKP761), ExbDΔ42-51 (RA1017/pKP762), or ExbDΔ52-61 (RA1017/pKP757).

Spheroplasts treated with DMSO (sph) or CCCP (sph + CCCP) were treated without (-) or with proteinase K for 2, 5, 10, or 15 min as described in Materials and Methods. The wild-type strain was included to confirm expression levels of the ExbD mutants compared to chromosomally-encoded ExbD, and only the 2 and 15 min time points were included for those samples. TCA precipitated samples were resolved on 11% or 15% SDS-polyacrylamide gels and immunoblotted with (A) TonB-specific monoclonal or (B) ExbD-specific polyclonal antibodies. Data shown are representative immunoblots from at least duplicate assays. Samples shown, immunoblotted with two antibodies, came from the same assay. “<” indicates an ~23kDa proteinase K resistant fragment of TonB. PK = proteinase K.

Fig. 3. The majority of inactive ExbD deletion mutants do not support formation of the TonB proteinase K resistant conformation. Spheroplasts were generated with a wild-type strain (W3110), a ΔexbBD, ΔtolQRA (RA1017) strain expressing plasmid-encoded ExbB with ExbDΔ12-21 (RA1017/pKP760), ExbDΔ22-31 (RA1017/pKP759), ExbDΔ32-41 (RA1017/pKP758), pKP756 (Δ62-71), pKP755 (Δ72-81), pKP754 (Δ82-91), pKP753 (Δ92-101), pKP752 (Δ102-111), pKP763 (Δ112-121), pKP764 (Δ122-131), or pKP724 (Δ132-141), and a ΔexbBD, ΔtolQRA (RA1017) strain. Spheroplasts treated with DMSO (sph) or CCCP (sph + CCCP) were treated without (-) or with proteinase K for 2 or 15 min as described in Materials and Methods. TCA precipitated samples were resolved on 11% SDS-polyacrylamide gels and immunoblotted with TonB-specific monoclonal. “>” indicates an ~23kDa proteinase K resistant fragment.
fragment of TonB. “*” indicates an ~28kDa TonB fragment. PK = proteinase K.

Fig. 4. 10-residue deletions alter known ExbD interactions and define a subdomain within the periplasmic carboxy terminus. A, Strains expressing chromosomally- or plasmid-encoded ExbD (W3110 and RA1017/pKP660, respectively) and ExbD deletion mutants [from left to right: RA1017/pKP761 (Δ2-11), pKP760 (Δ12-21), pKP759 (Δ22-31), pKP758 (Δ32-41), pKP762 (Δ42-51), pKP757 (Δ52-61), pKP756 (Δ62-71), pKP755 (Δ72-81), pKP754 (Δ82-91), pKP753 (Δ92-101), pKP752 (Δ102-111), pKP763 (Δ112-121), pKP764 (Δ122-131), or pKP724 (Δ132-141)] were crosslinked with formaldehyde as described in Materials and Methods. Samples were resolved on a 13% SDS-polyacrylamide gel and immunoblotted. ExbD was visualized with ExbD-specific polyclonal antibodies. Topological location of each deletion is indicated above each lane. Positions of molecular mass standards are indicated on the left. Identities of ExbD-specific crosslinked complexes and the ExbD monomer are indicated on the right. B, Strains expressing chromosomally- or plasmid-encoded ExbB, ExbD (W3110 and RA1017/pKP660, respectively) and ExbD Δ72-81 (RA1017/pKP755) and a ΔtonB strain expressing plasmid-encoded ExbB, ExbD (KP1503/pKP660) or ExbB, ExbD Δ72-81 (KP1503/pKP755) were crosslinked with formaldehyde and processed as above.

Fig. 5. Deletions removing portions of the ExbD TMD prevent CM insertion. A, Strains expressing chromosomally-encoded (RA1016) or plasmid-encoded (RA1017/pKP660) ExbD and ExbD deletion mutants (RA1017/pKP759 and RA1017/pKP758) were fractionated by sucrose density gradient fractionation, as described in Materials and Methods. TCA precipitated samples were resolved on 13% SDS-polyacrylamide gels and immunoblotted with ExbD-specific
polyclonal antibodies. Positions of soluble, cytoplasmic membrane (CM), and outer membrane (OM) fractions are indicated above. The faster migrating bands are degradation products. B, Spheroplasts were generated with a wild-type strain (W3110) or a ΔexbBD, ΔtolQRA (RA1017) strain expressing plasmid-encoded ExbB and ExbD (RA1017/pKP660), ExbDΔ22-31 (RA1017/pKP759), or ExbDΔ32-41 (RA1017/pKP758). Whole cell (WC), intact spheroplasts (sph), lysed spheroplasts (lysed sph), and collapsed pmf spheroplasts (sph +CCCP) were treated with or without proteinase K as described in Materials and Methods. TCA precipitated samples were resolved on 15% SDS-polyacrylamide gels and immunoblotted with ExbD-specific polyclonal antibodies. Data shown are representative immunoblots from at least duplicate assays. “+” or “−” above each lane indicates presence or absence, respectively, of added proteinase K. PK = proteinase K.

Fig. 6. Specific regions of ExbD mediate response to pmf and define functional interactions. Functional regions of ExbD identified in this study are highlighted on a topology diagram of full-length ExbD (left) and the solution structure of the ExbD periplasmic domain (2pfu, right). A functional unit of ExbD transmembrane residue D25 and the unstructured periplasmic region from residues 42-61 mediate conformational response of ExbD to pmf. The structured extreme carboxy terminus is important for interactions between the periplasmic domains of ExbD and TonB. Within this region, residues 92-121 form demonstrated homo- and heterodimeric interfaces and putative interactions with ExbB. The topology depiction is not drawn to scale and is not intended to reflect predicted tertiary structure. The image of the NMR structure was generated using Swiss-PdbViewer (10).
Table 1. Strains and plasmids used in this study.

<table>
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<tr>
<th>Strain or Plasmid</th>
<th>Genotype or Phenotype</th>
<th>Reference</th>
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Table 2: Spot titer assay results

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<th>Phenotype</th>
<th>Susceptibility&lt;sup&gt;a&lt;/sup&gt;</th>
<th>(\phi 80)</th>
<th>Colicin B</th>
<th>Colicin M</th>
<th>Colicin Ia</th>
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</tbody>
</table>

<sup>a</sup> Scored as the highest ten-fold dilution of bacteriophage \(\phi 80\) or five-fold dilution of a standard colicin preparation that provided an evident zone of clearing on a cell lawn.

<sup>b</sup> "T" indicates tolerance to undiluted colicin or phage (no clearing of the lawn).

The values of three platings are presented for each strain/plasmid and colicin or phage pairing.

Expression of ExbD mutants to near levels of chromosomally-encoded ExbD was verified by immunoblots with ExbD-specific antibodies (not shown).
**Table 3:** Transport of $^{55}$Fe-loaded ferrichrome

<table>
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<tr>
<th>Strain</th>
<th>Phenotype</th>
<th>Initial Rate of Transport</th>
<th>% Wild-type Activity</th>
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<td>KP1522</td>
<td>ΔExbBD, ΔTolQRA</td>
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<td>100</td>
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<td>KP1522/pKP1246</td>
<td>ExbDΔ2-11</td>
<td>654.7 ± 11.55</td>
<td>97</td>
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</table>

*Strains/plasmids indicated were assayed for ability to transport $^{55}$Fe-loaded ferrichrome as described in Materials and Methods. Plasmid-encoded ExbD mutants were induced with the following percentages of arabinose: pKP1194 = .005%, pKP1246 = .08%.

bPercent wild-type activity was recorded as the initial rate of transport of the mutant strain divided by the initial rate of transport of the wild-type strain (multiplied by 100). Rate of transport by the strain expressing ExbDΔ2-11 was also compared to the rate supported by plasmid-encoded wild-type ExbD (in parentheses). Expression levels for plasmid-encoded ExbD and ExbDΔ2-11 equal to chromosomally-encoded ExbD (W3110) levels were confirmed by Western blot with ExbD-specific polyclonal antibodies (data not shown).
I. Wrong or no ExbD-TonB interaction
No formaldehyde crosslink
Sensitive to proteinase K

Mutants:
ΔExbD
ExbD L132Q
TonB H20A

II. Pmf-independent ExbD-TonB interaction
No formaldehyde crosslink
Resistant to proteinase K

Mutants:
ExbD D25N

III. Pmf-dependent ExbD-TonB interaction
Formaldehyde crosslink★
Sensitive to proteinase K

Wild type ExbD

TonB
ExbD
CCCP
CM
Functional regions of ExbD:
- Residues D25 and 42-61—response to pmf
- Residues 62-141—assembly with TonB
- Residues 92-121—dimeric interfaces