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

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The TonB system of Gram-negative bacteria energizes the active transport of diverse nutrients through high-affinity TonB-gated outer membrane transporters using energy derived from the cytoplasmic membrane proton motive 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 proton motive force-independent to proton motive force-dependent interactions with TonB, catalyzing the conformational changes of TonB. A 10-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 to 61 facilitated the conformational response of ExbD to proton motive force. This region appears to be important for transmitting signals between the ExbD transmembrane domain and carboxy terminus. The carboxy terminus, encompassing periplasmic residues 62 to 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 proton motive force to TonB. Residues 92 to 121 were important for all three interactions previously observed for formaldehyde-cross-linked 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.

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 proton motive force (PMF) to TonB-gated transporters, energizing the active transport of nutrients into the periplasm. TonB-dependent substrates include iron-siderophore complexes, vitamin B_{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, 29, 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 His20 can be replaced with alanine with no loss of function. His20 serves a structural role and is not on a proton translocation pathway, since this position tolerates a nonprotonatable 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 a topology that is identical to that of TonB (239 amino acids), with a carboxy-terminal periplasmic domain (more than 60% of the protein), a single TMD, and a short cytoplasmic amino terminus. ExbD directs the conformational changes of the TonB periplasmic domain, which currently can 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 the rearrangement of the ExbD and TonB periplasmic domains such that they can be cross-linked 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 (30, 31).

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 the 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 to 121). The two di-

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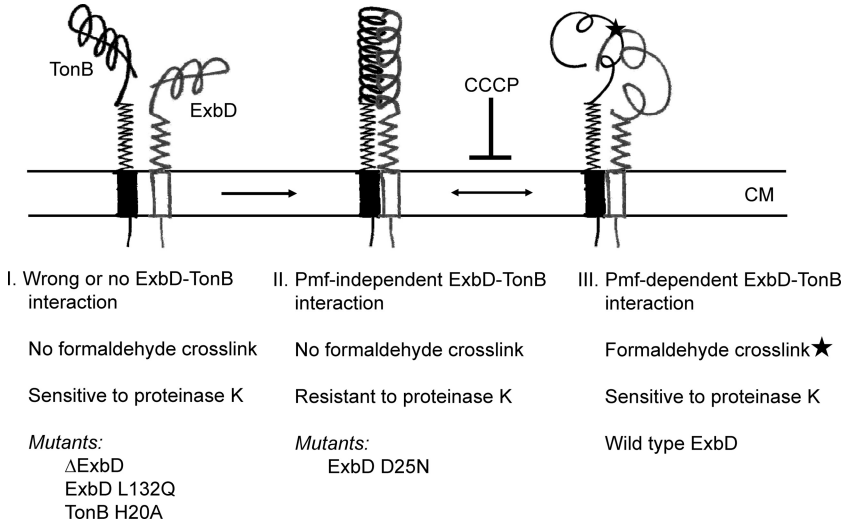


FIG 1 Model for initial stages in TonB energization. Three sequential stages in TonB energization in the cytoplasmic membrane (CM) are shown from left to right. ExbB, assumed to be present for all stages, is not shown. Black constructs with filled transmembrane domains represent TonB; gray constructs 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 the inability of TonB and ExbD to cross-link with formaldehyde. In stage II, the periplasmic domains of TonB and ExbD interact in a configuration that does not require the PMF. The 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 two-thirds of TonB and of ExbD. In stage III, the conformational relationship between the TonB and ExbD periplasmic domains has changed such that formaldehyde cross-linkable 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 the presence or absence of PMF acting as the toggle switch. (Reprinted from reference 32 with permission of the publisher.)

meric states require nearly the same set of contact residues in the ExbD periplasmic domain (32).

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 to 61 were required for TonB to progress to its energized stage III conformation but not for the 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 to 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. ExbD-TonB interactions within this subdomain are characterized in our accompanying article (32a).

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids used in this study are listed in Table 1. KP1522 was constructed by P1*vir* transduction of Δ*exbD*::*cam* from RA1021 into RA1016 (kind gifts from Ray Larsen), creating W3110, Δ*exbD*::*cam* Δ*tolQRA*.

A set of ExbD 10-amino-acid deletion mutants was constructed where the *exbB exbD* operon was located on the plasmid. Plasmids pKP724 and pKP761 through pKP764 were constructed by the in-frame deletion of 10 *exbD* codons using extra-long PCR as previously described (13). All were derivatives of pKP660 (30). 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*, the deletion of *exbB* left intact the initiating ATG plus the 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 the digestion of pKP999 (*exbD* in pPro24) and pBAD24 with NcoI. Fragments were separated by gel electrophoresis. The 4,542-bp fragment of pBAD24 and 506-bp fragment of pKP999, containing *exbD*, were purified by gel extraction and ligated together after the treatment of the vector fragment with Antarctic phosphatase. The 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 the expression of ExbD mutants at nearly native levels of ExbD: pKP660, no inducer; pKP761, 0.0001%; pKP760, 0.0003%; pKP759, 0.05% glucose (to repress basal levels of overexpression); pKP758, 0.0001%; pKP762, 0.0003%; pKP757, 0.001%; pKP756, 0.0025%; pKP755, 0.001%; pKP754, 0.004%; pKP753, 0.006%; pKP752, 0.008%; pKP763, 0.006%; pKP764, 0.004%; and pKP724, 0.002%.

For assays in 1× M9 and 37 μM Fe (with proteinase K accessibility, [^{55}Fe]ferrichrome uptake, formaldehyde cross-linking, and sucrose density gradient fractionation), the following percentages of arabinose were added at subculture to induce the expression of ExbD mutants at nearly native levels of ExbD: pKP660, no inducer; pKP761, 0.0008%; pKP760, 0.0008%; pKP759, 0.3% glucose (to repress basal levels of overexpression); pKP758, 0.002% glucose (to repress basal levels of overexpression); pKP762, 0.0006%; pKP757, 0.0007%; pKP756, 0.006%; pKP755, 0.008%; pKP754, 0.15%; pKP753, 0.18%; pKP752, 0.2%; pKP763, 0.18%; pKP764, 0.2%; and pKP724, 0.006%.

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
W3110	F⁻ IN(*rrnD-rrnE*)*1*	14
RA1016	W3110, Δ*tolQRA*	Ray Larsen
RA1017	W3110, Δ*exbBD*::*kan*, Δ*tolQRA*	20
RA1021	W3110, Δ*exbD*::*cam*	Ray Larsen
KP1503	GM1, *exbB*::Tn*10*, $tolQ_{am}$, Δ*tonB*::*kan*	31
KP1522	W3110, Δ*exbD*::*cam*, Δ*tolQRA*	Present study
Plasmids		
pBAD24	L-Arabinose inducible, pBR322 *ori*	11
pPro24	Sodium propionate (2-methyl citrate) inducible, pBR322 *ori*	26
pKP920	*exbB* in pBAD24	Present study
pKP999	*exbD* in pPro24	31
pKP660	*exbB*, *exbD* in pBAD24	31
pKP761	ExbB, ExbDΔ2-11	31
pKP760	ExbB, ExbDΔ12-21	Present study
pKP759	ExbB, ExbDΔ22-31	Present study
pKP758	ExbB, ExbDΔ32-41	Present study
pKP762	ExbB, ExbDΔ42-51	Present study
pKP757	ExbB, ExbDΔ52-61	Present study
pKP756	ExbB, ExbDΔ62-71	Present study
pKP755	ExbB, ExbDΔ72-81	Present study
pKP754	ExbB, ExbDΔ82-91	Present study
pKP753	ExbB, ExbDΔ92-101	Present study
pKP752	ExbB, ExbDΔ102-111	Present study
pKP763	ExbB, ExbDΔ112-121	Present study
pKP764	ExbB, ExbDΔ122-131	Present study
pKP724	ExbB, ExbDΔ132-141	Present study
pKP1194	*exbD* in pBAD24	Present study
pKP1246	ExbDΔ2-11	Present study
pKP1247	ExbDΔ12-21	Present study
pKP1248	ExbDΔ22-31	Present study
pKP1249	ExbDΔ32-41	Present study
pKP1250	ExbDΔ42-51	Present study
pKP1251	ExbDΔ52-61	Present study
pKP1252	ExbDΔ62-71	Present study
pKP1253	ExbDΔ72-81	Present study
pKP1254	ExbDΔ82-91	Present study
pKP1255	ExbDΔ92-101	Present study
pKP1256	ExbDΔ102-111	Present study
pKP1267	ExbDΔ112-121	Present study
pKP1258	ExbDΔ122-131	Present study
pKP1259	ExbDΔ132-141	Present study

Assays. Spot titer assays were performed as described previously (19, 35). Initial rates of [^{55}Fe]ferrichrome uptake were determined as described previously (22). Proteinase K assays and sucrose density gradient fractionation were performed as previously described (30, 32).

***In vivo* formaldehyde cross-linking.** Saturated overnight cultures were subcultured 1:100 into M9 minimal medium (as described above) supplemented with L-arabinose. At mid-exponential phase, cells were treated with formaldehyde as previously described (13). Cross-linked complexes were detected by immunoblotting with ExbD-specific polyclonal antibodies (12) or TonB-specific monoclonal antibodies (21). To normalize levels of ExbD monomer after cross-linking, the following ODmL (total amount of cells; obtained by multiplying the A_{550} of the culture by the volume of culture used) 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-carried *exbD* deletion mutants were expressed under the 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 the control of its native promoter. For activity assays, attempts were made to express all ExbD deletion proteins at levels equal to that of native, chromosomally encoded ExbD. Proteins with deletions in the amino terminus or TMD appeared to be stably expressed (see Fig. S1A and B in the supplemental material). However, 8 of the ExbD proteins with periplasmic deletions covering the region from 62 to 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 then was tested with plasmids encoding both *exbB* and *exbD* under the control of the arabinose promoter. With a concomitantly high level of ExbB expression, a subset of the ExbD proteins with periplasmic domain deletions (from residues 82 to 131) could be expressed at chromosomally encoded levels (see Materials and Methods; also see Fig. S1B in the supplemental material). 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 titers, 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). Thirteen 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 ^{55}Fe-ferrichrome at a rate near that of wild-type, plasmid-encoded ExbD (Table 3). Consistently with their insensitivity to phage and colicins, the other 13 deletion mutants did not support 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 (31). TonB and ExbD form an initial PMF-independent complex that renders both proteins resistant to exogenous proteinase K in spheroplasts. Proton motive force subsequently promotes the 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, which is indicative of the PMF-independent interaction of the ExbD and TonB periplasmic domains in stage II (Fig. 1). Like wild-type ExbD, ExbDΔ2-11 fully supported the formation of the TonB proteinase K-resistant conformation after col-

TABLE 2 Spot titer assay results

Strain	Phenotype	Susceptibility to[a]: ϕ80	Colicin B	Colicin M	Colicin Ia
W3110	Wild type	9,9,9	8,8,8	6,6,6	7,8,8
RA1017	ΔExbBD, ΔTolQRA	T,T,T[b]	T,T,T	T,T,T	T,T,T
RA1017/pKP660	ExbB, ExbD	7,7,7	5,5,5	4,4,4	6,6,6
RA1017/pKP761	ExbB, ExbDΔ2-11	7,7,7	4,4,4	4,4,4	6,6,6
RA1017/pKP760	ExbB, ExbDΔ12-21	T,T,T	T,T,T	T,T,T	T,T,T
RA1017/pKP759	ExbB, ExbDΔ22-31	T,T,T	T,T,T	T,T,T	T,T,T
RA1017/pKP758	ExbB, ExbDΔ32-41	T,T,T	T,T,T	T,T,T	T,T,T
RA1017/pKP762	ExbB, ExbDΔ42-51	T,T,T	T,T,T	T,T,T	T,T,T
RA1017/pKP757	ExbB, ExbDΔ52-61	T,T,T	T,T,T	T,T,T	T,T,T
RA1017/pKP756	ExbB, ExbDΔ62-71	T,T,T	T,T,T	T,T,T	T,T,T
RA1017/pKP755	ExbB, ExbDΔ72-81	T,T,T	T,T,T	T,T,T	T,T,T
RA1017/pKP754	ExbB, ExbDΔ82-91	T,T,T	T,T,T	T,T,T	T,T,T
RA1017/pKP753	ExbB, ExbDΔ92-101	T,T,T	T,T,T	T,T,T	T,T,T
RA1017/pKP752	ExbB, ExbDΔ102-111	T,T,T	T,T,T	T,T,T	T,T,T
RA1017/pKP763	ExbB, ExbDΔ112-121	T,T,T	T,T,T	T,T,T	T,T,T
RA1017/pKP764	ExbB, ExbDΔ122-131	T,T,T	T,T,T	T,T,T	T,T,T
RA1017/pKP724	ExbB, ExbDΔ132-141	T,T,T	T,T,T	T,T,T	T,T,T

[a] Scored as the highest 10-fold dilution of bacteriophage ϕ80 or 5-fold dilution of a standard colicin preparation that provided an evident zone of clearing on a cell lawn. The values of three platings are presented for each strain/plasmid and colicin or phage pairing. The expression of ExbD mutants to nearly the levels of chromosomally encoded ExbD was verified by immunoblottings with ExbD-specific antibodies (not shown).

[b] T indicates tolerance to undiluted colicin or phage (i.e., no clearing of the lawn).

lapsing PMF by the addition of the protonophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (Fig. 2A). ExbDΔ2-11 itself exhibited sensitivity to proteinase K by 15 min of treatment but stable resistance after PMF collapse, also similar to wild-type ExbD (Fig. 2B).

The majority of inactive ExbD deletion mutant proteins resulted in the 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 (31). Similar behavior was seen in the parent strain RA1017, which is deleted for *exbB* and *exbD* (Fig. 3).

The results with ExbDΔ42-51 and ExbDΔ52-61 were distinct

TABLE 3 Transport of ^{55}Fe-loaded ferrichrome

Strain	Phenotype	Initial rate of transport[a]	% Wild-type activity[b]
KP1522	ΔExbBD, ΔTolQRA	−4.167 ± 2.309	0
KP1522/pKP1194	ExbD	672.2 ± 15.97	100
KP1522/pKP1246	ExbDΔ2-11	654.7 ± 11.55	97

[a] Strains/plasmids indicated were assayed for the 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, 0.005%; pKP1246, 0.08%.

[b] Percent 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). That expression levels for plasmid-encoded ExbD and ExbDΔ2-11 were equal to chromosomally encoded ExbD (W3110) levels was confirmed by Western blotting with ExbD-specific polyclonal antibodies (data not shown).

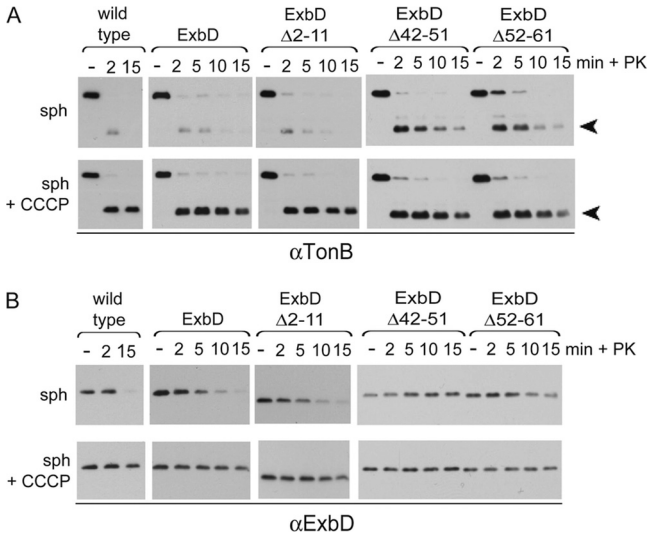


FIG 2 ExbDΔ42-51 and ExbDΔ52-61 fully support the proteinase K-resistant conformation of TonB that arises from PMF-independent interaction with ExbD but not PMF-dependent conformational changes. Spheroplasts were generated with a wild-type strain (W3110) or a Δ*exbBD* Δ*tolQRA* (RA1017) strain expressing plasmid-encoded ExbB with ExbD (RA1017/pKP660), ExbDΔ2-11 (RA1017/pKP761), ExbDΔ42-51 (RA1017/pKP762), or ExbDΔ52-61 (RA1017/pKP757). Spheroplasts treated with dimethyl sulfoxide (DMSO) (sph) or CCCP (sph + CCCP) were left untreated (−) or were treated with proteinase K for 2, 5, 10, or 15 min as described in reference 32. 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. Trichloroacetic acid (TCA)-precipitated samples were resolved on 11 or 15% SDS-polyacrylamide gels and immunoblotted with TonB-specific monoclonal (A) or ExbD-specific polyclonal (B) antibodies. Data shown are representative immunoblots from at least duplicate assays. The samples shown, which were immunoblotted with two antibodies, came from the same assay. The arrows indicate an ~23-kDa proteinase K-resistant fragment of TonB. PK, proteinase K.

from those for 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 min irrespective of PMF (Fig. 2A) (31). In both cases the mutant protein was more susceptible to proteolysis, but not fully degraded, by 15 min in the presence of PMF and maintained more stable resistance to proteinase K after the collapse of PMF. ExbDΔ42-51 and ExbDΔ52-61 themselves were resistant to proteinase K in spheroplasts under all conditions tested, similarly 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 min (Fig. 3). Although at a much lower level, this signal was PMF independent, like those for ExbDΔ42-51 and ExbDΔ52-61. Perhaps this initial assembly of TonB with the periplasmic domain of ExbDΔ12-21 was unstable or inefficiently formed. Overall, the regions of 12 to 21, 42 to 61, and 62 to 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 cross-links ExbD subunits to one another as

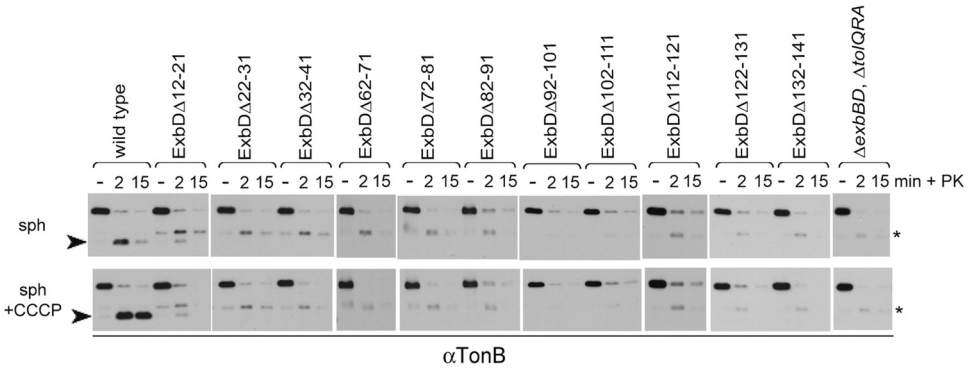


FIG 3 Majority of inactive ExbD deletion mutants do not support formation of the TonB proteinase K-resistant conformation. Spheroplasts were generated with a wild-type strain (W3110); a Δ*exbBD* Δ*tolQRA* (RA1017) strain expressing plasmid-encoded ExbB with ExbDΔ12-21 (RA1017/pKP760), ExbDΔ22-31 (RA1017/pKP759), 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 Δ*exbBD* Δ*tolQRA* (RA1017) strain. Spheroplasts treated with DMSO (sph) or CCCP (sph + CCCP) were left untreated or were treated with proteinase K for 2 or 15 min as described in reference 32. TCA-precipitated samples were resolved on 11% SDS-polyacrylamide gels and immunoblotted with TonB-specific monoclonal antibody. The arrow indicates an ~23-kDa proteinase K-resistant fragment of TonB. The asterisk indicates an ~28-kDa TonB fragment. PK, proteinase K.

well as to ExbB and TonB subunits (30). To determine which of those interactions were supported by the ExbD deletion proteins, their individual cross-linking profiles were analyzed. Formaldehyde is a conformation-sensitive cross-linking agent that forms a methylene bridge between specific reactive residues. This stringent requirement for close association means that while the absence of a cross-link 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 cross-linkable residues are no longer within cross-linking 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 that of wild-type ExbD (30) (Fig. 4A).

None of the 13 inactive ExbD deletion mutants formed a detectable cross-link to TonB, an interaction that, to date, appears to occur only through an active conformation of ExbD (30). Cases where a potential TonB-ExbD heterodimer was present based on a complex of similar molecular mass (ExbDΔ72-81, for example) were ruled out, because the cross-linking profile in the absence of TonB remained the same (Fig. 4B). ExbDΔ42-51 and ExbDΔ52-61 profiles, which clearly lacked the TonB-ExbD heterodimer, were also identical with and without TonB (data not shown). ExbDΔ12-21 exhibited decreased levels of cross-linking to ExbB and of homodimer formation. As expected, ExbDΔ22-31 and ExbDΔ32-41, each missing half of the proposed ExbD TMD, formed no detectable complexes. The likelihood that the 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 to 91 formed some formaldehyde cross-links with ExbB and formed ExbD homodimers (Fig. 4A). The 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 the 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 ExbDΔ52-61 mediated increased association with ExbB, especially compared to 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 to 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 ExbDΔ82-91 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 to 121 failed to form any of the expected complexes, including the cross-link 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 immunoblotting, suggesting that some interactions with ExbB remained (see Fig. S1B in the supplemental material). 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 cross-linked complex with an unknown protein (Fig. 4A) that is known to complex with wild-type ExbD (30). This unidentified complex was also observed for most of the other deletion proteins, each of which had been properly exported based on its ability to form complexes with ExbB and was not detected with either export-deficient ExbD TMD deletion mutant (see below).

ExbD mutants with deletions in the extreme carboxy terminus, ExbDΔ122-131 and ExbDΔ132-141, each formed homodimers and heterodimers with ExbB (Fig. 4A). The ExbDΔ122-131 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 to 43. Therefore, two ExbD deletion mutants, ExbDΔ22-31 and ExbDΔ32-41, each had partial potential TMDs. No formaldehyde cross-linked complexes were observed for either mutant, suggesting that these mutant proteins were not properly inserted in the CM. To directly test

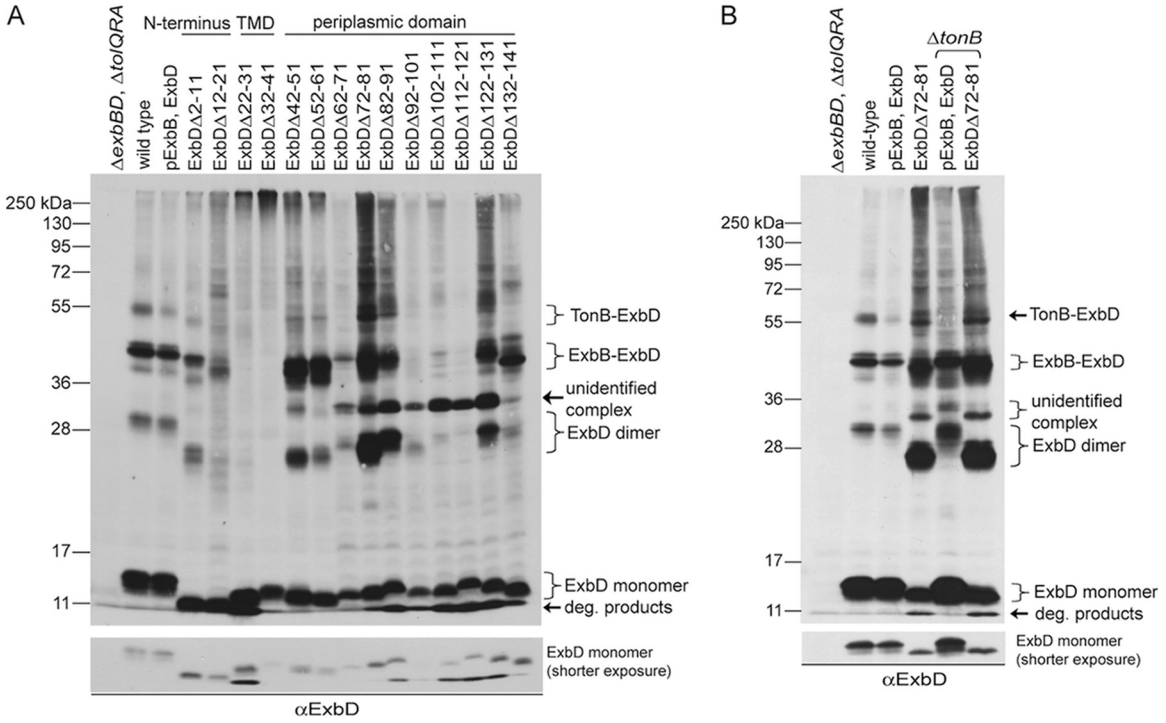


FIG 4 Ten-residue deletions alter known ExbD interactions and define a subdomain within the periplasmic carboxy terminus. (A) Strains expressing chromosomally or plasmid-encoded ExbD (W3110 and RA1017/pKP660, respectively) and ExbD deletion mutants [from left to right: RA1017/pKP761(Δ2-11), pKP760(Δ12-21), 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 cross-linked 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. The topological location of each deletion is indicated above each lane. Positions of molecular mass standards are indicated on the left. Identities of ExbD-specific cross-linked complexes and the ExbD monomer are indicated on the right. (B) Strains expressing chromosomally or plasmid-encoded ExbB and ExbD (W3110 and RA1017/pKP660, respectively), ExbB and ExbDΔ72-81 (RA1017/pKP755), and a Δ*tonB* strain expressing plasmid-encoded ExbB and ExbD (KP1503/pKP660) or ExbB and ExbDΔ72-81 (KP1503/pKP755) were cross-linked with formaldehyde and processed as described for panel A. deg., degradation.

their localization, strains expressing the TMD deletion mutants ExbDΔ22-31 and ExbDΔ32-41 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 the lysis of spheroplasts (Fig. 5B), which is indicative of cytoplasmic localization. A faint band of the stable degradation product of ExbDΔ22-31 was still present after 15 min 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 62 to 141 were important for the PMF-independent contacts between TonB and ExbD periplasmic domains, and the region from 42 to 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 phenotypes similar to those previously observed for ExbD-D25N in all assays, where TonB conformation remains stalled at stage II (Fig. 1) (30, 31). Thus, it appeared that the TMD and the region immediately following it were directly involved in the response of ExbD, and consequently TonB, to PMF. ExbD residues 44 to 66 are disordered in the nuclear magnetic resonance (NMR) solution structure of the periplasmic domain (7), and the same region from residues 45 to 66 was also predicted to be disordered by PONDR analysis (see Fig. S2 in the supplemental material). TonB residues 102 to 151 are disordered in the solution structure, with a region of residues ~35 to 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 to 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 to 141 is important for proper assembly with TonB. The proton motive 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

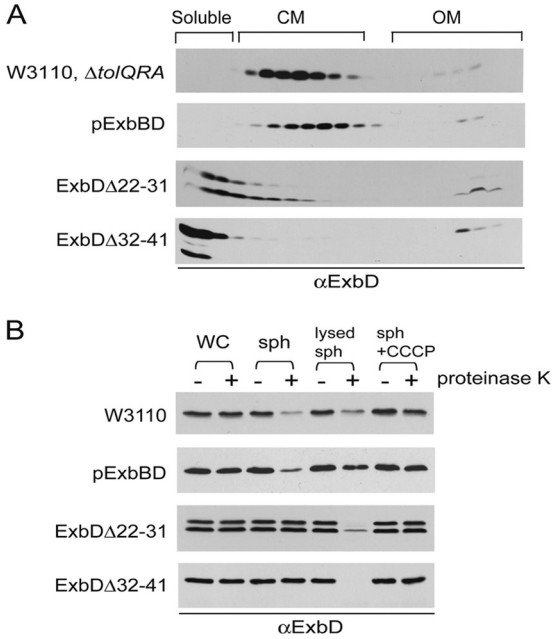
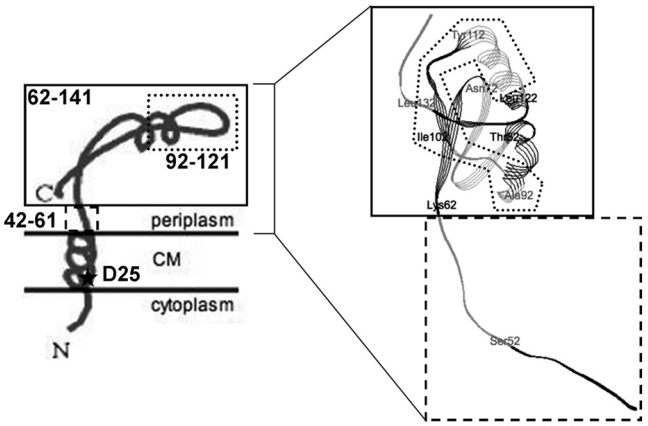


FIG 5 Deletions removing portions of the ExbD TMD prevent CM insertion. (A) Strains expressing chromosomally encoded (RA1016) or plasmid-encoded (RA1017/pKP660) ExbD and ExbD deletion mutants (RA1017/pKP759 and RA1017/pKP758) were fractionated by sucrose density gradient fractionation as described in reference 30. 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. The faster-migrating bands are degradation products. (B) Spheroplasts were generated with a wild-type strain (W3110) or a Δ*exbBD* Δ*tolQRA* (RA1017) strain expressing plasmid-encoded ExbB and ExbD (RA1017/pKP660), ExbDΔ22-31 (RA1017/pKP759), or ExbDΔ32-41 (RA1017/pKP758). Whole cells (WC), intact spheroplasts (sph), lysed spheroplasts (lysed sph), and collapsed PMF spheroplasts (sph + CCCP) were left untreated or were treated with proteinase K as described in reference 32. 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. A plus or minus above each lane indicates the presence or absence, respectively, of added proteinase K.



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

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 (Protein Data Bank code 2pfu; right). A functional unit of ExbD transmembrane residue D25 and the unstructured periplasmic region from residues 42 to 61 mediates the 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 to 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).

(31). The entire ExbD carboxy terminus from residues 62 to 141 appeared to be important for that initial assembly with TonB. All deletions within this region stalled TonB at stage I, indicating either the complete absence of TonB-ExbD periplasmic domain interactions or the lack of functionally important localized interactions. The 8 ExbD proteins with deletions involving residues 62 to 141 appeared to be proteolytically unstable, suggesting that deletions within this region had greater structural ramifications than those involving residues 42 to 61. Consistent with that idea, the carboxy-terminal deletions encompassed the region of defined tertiary structure (residues 64 to 133) in the ExbD periplasmic domain NMR structure (Fig. 6). However, the conformation of at least 5 deletions within this region, residues 62 to 91 and 122 to 141, was not so distorted as to prevent the formation of an ExbD homodimer, a known biologically relevant interaction, previously detected in complex with ExbB *in vivo*. The ExbD NMR structure requires significant conformational rearrangements to account for the *in vivo* dimer contacts (32). 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 the deletion of the last 10 amino acids of ExbD, Δ132-141, may be an effect of the removal of L132, which was previously shown to be important for ExbD activity and the assembly of ExbD and TonB periplasmic domains (2, 31). 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 cross-links. Three deletions within the ExbD carboxy terminus, spanning residues 92 to 121, prevented the formation of all three known ExbD formaldehyde cross-linked 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 to 121 region are involved in direct homodimer and TonB-ExbD heterodimer formation (32). The requirement of residues 92 to 121 for formaldehyde-cross-linked interaction with ExbB was unexpected, because the majority of ExbB residues are localized to the cytoplasm. There is currently no evidence for the 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 ExbDΔ52-61 exhibited increased formaldehyde cross-linking 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 paralogue 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, bidirectional signaling is proposed based primarily on crystal structures of the *Helicobacter pylori* His_6-MotB periplasmic domain containing various truncations of a linker region (residues 64 to 112) (33). It is currently unknown whether the ExbD region from 42 to 61 is important for TMD conformation or vice versa, although it is clear that the region is important for the 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, Δ12-21, are unclear. This region may be important for the proper or stable assembly of ExbD, because ExbDΔ12-21 exhibited reduced protein-protein interactions. ExbDΔ12-21 also failed to support the conformational response of TonB 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 the response of the ExbD and TonB periplasmic domains to PMF (16). Residues 12 to 17 do not appear to be essential for ExbD function because ExbDΔ4-15, ExbD-H16A, and ExbD-D17A retain activity (reference 2 and data not shown). Therefore, residues 18 to 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 to 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 the 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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