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Partial suppression of an *Escherichia coli* TonB transmembrane domain mutation (ΔV17) by a missense mutation in ExbB

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Summary

Active transport of vitamin B_{12} and Fe(III)-siderophore complexes across the outer membrane of *Escherichia coli* appears to be dependent upon the ability of the TonB protein to couple cytoplasmic membrane-generated protonmotive force to outer membrane receptors. TonB is supported in this role by an auxiliary protein, ExbB, which, in addition to stabilizing TonB against the activities of endogenous envelope proteases, directly contributes to the energy transduction process. The topological partitioning of TonB and ExbB to either side of the cytoplasmic membrane restricts the sites of interaction between these proteins primarily to their transmembrane domains. In this study, deletion of valine 17 within the amino-terminal transmembrane anchor of TonB resulted in complete loss of TonB activity, as well as loss of detectable *in vivo* crosslinking into a 59 kDa complex believed to contain ExbB. The ΔV17 mutation had no effect on TonB export. The loss of crosslinking appeared to reflect conformational changes in the TonB/ExbB pair rather than loss of interaction since ExbB was still required for some stabilization of TonBΔV17. Molecular modeling suggested that the ΔV17 mutation caused a significant change in the predicted conserved face of the TonB amino-terminal membrane anchor. TonBΔV17 was unable to achieve the 23 kDa proteinase K-resistant form in lysed sphaeroplasts that is characteristic of active TonB. Wild-type TonB also failed to achieve the proteinase K-resistant configuration when ExbB was absent. Taken together these results suggested that the ΔV17 mutation interrupted productive TonB–ExbB interactions. The apparent ability to crosslink to ExbB as well as a limited ability to transduce energy were restored by a second mutation (A39E) in or near the first predicted transmembrane domain of the ExbB protein. Consistent with the weak suppression, a 23 kDa proteinase K-resistant form of TonBΔV17 was not observed in the presence of ExbBA39E. Neither the ExbBA39E allele nor the absence of ExbB affected TonB or TonBΔV17 export. Unlike the *tonBΔV17* mutation, the *exbBA39E* mutation did not greatly alter a modelled ExbB transmembrane domain structure. Furthermore, the suppressor ExbBA39E functioned normally with wild-type TonB, suggesting that the suppressor was not allele specific. Contrary to expectations, the TonBΔV17, ExbBA39E pair resulted in a TonB with a greatly reduced half-life (≈10 min). These results together with protease susceptibility studies suggest that ExbB functions by modulating the conformation of TonB.

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Introduction

Gram-negative bacteria possess two membrane systems, separated by an aqueous compartment — the periplasmic space. The cytoplasmic (inner) membrane is a phospholipid bilayer, containing the proteins necessary to both generate and maintain a protonmotive force, and to tap this energy (or energy derived by hydrolysis of cytoplasmic ATP) for active transport. The outer membrane is an asymmetric lipopolysaccharide/phospholipid leaflet that represents a unique adaptation to life in a hostile environment. This outer membrane forms a diffusion barrier, protecting the cell from a variety of toxic agents, including some hydrophobic antibiotics and dyes, host-defence proteins such as lysozyme, and niche-related hazards like digestive enzymes and detergents. Molecules can traverse the outer membrane to enter the periplasmic space by three means (for a review see Nikaido and Saier, 1992): (i) small, hydrophilic nutrients passively diffuse through non-specific aqueous pores (formed by porin proteins such as OmpC and OmpF); (ii) diffusion of certain larger nutrients is facilitated through stereospecific pores such as LamB and Tsx; and (iii) Fe(III)-bearing siderophores and vitamin B_{12} are actively transported across the outer membrane via high-affinity outer membrane receptors. Active transport of nutrients across the outer membrane is energized by

cytoplasmic membrane-generated protonmotive force, and requires the cytoplasmic membrane protein, TonB (Frost and Rosenberg, 1975; Hantke and Braun, 1975; Bassford *et al.*, 1976; Hancock and Braun, 1976; Reynolds *et al.*, 1980; Bradbeer, 1993).

Amino acid sequences deduced from nucleotide sequences of the *tonB* genes of *Escherichia coli* (Postle and Good, 1983), *Salmonella typhimurium* (Hannavy *et al.*, 1990), *Serratia marcescens* (Gaisser and Braun, 1991), *Yersinia enterocolitica* (Koebnik *et al.*, 1993), *Klebsiella pneumoniae* (Bruske *et al.*, 1993), *Enterobacter aerogenes* (Bruske and Heller, 1993) and *Pseudomonas putida* (Bitter *et al.*, 1993) predict predominantly hydrophilic proteins with a potential membrane-spanning amino-terminal hydrophobic domain (for a review see Postle, 1993). Proteinase accessibility studies of TonB (Postle and Skare, 1988; Hannavy *et al.*, 1990) and topology studies of TonB–Bla and TonB–PhoA fusions (Hannavy *et al.*, 1990; Roof *et al.*, 1991) together demonstrate that TonB is anchored in the cytoplasmic membrane by an uncleaved hydrophobic amino-terminal signal sequence, with the remainder protruding into the periplasmic space. Results of genetic studies involving TonB suppressors of outer membrane receptor mutants (Heller *et al.*, 1988; Schöffler and Braun, 1989; Bell *et al.*, 1990) imply that TonB spans the periplasmic space to directly interact with outer membrane receptors. *In vivo* crosslinking studies provide direct evidence that TonB can physically interact with the outer membrane high-affinity enterochelin (also known as enterobactin) receptor FepA (Skare *et al.*, 1993). While these results are consistent with the role of TonB as an energy transducer, the mechanism of transduction is not yet clear.

Mutations in an unlinked locus, *exb* (Guterman and Dann, 1973; Hantke and Zimmerman, 1981), decrease TonB activity, indicating that auxiliary proteins are involved in energy transduction. The *exb* locus consists of two genes, *exbB*, followed by *exbD* (Eick-Helmerich and Braun, 1989). Associations between TonB and ExbB are suggested by *in vivo* chemical crosslinking (Skare *et al.*, 1993), and chemical half-life studies indicate the presence of ExbB is required for the protease resistance of both overexpressed (Fischer *et al.*, 1989) and chromosomally encoded (Skare and Postle, 1991) TonB. However, TonB turnover alone cannot account for the phenotype of *exbB* strains, since ExbB remains essential for efficient energy transduction in strains where TonB levels are stabilized by the absence of OmpT protease (Skare *et al.*, 1993). Thus, in addition to protecting TonB from endogenous proteases, ExbB appears to play a second, more direct role in energy transduction. It should be noted that the interpretation of these experiments is complicated by the fact that all investigations to date have used *exbB* mutations resulting from either large deletions (Guterman and Dann, 1973) or insertions (Hantke and Zimmerman, 1981). In these studies the potential contributions of ExbD, the downstream product of the *exb* operon, cannot be excluded. TonB can also interact in a limited way with the TolQ and R proteins, analogues of ExbB and D, that function primarily with TolA protein (Sun and Webster, 1987; Skare and Postle, 1991; Skare *et al.*, 1993; Braun and Herrmann, 1993).

ExbB is anchored in the cytoplasmic membrane by three transmembrane domains, with the bulk of ExbB occupying the cytoplasm (Kampfenkel and Braun, 1993a; Karlsson *et al.*, 1993b). Thus, the amino-terminal 12 amino acids and transmembrane domain of TonB (amino acids 13–32) are the most likely candidates for interaction with ExbB (Koebnik, 1993). Recent studies indicate that this region of TonB, beyond merely providing a membrane anchor, is essential for energy transduction. Substitution of the TonB transmembrane domain with the first transmembrane domain of either penicillin-binding protein 3 (Karlsson *et al.*, 1993a) or TetA (Jaskula *et al.*, 1994) prevents TonB activity, without hindering TonB export. The relevance of ExbB in this system is further indicated by the observation that the amino-terminal membrane anchor of TonB is required for *in vivo* chemical crosslinking to ExbB, but not for crosslinking to the other proteins with which TonB normally forms complexes, including the outer membrane receptor FepA (Jaskula *et al.*, 1994). The transmembrane domain is the only portion of TonB that shares significant sequence similarity with its crosstalk analogue, TolA (Levengood and Webster, 1989; Braun and Herrmann, 1993). Experimentally, replacement of the TonB transmembrane domain with the analogous domain of TolA results in a chimeric protein that can support suboptimal adsorption of bacteriophage ϕ80, dependent upon the presence of the ExbB/D analogues TolQ/R (Karlsson *et al.*, 1993a). These studies suggest that interactions between the transmembrane domains of TonB and ExbB are essential components of energy transduction.

In this study we characterize a Val-17 deletion mutation in the transmembrane domain of TonB and a corresponding A39E suppressor in or near the first transmembrane domain of ExbB. Characterization of interactions between these mutants and their wild-type counterparts extends previous observations to establish the importance of the transmembrane domains of these proteins and the role of ExbB in energy transduction. Molecular models are presented which suggest potential structural requirements for productive TonB/ExbB interaction, and data are presented that suggest TonB cycles between protease-sensitive and -resistant conformations in an ExbB-dependent manner.

Results

Isolation and characterization of tonBΔ*V17*

Spontaneous *tonB* mutants were selected for resistance to 0.25 μg ml^{-1} pirazmonam (Nikaido and Rosenberg, 1990)

as previously described (Larsen *et al.*, 1993). Examination of genomic DNA using *tonB*-specific primers and the polymerase chain reaction (PCR), and immunoblotting of whole-cell lysates with a TonB-specific monoclonal antibody (mAb) identified several strains that appeared to possess an intact *tonB* gene encoding full-length TonB protein (data not shown). Sequence analysis of one such strain, KP1127, indicated that *tonB* and its flanking regions (bp 279–1064; Postle and Good, 1983) were identical to wild type, except for the absence of three base pairs at positions 384–386 (data not shown). This small deletion removed the wobble base from codon 16, and the first two bases of codon 17, resulting in a silent change at codon 16 (Ser) and the loss of codon 17 (Val). The essential nature of this codon was revealed by phenotypic comparisons, where the *tonBΔV17* strain was indistinguishable from a *tonB*::*kan* strain (KP1032) with respect to resistance to bacteriophage ϕ80 and group B colicins, hypersecretion of enterochelin (indicative of iron starvation), and chromium sensitivity (Table 1).

Cytoplasmic membrane location of TonBΔ(V17) and interactions with ExbB

Because Val-17 was located within the transmembrane region of TonB, which also serves as the uncleaved export signal (Postle and Skare, 1988; Skare *et al.*, 1989; Hannavy *et al.*, 1990; Karlsson *et al.*, 1993a; Jaskula *et al.*, 1994), one possible explanation for the TonB$^-$ phenotype of the *tonBΔV17* mutant was export incompetence. To determine if TonBΔV17 was properly inserted into the cytoplasmic membrane, the proteinase K accessibility of mutant and wild-type TonB were compared (Fig. 1A). Both TonB and TonBΔV17 were susceptible to proteinase K degradation in cell sphaeroplasts, indicating that loss of Val-17 did not prevent export of TonB. Whole-cell controls indicate that the outer membranes of both strains were impermeable to proteinase K, while lysed sphaeroplast controls demonstrated that TonB from both strains was susceptible to proteinase K. Interestingly, a proteinase K-resistant degradation product was generated in the lysed sphaeroplast samples from wild-type TonB. This proteinase K-resistant species was not detectable in mutant TonBΔV17 samples (Fig. 1A) even upon extended lumigraphic exposure, suggesting that the two proteins had different conformations. Electrophoresis in 15% polyacrylamide resolved this degradation product into two distinct bands: a major species of 23 kDa, and a minor species of 26 kDa (data not shown).

Because the stability of chromosomally encoded TonB is dependent upon the presence of ExbB protein (Skare and Postle, 1991), and ExbB appears to function as a chaperone to stabilize newly synthesized cytoplasmic TonB (Karlsson *et al.*, 1993b), proteinase K accessibility studies were repeated in *exbB*::Tn*10* strains (Fig. 1B). The results of these studies were similar to those obtained in the presence of ExbB (Fig. 1A), except that the proteinase K-resistant degradation product from wild-type TonB was no longer apparent in lysed sphaeroplast samples of strains expressing either wild-type TonB or TonBΔV17. Identical results were obtained with GUC41, a Δ(*metC–exbBD–glc*) strain, excluding a role for a truncated ExbB fragment potentially expressed in *exbB*::Tn*10* strains (data not shown). These results indicated that ExbB was not required for TonB export, but suggested that ExbB was required for TonB to achieve the proteinase K-resistant conformation in lysed sphaeroplasts.

Because the proteinase K-resistant form of TonB did not appear in the *exbB* strain, it was possible that the similar lack of proteinase K-resistant TonBΔV17 in the *exbB*$^+$ strain might reflect the inability of TonBΔV17 to interact with ExbB. This possibility was examined by determining

Table 1. Phenotypes of *tonBΔV17* and *exbBA39E*.

Strain (relevant genotype)	Enterochelin[a]	Cr^{100} [b]	ϕ80[c]	Colicin Sensitivity[d]				
				Col B GID	D	Ia	K	A
W3110(*tonB*$^+$)	–	+	+	3.2×10^{-4}	+	+	+	+
KP1032(*tonB*::*kan*)	+	–	–	–	–	–	+	+
KP1127(*tonBΔVal-17*)	+	–	–	–	–	–	+	+
KP1131(*tonBΔVal-17, exbBA39E*)	+	+	+	–	–	–	+	+
KP1100(*tonB*$^+$, *exbBA39E*)	–	+	+	3.2×10^{-4}	+	+	+	+
KP1040(*tonB*$^+$, *exbB*::Tn*10*)	+	+	+	1.6×10^{-3}	±	+	+	+

a. '+' designates the hypersecretion of enterochelin, determined by presence of visible pigment surrounding colonies following overnight culture on LB. '–' indicates no hypersecretion.

b. '+' designates the ability to grow on plates containing 100 μg ml^{-1} chromium. '–' indicates no growth.

c. '+' designates inhibition of growth when cells were cross-streaked against 1.1×10^7 pfu ϕ80. '–' indicates complete resistance.

d. '+' designates complete inhibition of growth when cross-streaked against a particular colicin at a 1 : 10 dilution of crude preparation, '±' designates a partial inhibition of growth, '–' indicates complete resistance. Specific activities of colicins were not determined, but did vary when dilution series were examined (data not shown). In particular, the colicin D preparation had relatively low activity. For colicin B, the greatest dilution of colicin at which complete inhibition of growth (greatest inhibitory dilution: GID) occurred is shown.

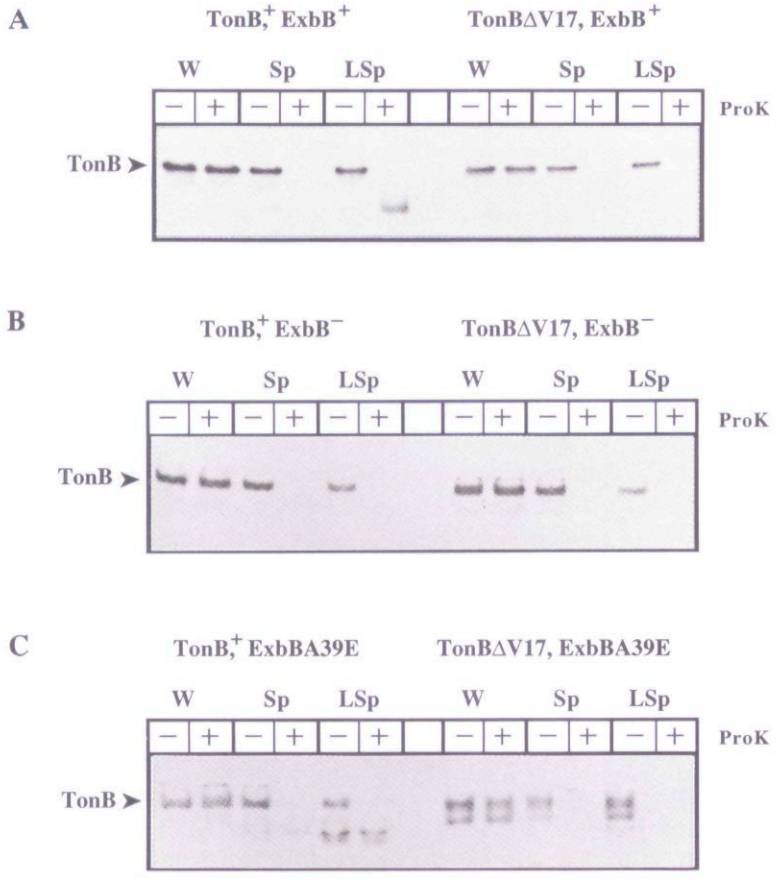


Fig. 1. Proteinase K accessibility of chromosomally encoded TonB in W3110 and mutant derivatives. Immunoblots of whole-cell (W), sphaeroplast (Sp) and lysed sphaeroplast (LSp) samples, treated with (+) or without (−) proteinase K are shown. Samples were resolved on 11% SDS–polyacrylamide gels and immunoblotted with 1 : 5000 mAb 4H4 (specific for an epitope between TonB residues 60 and 95; data not shown).
A. Sample size = 0.05 A_{550} cell equivalents. Left. W3110 (*tonB*$^+$, *exbB*$^+$); right, KP1127 (*tonBΔV17*, *exbB*$^+$).
B. Sample size = 0.10 A_{550} cell equivalents. Left, KP1040 (*tonB*$^+$, *exbB*::Tn*10*); right, KP1132 (*tonBΔV17*, *exbB*::Tn*10*).
C. Sample size = 0.10 A_{550} cell equivalents. Left, KP1100 (*tonB*$^+$, *exbBA39E*); right, KP1131 (*tonBΔV17*, *exbBA39E*). The position of full-length TonB is indicated by an arrow. All bands present on the lumirads are shown.

the half-lives of wild-type and mutant TonB in *exbB*$^+$ and *exbB*$^-$ strains (Fig. 2). In the presence of ExbB, the half-life of TonBΔV17 was similar to that of wild-type TonB (Fig. 2A). As with wild-type TonB, the half-life of TonBΔV17 was ExbB dependent, although to a lesser extent (Fig. 2B). These results suggested that TonBΔV17 continued to interact with ExbB, but this interaction was not sufficient to produce the proteinase K-resistant conformation detected in lysed sphaeroplasts.

The ability of TonBΔV17 to interact with ExbB was also examined by *in vivo* chemical crosslinking with formaldehyde (Fig. 3). We have previously shown that TonB forms a 59 kDa crosslinked complex that is absent in an *exbB*::Tn*10* strain. The apparent molecular mass of this complex is consistent with a composition of one ExbB and one TonB molecule (Skare *et al.*, 1993). Crosslinking studies with a mutant deleted for the proline-rich region of TonB suggest that the 59 kDa complex carries only one TonB molecule per complex (Larsen *et al.*, 1993). In Fig. 3, the *tonBΔV17* strain did not form the 59 kDa heterodimer. Interestingly, although TonBΔV17 did not support energy transduction-dependent processes, it still interacted with outer membrane receptors, evident by the formation of a TonBΔV17/FepA crosslinked complex, and with other, unidentified polypeptides present in the previously observed 43 kDa and 77 kDa crosslinked complexes (Skare *et al.*, 1993). This was consistent with observations regarding a TetA–TonB hybrid protein, where the first transmembrane domain of TetA substitutes for amino acids 1–33 of TonB. TetA–TonB is inactive, but can still crosslink to outer membrane receptors (Jaskula *et al.*, 1994).

Isolation and characterization of an exbB *suppressor of* tonB*ΔV17*

Phenotypic characterizations indicated that TonBΔV17 was inactive, perhaps because of its inability to interact productively with ExbB. To gain further insight, phenotypic revertants were isolated and characterized . Revertants of KP1127 (*tonBΔV17*) were selected on Cr^{100} plates, which primarily support TonB$^+$ phenotypes (Wang and Newton, 1970). Of four independently isolated chromium-resistant (Chr^R) revertants, three strains, exemplified by KP1131, were found to be partial *tonB*$^+$ revertants (Table 1). Like cells with wild-type TonB, these strains were sensitive to bacteriophage ϕ80 in cross-streaks but, like their mutant parent, retained resistance to group B colicins and hypersecreted enterochelin. P1 transductions indicated that the *chr*^{r} mutations were unlinked to TonB and tightly linked (>98%) to *metC*, the translational start of which

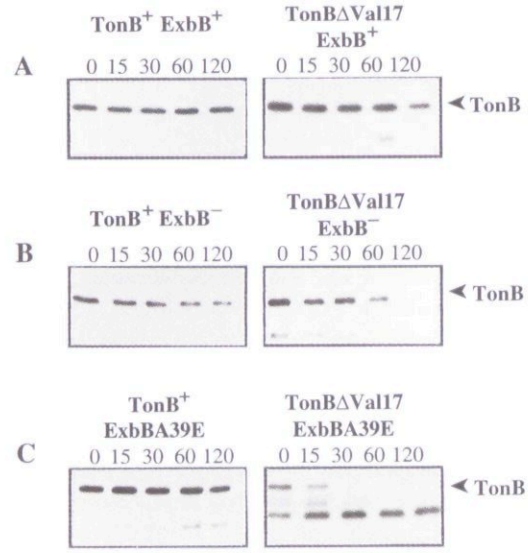


Fig. 2. Chemical stability of chromosomally encoded TonB in W3110 and mutant derivatives. Samples were collected at 0, 15, 30, 60, and 120 min following chloramphenicol addition and immunoblotted as in Fig. 1.
A. Sample size = 0.05 A_{550} cell equivalents. Left. W3110 (*tonB*$^+$, *exbB*$^+$); right, KP1127 (*tonBΔV17*, *exbB*$^+$).
B. Sample size = 0.10 A_{550} cell equivalents. Left. KP1040 (*tonB*$^+$, *exbB*::Tn*10*); right, KP1132 (*tonBΔV17*, *exbB*::Tn*10*).
C. Sample size = 0.10 A_{550} cell equivalents. Left. KP1100 (*tonB*$^+$, *exbBA39E*); right, KP1131 (*tonBΔV17*, *exbBA39E*). The position of full-length TonB is indicated by an arrow. All bands present on the lumirads are shown.

lies within 250 bp of *exbB* (Eick-Helmerich and Braun, 1989). Pools of *exbBD* plasmid clones were generated from the KP1127 parent and from each of the three suppressor strains, and sequences of the *exbB* and *exbD* genes were determined. Because each sequence was determined from a transformant pool, it was unlikely that the differences noted reflected *Taq* polymerase infidelity. The sequence of *exbBD* from KP1127 (considered to represent wild-type *exbBD*) was identical to that reported by Eick-Helmerich and Braun (1989), except that our strain encoded Gln(CAG) versus Leu(CTG) at codon 51 and a silent change at codon 200 (CGG versus CGC) of the *exbB* open reading frame. No differences were noted in the *exbD* open reading frame (data not shown). The sequence of *exbBD* from the KP1131 suppressor strain was identical to our wild type, except for a single-base-pair change at position 697, where a cytosine in the mRNA-like strand was replaced by an adenosine. This altered codon 39 of the ExbB open reading frame, resulting in the replacement of a hydrophobic Ala residue by a negatively charged Glu residue in the protein (data not shown). Identical changes were noted for the other two suppressors (data not shown).

Interaction of ExbBA39E with TonB and TonBΔV17

To evaluate the ability of ExbBA39E to interact with wild-type TonB, the allele was transduced into W3110 and the phenotype of the resultant strain (KP1100) compared with those of W3110 and an *exbB*::Tn*10* strain (KP1040, Table 1). The ExbBA39E allele had no detectable effect on the activity of wild-type TonB in either a W3110 (Table 1) or a GM1 (data not shown) background. A more quantitative measure of TonBΔV17 function and ExbBA39E suppression was obtained by examining the ability of strains to adsorb bacteriophage ϕ80 irreversibly (Fig. 4). The ability of wild-type TonB to promote irreversible ϕ80 adsorption was not significantly different between cells expressing the wild-type or the suppressor *exbB* allele. In contrast, the suppressor allele promoted irreversible ϕ80 adsorption by TonBΔV17 at low levels, which were similar to those observed in an *exbB* strain, where TonB activity is dependent upon crosstalk with the Tol system. It should

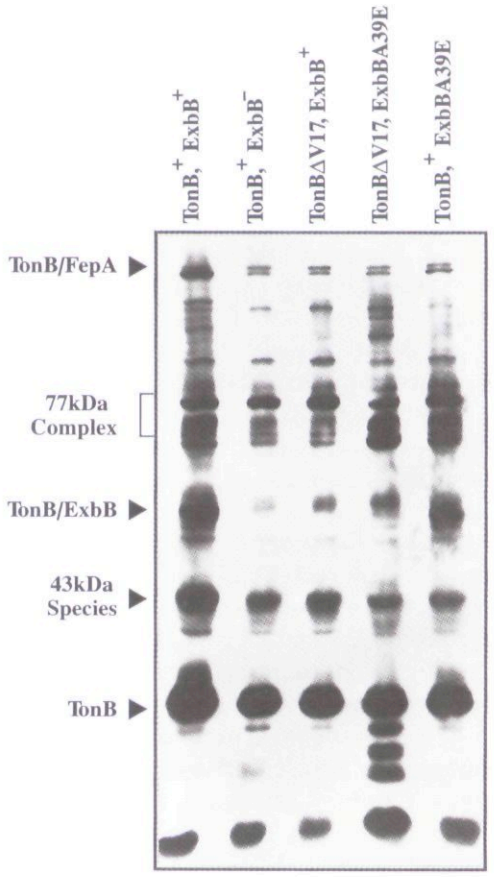


Fig. 3. Effect of ExbB on TonB crosslinking profiles. Crosslinked samples were prepared as described in the *Experimental procedures*, then 0.2 A_{550} cell equivalents were resolved on 11% SDS–polyacrylamide gels and immunoblotted as in Fig. 1. Major TonB-containing complexes are identified either by their composition or apparent molecular mass at the side of the panel. The positions of monomer TonB and TonBΔV17 are also indicated. The lower two-thirds of the gel are shown. From left to right: W3110 (*tonB*$^+$, *exbB*$^+$), KP1040 (*tonB*$^+$, *exbB*::Tn*10*), KP1100 (*tonB*$^+$, *exbBA39E*), KP1127 (*tonBΔV17*, *exbB*$^+$), KP1132 (*tonBΔV17*, *exbB*::Tn*10*), and KP1131 (*tonBΔV17*, *exbBA39E*).

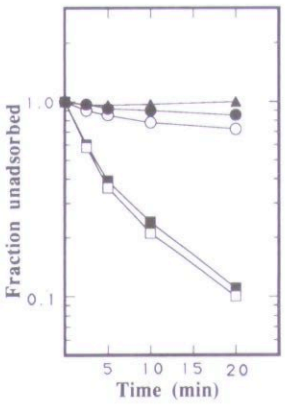


Fig. 4. Relative levels of TonB function in W3110 and mutant derivatives. Irreversible adsorption of ϕ80 was assayed as described in the *Experimental procedures*. Each point represents the mean of six independent assays. For each point, standard deviation was less than 0.1 log units. The higher the fraction unadsorbed, the lower the relative level of TonB function. ▲, *tonBΔV17*; □, *tonB*$^+$, *exbB*$^+$; ●, *tonB*$^+$, *exbB*::Tn*10*; ○, *tonBΔV17, exbBA39E*; ■, *tonB*$^+$, *exbBA39E*.

be noted that this similarity is coincidental, as the inability of TonBΔV17 to energize irreversible ϕ80 adsorption in the presence of either wild-type ExbB (Fig. 4) or in an *exbB* strain (not shown) indicated that TonBΔV17 could not engage in productive crosstalk with the Tol system.

Initial studies with TonBΔV17 suggested that this mutant interacted differently with ExbB than did wild-type TonB (Figs 1–3). Similar experiments were performed with strains expressing the *exbBA39E* allele. Like the results obtained with *exbB*$^+$ strains, both the wild type and TonBΔV17 were accessible to proteinase K in sphaeroplast preparations of *exbBA39E* strains (Fig. 1A versus 1C). ExbBA39E also supported the formation of the 23–26 kDa proteinase K-resistant degradation product in lysed sphaeroplasts from wild-type TonB, but not mutant TonBΔV17 (Fig. 1A versus 1C). Therefore the ability to form the proteinase K-resistant proteolytic degradation product in lysed sphaeroplast preparations correlated with the degree of TonB activity.

A further observation from these experiments was that the presence of ExbBA39E appeared to have a dramatic effect on the stability of TonBΔV17, as evidenced by the accumulation of proteolytic degradation products in whole-cell controls of the *tonBΔV17, exbBA39E* strain (Fig. 1C). This was confirmed when the half-life of TonB was examined in *exbBA39E* strains. Wild-type TonB had a similar half-life in the presence of either A39E suppressor or wild-type ExbB (Fig. 2C versus 2A), although the A39E suppressor strain did gradually accumulate low levels of a TonB degradation product. Degradation products of similar sizes also occurred in the presence of wild-type ExbB, but were only detectable by prolonged exposure of the immunoblots (data not shown). A greater difference between ExbB and ExbBA39E proteins was noted when the half-life of TonBΔV17 was examined. The presence of the ExbBA39E suppressor reduced the half-life of TonBΔV17 to approximately 10 min, with the resultant accumulation of a 28 kDa degradation product (Fig. 2A–C).

In vivo chemical crosslinking provided further comparisons between wild-type ExbB and the ExbBA39E suppressor (Fig. 3). Wild-type TonB was able to form a 59 kDa heterodimeric complex in the *exbBA39E* strain that was indistinguishable from the complex formed in the strain encoding wild-type ExbB, although the relative amount of complex was less. Identical crosslinking profiles were obtained for GM1, *exbBA39E* (data not shown). The TonBΔV17 mutant, which did not form a 59 kDa complex in an *exbB*$^+$ strain, nevertheless did form a novel 62 kDa complex in the *exbBA39E* strain. The absence of the 62 kDa species in *tonBΔV17* strains with either wild-type *exbB* (Fig. 3) or *exbB*::Tn*10* (data not shown) suggested that this band represented a TonBΔV17/ExbBA39E crosslinked heterodimer. TonBΔV17 was capable of forming all of the other formaldehyde crosslinked complexes normally seen with wild-type TonB, as was wild-type TonB in an *exbBA39E* background, although in both cases some differences were noted in the relative amounts of each complex (Fig. 3).

Discussion

The cytoplasmic membrane protein TonB is an energy transducer, which appears to couple cytoplasmic membrane protonmotive force to outer membrane active transport processes. The transmembrane domain is clearly important in this role, as the apparent sole connection of TonB to the energy source. Beyond merely anchoring TonB to an energy source and providing an export signal, the TonB transmembrane domain is required for energy transduction, since: (i) export-competent TonB hybrids with the amino-terminus and sole transmembrane domain provided by either penicillin-binding protein 3 (Karlsson *et al.*, 1993a) or TetA protein (Jaskula *et al.*, 1994) are inactive; (ii) replacement of the TonB transmembrane domain by that of TolA renders TonB activity primarily TolQ-, rather than ExbB-dependent (Karlsson *et al.*, 1993a), and (iii) cleavage at an engineered leader peptidase site inactivates TonB (Jaskula *et al.*, 1994). The transmembrane domain of TonB is very similar between species and, as previously noted, contains four residues also conserved in the transmembrane domain of TolA (Koebnik *et al.*, 1993). These residues are predicted to occupy the same face of a transmembrane α-helix, based on helical wheel analysis. Interestingly, two of these residues are divergent in *Pseudomonas putida* TonB (Fig. 5), the only TonB that cannot complement *E. coli tonB* strains (Hannavy *et al.*, 1990; Gaisser and Braun, 1991; Bitter *et al.*, 1993; Bruske

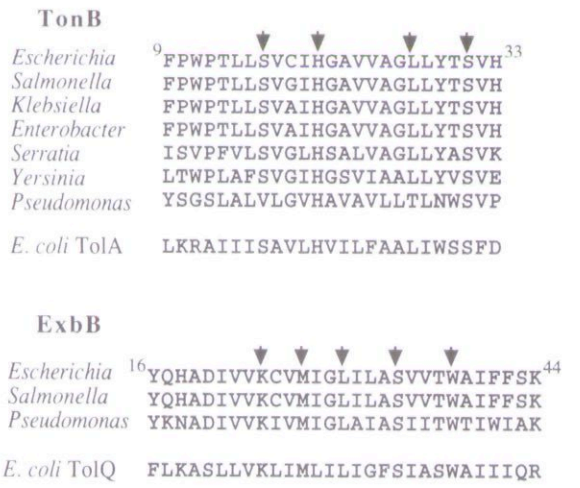


Fig. 5. Conservation of TonB and ExbB transmembrane domains. Predicted amino acid sequences of the amino-terminal region of *E. coli* TonB (residues 9–33) and the corresponding regions of TonB from other species (see text for references), compared to the analogous region of *E. coli* TolA (Levengood and Webster, 1989). Residues predicted to reside on the conserved α-helical face are indicated by arrows above the *E. coli* sequence (Koebnik, 1993). The lower portion of the panel presents a similar comparison between the amino-terminal regions of ExbB from *E. coli* (residues 16–44; Eick-Helmerich and Braun, 1989), *S. typhimurium* (Parks and Stauffer, 1989), and *P. putida* (Bitter *et al.*, 1993) and the analogous region of *E. coli* TolQ (Sun and Webster, 1987; Kampfenkel and Braun, 1993b). Residues predicted to reside on the conserved α-helical face are indicated by arrows above the *E. coli* sequence (Koebnik *et al.*, 1993).

and Heller, 1993; Bruske *et al.*, 1993; Koebnik *et al.*, 1993). In this study, we isolated a spontaneous deletion of Val17 in TonB that disrupted the relative positions of residues comprising the predicted conserved α-helix face of TonB. TonBΔV17 was unable to transduce energy, as evidenced by absolute resistance to ϕ80 and group B colicins.

Several possible explanations for the inactivity of TonBΔV17 were explored. The possibility that the TonB$^-$ phenotype of TonBΔV17 reflected its rapid degradation by endogenous envelope proteases was ruled out by the observation of similar half-lives for both TonB and TonBΔV17. The possibility that the deletion of Val17 prevented TonB export to the cytoplasmic membrane was ruled out by the observation that, like TonB, TonBΔV17 was completely accessible to proteinase K in sphaeroplasts. Proper export of TonBΔV17 was confirmed by the demonstration of *in vivo* chemical crosslinking of this mutant to the outer membrane protein FepA (Fig. 3). These results were not surprising, as in-frame deletion of valine from the predicted transmembrane domain did not significantly alter the overall hydrophobicity of the region.

The topological partitioning of TonB and ExbB to either side of the cytoplasmic membrane restricts the sites of interaction between these proteins primarily to their transmembrane domains. The possibility that ΔV17 affected or prevented the interaction of TonB with ExbB was tested. Like TonB, TonBΔV17 was more rapidly degraded in the absence of ExbB, suggesting that some interaction between TonBΔV17 and ExbB still existed. Proteinase K accessibility experiments further demonstrated that the absence of ExbB had no effect on the export of either TonB or TonBΔV17, suggesting that ExbB does not have an essential chaperone function. However the possibility that some of the TonB degradation that occurs in the absence of ExbB (Skare and Postle, 1991) takes place in the cytoplasm cannot be excluded.

The discovery of a suppressor for TonBΔV17 at or near the first ExbB transmembrane domain was consistent with the idea that TonB and ExbB interact through transmembrane domains. It also suggested why the Val17 deletion mutant was completely inactive: if TonB can no longer interact with ExbB, it should remove any possibility of interaction with TolQ, the crosstalk analogue of ExbB, as well. The absence of both ExbB and TolQ has been shown to render TonB completely inactive (Braun, 1989).

Previous results have shown that TonB can be crosslinked *in vivo* by formaldehyde into a 59 kDa complex that is absent from an *exbB::*Tn*10* strain (Skare *et al.*, 1993). In contrast to the results suggesting that the interaction of TonBΔV17 with ExbB was not impaired, the complete absence of detectable 59 kDa complex from *in vivo* crosslinking of TonBΔV17 suggested that the interaction had changed considerably. The easiest way to reconcile these apparently contradictory results would be if the ΔV17 mutation resulted in a conformational shift of the TonBΔV17 and wild-type ExbB relative to one another, such that fromaldehyde-reactive residues were no longer close enough to allow methyl bridge formation. The hypothesis of conformational shifts is supported by the observation of a proteinase K-resistant species from lysed sphaeroplasts of *tonB*$^+$, but not *tonBΔV17* strains, indicating that the two proteins had achieved different conformations. These experiments also provide the first direct evidence for activity-specific conformational changes in TonB. The significance and source of the proteinase K-resistant species will be addressed later in the discussion.

In the hope of understanding more about the effects of the ΔV17 mutation, suppressors were isolated and mapped. Three identical, but independently isolated, suppressors of TonBΔV17 were mapped to the *exb* locus. Each resulted in an Ala → Glu substitution at amino acid 39 of the predicted ExbB open reading frame. The *exbBA39E* allele had no effect on activity, export, or stability of wild-type TonB, suggesting that it was not an allele-specific suppressor. The *exbBA39E* allele did, however, restore detectable TonB activity to *tonBΔV17* strains. The low level of TonB activity in these strains may reflect the observed rapid degradation of TonBΔV17 in the presence of ExbBA39E, as well as incomplete suppression.

The presence of the suppressor in combination

with TonBΔV17 also resulted in the appearance of a crosslinked complex at 62 kDa. Together, the absence of the 59 kDa complex in TonBΔV17 and its restoration (albeit at a slightly higher apparent molecular mass of 62 kDa) in strains expressing both TonBΔV17 and ExbBA39E, strongly suggested that the 59 kDa complex observed by *in vivo* crosslinking of wild-type TonB consists of both TonB and ExbB. In that case, the suppressor mutation would probably be a conformational change in ExbB that now concomitantly allows crosslinking to occur and some TonB to be activated for energy transduction. TonBΔV17 was predicted to have undergone a significant shift in residues that could be crosslinked to ExbB. Thus the slightly higher apparent molecular mass of the complex may be due to crosslinking at different sites, as previously observed with the Tar receptor system (Pakula and Simon, 1992). The conformational change in ExbBA39E was not so severe as to prevent normal crosslinking to wild-type TonB, although the amount of heterodimer generated was less.

Six amino acids (Lys, His, Trp, Tyr, Arg, and Cys) have side-groups that can engage in formaldehyde-mediated crosslinking (reviewed by Means and Feeney, 1971). Because formaldehyde crosslinking occurs when a methyl bridge forms between reactive residues, formation of crosslinks is dependent upon proximity, and can serve as a probe for close molecular interactions, as first established for periplasmically localized polypeptides in the histidine transport system of *Salmonella typhimurium* (Prossnitz *et al.*, 1988). We have demonstrated that *E. coli* TonB can be crosslinked *in vivo* by formaldehyde to form four major protein complexes, including a 195 kDa complex containing the outer membrane protein FepA and a 59 kDa species dependent upon the presence of ExbB (Skare *et al.*, 1993). The observation that a TrpC–TonB fusion protein lacking residues 1–12 (the cytoplasmic domain) of TonB can form a 59 kDa heterodimer with ExbB when crosslinked (R. Larsen and K. Postle, unpublished) suggests that formation of this complex is not dependent upon the cytoplasmic domain of TonB. Similarly, loss of crosslinking to ExbB (but not to FepA or other polypeptides) when the TonB transmembrane domain is replaced by the transmembrane domain of TetA suggests that periplasmically located residues do not mediate TonB/ExbB crosslinking (Jaskula *et al.*, 1994). Thus, consistent with the results of this study, the transmembrane domain of TonB appears to be essential for crosslinking with ExbB.

Only four TonB residues within or near the TonB transmembrane domain (Cys-18, His-20, Tyr-29 and His-33) are formaldehyde-reactive and could potentially engage in crosslinking with ExbB. Of these, Cys-18 can be excluded, since *Salmonella typhimurium* TonB (where a non-reactive Gly residue replaces Cys-18 — the only distinct site in their predicted transmembrane domains) crosslinks normally with ExbB (R. Larsen and K. Postle, unpublished). Four formaldehyde-reactive residues occur in the predicted first transmembrane domain of ExbB (His-19, Lys-25, Cys-26, and Trp-38). The other two predicted transmembrane domains of ExbB are devoid of formaldehyde-reactive residues, apart from a single Trp (predicted domain 2, residue 150). Our data do not exclude the possibility that formaldehyde-mediated TonB/ExbB complexes form through the reactive residues lying near the membrane/periplasm interface, but both the TonB and the ExbB suppressor mutations affect regions near the membrane/cytoplasm interface of the predicted transmembrane helices, where each protein has a single formaldehyde-reactive residue (TonB His-20, ExbB Trp-38). The proximity of these reactive residues to the mutation sites and the altered crosslinking profiles of the mutants support the hypothesis that TonB/ExbB interaction occurs through the conserved faces of their respective transmembrane helices (Koebnik, 1993).

In the absence of crystallographic data, representations of TonB and ExbB transmembrane domains can only be approximations, but such estimates can provide the basis

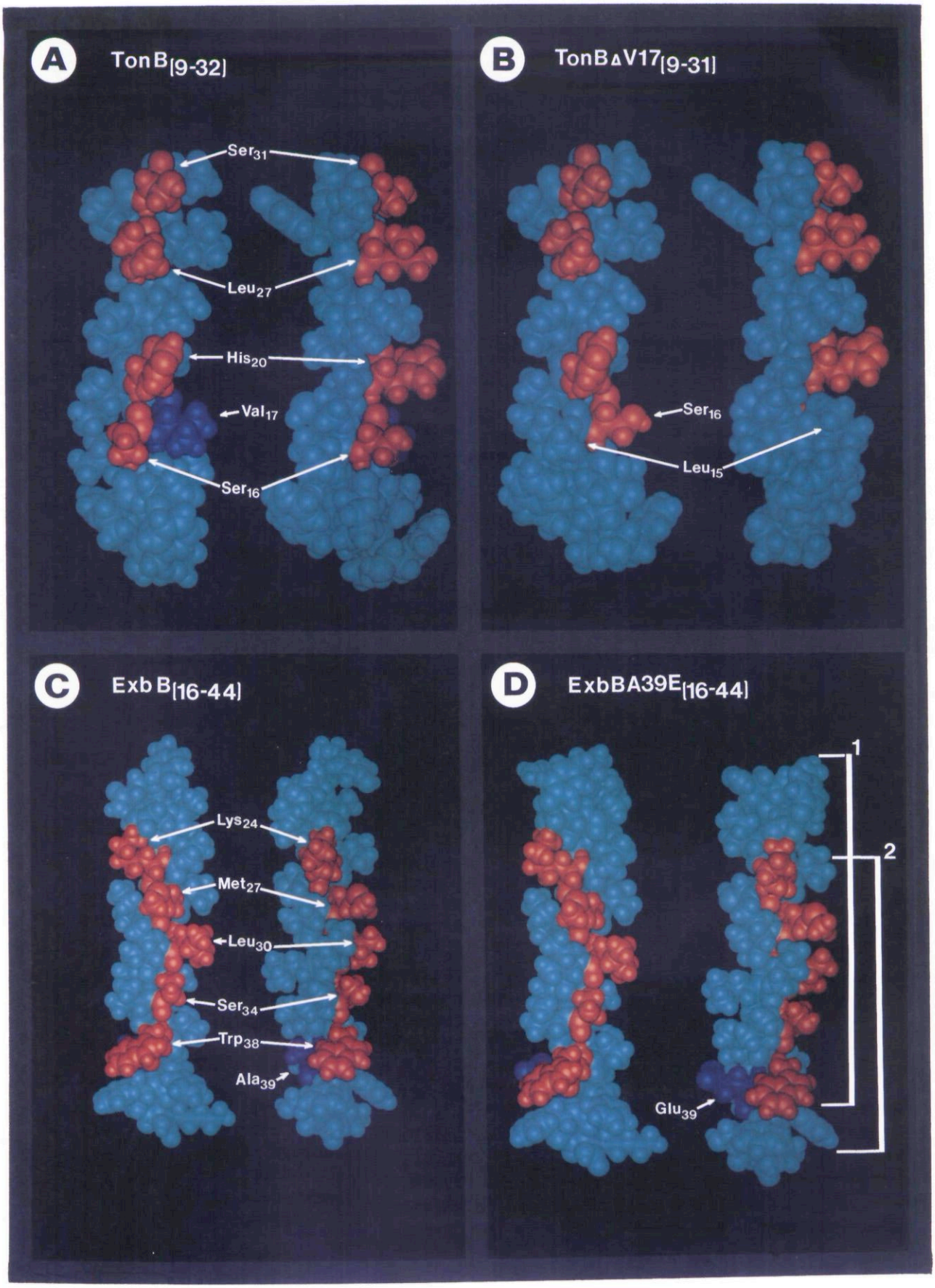
Fig. 6. Predicted structures for TonB, TonBΔV17, ExbB and ExbBA39E amino-terminal transmembrane domains. Structures represent thermodynamically minimized α-helical models (3.6 residues/turn, 5.4 Å pitch) generated by the BatchMin (V3.1) algorithm (Mohamadi *et al.*, 1990). Potential transmembrane sections were identified by correlating predicted windows of average hydrophobicity (Kyte and Doolittle, 1982) with topologic data (Postle and Skare, 1988; Roof *et al.*, 1991; Kampfenkel and Braun, 1993a; Karlsson *et al.*, 1993b). For TonB, these studies predict a 21-residue region (residues 12–32); and for ExbB, two alternative regions of either 20 (residues 25–44) or 25 (residues 16–40) residues. Because transmembrane boundaries are not specifically defined, inclusive regions were modelled. Each panel presents two views of a modelled structure: on the left, a direct view of the conserved face is shown; on the right, an 80–90° rotation of the structure. All structures are oriented such that the portion proximal to the cytoplasmic face of the membrane is at the bottom of the panel, while the portion proximal to the periplasmic face of the membrane is at the top of the panel. The calculated minimal free energy (ΔG°) is provided for each structure according to the legend discription.

A. Wild-type TonB, residues 9–32 ($\Delta G^\circ = -190.1\ kJ\ mol^{-1}$). Residues defining the conserved face are Ser-16, His-20, Leu-27, and Ser-31, all indicated in red. Val-17, deleted in the mutant TonB, is indicated in dark blue.

B. TonBΔV17, residues 9–31 (corresponding to 9–32 in the wild type; $\Delta G^\circ = -12.7\ kJ\ mol^{-1}$). The conserved, displaced residue Ser-16 is indicated, as is Leu-15, which occupies the region on the conserved face previously occupied by Ser-16 in the wild type.

C. Wild-type ExbB, residues 16–44 ($\Delta G^\circ = -755.4\ kJ\ mol^{-1}$). Residues defining the conserved face are Trp-38, Ser-34, Leu-30, Met-27, and Lys-24, indicated in red. Ala-39, altered in the mutant ExbB, is indicated in dark blue.

D. ExbBA39E, residues 16–44 ($\Delta G^\circ = -807.7\ kJ\ mol^{-1}$). The result of the A39E missense mutation (Glu-39) is indicated in dark blue. Two sets of predicted transmembrane domain boundaries are indicated on the right of the panel: Boundary 1, residues 16–40 (Kampfenkel and Braun, 1993a); Boundary 2, residues 25–44 (Karlsson *et al.*, 1993b).


A
TonB[9-32]
Ser31
Leu27
His20
Val17
Ser16
B
TonBΔV17[9-31]
Ser16
Leu15
C
ExbB[16-44]
Lys24
Met27
Leu30
Ser34
Trp38
Ala39
D
ExbBA39E[16-44]
1
2
Glu39

for testable hypotheses. Helical wheel representations of predicted transmembrane domains have been used to identify potential sites of TonB/ExbB interaction (Koebnik, 1993). Helical wheel representations of our mutants suggested distortion of these sites (not shown). To examine this possibility more rigorously, thermodynamically minimized space-filling models of predicted α-helices were generated (Fig. 6).

The deletion of Val17 altered the predicted configuration of the conserved α-helical face of TonB in an area clearly within the transmembrane region (Fig. 6, panel A versus B). A polar Ser residue (position 16) was displaced by a bulky hydrophobic residue (Leu-15) adjacent to His-20. This was consistent with our crosslinking experiments, and with the reported impairment of TonB energy transduction by a His → Arg substitution at this position (Traub *et al.*, 1993), and suggested that the relationship between His-20 and Ser-16 was essential to energy transduction. Interestingly, the Ala→Glu substitution at codon 39 did not distort the predicted conserved α-helical face of ExbB Fig. 6, panel C versus D). This was consistent with our data suggesting that suppression of TonBΔVal-17 by ExbBA39E was not allele specific. Because suppression did not involve a distortion of the conserved α-helical face of ExbB that is predicted to complement the conserved α-helical face of TonB (Traub *et al.*, 1993), it is unlikely that these regions are the sole sites of TonB/ExbB interaction.

We hypothesize that TonB activity and stability are conformation dependent, and that TonB conformation is mediated by signals from ExbB. This model is similar to the Mal system, where protease resistance of the periplasmically exposed MalF protein is dependent upon conformational changes imposed by MalG and a cytoplasmic protein, MalK (Traxler and Beckwith, 1992). In the TonB model, the ΔVal-17 mutation affects the ability of TonB to respond to ExbB-generated signals that mediate conformational changes in TonB. The formation of proteinase K-resistant degradation products from TonB, but not TonBΔV17, in lysed sphaeroplasts preparations supported the contention that wild-type TonB can achieve protease-resistant conformations that mutant TonB cannot. Proteinase K-resistant forms occurred only in lysed sphaeroplast preparations from cells expressing functional forms of ExbB, suggesting that the ability of TonB to achieve this conformation was dependent upon the presence of ExbB. The proteinase K-resistant conformation was also achieved in the presence of ExbBA39E, again suggesting that suppression was not allele specific. Such a mechanism would also explain how the predominantly cytoplasmically exposed ExbB can stabilize periplasmically exposed TonB. Further evidence for conformational changes comes from *in vivo* crosslinking patterns obtained with *tonBΔV17* strains.

The mechanism by which the A39E mutation suppresses the TonBΔV17 mutant is unclear. Several lines of evidence suggest that it is unlikely that the mutation merely alters the conformation of ExbB such that it now 'fits' TonBΔV17. First, the A39E mutation did not appreciably alter the predicted structure of the ExbB transmembrane α-helix (Fig. 6). However, the A39E mutation did occur adjacent to Trp-38, and may have altered that residue's ability to participate in crosslinking with TonB. Second, both suppressor and wild-type ExbB alleles equally supported wild-type TonB activity (Table 1, Fig. 4). It is difficult to envisage a structural alteration of the ExbB transmembrane domain that could accommodate the effects of the Val-17 deletion and still conform to the requirements of wild-type TonB. Third, ExbB is still required for stability of TonBΔV17, indicating that the predicted deformation of the TonB α-helix did not prevent this facet of TonB/ExbB interaction. Finally, it is not entirely clear that the suppressor lies within the ExbB transmembrane domain. Because the first transmembrane domain of ExbB is ill-defined (Fig. 6), the suppressor mutation could be cytoplasmically localized, and may not directly interact with the mutated transmembrane domain of TonB. As it appears unlikely that ExbBA39E functions in an allele-specific manner, we must consider alternative explanations for the observed suppression.

If the suppressor did not affect the way TonB and ExbB interact, it may function by altering the ability of TonB to either receive or to respond to an ExbB signal to undergo a conformational change. A mutation which locked ExbB in a signal-propagating conformation, or provided an alternative route for signal propagation, could circumvent the blocked response of TonBΔV17. For example, the ΔVal-17 mutation may function by increasing the thermodynamic requirements of TonB transition between conformations. More frequent or more intense signals from ExbB could overcome this barrier sufficiently to support TonB activity. Alternatively, the ExbB signal could be propagated by a different route, perhaps via a salt bridge involving the mutant Glu residue of ExbBA39E and one or more cytoplasmically exposed Arg residues of TonB. Such mechanisms are at present highly speculative, but the intriguing topology of ExbB may suggest some clues. The majority of ExbB is cytoplasmically exposed (Kampfenkel and Braun, 1993a), and the conservation of its cytoplasmic domain between *E. coli* and *P. putida* is much greater than that of the periplasmic domain of TonB from these species (Eick-Helmerich and Braun, 1989; Bitter *et al.*, 1993). This conservation could reflect sequence constraints owing to interaction of ExbB with other, equally conserved proteins. It is possible the phenotype of the suppressed mutant did not reflect the ability of ExbB to interact with TonB, but rather, the direction and intensity of that interaction. In this scenario, a suppressor mutation would not be restricted to the transmembrane domain. In that case, it should be possible

to isolate ExbB point mutations in the cytoplasmic domain that adversely affect TonB activity.

Experimental procedures

Bacterial strains and plasmids

Bacteria and plasmids used are listed in Table 2. All bacteria are derivatives of *E. coli* K-12. Strain KP1127 is a spontaneous pirazmonam-resistant mutant of W3110, selected by plating log-phase cells on Luria–Bertani (LB) containing 0.25 μg ml^{-1} pirazmonam. Strain KP1131 was isolated as one of four independent spontaneous revertants of KP1127, capable of growth in the presence of 100 μM Cr^{3+}. KP1134 was constructed by transducing KP1131 with a P1 lysate (Miller, 1972) from CAG18475 (*metC*::Tn*10*), and screening tetracycline-resistant recombinants for retention of the revertant phenotype and methionine auxotrophy. KP1100 and KP1108 were constructed by transducing W3110 and GM1, respectively, with a P1 lysate from KP1134. Because the TonB phenotypes were unaltered, tetracycline-resistant recombinants were confirmed by P1 transduction of the revertant phenotype to KP1127 to restore suppression. The *exbB*::Tn*10* allele from H1388 was transduced into W3110 and KP1127, resulting in KP1040 and KP1132, respectively. Transduction of the *exbB*::Tn*10* allele was selected by resistance to tetracycline and confirmed by testing for increased resistance to colicin B in the case of KP1040. For KP1132, which was already TonB$^-$, P1 transduction of the *exbB* phenotype back to W3110 confirmed the existence of the *exbB*::Tn*10* allele. KP1032 was generated from W3110 by selecting for recombination with a *tonB*::Kan^R gene on plasmid pKP172 (Postle, 1990). After selection by φ80vir and colicin B in the presence of 100 μg ml^{-1} ampicillin, the homogenotes were grown in the absence of ampicillin and screened for loss of the plasmid. The construction was confirmed by P1 transduction, resulting in 100% linkage between the TonB$^-$ and Kan^R phenotypes.

Media

Strains were maintained on LB agar. Liquid cultures were grown with aeration at 37°C in M9 minimal salts (supplemented with 0.4% glucose, 40 μg ml^{-1} tryptophan, 0.5 μg ml^{-1} $FeCl_3 \cdot 6H_2O$, 0.4 μg ml^{-1} thiamine, 0.1 mM $MgSO_4$, 0.5 mM $CaCl_2$ and either 0.2% casamino acids or defined amino acids (40 μg ml^{-1}, except Tyr — 0.4 μg ml^{-1}; Asp, Glu — 30 μg ml^{-1})) except where indicated. Chromium agar (Cr^{100}) consisted of 1.5% agar in M9 minimal salts supplemented with 0.3% vitamin-free casamino acids, 0.2% glucose, 4.0 μg ml^{-1} thiamine, 10.0 mM $MgSO_4$, and 100 μM $CrCl_3$. Antibiotics were used in LB agar plates (where indicated) at the following concentrations: pirazmonam at 0.25 μg ml^{-1}; ampicillin at 100 μg ml^{-1}; kanamycin at 50 μg ml^{-1}; tetracycline at 20 μg ml^{-1}. LB/XG plates were prepared by spreading 40 μl of dimethylformamide containing 25 mg ml^{-1} Xgal on LB plates 1–2 h prior to use.

Chemicals and reagents

Colicins and anti-TonB mAbs were prepared as previously described (Larsen *et al.*, 1993; Skare *et al.*, 1993). Pirazmonam (Batch NN010NA) was the generous gift of Bristol–Meyers Squibb, Inc. Media components were purchased from Difco Laboratories. Enhanced chemiluminescence (ECL) immunoblot kits and horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin were purchased from Amersham Inc. Immobilon-P was purchased from Millipore Corp. Acrylamide was purchased from Fisher Biotech., and bisacrylamide from Bio-Rad Laboratories. SDS was purchased from Schwartz/Mann. Formaldehyde was purchased from J. T. Baker, Inc. Sequenase and Sequenase Kit reagents (Version 2.0) were purchased from United States Biochemical Corp. Oligonucleotides used in PCRs were synthesized by Genosys Biotechnologies, Inc. and by Midland Certified Reagent Company. Thermalase was purchased from International Biotechnologies, Inc. Gelase was purchased from Epicenter Technologies. TA cloning kits were purchased from InVitrogen Corp. Proteinase K and PMSF were purchased from Boehringer Mannheim. All other reagents were purchased from Sigma Chemical Co.

Table 2. *E. coli* strains and plasmids.

Strain/Plasmid	Relevant genotype	Source/Reference
E. coli strain		
INVαF′	*recA1 hsdR17 supE44 relA1 lacZΔM15 Δ(lacZYA–argF) deoR*$^+$ F′	InVitrogen
W3110	F$^-$ IN(*rrnD–rrnE*)1	Hill and Harnish (1981)
GM1	*Δ(arg–lac)U169 relA thi F′pro lac*	Sun and Webster (1986)
H1388	*aroB pro lac malT tsx thi exbB*::Tn*10*	Hantke and Zimmerman (1981)
GUC41	*thr-1 leuB6 tonA 21 lacY1 supE44 thi-1 Δ(exbB–metC)*	Guterman and Dann (1973)
CAG18475	*metC*::Tn*10*	Singer *et al.* (1989)
KP1032	W3110 (*tonB*::Kan)	This work
KP1127	W3110 (*tonBΔV17*)	This work
KP1131	KP1127 (*exbBA39E*)	This work
KP1134	KP1131 (*metC*::Tn*10*)	This work
KP1132	KP1127 (*exbB*::Tn*10*)	This work
KP1040	W3110 (*exbB*::Tn*10*)	This work
KP1100	W3110 (*exbBA39E*)	This work
KP1108	GM1 (*exbBA39E*)	This work
Plasmid		
pCRII	*kan amp lacZα*	InVitrogen
pKP172	*tonB*::Kan derivative of pKP925	Postle (1990)
pKP299	pACYC184 (*tonB*$^+$)	Larsen *et al.* (1993)
pKP310	pCRII (*tonBΔV17*)	This work
pKP311	pCRII (*exbB*$^+$)	This work
pKP312	pCRII (*exbBA39E*)	This work

Electrophoretic analysis of TonB protein

Cells grown in M9 medium were harvested at $A_{550} = 0.4$ (determined on a Spectronic 20 with a path length of 1.5 cm) by transferring 0.5-ml volumes to 0.5 ml of cold 10% (w/v) trichloroacetic acid (TCA) and incubating on ice for 15 min. Samples were centrifuged, and pellets washed once in 1.0 ml of 10 mM Tris-HCl (pH 8.0), then solubilized by incubation at 97°C for 5 min in concentrated 2× sample buffer

(Laemmli, 1970). Samples equivalent to 0.05 A_{550} (ml) of whole cells were subjected to electrophoresis on 11% SDS–polyacrylamide gels and the resolved proteins electrotransferred to Immobilon-P PVDF membranes. Immunoblot analyses were subsequently performed using TonB-specific mAbs and enhanced chemiluminescence, as previously described (Larsen *et al.*, 1993).

To examine the steady-state half-life of TonB in various strains, cells were grown in supplemented M9 to $A_{550} = 0.4$, at which point chloramphenicol was added to a final concentration of 100 $\mu g\,ml^{-1}$ to halt protein synthesis. Samples were removed at 0, 15, 30, 60 and 120 min after chloramphenicol addition, precipitated with TCA, and processed as above. Following immunoblot analysis, membranes were stained with Coomassie brilliant blue to verify equivalent loading of all lanes.

Proteinase K accessibility experiments were performed essentially according to the method of Randall and Hardy (1986), except that whole cells (grown in supplemented M9 without Fe) and sphaeroplasts (generated as described by Postle and Skare (1988)) were centrifuged for 5 min rather than 40 s. Samples were suspended in 50 mM Tris-acetate (pH 8.2), 20 mM $MgSO_4$, divided into two aliquots, and incubated with or without proteinase K (25 $\mu g\,ml^{-1}$) for 20 min at 4°C, treated for 2 min with 1 mM PMSF, then TCA-precipitated and processed as above.

Formaldehyde-mediated *in vivo* crosslinking was performed essentially as described by Skare *et al.* (1993). Cells were grown to log phase at 37°C in supplemented M9 lacking Fe, harvested and suspended in 0.1 M sodium phosphate buffer (pH 6.8) to 0.5 A_{550}, with formaldehyde subsequently added to a final concentration of 1.0% (v/v). Samples were incubated at room temperature (approx. 22°C) for 15 min, centrifuged, pellets suspended in 2× concentrated sample buffer and solublized at 60°C (rather than 37°C) for 10 min. Samples equivalent to 0.2 A_{550} (ml) of whole cells were resolved by SDS/11% polyacrylamide gel electrophoresis, with crosslinked species detected by immunoblot analysis.

Analyses of degradation products were performed as above, except that samples were resolved on 15% SDS–polyacrylamide gels (rather than 11% gels).

ϕ80 Adsorption assay

Assays were performed as previously described (Larsen *et al.*, 1993). Briefly, cells were grown at 37°C in supplemented M9 to $A_{550} = 0.4$. Cells were pelleted, suspended in 0.1 vol of 5.0 mM $CaCl_2$ containing ϕ80 at a multiplicity of infection of 1.0, and incubated at 37°C without agitation. Samples of 10 µl were harvested at selected time points, diluted in 1.0 ml LB containing 0.5% $CHCl_3$, and vortexed vigorously to release reversibly bound phage. Cells were removed by centrifugation and supernatants titred to quantify unadsorbed phages.

Amplification, cloning, and analysis of PCR products

Chromosomal DNA was prepared as described by Silhavy *et al.* (1984). Amplimers of *tonB* were synthesized by the PCR using the primers and method previously described (Larsen *et al.*, 1993). Amplimers of *exbBD* were similarly generated using oligomers based on the published sequence (Eick-Helmerich and Braun, 1989): oKP144 (5′-TTG TCC GCC ATG TCG GGA-3′), bases 319–336 of the mRNA-like strand; oKP147 (5′-TCA GGA ATC GAG CTA CGT CAC CGC-3′), bases 2081–2058 of the non-mRNA-like strand. Amplimers were gel-purified and ligated into pCRII plasmids as described in the TA cloning kit instruction manual (K2000-01). The products were used to transform competent INVαF′ *E. coli*, and the resultant transformants selected on LB/XG plates supplemented with kanamycin and ampicillin.

Sequencing was performed on double-stranded plasmid DNA by the dideoxy method of Sanger *et al.* (1977), using a Sequenase Kit (Version 2.0) and a set of *tonB*- or *exbBD*-specific primers. For *tonB*, sequences were determined from an equimolar pool of four independently isolated plasmids. For *exbBD*, plasmids were prepared from pools of 23–72 transformants and sequences determined.

Computer modelling

All sequence analyses were performed on the WSU VAXS system. Sequence comparisons, alignments, and helical wheel predictions were performed using programs in the GCG software package (Devereux *et al.*, 1985). Spacefilling models of potential transmembrane regions were developed by volume mapping with the MACROMODEL Program set from predicted minimal energy thermodynamic α-helical structures in chloroform (to mimic a hydrophobic environment) generated with BatchMin (V3.1) algorithms (Mohamadi *et al.*, 1990).

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