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The conserved proline-rich motif is not essential for energy transduction by *Escherichia coli* TonB protein

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Summary

TonB protein functions as an energy transducer, coupling cytoplasmic membrane electrochemical potential to the active transport of vitamin B_{12} and Fe(III)–siderophore complexes across the outer membrane of *Escherichia coli* and other Gram-negative bacteria. Accumulated evidence indicates that TonB is anchored in the cytoplasm, but spans the periplasmic space to interact physically with outer membrane receptors. It has been presumed that this ability is caused by a conserved $(Glu–Pro)_n$–$(Lys–Pro)_m$ repeat motif, predicted to assume a rigid, linear conformation of sufficient length to reach the outer membrane. Based on *in vitro* studies with synthetic peptides and purified FhuA outer membrane receptor, it has been suggested that this region contains a site that directly binds outer membrane receptors and is essential for energy transduction. We have found a TonB lacking the $(Glu–Pro)_n$–$(Lys–Pro)_m$ repeat motif (TonBΔ(66–100)). TonBΔ(66–100) is fully capable of irreversible φ80 adsorption, except under physiological circumstances where the periplasmic space is expanded. Based on the ability of TonBΔ(66–100) to interact with outer membrane receptors and components of the energy transduction apparatus under normal physiological conditions, it is evident that the TonB proline-rich region has no role in energy transduction other than to provide a physical extension sufficient to reach the outer membrane.

Introduction

TonB-dependent transport systems of *Escherichia coli* and presumably other Gram-negative organisms provide for the uptake of vitamin B_{12}, iron-bearing siderophores, group B colicins, and infection by bacteriophages T1 and φ80 (Matsushiro, 1963; Davies and Reeves, 1975; Frost and Rosenberg, 1975; Hantke and Braun, 1975; Pugsley and Reeves, 1976; Bassford *et al.*, 1977). TonB functions in these systems as an energy transducer, coupling cytoplasmic membrane proton motive force to high-affinity outer membrane receptors, facilitating active transport (Bradbeer, 1993). In addition to TonB, energy transduction requires several auxiliary proteins, including ExbB, potentially ExbD, and a putative proton translocator. Several models for the mechanism of TonB-dependent energy transduction have been proposed (for reviews see Kadner, 1990; Postle, 1990a; 1993; Bradbeer, 1991; Braun *et al.*, 1991), but detailed understanding of the process remains a distant goal.

Amino acid sequences deduced from nucleotide sequences of *tonB* genes from *E. 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) and *Enterobacter aerogenes* (Bruske and Heller, 1993) predict predominantly hydrophilic proteins with a potential membrane-spanning amino-terminal hydrophobic domain. A second, carboxy-terminal hydrophobic domain is predicted only for the *E. coli* and *S. typhimurium* TonB. Proteinase accessibility studies with intact sphaeroplasts (Postle and Skare, 1988; Hannavy *et al.*, 1990), and fusion protein studies (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 a majority of the protein (including the carboxy terminus) occupying the periplasmic space.

The cytoplasmic membrane location of TonB, the existence of mutations in TonB that weakly suppress mutations in a consensus motif (termed the TonB-box) shared by TonB-dependent outer membrane receptors (Heller *et al.*, 1988; Schoffler and Braun, 1989; Bell *et al.*, 1990), and the ability of synthetic TonB-box pentapeptide to inhibit TonB activity (Tuckman and Osburne, 1992) all imply physical interactions between TonB and TonB-dependent outer membrane receptors. Direct biochemical evidence for TonB interaction with outer membrane receptors came from studies using chemical cross-linking (Skare *et al.*, 1993). In that study, *in vivo* formaldehyde cross-linking generated a 195 kDa complex recognized

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by monoclonal antibodies (mAbs) specific for either TonB or FepA, and absent from *fepA* and *tonB* strains.

The predicted amino acid sequences of TonB protein from the Enterobacteriaceae examined all include a proline-rich region, consisting of $(Glu–Pro)_n$ and $(Lys–Pro)_m$ repeats, separated by a short spacer. This region is quite divergent in *Pseudomonas putida* TonB, which shares only about 28% identity with *E. coli* TonB and, unlike TonB from other species, cannot complement *tonB E. coli* (Bitter *et al.*, 1993). Nuclear magnetic resonance (NMR) studies of a synthetic polypeptide representing $(Glu–Pro)_n$–$(Lys–Pro)_m$ repeat motif of *S. typhimurium* TonB predicted an extended constrained conformation which could extend up to 100 Å, and presumably span the periplasmic space to directly contact outer membrane receptors (Evans *et al.*, 1986). Subsequent NMR studies (Brewer *et al.*, 1990) found that this synthetic polypeptide and a shorter polypeptide corresponding to the $(Lys–Pro)_6$ repeat could bind to a purified *E. coli* outer membrane ferrichrome receptor (FhuA), at a site exclusive of the receptor's TonB-box. The authors (Hannavy *et al.* 1990) incorporated this information into a model originally proposed by Holroyd and Bradbeer (1984) to suggest that TonB protein provides a mechanical linkage through which protein conformational changes transmit cytoplasmic membrane-derived energy to outer membrane receptors via an interaction between the proline-rich motif of TonB and a region of the receptor distinct from the TonB-box. This hypothesis was indirectly addressed in a study that attempted to dissect functional domains of TonB (Traub *et al.*, 1993). In that study, a plasmid-encoded TonB point mutation (replacement of Lys-91 by Glu within the $(Lys–Pro)_m$ repeat) and a plasmid-encoded *E. coli/Serratia* chimeric TonB bearing two $(Glu–Pro)_n$–$(Lys–Pro)_m$ repeat motifs were used to complement *tonB E. coli* strains. Resultant phenotypes were interpreted to suggest that the mutations did not alter TonB activity.

In the present study, a spontaneous *E. coli tonB* mutant that encodes a TonB lacking the proline-rich motif is described. Phenotypic characterization of this mutant demonstrates that the $(Glu–Pro)_n$–$(Lys–Pro)_m$ repeat motif is not essential for functional interaction with FhuA or other outer membrane receptors, but does contribute to the ability of TonB to span the periplasm. The implications of these findings concerning the mechanism of TonB function are discussed.

Results

Isolation and characterization of tonBΔ(66–100)

To facilitate the identification of structural features essential to TonB function, we have isolated mutants in several genetic backgrounds with leaky or absent TonB function, and for some, corresponding revertants and suppressors. In this study, spontaneous *tonB* mutants were selected by plating log phase MC4100 on LB containing 0.25 μg ml^{-1} pirazmonam, a TonB-dependent catechol monobactam (Nikaido and Rosenberg, 1990). Complementation of isolated pirazmonam-resistant derivatives with the TonB-encoding plasmid pKP299 confirmed a subset to be *tonB* strains. Revertants of *tonB* strains were subsequently selected on Cr^{100} plates, which support only $TonB^+$ phenotypes (Wang and Newton, 1970). Mutants and corresponding revertants were examined by several criteria. Polymerase chain reaction (PCR) analysis revealed that some $TonB^-$ phenotypes represented insertion events which interrupted *tonB*, with revertants resulting from excision of the inserted element. In most cases, excision restored *tonB* to wild-type size. A more informative event occurred in an insertion mutant hereafter referred to as KP1123. Restriction mapping of PCR products revealed that KP1123 contained an insertion element of about 1.3 kb between base pairs 431 and 653 of *tonB*. Two KP1123 revertants were isolated, one (KP1124) with a restriction enzyme profile indistinguishable from wild-type *tonB*, the other (KP1125) missing both the insertion element and *tonB* DNA sufficient to encode 35–37 residues of TonB (data not shown). Because this deletion mutant retained TonB function, it was characterized further.

Sequence determination revealed that KP1125 *tonB* and its flanking regulatory regions were identical to the wild type (corresponding to base pairs 245–1109 of the wild type), save for the absence of 105 base pairs (532–636), encoding a stretch of 35 amino acids which contains the $(Glu–Pro)_n$–$(Lys–Pro)_m$ repeat motif (specifically, residues 66–100, where Pro-65 represents either Pro-65 or Pro-100 of the wild type: Figs 1 and 2). Immunoblot analysis revealed that the TonB encoded by KP1125 migrated with an apparent molecular mass of 23 kDa, consistent with a sequence-determined molecular mass of 22.2 kDa (see Figs 4–6 for examples).

Function of TonBΔ(66–100)

The use of chromium resistance for isolation of the TonBΔ(66–100), KP1125 revertant suggested that it was functional. Similarly, while the *tonB*::IS mutant KP1123 hypersecreted enterochelin — indicative of iron starvation — the *tonBΔ(66–100)* revertant did not. Nevertheless, relative amounts of TonB were slightly higher for KP1125 in immunoblots (for example, Fig. 4), suggesting the possibility of compensatory overexpression. To estimate levels of TonBΔ(66–100) function, sensitivities of KP1125 to colicins and bacteriophage ϕ80 were compared. Like its parent MC4100, KP1125 was sensitive to ϕ80. Unlike the parent, it was slightly more resistant to the TonB-dependent colicins B and D (Table 1). As expected, the *tonB*::IS

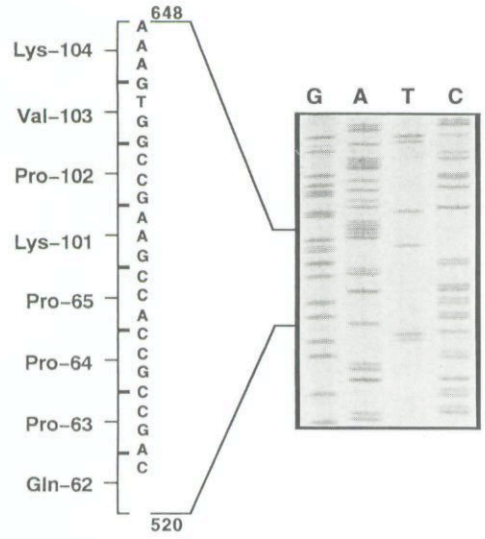

Fig. 1. Partial sequence of the coding strand of *tonBΔ(66–100)*, depicting the region affected by IS excision. Base and predicted amino acid residue positions correspond to wild-type sequences (Postle and Good, 1983).

strain KP1123 was indistinguishable from the *Δ(trp–tonB–ana)* strain KP1039, and the revertant lacking the insertion, KP1124, was identical to MC4100. All strains were equally sensitive to a panel of TonB-independent A group colicins (Table 1). To verify that the phenotype of KP1125 reflected only the Δ(66–100) mutation, KP1125 *tonB* was transduced into W3110 and GM1. The resultant strains (KP1096 and KP1097, respectively), like KP1125, were slightly more resistant to colicin B than their isogenic wild-type parents, indicating that the phenotypic differences noted were solely the product of the *tonBΔ(66–100)* allele (Table 1). The mild enhancement of colicin B resistance in *tonB*Δ(66–100) strains suggested subtle impairment of TonB function. A more quantitative measure of TonB function was obtained by comparing the ability of strains to irreversibly adsorb bacteriophage ϕ80. Consistent with the colicin assays, irreversible adsorption of phage was not evident in the original insertion mutant KP1123, or in KP1039 (*Δ(trp–tonB–ana)*). Surprisingly, irreversible ϕ80 adsorption was only mildly compromised in the TonBΔ–(66–100) strain KP1125, relative to its MC4100 parent (Fig. 3).

Interaction of TonBΔ(66–100) with other proteins

Relative chemical stability was evaluated by immunoblots comparing levels of TonBΔ(66–100) with those of wild-type TonB following the cessation of protein synthesis (Fig. 4). As shown in Fig. 4A, both wild-type and TonBΔ(66–100) had chemical half-lives greater than 60 min. This stability was dependent on the presence of a functional *exbB* gene, as demonstrated in Fig. 4B. In the absence of functional ExbB, the stabilities of both wild-type TonB and TonBΔ(66–100) were reduced, suggesting that TonB(66–100) can interact with ExbB. Phenotypically, both *exbB* derivatives retained low levels of TonB-dependent functions (both ϕ80 and colicin B sensitivity; data not shown), suggesting that TonBΔ(66–100) was capable of cross-talk with TolQ (Braun and Herrmann, 1993).

Our previous studies found that physical interactions

Table 1. Function of TonBΔ(66–100).

Strain (relevant genotype)	Entero-chelin[a]	Cr^{100b}	ϕ80[c]	Colicin Sensitivity[d]					
				Col B GID	D	Ia	K	A	N	E2
MC4100 (*tonB*$^+$)	–	+	+	3.2×10^{-4}	+	+	+	+	+	+
KP1039 (*Δ(trp–tonB–ana)*)	+	–	–	–	–	–	+	+	+	+
KP1123 (*tonB*::IS)	+	–	–	–	–	–	+	+	+	+
KP1124 (*tonB*$^+$)	–	+	+	3.2×10^{-4}	+	+	+	+	+	+
KP1125 (*tonBΔ(66–100)*)	–	+	+	1.6×10^{-3}	±	+	+	+	+	+
W3110 (*tonB*$^+$)	–	+	+	3.2×10^{-4}	ND	ND	ND	ND	ND	ND
KP1096 (W3110–*tonBΔ(66–100)*)	–	+	+	1.6×10^{-3}	ND	ND	ND	ND	ND	ND
GM1 (*tonB*$^+$)	–	+	+	3.2×10^{-4}	ND	ND	ND	ND	ND	ND
KP1097 (GM1–*tonBΔ(66–100)*)	–	+	+	1.6×10^{-3}	ND	ND	ND	ND	ND	ND

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 $\mu g\,ml^{-1}$ 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. 'ND' = not determined. 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 (GID) occurred is shown.

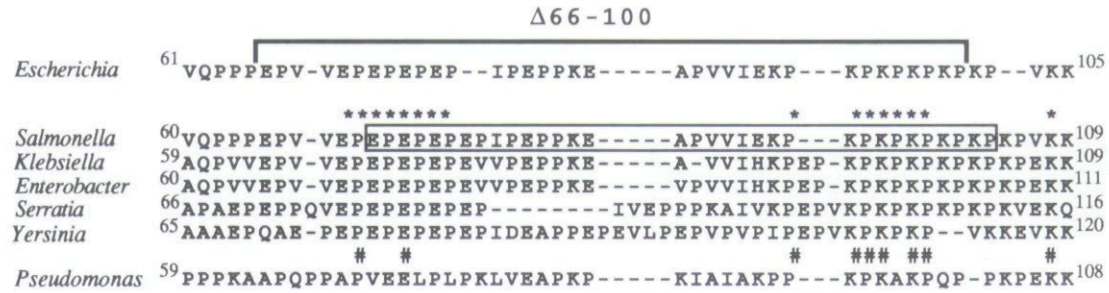

Fig. 2. Comparison of TonB $(Glu–Pro)_n$–$(Lys–Pro)_m$ repeat motif from the enterics *Escherichia coli* (Postle and Good, 1983), *Salmonella typhimurium* (Hannavy *et al.*, 1990), *Serratia marcescens* (Gaisser and Braun, 1991), *Yersinia enterocolitica* (Koebnik *et al.*, 1993), *Klebsiella pneumoniae* (Bruske *et al.*, 1993) and *Enterobacter aerogenes* (Bruske and Heller, 1993) and the corresponding region from *Pseudomonas putida* (Bitter *et al.*, 1993). One of several possible alignments for the region is presented. The region deleted in KP1125 (Δ66–100) is indicated in the *Escherichia* sequence, and the region corresponding to the synthetic peptide examined by Evans *et al.* (1986) and Brewer *et al.* (1990) is indicated in the *Salmonella* sequence. Residues absolutely conserved among enterics are indicated by '*', immediately above the *Salmonella* sequence; residues where this conservation extends to *Pseudomonas* are indicated by '#', immediately above the *Pseudomonas* sequence.

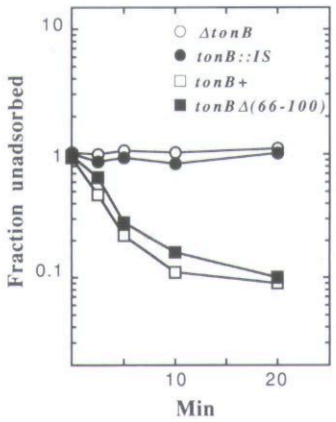

Fig. 3. Relative levels of TonB function of MC4100 and mutant derivatives. Irreversible adsorption of φ80, assayed as described in the *Experimental procedures*. Each point represents the mean of three independent assays. The higher the fraction unadsorbed, the lower the relative level of TonB function.

between TonB and other proteins were demonstrable by *in vivo* formaldehyde-mediated chemical cross-linking (Skare *et al.*, 1993). In the present study, this approach was used to determine whether TonBΔ(66–100) participated in similar interactions (Fig. 5). *In vivo* chemical cross-linking of KP1125 produced TonB-specific complexes similar to, albeit more rapidly migrating than, those produced with MC4100. The most prominent TonB-specific complexes of KP1125 included species of 180, 58, 50, and 29 kDa, versus species of 195, 77, 59, and 43.5 kDa for MC4100. One cross-linked species (50 kDa) was absent from samples of KP1125, *exbB*::Tn*10* (KP1102), corresponding to one species (59 kDa) similarly absent from samples of MC4100, *exbB*::Tn*10* (KP1044). *exbB*::Tn*10* strains are presumably missing both ExbB (26 kDa) and the downstream protein encoded by the operon, ExbD (15.5 kDa). For the TonBΔ(66–100) strain, the 50 kDa cross-linked species is of sufficient size to accommodate one TonB and one ExbB; however, the actual molecular composition of this species has not been determined. One species (180 kDa) was absent from cross-linked samples of KP1125, *fepA*::*kan* (KP1113), corresponding with the MC4100 195 kDa species absent from cross-linked samples of MC4100, *fepA*::*kan* (KP1113). Together, these results confirm that, like wild-type TonB, TonBΔ(66–100) physically interacts with ExbB, FepA, and other proteins.

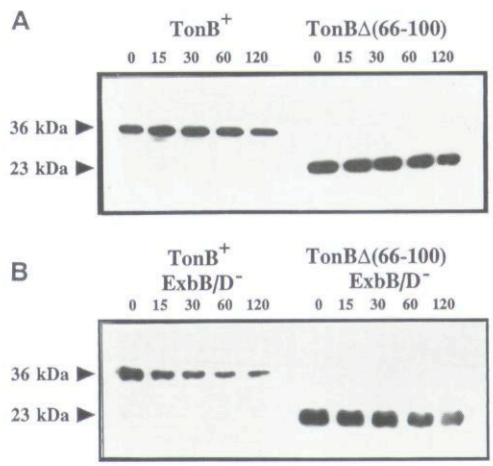

Fig. 4. Chemical stability of chromosomally encoded TonB in wild-type MC4100 and mutant derivatives. Western blots of TCA-precipitated samples from 0.05 OD_{550} cell equivalents at 0, 15, 30, 60, and 120 min post chloramphenicol addition. Resolved on SDS–11% polyacrylamide gels, electrotransferred and probed with 1:5000 mAb 4F1 (specific for an epitope between TonB residues 125 and 195, data not shown). Positions of the 36 kDa wild-type TonB and 23 kDa TonBΔ(66–100) proteins are indicated by arrows.

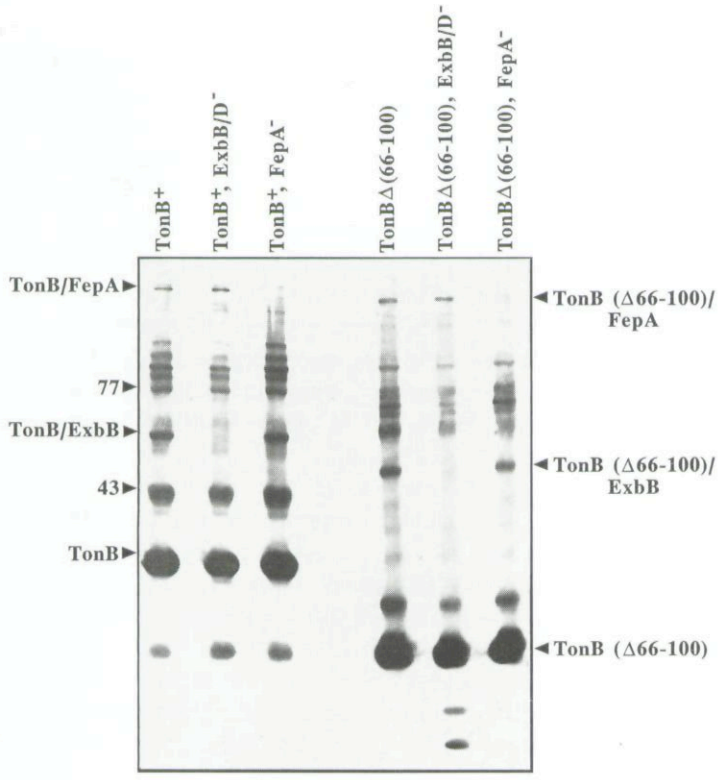

Fig. 5. *In vivo* cross-linking of Δ(66–100) and wild-type TonB. Cross-linked samples were loaded as 0.2 OD_{550} cell equivalents and resolved on SDS–11% polyacrylamide gels, electrotransferred and probed with 1:5000 mAb 4F1. Major complexes are identified either by their composition or apparent molecular weight at the sides of the panel. The positions of monomer TonB and TonB Δ(66–100) are also indicated.

Function of the proline-rich region

Since deletion of the proline-rich motif failed to alter the overall activity of TonB, the possibility that the proline-rich region serves only to extend the reach of TonB to the outer membrane was tested. TonB function was assayed under conditions where the distance between the cytoplasmic and outer membranes was varied (Fig. 6). When grown and assayed at either low or high osmolarity, the ability to adsorb φ80 irreversibly did not differ between MC4100 and KP1125 (Fig. 6A). Similarly, a shift from high to low osmotic conditions (with a resultant compression of the periplasm (Stock *et al.*, 1977)) did not provide for differential irreversible adsorption of φ80. However, when cells were shifted from low to high osmotic conditions (with a resultant expansion of the periplasm), MC4100 retained the ability to adsorb phage irreversibly, whereas irreversible phage adsorption by KP1125 was significantly compromised (Fig. 6B). Because the relative amounts of steady-state TonB did not vary significantly between high and low osmotic growth conditions (Fig. 6C), this difference appears to reflect an inability of TonBΔ(66–100) to span an expanded periplasmic space.

It should be noted that in this study the phenotypic differences between cells bearing TonBΔ(66–100) and cells bearing wild-type TonB were subtle. The results presented above emphasize the need to consider levels of *tonB* expression when characterizing mutants which retain some TonB function.

Discussion

TonB protein functions as an energy transducer, coupling cytoplasmic membrane proton motive force to outer membrane active transport processes. While the precise mechanism by which this is effected remains unknown, the predicted structure of the $(Glu–Pro)_n$–$(Lys–Pro)_m$ repeat motif, its *in vitro* ability to bind an outer membrane receptor (FhuA), and conservation between a variety of Gram-negative enterics have lead to the presumption that it has an essential role in TonB function (Evans *et al.*, 1986; Brewer *et al.*, 1990; Hannavy *et al.*, 1990; Postle, 1990). In the present study, the loss of an insertion element interrupting a wild-type *tonB* resulted in an allele, *tonBΔ(66–100)*, that encodes a TonB lacking all but the final Lys–Pro of the $(Glu–Pro)_n$–$(Lys–Pro)_m$ motif (Fig. 2). The deletion may have been caused by imprecise excision of the element or its removal owing to homologous recombination in this highly repetitive region. The migration of TonBΔ(66–100) in SDS–polyacrylamide gels was consistent with the calculated molecular mass of 22.2 kDa. This verified the previous assumption that the proline-rich region was responsible for the anomalous migration characteristics of TonB (Postle and Reznikoff, 1979; Postle and Good, 1983; Traub *et al.*, 1993). Surprisingly, TonBΔ(66–100) strains were able to perform a normal range of TonB-dependent processes, providing an opportunity to more critically examine the role of the $(Glu–Pro)_n$–$(Lys–Pro)_m$ repeat motif in TonB function.

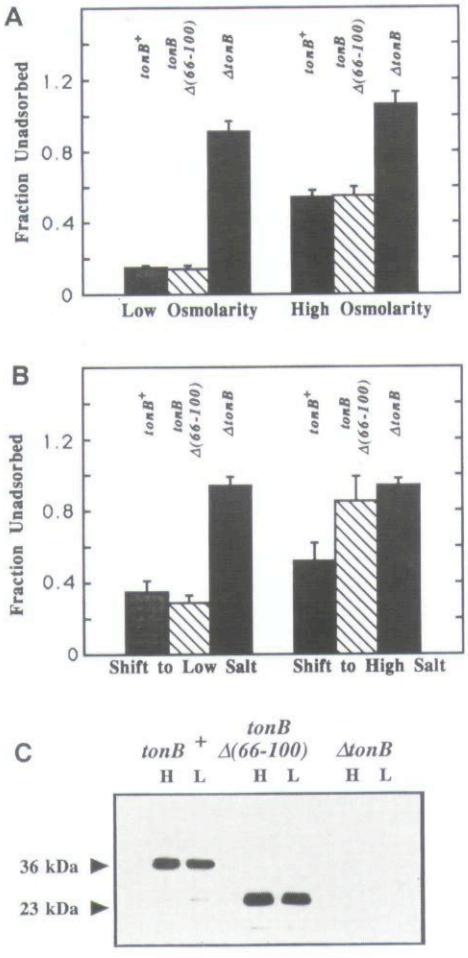

Fig. 6. TonB function of MC4100 and KP1125 under varied osmotic conditions.

A. Irreversible ϕ80 adsorption under conditions of low or high osmolarity. Values represent the fraction of ϕ80 unadsorbed (mean of three replications, error bars denote 1 standard deviation) following a 5 min incubation.

B. Irreversible ϕ80 adsorption following osmotic perturbation. Values presented as for (A).

C. Relative steady-state levels of TonB grown in high (H) or low (L) osmolarity medium. Western blot of 0.05 OD_{550} cell equivalents, probed with mAb 4F1 as per Fig. 3.

Because infection by a single bacteriophage will result in cell death, resistance to ϕ80 is a very sensitive indicator for the absence of TonB function, but lacks resolution. Conversely, irreversible adsorption of ϕ80 provides high-resolution evaluation of TonB activity levels. For example, irreversible adsorption of ϕ80 clearly distinguishes *exbB* mutations from *exbB*$^+$ and *tolQ* strains (Skare and Postle, 1991), whereas endpoint dilution assays with ϕ80 do not (Braun, 1989). In this study, ϕ80 sensitivity merely indicated that TonBΔ(66–100) was functional. However, the ability of TonBΔ(66–100) to mediate the irreversible adsorption of ϕ80 in a manner similar to wild-type TonB (Fig. 3) demonstrates a high level of TonB activity, and indicates that the reported *in vitro* binding of synthetic $(Glu–Pro)_n–(Lys–Pro)_m$ to FhuA (Brewer *et al.*, 1990) did not reflect a functional TonB–FhuA interaction. Similarly, sensitivity to group B colicins indicates that the $(Glu–Pro)_n–(Lys–Pro)_m$ repeat motif is not essential for the interaction of TonB with other outer membrane receptors.

Resistance to chromium and lack of enterochelin hypersecretion demonstrate that cells with TonBΔ–(66–100) are maintaining adequate levels of iron, however immunoblots revealed that TonBΔ(66–100) was present at slightly higher steady-state levels than seen for wild-type TonB. Because expression of *tonB*, even under aerobic conditions, is regulated on the basis of iron availability (Postle, 1990b), these results raised the possibility that TonBΔ(66–100) might transport iron-bearing siderophores less efficiently than wild-type TonB. Further, the slightly higher expression of TonBΔ(66–100), coupled with the slightly greater B group colicin resistance of TonBΔ(66–100) strains suggested that the specific activity of TonBΔ(66–100) was less than that of wild-type TonB.

One potential reason for the slightly reduced specific activity of TonBΔ(66–100) was the possibility that the proline-rich region participated directly in the interaction of TonB with other, auxiliary proteins. Mutations in the *exbB* gene have a leaky *tonB* phenotype (Hantke and Zimmerman, 1981) and chromosomally encoded TonB is chemically labile in the absence of ExbB (Skare and Postle, 1991). We found that, like wild-type TonB, the Δ(66–100) mutant appears to be stable in the presence of ExbB and destabilized by the absence of ExbB, although not to the same extent. The increased stability in exbB strains for TonBΔ(66–100) may be caused by loss of a key protease-susceptible site(s). This indicates the $(Glu–Pro)_n–(Lys–Pro)_m$ repeat motif is not involved in the stabilization of TonB. Further, the finding that *exbB, tonBΔ(66–100)* cells were capable of some TonB-dependent function indicates the $(Glu–Pro)_n–(Lys–Pro)_m$ repeat motif is not involved in molecular crosstalk with TolQ (Braun and Herrmann, 1993). These results are consistent with the proposed topology of ExbB, which is anchored in the cytoplasmic membrane and protrudes into the cytoplasm (Kampfenkel and Braun, 1993).

Additional evidence for a lack of $(Glu–Pro)_n–(Lys–Pro)_m$ repeat motif involvement in TonB–ExbB interaction came from chemical cross-linking studies. Cross-linked complexes incorporating TonBΔ(66–100) were detected which correlated with all of the major cross-linked species formed with wild-type TonB (Skare *et al.*, 1993). This correlation, along with the non-anomalous migration of TonBΔ(66–100), confirms the previous identification of a TonB–FepA complex and strengthens the argument that the 59 kDa wild-type (50 kDa TonBΔ(66–100)) species is a TonB–ExbB dimer. Interestingly, these experiments

revealed that none of the major crosslinked complexes represented TonB homodimers, since the apparent molecular mass for all major complexes of TonBΔ–(66–100) was only 9–19 kDa less than the corresponding wild-type complexes. Had any of these complexes been TonB dimers, the predicted molecular mass difference would be approximately 28 kDa. These results indicate that the $(Glu–Pro)_n$–$(Lys–Pro)_m$ is not necessary for formation of the TonB-containing intermolecular complexes detected by chemical crosslinking, and does not play a direct role in the interactions of TonB with these molecules.

The above results suggest that deletion of the $(Glu–Pro)_n$–$(Lys–Pro)_m$ repeat did not interfere with the ability of TonB to engage in essential interactions with other proteins of the energy transduction complex. It was therefore likely that the minor reduction of TonB activity noted for KP1125 and its derivatives reflected an impediment of TonBΔ(66–100) interaction with the outer membrane. This possibility was addressed by assaying TonB-dependent function under physiological conditions that altered periplasmic volume, thus changing the periplasmic distance TonB was required to span (Stock *et al.*, 1977). The activities of mutant and wild-type TonB were similar under conditions of normal or reduced periplasmic volume, however the activity of TonBΔ(66–100) was significantly diminished under conditions of expanded periplasmic volume. The simplest interpretation of this observation is that absence of the $(Glu–Pro)_n$–$(Lys–Pro)_m$ repeat results in a shorter TonB molecule which, under normal physiological conditions, can still reach outer membrane receptors a significant amount of the time, but is unable to traverse an extended periplasmic space. The differential activity of wild-type and TonBΔ(66–100) under these conditions strengthens the argument (Postle, 1990a) that TonB probably does not work through pre-existing membrane adhesion sites (Bayer, 1979).

The results of this study are consistent with the model for TonB function originally proposed by Holroyd and Bradbeer (1984), and the prediction that the $(Glu–Pro)_n$–$(Lys–Pro)_m$ repeat motif assumes a conformation that facilitates the extension of TonB across the periplasmic space (Evans *et al.*, 1986). The assertion that this motif directly interacts with outer membrane proteins (Brewer *et al.*, 1990; Hannavy *et al.*, 1990) was not substantiated. The evidence presented in this study suggests that the proline-rich region serves only to provide a physical extension of the TonB molecule, allowing it to traverse the periplasm. Thus, by default, it seems apparent that TonB interacts with outer membrane proteins through its carboxy terminus. Two alternative carboxy-terminal regions of TonB that could directly interact with outer membrane receptors are (i) the motif that includes Glu-160: the site where *tonB* suppressors of TonB-box mutations in outer membrane receptors occur (Heller *et al.*, 1988; Schoffler and Braun, 1989; Bell *et al.*, 1990), and (ii) the extreme carboxy-terminal region of TonB. The ability of a synthetic pentapeptide corresponding to the TonB-box consensus to inhibit TonB function suggests this region is involved with TonB interaction (Tuckman and Osburne, 1992); however, the possibility that the TonB-box is not periplasmically exposed (Murphy *et al.*, 1990) and the recent description of a TonB-dependent outer membrane receptor of *Pseudomonas putida* (PupB) that lacks an identifiable TonB-box suggests that such interactions are not essential for TonB function (Koster *et al.*, 1993). Conversely, the extreme carboxy-terminal region of TonB is required for function (Roof *et al.*, 1991; Anton and Heller, 1991). Carboxy-terminal TonB deletion mutants do not exhibit a dominant negative phenotype (Anton and Heller, 1993), whereas a TonB cleavage mutant which releases the carboxy-terminal region into the cytoplasm does (J. C. Jaskula and K. Postle, unpublished observations), suggesting that this region directly interacts with outer membrane receptors. Examination of the deduced carboxy-terminal sequences of various TonBs predicts that a possible amphipathic α-helix exists at the carboxy terminus of TonB (amino acid (aa) 202–220) in a region highly conserved among enteric species, but significantly divergent in *Pseudomonas* (Fig. 7). This region could mediate interactions between the carboxy-terminal region of TonB and the outer membrane. If so, the absence of this region could in part explain why, unlike the TonB of enteric species, *P. putida* TonB cannot function in *E. coli* (Bitter *et al.*, 1993).

The TonB system shares numerous characteristics with the analogous Tol system, a second, outer membrane transport system exploited by group A colicins and some bacteriophages (Webster, 1991). TonB can engage in crosstalk with certain Tol system components (Braun, 1989), probably via a transmembrane region conserved

```
                  *  *    *  **  *
Escherichia       ANMFEREVKNAMRRWRYE

Salmonella        ANMFEREVKNAMRKWRYE
Klebsiella        ANMFEREVKSAMRRWRYQ
Enterobacter      ANMFERDVKTAMRKWRYE
Serratia          RNMFEREVKQAMRKWRYE
Yersinia          RNTFEREVKQVMRKWRFE

Pseudomonas       SAALDRATLEMIRR-AGT
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Fig. 7. Comparison of predicted peptide sequence of the *E. coli* TonB region (aminmo acid (aa) 202–219) that may assume the configuration of an amphipathic helix, with the corresponding regions from the TonBs of other enterics and *P. putida*. *E. coli* residues predicted to form the hydrophobic face of the helix are indicated by '*'. The alignment of the *P. putida* sequence relative to *E. coli* is identical to that used by Bitter *et al.* (1993).

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

Strain/ Plasmid	Relevant properties	Source/Reference
E. coli strain		
INVαF'	*recA1 hsdR17 supE44 relA1 lacZΔM15 F'*	InVitrogen
MC4100	*araD 139 rpsL150 deoC1 ptsF25rbsR flbB5301 Δ(arg–lac)U169 relA thi*	Casadaban (1976)
W3110	F^- IN(*rrnD–rrnE*)1	Hill and Harnish (1981)
GM1	*Δ(arg–lac)U169 relA thi F'pro lac*	Sun and Webster (1986)
KP1092	GM1 (*fepA::kan*)	Skare *et al.* (1993)
CH483	*Δ(trp–tonB–opp–galU–ana)*	C. Higgins
H1388	*aroB pro lac malT tsx thi exbB*::Tn*10*	Hantke and Zimmerman (1981)
CAG5054	*trpB*::Tn*10*	Singer *et al.* (1989)
KP1123	MC4100 (*tonB*)	This work
KP1124	KP1123 *tonB*$^+$	This work
KP1125	KP1123 (*tonB(Δ66–100)*)	This work
KP1126	KP1125 (*trpB*::Tn*10*)	This work
KP1044	MC4100 (*exbB*::Tn*10*)	This work
KP1102	KP1125(*exbB*::Tn*10*)	This work
KP1039	MC4100 (*Δ(trp–tonB–opp–galU–ana)*)	This work
KP1096	W3110 (*trpB*:Tn*10 tonBΔ(66–100)*)	This work
KP1097	GM1 (*trpB*::Tn*10 tonBΔ(66–100)*)	This work
KP1112	MC4100 (*fepA::kan*)	This work
KP1113	KP1125 (*fepA::kan*)	This work
Plasmid		
pCRII	*kan amp lacZα*	InVitrogen
pES3	*colB*	V. Braun
pColA-CA31	*colA*	A. P. Pugsley
pColIa-CA53	*colIa*	A. P. Pugsley
pColE2-P9	*colE2*	A. P. Pugsley
pColK-K235	*colK*	A. P. Pugsley
pColN-284	*colN*	A. P. Pugsley
pKP299	pACYC184 (*tonB*$^+$)	This work
pKP309	pCRII (*tonB(Δ66–100)*)	This work

between TonB and its analogue, TolA (Koebnik *et al.*, 1993). A major structural feature of TolA is a long (262 residue), central α-helical region, which allows TolA to span the periplasmic space (Levengood et al., 1991). While TonB lacks a homologous region (Postle and Good, 1983), it appears that the proline-rich region has evolved to serve the same function.

Experimental procedures

Chemicals and reagents

Pirazmonam (batch NN010NA) was the generous gift of Bristol-Meyers Squibb, Inc. Anti-TonB monoclonal antibodies (mAbs) were prepared in female BALB/c mice as previously described (Skare *et al.*, 1993). Media components were purchased from Difco Laboratories. Enhanced chemiluminescence (ECL) immunoblot kit and horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin were purchased from Amersham Inc. Formaldehyde was purchased from J.T. Baker, Inc. Immobilon-P was purchased from Millipore Corp. Acrylamide was purchased from Fisher Biotech. Bis-acrylamide was purchased from Bio-Rad Laboratories. SDS was purchased from Schwartz/Mann. Sequenase and Sequenase Kit reagents (Version 2.0) were purchased from United States Biochemical Corp. TA cloning kits were purchased from InVitrogen Corp. Thermalase was purchased from International Biotechnologies, Inc. The oligonucleotides oKP125 and oKP126 were synthesized by Genosys Biotechnologies, Inc. Gelase was purchased from Epicenter Technologies. All other reagents were purchased from Sigma Chemical Co.

Bacterial strains and plasmids

The principal bacteria and plasmids used are listed in Table 2. All bacteria are derivatives of *E. coli* K-12. Strain KP1123 is a spontaneous pirazmonam-resistant mutant of MC4100, selected by plating log-phase cells on LB containing 0.25 μg ml^{-1} pirazmonam. Strains KP1124 and KP1125 were subsequently isolated as spontaneous revertants of KP1123 capable of growth in the presence of 100 μM Cr^{3+}. Strain KP1126 was constructed by infecting KP1125 with a P1 lysate (Miller, 1972) from CAG5054 (*trpB*::Tn*10*) and screening tetracycline-resistant recombinants by immunoblot analysis for retention of the KP1125 *tonB* allele. KP1096 and KP1097 were constructed by infection of W3110 and GM1, respectively, with a P1 lysate from KP1126. Transduction of the KP1125 *tonB* allele was selected by resistance to tetracycline and confirmed by immunoblot analysis. KP1044 and KP1102 were constructed by transduction using a P1 lysate from H1388 to infect MC4100 and KP1125, respectively. Transduction of the *exbB*::Tn*10* allele was selected by resistance to tetracycline and confirmed by testing for increased resistance to colicin B. KP1112 and KP1113 were constructed by transduction using a P1 lysate from KP1092 to infect MC4100 and KP1125, respectively. Transduction of the *fepA*::*kan* allele was selected by resistance to kanamycin and confirmed by testing for resistance to colicin B. KP1039 (MC4100 Δ(*trp–tonB–opp–galU–ana*)) was constructed by transduction from CH483 (a kind gift from C. F. Higgins) with selection for simultaneous resistance to colicin B and φ80, and screening for tryptophan auxotrophy.

Media

Strains were maintained on LB agar. Liquid cultures were grown with aeration at 37°C in either LB broth, M9 minimal salts (supplemented with 0.4% glucose, 0.2% casamino acids, 40 μg ml^{-1} tryptophan, 0.5 μg ml^{-1} $FeCl_3$•$6H_2O$, 0.4 μg ml^{-1} thiamine, 0.1 mM $MgSO_4$, and 0.5 mM $CaCl_2$); low-osmolarity medium (1.0 mM KH_2PO_4, 1.5 mM $(NH_4)_2SO_4$, 0.08 mM $MgCl_2$, supplemented with 0.4% glucose, 0.2% casamino acids, 40 μg ml^{-1} tryptophan, 0.4 μg ml^{-1} thiamine, and 0.5 mM $CaCl_2$); or high-osmolarity medium (low-osmolarity medium, supplemented with 300 mM NaCl). Chromium agar (Cr^{100}) consisted of 1.5% agar in M9 minimal salts supplemented with 0.3% 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} 5-bromo-4-chloro-3-indolyl-β-D-galactoside on LB plates 1–2 h prior to use.

Preparation of colicins

Crude preparations of colicins were made essentially as described by Pugsley and Reeves (1977). Briefly, colicin production was induced by addition of mitomycin C to a final concentration of 1 μg ml^{-1} to log-phase cells containing colicin-encoding plasmids (Table 2). Induced cells were incubated and processed as described, with sonication at 5–10 20 s intervals. Preparations were stored at –70°C until use.

Electrophoretic analysis of TonB protein

Cells grown in M9 medium were harvested at OD_{550} = 0.4 by transferring 0.5 ml volumes to equal volumes of cold 10% (w/v) trichloroacetic acid (TCA) and incubating on ice for 15 min. Samples were centrifuged, and pellets washed once in 1 ml of 10 mM Tris-HCl, pH 8.0, then solublized by incubation at 97°C for 5 min in concentrated 2× sample buffer (Laemmli, 1970). Precipitation with TCA prior to solublization is essential for determining steady-state levels of TonB, preventing the proteolytic degradation of TonB that normally occurs in samples prepared for electrophoresis (Skare *et al.*, 1993). Samples equivalent to 0.05 OD_{550} per ml of whole cells were subjected to SDS–11% polyacrylamide gel electrophoresis and the resolved proteins electrotransferred to Immobilon-P PVDF membranes. Immunoblot analyses were subsequently performed using TonB-specific mAb and enhanced chemiluminescence, as previously described (Skare *et al.*, 1993).

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

Formaldehyde-mediated *in vivo* cross-linking was performed as previously described (Skare *et al.*, 1993). Briefly, log-phase cells were suspended in 0.1 M sodium phosphate buffer, pH 6.8, to 0.5 OD_{550} per ml and formaldehyde added to a final concentration of 1.0% (v/v). Samples were incubated at room temperature (approximately 22°C) for 15 min, centrifuged, pellets suspended in 2× concentrated sample buffer and solublized at 60°C for 10 min. Samples equivalent to 0.2 OD_{550} per ml of whole cells were resolved by SDS–11% polyacrylamide gel electrophoresis, with cross-linked species detected by immunoblot analysis.

φ80 adsorption assay

Assays were performed essentially as described by Skare and Postle (1991), with the following modifications. Cells were grown at 37°C in liquid culture to OD_{550} = 0.4. Cells were pelleted, suspended in 0.1 volumes of medium containing 5.0 mM $CaCl_2$ and φ80 at an M.O.I. of 0.5–1.0 (instead of 5.0 mM $CaCl_2$ alone, with subsequent addition of φ80), 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 pelleted by centrifugation and supernatants titred to quantify unadsorbed phage.

Amplification, cloning, and analysis of PCR products

Chromosomal DNA was prepared as described by Silhavy *et al.* (1984). All nucleotide positions are based on the 1697 bp fragment sequenced by Postle and Good (1983). Amplimers of *tonB* were synthesized by PCR, using primers specific for regions flanking the *tonB* gene (oKP125 (5'-CACTGATCCTGATCGTCTTGCCTTA-3'), bases 244–268 of the sense strand; oKP126 (5'-AGTATGTCGCGGTTGATCCTGAAGG-3'), bases 1148–1124 of the antisense strand). Amplifications were performed by standard methods, with resultant amplimers analysed directly or gel purified for cloning. Purified amplimers were ligated into pCRII plasmids as described in the TA cloning kit instruction manual (K2000-01), products used to transform competent INVaF' *E. coli*, and transformants selected on LB/XG plates supplemented with kanamycin and ampicillin.

Sequencing was performed on plasmid DNA by the dideoxy method of Sanger *et al.* (1977), using a Sequenase Kit (Version 2.0) and a set of *tonB*-specific primers. Initial sequence data were obtained from an equimolar pool of four independently isolated plasmids. Subsequently, one plasmid (pKP309) was selected for further use, and sequenced as above.

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