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Evidence for a TonB-dependent energy transduction complex in *Escherichia coli*

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Summary

Escherichia coli **TonB protein is required for the active transport of vitamin B_{12} and Fe(III)-siderophore complexes across the outer membrane, infection by bacteriophages T1 and Φ80, and sensitivity to B-group colicins. TonB appears to function as an energy transducer in these processes, coupling cytoplasmic membrane electrochemical potential to receptors in the outer membrane. Previous reports have demonstrated that chromosomally encoded TonB is functionally unstable in the absence of protein synthesis (half-life ≈15–30 minutes) and have shown that plasmid-encoded, overexpressed TonB is chemically unstable (half-life ≈5 minutes). In contrast, this study has shown that chromosomally encoded TonB was chemically stable for greater than 90 minutes while maintaining its functional instability. These data suggest that proteolytic degradation of TonB protein is not the basis of its functional instability. Auxiliary proteins such as ExbB also play a role in TonB-dependent energy transduction. In this study, we have shown that the chemical half-life of chromosomally encoded TonB in an** ***exbB*****::Tn*****10*** **mutant was reduced at least 18-fold, suggesting that TonB is a part of a cytoplasmic membrane complex that includes, at the minimum, ExbB. These results also suggest that the chemical instability of plasmid-encoded TonB resulted when the TonB/ExbB ratio was too high and are consistent with previous observations that plasmid-encoded ExbB can stabilize plasmid-encoded TonB. The** ***exbB*** **mutation also resulted in a significant decrease in TonB function as measured by the ability of cells to adsorb bacteriophage Φ80. In a previous study, strains carrying a** ***tolQ*** **nonsense mutation in combination with an** ***exbB*** **mutation were observed to mimic a** ***tonB*** **phenotype, suggesting that either ExbB or TolQ can alternatively activate TonB. In contrast, we have shown that neither the chemical half-life nor the function of TonB are affected by the** ***tolQ*** **mutation, and thus, unlike ExbB, TolQ plays a minimal role in TonB-dependent processes.**

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Introduction

In *Escherichia coli*, Fe(III)-siderophore complexes, vitamin B_{12}, and group-B colicins, as well as infection by bacteriophages T1 and Φ80, exhibit a common requirement for cytoplasmic membrane energy and TonB protein in order to traverse the outer membrane via specific outer membrane receptors (Matsushiro, 1963; Davies and Reeves, 1975; Frost and Rosenberg, 1975; Hantke and Braun, 1975; Pugsley and Reeves, 1976; Bassford *et al.*, 1977). TonB is postulated to function as an energy transducer in those processes, coupling cytoplasmic membrane energy to active transport across the outer membrane (for reviews, see Postle, 1990b; Kadner, 1990; Bradbeer, 1991). Although various models for the mechanism of TonB-dependent energy transduction have been presented (Hancock and Braun, 1976; Reynolds *et al.*, 1980; Holroyd and Bradbeer, 1984; Hannavy *et al.*, 1990), the details of this process are poorly understood.

The amino acid sequence deduced from the nucleotide sequence of the *tonB* gene predicts a predominantly hydrophilic protein with two potential membrane-spanning domains (Postle and Good, 1983; Postle and Skare, 1988). Protease accessibility studies in intact sphaeroplasts (Postle and Skare, 1988; Hannavy *et al.*, 1990) together with *tonB–phoA* (Roof *et al.*, 1991) and *tonB–bla* fusion studies (Hannavy *et al.*,1990) indicate that TonB is anchored in the cytoplasmic membrane by an uncleaved hydrophobic amino-terminal signal sequence, with the remainder of the protein (including the carboxy-terminal hydrophobic region) protruding into the periplasmic space. The localization of TonB, the biochemical interaction of a proline-rich region of TonB with purified FhuA protein (Brewer *et al.*, 1990), and the existence of mutations in TonB that suppress defects in outer membrane receptors (Heller *et al.*, 1988; Schoffler and Braun, 1989; Bell *et al.*, 1990) all suggest that TonB interacts physically with TonB-dependent outer membrane receptors.

In addition to TonB, auxiliary proteins are required for energy transduction between membranes. The best characterized is ExbB, a 26 kDa envelope protein (Hantke and

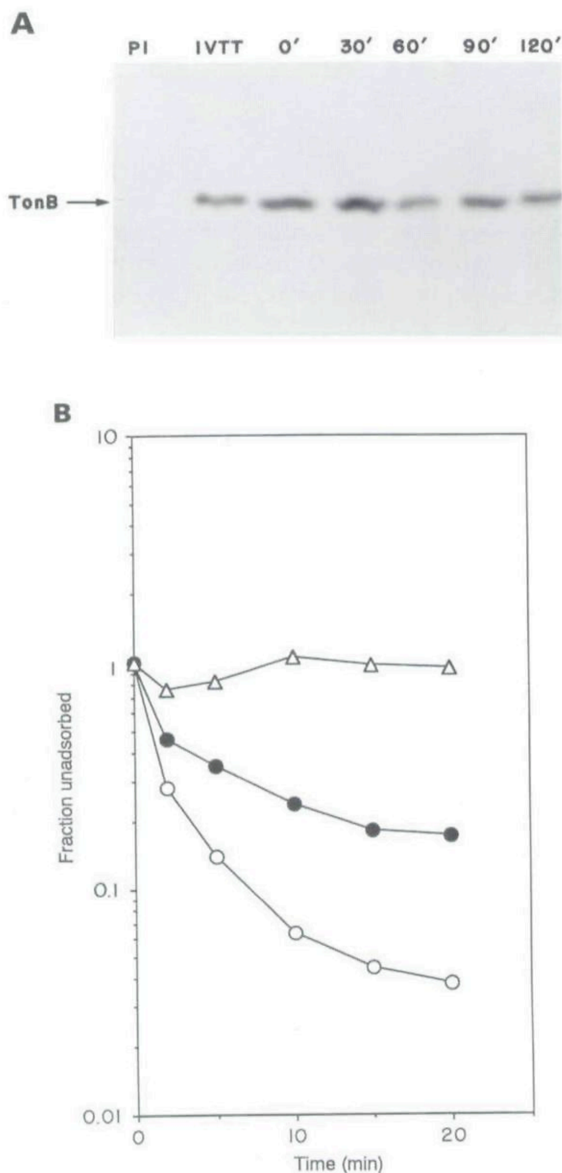

Fig. 1. Chemical and functional stability of chromosomally encoded TonB in W3110.
A. An autoradiogram of [^{35}S]-methionine-labelled, immunoprecipitated TonB from strain W3110 is shown. Cells were pulse-labelled for 1 min and chased with unlabelled methionine for the times indicated as described in the *Experimental procedures*. PI indicates a 0 min sample immunoprecipitated with pre-immune sera. IVTT indicates TonB encoded by plasmid pKP166, labelled *in vitro*, and included on the autoradiogram as a marker. The position of TonB is indicated by an arrow.
B. Irreversible adsorption of bacteriophage Φ80 was assayed as described in the *Experimental procedures*. Briefly, bacteria and bacteriophages were incubated together at 37°C and, at the times indicated, samples were removed and diluted to remove reversibly adsorbed bacteriophages. Bacteriophages remaining in the supernatant were titered and the results expressed as the fraction of unadsorbed bacteriophage. The higher the fraction unadsorbed, the lower the relative level of TonB function. ○, W3110; ●, W3110 incubated with chloramphenicol one hour prior to assay; △ KP1026 (a *tonB*::Kan^R derivative of W3110).

Zimmerman, 1981). The deduced amino acid sequence of ExbB protein has been determined and is consistent with its localization in the cytoplasmic membrane (Eick-Helmerich and Braun, 1989). Like TonB, ExbB is predicted to protrude into the aqueous periplasm. Mutations in *exbB* result in reduced, but not entirely absent, levels of TonB-dependent processes (Hantke and Zimmerman, 1981). Other candidates for auxiliary proteins include TolQ (26% amino acid identity to ExbB with 79% similarity amongst the remaining amino acids), which is required for group-A colicin sensitivity (Sun and Webster, 1987), ExbD, a 15.5 kDa cytoplasmic membrane protein genetically linked to *exbB* (Eick-Helmerich and Braun, 1989), and TolR (25% amino acid identity to ExbD with 70% similarity amongst the remaining amino acids; Eick-Helmerich and Braun, 1989; Sun and Webster, 1987). Simultaneous mutations in *exbB* and *tolQ* mimic a *tonB*$^-$ phenotype (Braun, 1989).

The functional half-life of TonB activity in the absence of protein synthesis is 15–30 minutes (Bassford *et al.*, 1977; Kadner and McElhaney, 1978). Apparently consistent with that observation, the chemical half-life of plasmid-encoded TonB is approximately 5 minutes (Postle and Skare, 1988; Fischer *et al.*, 1989). Experiments to determine the chemical half-life of chromosomally encoded TonB have been previously hampered by the inability to detect it. In this report we have shown by immunoprecipitation of pulse-labelled proteins that, in contrast with plasmid-encoded TonB, chromosomally encoded TonB was chemically stable. In addition, we examined the effects of mutations in *exbB* and *tolQ* on TonB chemical stability and function. Results from those experiments provided evidence that TonB was part of an energy-transduction complex that included ExbB. The results also indicated that, compared with ExbB, the role of TolQ in TonB-dependent energy transduction was minor.

Results

Chromosomally encoded TonB protein is chemically stable

Earlier studies have suggested that TonB is both functionally (Bassford *et al.*, 1977; Kadner and McElhaney, 1978) and chemically (Postle and Skare, 1988; Fischer *et al.*, 1989) unstable. We wanted to determine whether the chemical half-life of chromosomally encoded TonB was equally short. A typical pulse-chase analysis of chromosomally encoded TonB is shown in Fig. 1A. Immune sera specifically immunoprecipitated chromosomally encoded TonB, pre-immune sera did not, and, as expected (Postle and Skare, 1988), the *in vivo* labelled, immunoprecipitated TonB comigrated with TonB labelled *in vitro*. The

Table 1. Reversible adsorption of Φ80.

Bacterial strain	Fraction Φ80 unadsorbed
W3110	0.37 ± 0.03
W3110+CAM	0.36 ± 0.05
KP1026	0.38 ± 0.06
KP1026+CAM	0.36 ± 0.05
KP1026+Fe	0.53 ± 0.04
GM1	0.30 ± 0.12
GM1+CAM	0.30 ± 0.07
KP1037	0.18 ± 0.02
KP1037+CAM	0.14 ± 0.02
TPS13	0.30 ±.0.06
TPS13+CAM	0.31 ± 0.06
KP1038	0.29 ± 0.02
KP1038+CAM	0.27 ± 0.05

Assays were conducted as described in the *Experimental procedures*. Briefly, bacteria and bacteriophages were incubated together for 20 min at 4°C and the bacteria with reversibly adsorbed bacteriophages were pelleted. The supernatants were titred and expressed as the fraction of unadsorbed bacteriophages. 'CAM' indicates that the bacteria were incubated for one hour in chloramphenicol prior to assay. 'Fe' indicates that the bacteria were grown in the presence of 100 μM Fe.

labelled band corresponding to full-length TonB was not immunoprecipitated in a *tonB*::Kan^R strain (data not shown). However, in contrast with plasmid-encoded TonB, chromosomally encoded TonB in W3110 was stable for over two hours at 37°C. In separate experiments (data not shown), the presence or absence of either 10 mM dinitrophenol, 0.5 mM $CaCl_2$, 50 nM vitamin B_{12}, or 200 μM dipyridyl did not alter the chemical half-life of TonB shown in Fig. 1. Elimination of chloramphenicol from the chase also had no effect on TonB stability, indicating that continued protein synthesis did not lead to proteolysis of chromosomally encoded TonB.

Chromosomally encoded TonB activity is functionally unstable

All TonB-dependent processes studied to date decay with similar kinetics following the addition of protein synthesis inhibitors (Kadner and McElhaney, 1978), suggesting that continued protein synthesis is required for TonB function. To determine if TonB activity was functionally unstable under the conditions of the pulse-chase experiments described above, we compared the ability of cells to irreversibly adsorb bacteriophage Φ80 in the presence and absence of a protein synthesis inhibitor. Infection of *E. coli* by Φ80 requires both FhuA (the outer membrane receptor for Φ80, Wayne and Neilands, 1975) and TonB (Matsushiro, 1963). Initially, Φ80 binds reversibly to FhuA in an energy-independent, TonB-independent fashion. In order to proceed to the second (irreversible) step in adsorption, both TonB and cytoplasmic membrane energy are required (Hancock and Braun, 1976). Therefore irreversible infection of *E. coli* by bacteriophage Φ80 serves as a means of assessing relative levels of functional TonB. Briefly, the assay involves infection with Φ80, dilution of infected cells to liberate the reversibly adsorbed phages, centrifugation of the bacteria, and titering of the supernatant to quantify the phages that did not adsorb irreversibly. As expected, a *tonB* mutant (KP1026) was unable to adsorb Φ80 irreversibly (Fig. 1B). However, after 20 minutes approximately 96% of the phages were irreversibly adsorbed to the *tonB*$^+$ strain, W3110. When protein synthesis was terminated by addition of chloramphenicol one hour prior to infection, the amount of irreversibly adsorbed Φ80 after 20 minutes was reduced approximately fivefold.

In order to demonstrate that the reduction in irreversible phage adsorption was TonB-specific, we assayed reversible, FhuA-specific adsorption by incubating *E. coli* with Φ80 at 4°C (Table 1). Under these conditions, W3110, as well as all other strains used in this study, exhibited only reversible adsorption similar to the *fhuA*$^+$, *tonB*$^-$ control, KP1026. The amount of adsorption decreased when iron was added, suggesting that expression of the outer membrane receptor, FhuA, was repressed. In addition, incubation with chloramphenicol prior to assay did not alter the efficiency of Φ80 reversible adsorption. These results indicated that FhuA was neither functionally nor chemically labile under the conditions of this assay and suggested that the reduction in irreversible adsorption of Φ80 was due to a decrease in the intracellular pool of functional TonB.

TonB is chemically stabilized by ExbB

Mutants in *exbB* show reduced but not entirely abolished levels of several TonB-dependent processes (Hantke and Zimmerman, 1981). Recent work has also shown that overproduced TonB can be chemically stabilized by the concomitant overproduction of ExbB (Fischer *et al.*, 1989). To compare the effects of mutations in *exbB* and *tolQ* (next section) on the chemical half-life of chromosomally encoded TonB, the chemical half-life of chromosomally encoded TonB was determined in the parental strain GM1. The half-life of 90 minutes (Fig. 2A) was slightly less than that observed in W3110 (>120 minutes), but was nonetheless longer than the cell doubling time in supplemented M9 media (approximately 45 minutes). TonB activity in GM1 (Fig. 3) was also functionally unstable in the absence of protein synthesis (eightfold decrease following 20 minutes of irreversible Φ80 adsorption). In the *exbB*::Tn*10* derivative of GM1 (KP1037), the chemical half-life of TonB was significantly decreased to between 2 and 5 minutes (Fig. 2B). The same half-life was also observed in the W3110 *exbB*::Tn*10* derivative (data not shown). Since the Φ80 adsorption assay can be used to evaluate relative levels of functional TonB, we

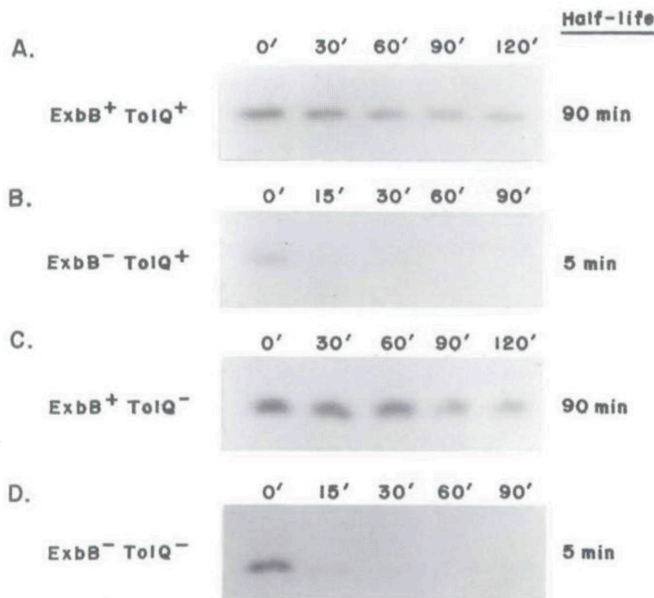

Fig. 2. Chemical stability of chromosomally encoded TonB in GM1 and mutant derivatives. An autoradiogram of [^{35}S]-methionine-labelled, immunoprecipitated TonB from GM1 (A, wild-type), KP1037 (B, *exbB*::Tn*10*), TPS13 (C, *tolQ*), and KP1038 (D, *exbB*::Tn*10*, *tolQ*). Cells were pulse-labelled for 1 min and chased with unlabelled methionine and chloramphenicol for the times indicated, and the samples immunoprecipitated as described in the *Experimental procedures*. Relevant genotypes and an estimation of the chemical half-life of chromosomally encoded TonB are indicated.

also determined the effect of the *exbB*::Tn*10* mutation on levels of Φ80 adsorption after 20 minutes. Consistent with the short chemical half-life of TonB observed in that strain, TonB activity was reduced 18-fold in the *exbB*::Tn*10* strain relative to the its parent, GM1. Thus the *exbB*::Tn*10* mutation affected both the chemical stability and the function of TonB.

TonB is not chemically stabilized by TolQ

Mutations in *tolQ* render cells insensitive to several group-A colicins and filamentous bacteriophages. In contrast with single mutants in either *tolQ* or *exbB*, an *exbB, tolQ* double mutant, like a *tonB* mutant, is completely resistant to undiluted colicin B and Φ80 (Braun, 1989). If, as previously proposed (Braun, 1989; Braun *et al*., 1991), TolQ and ExbB are interchangeable alternatives in TonB-dependent processes, the absence of TolQ might have an effect comparable to the absence of ExbB on the chemical half-life of TonB. However, the *tolQ* mutation had no effect on the chemical stability of chromosomally encoded TonB (compare Fig. 2 panels A and C). Consistent with that result, in the *exbB, tolQ* double mutant, chromosomally encoded TonB was proteolytically degraded with a half-life apparently characteristic of the *exbB* mutation alone (Fig. 2D). The double mutant still synthesized TonB but, as shown in Fig. 4, it was completely inactive.

The results of Φ80 adsorption by the *tolQ* strain indicated that it adsorbed Φ80 as effectively as its GM1 parent (Fig. 4). Thus the *tolQ* mutation had no effect on either the chemical half-life or the function of TonB. As expected, the *exbB, tolQ* double mutant did not irreversibly adsorb Φ80 (consistent with its TonB$^-$ phenotype).

Discussion

TonB protein appears to function as an energy transducer, coupling the cytoplasmic membrane proton-motive force to active transport across the outer (and essentially non-energized) membrane. While the mechanism of energy transduction is unknown, chromosomally encoded

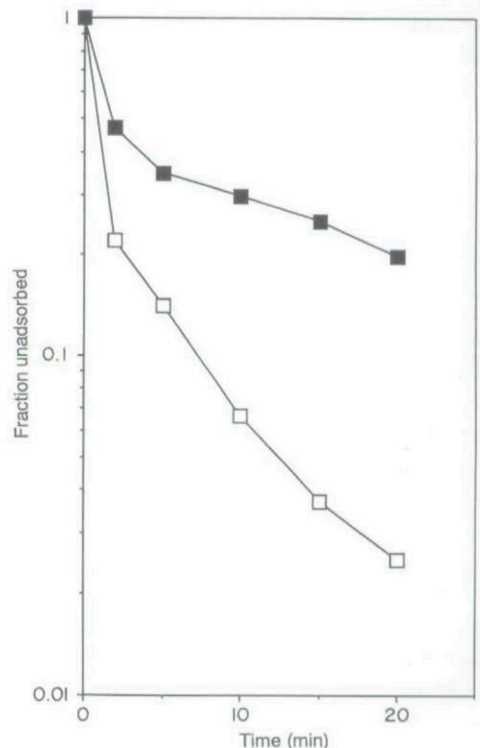

Fig. 3. Functional stability of chromosomally encoded TonB in GM1. Assays to assess irreversible adsorption of Φ80 were conducted as described in Fig. 1B and the *Experimental procedures*. □, GM1; ■, GM1 incubated with chloramphenicol one hour prior to assay.

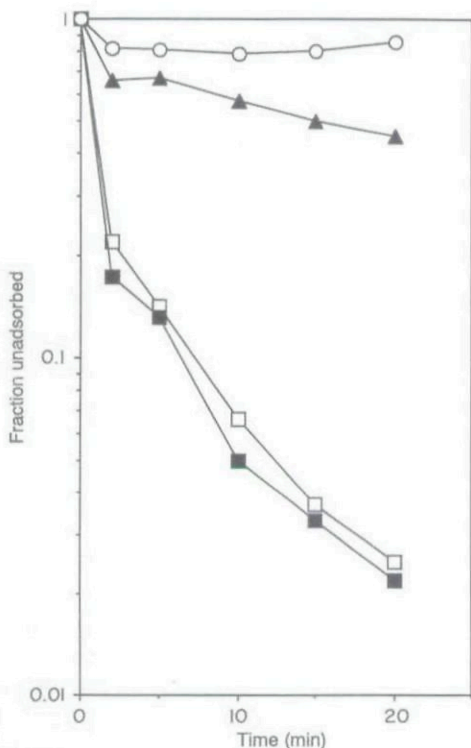

Fig. 4. Relative levels of TonB function in GM1 and mutant derivatives. Irreversible adsorption of bacteriophage Φ80 was assayed as described in Fig. 1, (lower part) and the *Experimental procedures.* □, GM1 (parent); ■, TPS13 (*tolQ*); ▲, KP1037 (*exbB*::Tn*10*); O, KP1038 (*exbB*::Tn*10*, *tolQ*).

TonB has previously been demonstrated to have a short functional half-life in the absence of continued protein synthesis, suggesting that some aspect of energy transduction was chemically unstable. In those studies, strains carrying a *tonB*(am) mutation and a temperature-sensitive amber suppressor were shifted to the non-permissive temperature and their rates of vitamin B_{12} transport, which decayed rapidly, were determined (Bassford *et al.*, 1977). Subsequently it was demonstrated that inhibition of protein synthesis resulted in the decay of all TonB-dependent processes (Kadner and McElhaney, 1978). In both studies, TonB activity decayed with a half-life of 15–30 minutes. The data concerning the somewhat shorter chemical half-life of plasmid-encoded TonB are in apparent agreement with observations on the functional half-life (Postle and Skare, 1988; Fischer *et al.*, 1989). However, recent observations that overexpression of nearly all proteins believed to play roles in TonB-dependent processes—ExbB (Fischer *et al.*, 1989), FhuA (Günter and Braun, 1990), and TolQR (Braun *et al.*, 1991)—results in the chemical stabilization of plasmid-encoded TonB suggests that it would be more appropriate to analyse the chemical half-life of chromosomally encoded TonB. In addition, a negative gene dosage effect is observed in many *tonB* plasmid clones (Mann *et al.*, 1986).

Here we show that chromosomally encoded TonB is chemically stable but functionally unstable in two strains of *E. coli*. In W3110, which exhibits a doubling time of 70 minutes (data not shown) in the medium used in these experiments, the chemical half-life was greater than two hours (Fig. 1A) and the decrease in the ability to adsorb Φ80 after exposure to chloramphenicol for one hour was approximately fivefold (Fig. 1B). In GM1, which exhibits a doubling time of 45 minutes (data not shown), the chemical half-life was approximately 90 minutes (Fig. 2A) and the decrease in the ability to adsorb Φ80 after exposure to chloramphenicol for one hour was approximately eightfold (Figure 4). The differences between strains may be due to differences in their growth rates. When cells are growing more slowly, the effects of chloramphenicol on decay of TonB function might be less apparent and the chemical decay of TonB might be decreased. Nevertheless, in neither case was the chemical half-life of TonB capable of accounting for the degree of functional turnover observed after exposure to chloramphenicol for one hour. Since it is clear that chromosomally encoded TonB is chemically stable, a reasonable explanation for the functional instability in the absence of protein synthesis is that TonB or some component interacting with TonB can be activated or inactivated by reversible chemical modification, and that some molecule (other than TonB) involved in energy transduction has a 15–30 minute chemical half-life.

These results seem to contradict earlier studies in which TonB synthesis was halted by shifting a *tonB*(am), *supD*(ts) strain to the non-permissive temperature (Bassford *et al.*, 1977). After the temperature shift, TonB-dependent rates of vitamin B_{12} transport rapidly decayed. Our finding that TonB is chemically stable appears to be contradictory. An alternative explanation could, however, rest in the shift from synthesis of full-length TonB to the synthesis of truncated, non-functional TonB. It is possible that the TonB amber fragment could somehow interfere with TonB-dependent energy transduction: we are currently testing this possibility.

Mutations in the *exbB* gene have a leaky *tonB* phenotype (reduced but not entirely absent levels of TonB-dependent processes; Hantke and Zimmerman, 1981). Because an additional distal gene, *exbD*, is probably transcribed as part of the *exbB* operon, existing chromosomal mutations do not distinguish between loss of ExbB and loss of ExbD. Thus the *exbB*::*Tn*10 phenotype may be due to loss of both proteins or ExbD alone. Both *exbB* and *exbD* appear to encode cytoplasmic membrane proteins (Eick-Helmerick and Braun, 1989) whose transcriptional regulation is similar to that of *tonB* (Braun, 1985; Postle,

1990a). Overproduction of ExbB stabilizes plasmid-encoded TonB, but overproduction of ExbD does not. In addition, overproduction of ExbB cannot compensate for a *tonB* mutation, but overproduction of TonB can compensate for an *exbB*::Tn*10* mutation (Fischer *et al.*, 1989).

The short chemical half-life (≈5 minutes) we observed for chromosomally encoded TonB in *exbB*::Tn*10* strains was significantly shorter than its chemical half-life in wild-type strains (>90 minutes). These results suggest that a proper ratio of TonB and ExbB must exist to protect otherwise protease-sensitive sites in TonB, and they imply that TonB and ExbB, at the minimum, form an energy-transduction complex within the cytoplasmic membrane. Since the uncoupler dinitrophenol had no effect on the chemical half-life of TonB, cytoplasmic membrane energy was apparently not required for association. The chemical instability of plasmid-encoded TonB resulted when the TonB:ExbB ratio was too high. Once that ratio fell to normal levels, a significantly longer half-life was observed, as demonstrated by the essentially biphasic nature of the decay, whereby residual TonB persists to the end of the chase period (Postle and Skare, 1988; J. Skare, unpublished). Other examples of the chemical instability of overproduced membrane proteins include MotB and SecY (Wilson and Macnab, 1990; Matsuyama *et al.*, 1990): as with TonB, overproduction of either protein alone results in its rapid proteolytic degradation. However, if MotA is overproduced with MotB, or if SecE is overproduced with SecY, both are rendered chemically stable. Such studies have been used to suggest physical interactions between either MotA and MotB or SecE and SecY. Thus proteolytic degradation of individual membrane proteins appears to result from their unbalanced overexpression relative to the proteins with which they form a complex. These observations may reflect a common motif for membrane proteins found in complexes that extend into the protease-rich periplasm. Our results also suggest an explanation for the *exbB/D* phenotype: reduced quantities of TonB protein result in reduced levels of TonB activity. The actual role of ExbB in energy transduction, however, will be difficult to assess as long as its absence results in significantly decreased levels of TonB protein.

Previous results have shown that TolQ, which is involved in sensitivity to A-group colicins, and ExbB exhibit similarity at the nucleotide and amino acid sequence level (Eick-Helmerich and Braun, 1989). Subsequent analysis of an *exbB*, *tolQ* double mutant indicated that it mimics a *tonB* mutant, and led to the hypothesis that TolQ and ExbB could serve alternatively in TonB-dependent energy transduction (Braun, 1989). In those studies it was not possible to achieve significant differentiation of the effects of an *exbB* mutation from a *tolQ* mutation. Both appeared to be as sensitive to bacteriophage Φ80 as the parental strain in a spot titre assay. In contrast, our results using a Φ80 adsorption assay demonstrated that while a mutation in *exbB* significantly reduced TonB activity, a mutation in *tolQ* had no effect. These observations are consistent with the fact that no *tolQ* mutations have been reported to arise from selections for either *tonB* or *exbB* mutants.

Braun *et al.* (1991) also demonstrate that, like overexpressed ExbB or FhuA, overexpressed TolQ can chemically stabilize overexpressed TonB. If TolQ protein stabilizes TonB protein, one would predict that the absence of TolQ would, like the absence of ExbB, result in the rapid degradation of chromosomally encoded TonB. Contrary to that expectation, our results demonstrate that the absence of TolQ has no effect on the chemical half-life of chromosomally encoded TonB protein. Our results are also consistent with the lack of an observable effect of *tolQ* mutations on TonB function. Since TonB-dependent energy transduction is probably a finely balanced multicomponent system, results in which only a subset of the components is overproduced may not accurately reflect the normal *in vivo* interactions.

In this study, an *exbB, tolQ* mutant still synthesized TonB protein that is completely inactive in terms of Φ80 adsorption. It may be that the proteolytic fragments of TonB generated in an *exbB* strain can completely inhibit energy transduction, but only in a *tolQ* strain. It may be that TolQ participates in energy transduction, but in more subtle ways than ExbB. While it is important to understand that the lack of a *tolQ* effect on TonB chemical stability or function is not proof of a lack of physical interaction with TonB, our results do indicate that ExbB and TolQ are not interchangeable proteins. We are currently pursuing a deeper understanding of the role of ExbB in energy transduction that may ultimately reveal the source of the TolQ effect.

Experimental procedures

Bacteria, plasmids, and media

E. coli strains W3110 (F^- IN(*rrnD–rrnE)1*)), KP1026 (W3110·λRS205(*tonB*168), *tonB::Kan*^{R}; Postle, 1990a), GM1 (*ara*, Δ(*pro–lac*), *thi* ,F', *pro*, *lac*; Sun and Webster, 1986), TPS13 (GM1 carrying a nonsense mutation in *tolQ*; Sun and Webster, 1986), KP1037 (GM1 *exbB*::Tn*10*), KP1038 (TPS13 *exbB*::Tn*10*), and H1388 (*aroB*, Δ(*pro–lac*), *malT*, *thi*, *tsx*, *exbB*::Tn*10;* Hantke and Zimmerman, 1981) were used in this study. Strains KP1037 and KP1038 were constructed using a P1 lysate (Miller, 1972) from H1388 to infect GM1 and TPS13, respectively. Transduction of the *exbB*::Tn*10* allele was confirmed by resistance to 20 μg ml^{-1} tetracycline and decreased sensitivity to colicin B relative to GM1. Bacteriophage $\Phi80_{vir}$ was used in all experiments.

Plasmid pES3 was generously provided by Volkmar Braun (Pressler *et al.*, 1986). Plasmid pKP166 was constructed by cloning the 1697bp *Hin*cII fragment containing *tonB* into

M13mp7 (Messing *et al.*, 1981). The resulting plasmid was digested with *Eco*RI and the 1730 bp *tonB*+ fragment cloned into the unique *Eco*RI site in pKP924 (Skare *et al.*, 1989) such that *tonB* is regulated by both the *trp* promoter and its own promoter.

All strains were maintained on Luria-Bertani (LB) or YT agar (Miller, 1972). Liquid cultures were grown at 37°C in LB broth or M9 minimal salts lacking methionine as described by Postle and Skare (1988), except that the minimal medium used in these studies also contained 0.5 mM $CaCl_2$ (to be referred to as labelling media for the remainder of this report).

Preparation of colicin B

A crude preparation of colicin B was prepared according to Pugsley and Reeves (1977), with minor modifications. Colicin B production was induced by the addition of mitomycin C (Sigma Chemicals) to a final concentration of 1 μg ml^{-1} in cells containing pES3. Cells were incubated and processed as described with sonication at five 20-s intervals. The colicin preparation was stored at −70°C.

Labelling and processing of whole cells

Cells were grown in labelling medium, pulse-labelled (Postle and Skare, 1988) and converted to sphaeroplasts (Hardy *et al.*, 1988) as described previously, except that labelled cells were simultaneously chased with a 660-fold molar excess of unlabelled methionine and with chloramphenicol at a final concentration of 100 μg ml^{-1}. The final sphaeroplast pellet was suspended in 25 μl of Laemmli sample buffer (Laemmli, 1970), heated at 95–100°C for 5 min, and immunoprecipitated using antiserum obtained from rabbits immunized with Incomplete Freund's Adjuvant (Sigma Chemicals) containing purified TrpC–TonBG26D protein (Skare *et al.*, 1989).

Immunoprecipitation

TonB was immunoprecipitated from [^{35}S]-methionine-labelled protein lysates essentially as described by Postle and Skare (1988), except for the following alterations. The 25 μl protein lysate was diluted in 750 μl of RIPA buffer (Postle and Skare, 1988) and centrifuged at 30 000 × *g* for 20 min (4°C) to pellet the insoluble material. The cleared lysate was incubated with 5 μl of pre-immune or post-immune sera and incubated on ice for one hour. The immune complexes were collected by addition of 200 μl of a 25% slurry of Protein A Sepharose (Pierce) and agitated continuously for 30 min at 4°C. The final wash of the Sepharose beads was removed with a 26-gauge Hamilton syringe. The remaining beads were suspended in 15 μl of sample buffer (Laemmli, 1970), heated at 95–100°C for 5 min, and the entire volume removed with the 26-gauge Hamilton syringe and then loaded onto an sodium dodecyl sulphate 11%-polyacrylamide gel. The samples were electrophoresed, the gel treated with Autofluor (National Diagnostics), dried at 80°C, and exposed to XAR-5 film at −70°C for 2–7 d.

Irreversible adsorption of Φ80

Assays were performed essentially as described (Kadner and McElhaney, 1978). Briefly, cells (20 ml volume) were grown in labelling medium in duplicate at 37°C to an OD_{550} of 0.4. One culture was then harvested by centrifugation and suspended in 500 μl of 5 mM $CaCl_2$ and infected with Φ80 at a multiplicity of infection of 0.25–1.0. Chloramphenicol at either 100 or 200 μg ml^{-1} was added to the duplicate culture and incubation at 37°C was continued with shaking for an additional hour. The cells were then harvested and infected as described above. The infected cells were incubated at 37°C without agitation and 10 μl volumes removed at 0, 2, 5, 10, 15, and 20 min post-infection and diluted in 1 ml of LB with 0.5% $CHCl_3$ and vortexed vigorously to release reversibly bound bacteriophages. The cells were pelleted by centrifugation and the supernatants titered to determine the amount of unabsorbed Φ80 present. Results represent averages of at least six independent assays.

Reversible adsorption of Φ80

Cells were grown and infected as described in the previous section except that the adsorption of Φ80 was conducted for 20 min at 4°C to prevent temperature-dependent, irreversible adsorption of Φ80 while maintaining temperature-independent, reversible adsorption (Hancock and Braun, 1976; Garen and Puck, 1951; Garen, 1954). Infection at 4°C does not alter reversible binding at the Φ80 outer membrane receptor FhuA but eliminates TonB-dependent, irreversible adsorption (Hancock and Braun, 1976). The assay was conducted by pelleting cells with reversibly bound bacteriophages and titering the supernatants to determine the amount of unadsorbed Φ80 remaining. Results presented represent averages of triplicate cultures, each of which was assayed in triplicate.

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