TonB System, In Vivo Assays and Characterization

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Abstract

The multiprotein TonB system of Escherichia coli involves proteins in both the cytoplasmic membrane and the outer membrane. By a still unclear mechanism, the proton-motive force of the cytoplasmic membrane is used to catalyze active transport through high-affinity transporters in the outer membrane. TonB, ExbB, and ExbD are required to transduce the cytoplasmic membrane energy to these transporters. For E. coli, transport ligands consist of iron-siderophore complexes, vitamin B12, group B colicins, and bacteriophages T1 and ϕ80. Our experimental philosophy is that data gathered in vivo, where all known and unknown components are present at balanced chromosomal levels in the whole cell, can be interpreted with less ambiguity than when a subset of components is overexpressed or analysed in vitro. This chapter describes in vivo assays for the TonB system and their application.

Introduction

In Gram-negative bacteria, the TonB system is responsible for energizing transport events at the outer membrane using the proton-motive force of the cytoplasmic membrane (reviewed in Braun, 2003; Faraldo-Gomez and Sansom, 2003; Postle and Kadner, 2003; Wiener, 2005). In the Escherichia coli K-12 cytoplasmic membrane, TonB exists in a complex with two integral cytoplasmic membrane (CM) proteins, ExbB and ExbD. At the outer membrane (OM), TonB directly contacts and transduces energy to outer membrane active transporters (FepA, FecA, FhuA, FiuA, Cir, and FhuE for the transport of iron–siderophores; BtuB for the transport of vitamin B12). TonB-gated transporters (TGTs) consist of 22-stranded β barrels with the lumen of each barrel occluded by a ~150 residue amino-terminal domain, known as the cork. The function of TonB appears to involve release or rearrangement of the cork, and its associated bound ligand, from the barrel into the periplasmic space (Ma et al., 2007; Devanathan and Postle, unpublished results). From there, siderophores or vitamin B12 are captured by binding proteins and delivered to ABC transporters in the cytoplasmic membrane. The TGTs also serve as outer membrane receptors for a variety of bacteriophages and protein toxins called colicins (reviewed in Braun et al., 2002).
In vitro study of the TonB system has been limited by an inability to fully reconstitute the system. The amino terminus of TonB is essential for its activity (Jaskula et al., 1994; Larsen and Postle, 2001; Larsen et al., 1999); however, it has not yet been possible to purify full-length TonB with the amino terminus intact. Nonetheless, the TonB carboxy terminus (~90 residues) has been crystallized and its nuclear magnetic resonance (NMR) structure determined, both alone (Chang et al., 2001; Kodding et al., 2005; Sean Peacock et al., 2005) and in the presence of TGTs (Pawelek et al., 2006; Shultis et al., 2006). Although the structures have provided important food for thought, the correlation between in vitro and in vivo results is not good, suggesting that the most meaningful and unambiguously interpretable data will come from in vivo studies for the time being. For just one example out of several, while in vivo studies identify the five aromatic amino acids of the TonB carboxy terminus as virtually the only residues playing a role in recognition of TGTs, in all of the crystal/NMR structures solved to date, the side chains of the aromatic residues are buried (reviewed in Postle and Larsen, 2007; Wiener, 2005).

Selection For and Against the tonB Gene

The tonB gene is unusually versatile in that both mutant and wild-type tonB can be directly selected. Mutations are selected by resistance to bacteriophage and colicins or by resistance to TonB-dependent antibiotics (Hantke and Braun, 1978; Larsen et al., 1993; Nikaido and Rosenberg, 1990). Selection against tonB mutants uses chromium (Larsen et al., 1994; Wang and Newton, 1969a).

Selecting Against tonB

Strains lacking tonB are highly pleiotropic, with phenotypes that include a lack of high-affinity iron transport, lack of vitamin B12 transport, tolerance to group B colicins, and tolerance to bacteriophages T1 and ø80. It is worth remembering that, due to overall iron starvation, other aspects of E. coli physiology for tonB strains are abnormal as well. For example, enzymes involved in aromatic amino acid synthesis are derepressed because most aromatic amino acid pathway substrates are being diverted to the synthesis of enterochelin (McCray and Herrmann, 1976). Similarly, under iron starvation conditions, certain tRNAs are undermodified such that their impaired function is capable of deattenuating the tryptophan operon (Buck and Griffiths, 1982).

Selection with a single TonB-dependent bacteriophage or colicin will yield mutations that inactivate either the receptor for that phage or TonB. Mutations are not generally recovered in ExbB or ExbD, primarily because
TolQ and TolR proteins can replace their function sufficiently that the cells will be killed by the selective agents (Bradbeer, 1993; Braun, 1989; Skare et al., 1993). Selection in a TolQ/R background is complicated by the compromised state of the OM and the compensatory secretion of capsular material that characterizes these strains. Secretion of capsular material can itself confer physical protection against phage. Selection with multiple TonB-dependent bacteriophage or colicins that recognize different TGTs (e.g., colicin B, which uses FepA as a receptor together with bacteriophage ϕ80, which uses FhuA as receptor) specifically targets mutations to tonB. Selections for tonB mutants have dropped into disuse largely because the occurrence of knockout missense mutants has been limited to the amino-terminal TonB transmembrane domain, with the majority of those recovered involving insertions, large deletions, or nonsense mutations. A note of caution about using bacteriophage T1: Although bacteriophage T1 is a TonB-dependent bacteriophage, its use is not advised except under special containment conditions. The extraordinary stability of this phage has led to the maxim: “if you are using T1 in your lab, pretty soon everyone in the building will be using T1”—not a good way to maintain collegial bridges.

Selecting for TonB⁺

Even though iron is an essential nutrient, tonB strains can grow without problem because of the degree to which iron contaminates chemicals/growth media and the apparent existence of a low-affinity iron uptake system. However, because chromium competes with the low-affinity iron uptake system, tonB strains are unusually sensitive to chromium (Wang and Newton, 1969a). Thus resistance to chromium can serve as a selection for wild-type TonB system function. This selection has been used successfully to obtain second site exbB suppressors of tonB missense mutations (Larsen et al., 1994).

To select for TonB⁺ phenotypes, a culture of tonB cells is grown to saturation, washed twice in 1× M9 salts, and plated on chromium agar plates (1.5% Difco agar in M9 minimal salts medium supplemented with 0.3% vitamin-free casamino acids, 0.2% glucose, 4.0 μg ml⁻¹ thiamine, 10 mM MgSO₄, and 100 μM CrCl₃). In the early days, growth of tonB strains in minimal media required the addition of citrate, even though E. coli does not use citrate as a carbon source and a tonB strain cannot transport ferric-citrate. This was most likely due to chromium contamination in the media and is no longer a problem (Wang and Newton, 1969b). It is important to use vitamin-free casamino acids and other chemicals with the lowest Fe contamination possible for this selection to work, as supplemental iron allows growth regardless of tonB status.
Precautions for Experiments Where TonB System Proteins Are Expressed from Plasmids

Mutations in genes are most easily generated and mobilized in plasmids. In a multicomponent system, it is often most straightforward to assay mutant proteins expressed from plasmids in strains where the cognate gene has been knocked out (Datsenko and Wanner, 2000). However, when this approach is taken to study the TonB system, it is important to ensure that only chromosomal levels of all components are expressed, from either the chromosome or a regulatable promoter.

*TonB and ExbD Exhibit Dominant-Negative Gene Dosage Effects*

In the initial efforts to clone *tonB* (early 1980s), three research groups (Higgins, Kadner, and Postle) all had made the same discovery, a fact that became apparent once notes were informally compared: it was impossible to select TonB plasmid clones by growth on chromium plates in a *tonB* background (the standard selection for wild-type TonB). This phenomenon was later described more fully where it was noted that overexpression of TonB from multicopy plasmids caused a dominant decrease in detectable TonB activity (Mann et al., 1986). TonB was shown to be unstable in the absence of ExbB also thus supplying the rationale for dominant negativity (Fischer et al., 1989; Skare and Postle, 1991). When TonB is overexpressed, there is insufficient ExbB to stabilize the excess TonB. The proteolyzed TonB peptides become competitive at sites where wild-type TonB would normally interact (Howard et al., 2001; Jaskula et al., 1994). It is therefore important to maintain balanced chromosomal levels of TonB, ExbB, and ExbD (Higgs et al., 2002a) due to the requirement for ExbB to stabilize both ExbD and TonB. Indeed ExbD also exhibits dominant-negative gene dosage, while ExbB does not (Bulathsinghala and Postle, unpublished observations). Chromosomal levels of expression can be accomplished by the use of genes expressed from the pBAD promoter (Guzman et al., 1995).

*One or More Proteins in the TonB System Remain to be Identified*

One might wonder whether overexpression of TonB, ExbB, and ExbD proteins simultaneously could lead to interpretable data about the function(s) of the complex. Such an approach is impeded by the apparent existence of at least one unidentified protein in the system. Even though TonB is a stable protein (*t*\(_{1/2}\) = 90–120 min), in the absence of continued protein synthesis TonB activity decays with a half-life of 15–20 min (Kadner and McElhaney, 1978; Skare and Postle, 1991). The other known proteins of the TonB system, ExbB, ExbD, and the outer membrane transporter FepA, are chemically stable for over 90 min when expressed at normal chromosomal
levels (Skare and Postle, 1991; unpublished results). Proton-motive force, which energizes the TonB system, remains undisturbed when protein synthesis is inhibited (Skare and Postle, unpublished results). The conclusion is that there must be an unidentified protein of short half-life that participates in energizing TonB. The unidentified protein has not turned up in selections, leading to speculation that this unstable protein has either redundant function or is essential for cell growth. Either way, until the unidentified protein has been fully characterized, there is a possibility that simultaneous overexpression of ExbB, ExbD, and TonB might exceed the capacity of the unidentified protein. Where TonB system protein levels are not chromosomal, skewed assay results cannot be excluded.

Phenotypic Assays for the TonB System

Because the TonB system is involved in signal transduction (with the signal being energy), several of the assays for its activity are of necessity indirect and based on the role that TonB plays in the transport of ligands and colicins, and irreversible phage adsorption (Hancock and Braun, 1976). Different assays have different windows of sensitivity (Fig. 1). Spot titers of phage or colicin preparations on lawns of bacteria are used to estimate the level of TonB activity in a given strain. TonB activity is also used as an indirect measure of ExbB and ExbD activity (Held and Postle, 2002). As few as 1 TonB molecule per cell can be detected by vitamin B$_{12}$-dependent growth in metE strains or by sensitivity to bacteriophage ø80. Less sensitive but more discriminating, relative rates of iron transport can distinguish between 0 and 100% TonB activity. Disk assays for siderophore utilization are unreliable because less TonB can result in larger zones of growth due to diffusion of the siderophore in the plates (Larsen et al., 2003a). Given their variations in sensitivity, it is recommended that an entire panel of assays be applied to each mutant. Because tonB strains are iron stressed, they hyperexcrete the siderophore enterochelin (also known as enterobactin). The excess enterochelin can be readily detected on chrome-azurol-S plates (Schwyn and Neilands, 1987), but is also apparent in turning both liquid and solid media slightly pink, the color of Fe-enterochelin. With each of these assays, TonB (or ExbB or ExbD) can be classed as fully active, partially active, or completely inactive; however, little is revealed about the reasons for the inactivity.

Colicin/Phage Sensitivity by Spot Titer Assays

If a plasmid-encoded TonB system protein is being assayed, the first step is to determine the inducer concentration required for chromosomal levels of expression by immunoblot (described further later). TonB system mutants
are often somewhat unstable and require increased concentrations of inducer relative to the wild type to achieve chromosomal levels of expression. The basic procedure is to subculture a saturated overnight LB culture of the strain to be tested at 1:100 in T-broth with appropriate antibiotics and inducer (arabinose in our experiments). When the culture reaches midexponential phase ($A_{550} \approx 0.4$ on a Spectronic 20 with a 1.5-cm path length), 3.0 ml tempered molten T-top agar (50–60°C) is added to 200 $\mu$l cells in a sterile 13-mm glass tube. The tube is vortexed rapidly but gently and the contents quickly poured onto a T-plate equilibrated at room temperature. *Note:* Antibiotics and inducer identical to those in the liquid culture have been previously added to both the T-plates and the T-top agar.

Just before the cells are combined with molten agar, an aliquot is precipitated with an equal volume of cold 20% (w/v) trichloroacetic acid (TCA) for later analysis by immunoblot to confirm that the proteins in question are expressed at chromosomal levels. Cells in TCA are incubated on ice for at least 15 min, centrifuged in a microfuge, and the supernatant aspirated.

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**FIG. 1.** Comparison of TonB phenotypic assays. Cells expressing wild-type TonB protein at 100, 12, or 0.4% of chromosomally encoded levels were evaluated in phenotypic assays. The black region depicts the range of TonB over which a given assay can discriminate. For example, while transport assays can discriminate between ~10 and 100% TonB activity, they cannot tell the difference between something less than 10% activity and zero activity. The transition from black to gray identifies regions where the exact end point is not clear because more intermediate levels of TonB were not tested. The enterochelin hypersecretion assay lacks a gray zone because it is insensitive and unsuitable for use in all but the broadest phenotypic characterizations (e.g., is the TonB system active or not?). Note that the 0.4% level corresponds approximately to 1 TonB per cell under iron-replete conditions. Figure adapted from Postle and Larsen (2004) and reprinted with permission.
Pellets from 0.5 ml of cells are suspended in 25 μl 1 M Tris-HCl at pH 7.8 and 25 μl 2× gel sample buffer. We have learned that without TCA precipitation, TonB and ExbD proteins are proteolytically degraded at some point during lysis and boiling in Laemmli gel sample buffer (Laemmli, 1970; Skare and Postle, 1991).

After the T-top agar has solidified undisturbed for at least 5 min, 10-μl aliquots of diluted phage or colicin are spotted onto the plates. If the highest dilution is spotted first, the need to change to a fresh tip after spotting a given dilution can be avoided. Plates are incubated upright at 37°C for 18 h and results recorded as the highest dilution at which clearing is evident. These assays are performed in triplicate.

Notes on dilution: Each agent must be diluted beyond its capacity to clear a lawn such that at least one dilution that does not clear the lawn is tested. Bacteriophage φ80 is usually diluted 10-fold in sterile λ-Ca²⁺ buffer and, as an alternative, can be fully titered to determine relative plating efficiencies (Larsen et al., 2003a). Colicins are usually diluted 5-fold in λ-Ca²⁺ buffer. It is generally a good idea to make fresh dilutions for each assay; in the case of colicin M, it is imperative due to its instability. Colicin M can be stabilized by the addition of 0.1% Triton X-100. If other diluents are used, such as 1× M9 minimal salts, it is important to remember that colicin M requires Ca²⁺ (Schaller et al., 1981). TonB strains should show no evident clearing even with undiluted colicin.

λ-Ca²⁺ buffer: 1.0 ml 1 M Tris-HCl, pH 7.9 (10 mM), 2.0 ml 1 M MgSO₄ (20 mM), 1.0 ml 0.5 M CaCl₂ (5 mM), 96 ml distilled H₂O. Note: This can be made from sterile solutions added to sterile bottles or prepared nonsterilely and autoclaved.

Colicin Preparation

Colicins are protein toxins synthesized by some strains of E. coli to kill sensitive strains in what could be considered intraniche warfare. TonB-dependent group B colicins are generally large proteins consisting of three domains: a receptor-binding domain where the TGTs serve as receptors, a translocation domain that facilitates movement of the colicin across the OM via the transporter/receptor, and a toxic domain that determines how the unfortunate E. coli will be killed. The means of death are many and varied and have been reviewed in Braun et al. (2002) and Cao and Klebba (2002).

Colicins are encoded on plasmids and expressed from SOS promoters. Thus colicin expression is induced in late exponential phase (A₅₅₀ = 1.0) by the addition of mitomycin C to a final concentration of 1 μg ml⁻¹.
Because mitomycin C is carcinogenic, gloves should be worn and care taken to avoid direct contact with media that contain it. Cells are incubated for 4 h with shaking (or less if lysis occurs), collected by centrifugation, suspended in 1/10 volume of 1× M9 medium, and lysed by two passages through a French press at ~20,000 psi. The lysate is centrifuged to remove unbroken cells and debris (20 min at ~11,000 g) and the supernatant is filtered through 0.45-μm filters to sterilize (procedure adapted from Pugsley and Reeves, 1977). Aliquots can be frozen at −20°C until use. Special handling is needed for colicin M, which is unstable when diluted (Schaller et al., 1981). Colicin Ia is expressed from pColIa-CA53 (Davies and Reeves, 1975), colicin D is expressed from pColD-CA23 (Davies and Reeves, 1975), colicin M is expressed from pTO4 (Olschlager et al., 1984), and colicin B is expressed from pES3 (Pressler et al., 1986).

**Vitamin B<sub>12</sub> Nutritional Assay**

Unlike iron, vitamin B<sub>12</sub> (cyanocobalamin) is not a required nutrient. Nonetheless, it fits the profile of TonB-dependent transport ligands by being sufficiently large, scarce, and important that it is actively transported by TGT.

There are two methionine synthases in *E. coli*, MetE and MetH. It is advantageous for *E. coli* to use MetH (if it can obtain vitamin B<sub>12</sub>) because the MetH turnover rate is 100 times faster than that of MetE (Whitfield et al., 1970). Because MetH is vitamin B<sub>12</sub> dependent, a nutritional requirement for vitamin B<sub>12</sub> becomes apparent in *metE* strains grown in the absence of methionine. These growth assays, like sensitivity to bacteriophage φ80, are capable of detecting very low level of TonB activity (Larsen et al., 2003a).

Vitamin B<sub>12</sub>-dependent *metE* strains are grown to midexponential phase in M9 minimal medium, supplemented as in the iron transport assays described later, with the exception that the final growth medium contains ~90 μM FeCl₃ • 6H₂O. The cells are harvested in midexponential phase and washed twice in unsupplemented 1× M9 salts. Two hundred microliters of cells is added to 5 ml melted methionine-free top agar (0.75%) in an M9 base supplemented with defined amino acids (Gerhardt et al., 1994). The top agar also contains 0.01% tetrazolium to allow enhanced detection of growth zones. The top agar is allowed to solidify on methionine-free supplemented plates (1.5% agar), and sterile disks containing 5 μl of 100 or 500 nM freshly prepared vitamin B<sub>12</sub> or, as a control, 27 mM methionine are placed in triplicate on the solidified top agar. Growth zones are measured 18 h postinoculation. The larger the zone around the vitamin B<sub>12</sub>-containing disks, the more TonB activity. *tonB* strains cannot grow except around the disk containing methionine.
Overview. Colicin and phage sensitivity assays develop over a 12- to 18-h time span and are thus very sensitive to low levels of TonB system activity. In contrast, assays to determine relative rates of iron-siderophore or vitamin B$_{12}$ transport take place over a short time frame and are thus able to discriminate in the range between ~10 and 100% TonB activity where colicin and phage sensitivity assays are less useful.

Ferrichrome crosses the OM through the TonB-gated transporter known as FhuA. This siderophore produced by the fungus *Ustilago sphaerogena* is, unlike vitamin B$_{12}$, available commercially (Sigma) and, unlike enterochelin—the siderophore that is native to *E. coli*—is very stable. The ferrichrome transport protocol described here has been adapted (Koster and Braun, 1990) and is also described in Larsen and Postle (2001). The best strains to use for Fe-ferrichrome transport assays might be those mutated in the *aroB* locus to prevent synthesis of enterochelin precursors that could serve as alternative siderophores (Hantke, 1990). If necessary, the *aroB* locus can be transferred into strains by P1 transduction from strain CAG18450 [MG1655 *zhf-50::Tn10*] (Higgs *et al.*, 2002b; Singer *et al.*, 1989). The *aroB* strain is grown overnight in 5 ml LB at 37°C and subcultured 1:100 into 10 ml 1× M9 medium (see later) with higher levels of iron (~100 μM). However, *aroB* strains generally grow poorly in minimal medium no matter how much iron is added, and if *aroB* strains are used instead, it does not appear to affect the results significantly. Both enterochelin and ferrichrome require the TonB system for transport into cells. Furthermore, it seems reasonable to assume that the degree of exchange of $^{55}$Fe between enterochelin and ferrichrome taking place during the short time course of the transport experiment is limited. For specific investigations on the ferrichrome transporter FhuA, *aroB* strains should probably be employed.

Another assay provides discrimination among TonB system levels similar to the Fe-ferrichrome transport assay, but is not nearly as straightforward. Bacteriophage φ80 adsorbs to *E. coli* in two steps: an initial reversible adsorption that is energy independent, followed by irreversible adsorption that is TonB system dependent (Hancock and Braun, 1976). The basic experiment is to add φ80 to a sensitive strain and, at various times afterward, withdraw samples that are vortexed vigorously to remove unadsorbed phage and then centrifuged. Supernatants representing the unadsorbed phage population are then titered and the rate of adsorption determined (Skare and Postle, 1991). Because this assay is significantly more work than transport assays and more difficult to standardize, it has largely fallen into disuse.
**55Fe-Ferrichrome Transport Assay Protocol**

1. After growth of a 10-ml culture to \( A_{550} = 0.5 \) (midexponential phase) at 37°C in side-arm flasks with vigorous aeration, cells are centrifuged at room temperature and suspended in an equal volume of 1× M9 salts, 0.4% glucose, and 0.1 mM nitrilotriacetate to block the nonspecific low-affinity iron uptake system. The suspended cells are incubated in disposable 50-ml tubes with shaking at 30°C for 5 min to adapt to the new lower temperature. At 37°C transport rapidly becomes nonlinear.

2. Immediately prior to initiation of transport, a 0.5-ml sample is removed and precipitated with TCA preparative to immunoblot analysis to confirm chromosomal levels of expression.

3. While shaking continues at 30°C, transport is initiated by the addition of 150 pmol 55Fe-ferrichrome per milliliter of cells. Each tube is vortexed briefly and triplicate 0.5-ml samples are harvested by filtration onto Whatman G/FC glass microfiber filters in a vacuum manifold.

4. Each sample is rapidly washed three times with 5 ml 0.1 M LiCl, and the filters are placed on blotting paper to dry. This is the 1-min sample. Additional triplicate samples are taken at 4, 7, and 10 (sometimes also 13 and 16) min. After drying, filters are placed in scintillation vials with 3 ml scintillant. Filters are counted in a liquid scintillation counter, and relative rates of transport are determined from the raw counts. Data can be reported as cpm per 1 \( \times 10^8 \) cells or per \( A_{550} \) ml. Because 55Fe is a low-energy X-ray emitter, it can be counted in a 0-400 window using a Beckman LS6500 multipurpose liquid scintillation counter. In our experiments, there is less variation between triplicate samples if the filters are incubated overnight in scintillant prior to counting.

5. TCA-precipitated samples are resolved on 11% SDS-PAGE and immunoblotted for the protein of interest to confirm that the assayed cultures were all expressing near-chromosomal levels of proteins in question (e.g., TonB mutants). Because ferrichrome transport is an indirect assay of TonB system functioning, mutations in TonB, ExbB, and ExbD can all be assayed.

**Growth Medium Recipe.** For 100 ml 1× M9 medium (all solutions are sterile):

- 10 ml 10× M9 salts (Shedlovsky and Brenner, 1963)
- 1 ml 50% glycerol (if using arabinose to induce gene expression from arabinose promoter on a plasmid) or 2 ml 20% glucose
- 2 ml 20% casamino acids, preferably lowest iron contamination available
- 1 ml 4 mg/ml L-tryptophan (missing from casamino acids)
200 μl vitamin B₁ at 2 mg/ml
100 μl 1 M MgSO₄
100 μl 0.5 M CaCl₂
50 μl FeCl₃ • 6H₂O at 1 mg/ml (1.85 μM)
85 ml sterile dH₂O
Antibiotics as needed

Preparation of ⁵⁵Fe-Ferrichrome

1. Prepare 1 mM iron-free ferrichrome (FW = 687.7) stock using double-distilled water as solvent. The 1 mM ferrichrome solution can be frozen at −20° and used repeatedly if stored in a container that protects it from light. (Dissolving the entire contents of the bottle of ferrichrome [Sigma F8014] allows for easy storage and eliminates the need to weigh out tiny amounts.)

2. Dilute ⁵⁵FeCl₃ to 150 μM with 0.5 M HCl. Because the nuclide is provided in small volumes, the expanded volume also reduces losses due to evaporation. Specific activity of the ⁵⁵FeCl₃ as it comes from the supplier can range from 20 to 120 mCi/mg. The half-life of ⁵⁵Fe is 2.7 years.

3. Add 5 μl 1 mM ferrichrome and 5 μl 150 μM ⁵⁵FeCl₃ to 240 μl 10 mM HCl. The final concentration is 20 μM ferrichrome loaded with ⁵⁵FeCl₃ with a 6.7:1 excess of ferrichrome.

4. Incubate the ⁵⁵Fe-ferrichrome solution at 37° for 15 min and place on ice for the remainder of the experiment.

Typical results in this assay are that the rate of transport remains linear over the time course, with 10,000 to 20,000 cpm incorporated by 10 min. Strains lacking TonB bind only a few hundred counts per minute, an amount that remains unchanged over the course of the assay.

Mechanistically Informative Assays

Mechanistically informative TonB system assays generally require the use of antisera directed against the component proteins to determine steady-state levels of mutant proteins, localize proteins, analyze assembly, and detect conformational changes. Even the phenotypic assays described earlier require knowledge of steady levels of mutant proteins in order to interpret the results unambiguously. To generate specific antisera, we obtained purified TonB as inclusion bodies of a hybrid TrpC-TonB-G26D protein that carries a signal sequence mutation (Skare et al., 1989) and, as technologies improved, were also able to purify His-tagged versions of ExbB and ExbD. Overexpressed OM transporters were generous gifts from colleagues (Clive Bradbeer and
determine its crystal structure (Hilsenbeck et al., 2004). We generated monoclonal antibodies directed against TonB as part of a collaboration (Larsen et al., 1996) and made the remaining antisera ourselves.

To obtain high specificity antisera with low cross-reactivity, we pre-screen several rabbits for the lowest possible level of cross-reactivity in their preimmune sera. Selected rabbits are each surgically implanted subcutaneously by staff veterinarians with a golf ball-sized whiffle ball, which serves as a chamber through which antigen can be injected and red blood cell-free immune serum can be retrieved (Hillam et al., 1974; Ried et al., 1990). The proteins used as antigens do not need to be especially pure—the only important criterion is that they be free of contaminants at the same molecular mass as the antigen on the SDS gel from which they will be excised. After confirming that a purified protein band on a one-dimensional gel has no extraneous proteins (using two-dimensional electrophoresis), the band containing the antigen is excised, emulsified, and injected without any adjuvant other than the polyacrylamide gel itself. The band of interest is identified by Coomassie stain, which does not need to be removed. Freund’s adjuvant should not be used due to the deleterious effect it has on the animals and the generation of many cross-reactive immunoglobulin species in the resulting antiserum.

In Vivo Formaldehyde Cross-Linking

Formaldehyde can cross both OM and CM (it efficiently cross-links GroEL protein in our experiments). The degree of cross-linking can be controlled by time, temperature, and pH. Cross-links are broken by boiling for 5 to 10 min (Prossnitz et al., 1988; Skare et al., 1993). Monomeric formaldehyde inserts a CH2 group between two reactive residues (K, Y, H, C \(\rightarrow\) R, W) and is thus a probe for close protein contacts through diverse side chains (Means and Feeney, 1971). Failure to observe a cross-linked complex is not evidence that protein–protein interaction is not occurring elsewhere between noncross-linkable residues. In addition, detectable cross-linked complexes might not represent the entire set of interactions with other proteins. Protein–protein interactions could occur in which the correct amino acids are not positioned correctly to allow cross-linking or the interactions may be too transient to trap efficiently.

In vivo, TonB can cross-link to OM proteins Lpp, OmpA, and FepA and to CM proteins ExbB and ExbD (Higgs et al., 1998, 2002b; Skare et al., 1993). The OM TonB complexes appear to represent interactions with nonenergized TonB (Ghosh and Postle, 2005). Cross-links between ExbB and TonB assay accurately whether correct transmembrane domain relationships have
been maintained (Larsen et al., 1994). In addition, *in vivo* formaldehyde cross-linking detects oligomeric forms of ExbB and ExbD (Higgs et al., 1998, 2002b) and can be used to rule out assembly defects as the cause of mutant phenotypes. Cross-linked complexes are detected by immunoblot and manifest as specific bands with molecular masses that are larger than the monomer.

ExbB/ExbD and especially TonB have a low abundance in the cell. Under conditions of extreme induction (knockout of the Fur transcriptional repressor) the highest levels of TonB achieved are $1350 \pm 400$ copies per cell (Higgs et al., 2002a). Because formaldehyde cross-linking is inefficient (Skare et al., 1993), it is not currently feasible to purify cross-linked complexes of proteins expressed at chromosomal levels and subject them to mass spectrometric analysis to identify sites of interaction.

For *in vivo* cross-linking, 1 ml of midexponential phase cells ($A_{550} = 0.5$) is harvested by centrifugation for 5 min in a microcentrifuge at room temperature. The cell pellet is suspended in 938 $\mu$l 10 mM phosphate buffer, pH 6.8. Tris or any buffer with free amino groups should not be used. These experiments should be carried out in a hood and wearing protective gloves to avoid contact with formaldehyde, a probable carcinogen that is very toxic by inhalation, ingestion, or skin contact. To initiate cross-linking, 62 $\mu$l of 16% monomeric $p$-formaldehyde (final concentration = 1.0%) is added to the suspended cell pellet with vortexing and the incubation is continued at room temperature for 15 min in the hood. Monomeric $p$-formaldehyde is obtained in sealed glass ampoules from Electron Microscopy Services (215-646-1566), web site www.emsdiasum.com. It can be stored at room temperature for 1 month in a small brown vial after opening. Treated cells are collected by centrifugation for 5 min at room temperature in a microcentrifuge. Pellets are suspended in 50 $\mu$l 1 × Laemmli gel sample buffer (Laemmli, 1970) and heated to 60° for 5 min. Control samples may be boiled for 5 to 10 min to break cross-links. Samples may be frozen at $-20°$ but best results seem to arise from immediate analysis by SDS-polyacrylamide gel electrophoresis.

**Sucrose Equilibrium Density Gradient Fractionation**

There are many protocols available for fractionation of *E. coli* membranes by sucrose density gradient fractionation. The method we use (Osborn et al., 1972) is briefly summarized here with the TonB-specific features noted. Cells are grown to $A_{550} = 0.5$, chilled to 4°, and 14 $A_{550}$ ml are harvested, suspended in 2 ml 10 mM HEPES, pH 7.8, and lysed by two passes through a French press at 20,000 psi. Unbroken cells are removed by low-speed centrifugation (600g for 5–10 min). It is especially important to keep the
g force well below the 13,000 g for 15 min that will pellet OM efficiently (Nikaido, 1994). Eight hundred fifty microliters of the lysate is loaded onto the top of a 30–56% sucrose gradient for centrifugation in a MLS-50 swinging bucket rotor in a Beckman Optima Max Ultracentrifuge at 50,000 rpm (238,000g) for 6 h. Although this protocol can be scaled up for larger swinging bucket rotors, such as the SW40 (Letain and Postle, 1997), the tiny MLS-50 swinging bucket rotor is now used because it has a more advantageous clearing factor (k). The decreased centrifugation times help limit proteolysis of TonB system proteins, especially the mutant variants that can be unstable even in whole cells. The gradient is prepared by layering sucrose solutions in 10 mM HEPES, pH 7.8, beginning with a small volume of 56% (w/w) sucrose (0.2 ml) and adding successive layers of 50, 45, 40, 35, and 30% (w/w) sucrose (0.8 ml for each layer). Flotation experiments are performed by adjusting the lysate or a fraction from a previous gradient to 56% sucrose and loading it on a small cushion of 61% sucrose, with layers of 56%, 50%, 45%, and so on sucrose layered on top (Letain and Postle, 1997). Fractions are collected (0.25 ml), and 100 μl is immediately precipitated with an equal volume of 4°C 20% TCA for immunoblot analysis with the remainder frozen for subsequent determination of specific gravity and NADH oxidase activity. The TCA-precipitated pellet is suspended in 50 μl gel sample buffer (Laemmli, 1970), and 15 μl is resolved on a 1.0-mm-thick slab gel with 20 wells. TonB is detected in both the CM and the OM (Larsen et al., 2003b; Letain and Postle, 1997). Established markers are used to identify fractions containing the CM and the OM. NADH oxidase activity (Osborn et al., 1972) measured in each fraction identifies the CM fractions typically found at a sucrose density of 1.16–1.18. The distinctive pattern of highly expressed OM proteins OmpF and OmpA in the stained immunoblot between 35 and 40 kDa identifies the OM fractions typically found at a sucrose density of 1.22–1.25 (Letain and Postle, 1997; Osborn et al., 1972). Polyvinylidene fluoride (PVDF) membranes used for the immunoblot analysis are stained following chemiluminescent detection of proteins of interest in 0.1% (w/v) Coomassie brilliant blue R, 50% methanol, 7% glacial acetic acid and destained in the same solution but without the Coomassie blue.

In addition to the location of the OM fractions, the characteristic pattern seen on the stained PVDF membranes also provides information about how well the sucrose density gradient formed. Even though protein is denser than lipid or any biological membrane, most of the soluble proteins will be located in the top fractions of the gradient because of their small size and the viscosity of the sucrose. Large protein complexes such as ribosomes will be found at increasingly higher sucrose densities as the run times
increase. Given enough time, they will pellet at the bottom of the gradient. For this reason, initial characterizations of a protein as being membrane associated are best confirmed by flotation in a sucrose density gradient.

**Pmf-Responsive Conformational Changes**

The amino terminal ~140 amino acids of TonB can be protected from proteinase K added exogenously to intact spheroplasts if two conditions are met: (1) ExbB, ExbD, TonB, and the pmf were all intact at the time the spheroplasts were produced and (2) pmf is subsequently collapsed by protonophores DNP or CCCP (Larsen *et al.*, 1994, 1999). The result is a ~25-kDa proteinase K-resistant fragment that derives from the TonB amino terminus (Fig. 2). In the absence of ExbB/D or if the TonB transmembrane domain carries a mutation that inactivates TonB, such as TonB-ΔVal17, TonB-His20Tyr, or TonB-Ser16Leu, the addition of proteinase K following treatment with the protonophore CCCP results in complete degradation of the TonB. The TonB carboxy terminus is not required for the pmf-responsive conformational change that leads to proteinase K resistance. The proteinase K-resistant fragment is formed by as few as TonB amino acids 1–168 (full-length TonB is 239 amino acids). (Smaller amino-terminal TonB fragments have not been tested.) Formation of the proteinase K-resistant fragment serves as an indicator of overall potential for TonB to be energized or otherwise managed by ExbB/D.

![Figure 2](image_url)

**Fig. 2.** TonB conformations differ *in vivo* depending on whether pmf is present or absent. The position of full-length TonB is indicated by an arrow. The asterisk indicates the position of the proteinase K-resistant fragment indicative of prior TonB energization. WC, whole cells; Sph, spheroplasts; CCCP/Sph, spheroplasts pretreated with protonophore CCCP to collapse the pmf prior to proteinase K treatment. Strain backgrounds in which experiments were performed are indicated above each set. The proteinase K-resistant fragment does not form when TonB carries a mutant transmembrane domain (ΔVal17) or if ExbB/D is absent. Figure adapted from Larsen *et al.* (1999) and reprinted with permission.
Escherichia coli can be converted to spheroplasts when the OM is loosened, the peptidoglycan largely degraded exogenously by added lysozyme, and the intact cytoplasmic membrane stabilized by osmoprotectants according to several protocols with small variations (Randall and Hardy, 1986; Witholt et al., 1976). We follow the Randall and Hardy (1986) procedure with minor modifications. Recipes for spheroplasting buffers are shown in Table I.

Bacteria are grown in M9 minimal salts with casamino acids medium described earlier to $A_{550} = 0.5$ and six 1-ml aliquots are pelleted in microcentrifuge tubes. If strains are lacking ExbB/D or are aroB it may be necessary to increase the amount of Fe added to ~90 µM. While cells are growing, the lysozyme solution (2 mg/ml in distilled H2O) and proteinase K solution can be made. (For proteinase K, start with 2 mg/ml—the precise amount may need to be titrated in the assay as too much will also degrade the “proteinase K-resistant fragment.”) All solutions should be well chilled before beginning the experiment and all cell samples should remain on ice throughout.

1. Tubes 1 + 2: suspend in 500 µl buffer 1, place on ice. These are whole cells.
2. Tubes 3–6: suspend in 250 µl buffer 2 by gently pipetting the buffer over the pellet repeatedly. This is key.
3. Tubes 3–6: add 20 µl freshly prepared lysozyme at 2 mg/ml in dH2O.
5. Tubes 3–6: incubate on ice 5 min with tops off for subsequent addition of Mg2+.
6. Tubes 3–6: add 10 µl 1.0 M MgSO4 to stabilize the spheroplasts. Mix gently.

### TABLE I

<table>
<thead>
<tr>
<th>Buffer</th>
<th>H2O</th>
<th>1 M TrisAc, pH 8.2</th>
<th>2 M sucrose</th>
<th>1 M MgSO4</th>
<th>500 mM EDTA</th>
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<tr>
<td>#1</td>
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<td>1.25</td>
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</tr>
<tr>
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<td>—</td>
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</tr>
<tr>
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<td>2.0</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>#4</td>
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<td>2.0</td>
<td>1.25</td>
<td>0.2</td>
<td>—</td>
</tr>
<tr>
<td>#5</td>
<td>9.5</td>
<td>0.5</td>
<td>—</td>
<td>—</td>
<td>0.05</td>
</tr>
</tbody>
</table>

*Buffers are made in 10-ml aliquots using distilled deionized H2O. Volumes are in milliliters.
8. Tubes 3–6: aspirate supernatants but leave just a bit in the tube.
9. Tubes 3 + 4: suspend in 0.5 ml buffer 4 by gently pipetting just above the pellet. This is key. Tubes 3 + 4 are intact spheroplasts.
10. Tubes 5 + 6: suspend in 500 µl buffer 5 and vortex very well. Add 10 µl 1 M MgSO₄. These are lysed spheroplasts.
11. Tubes 2, 4, and 6: add 6.3 µl of proteinase K solution at 2.0 mg/ml (final concentration 25 µg/ml). Mix by pipetting up and down; when all three are done, mix gently and tap to get all liquids to the bottom of the tube. Incubate on ice for 15 min.
12. All tubes: add 5 µl freshly prepared 100 mM phenylmethanesulfonyl fluoride to inactivate the proteinase K.
13. All tubes: add 500 µl chilled 20% TCA and analyze samples by immunoblot.

As an alternative to the lysed spheroplasts, at step 10, tubes 5 and 6 are suspended in 500 µl buffer 4 and carbonyl cyanide m-chlorophenylhydrazone (CCCP; 50 µM) or dinitrophenol (DNP; 10 mM) is added. Control experiments require two additional tubes of unlysed spheroplasts with solvent for each protonophore added (ethanol or dimethyl sulfoxide, respectively). Because lysis of spheroplasts can also collapse the CM proton gradient (Larsen et al., 1994), it is essential to monitor the integrity of spheroplasts treated with protonophores. For this control, cells are grown to early exponential phase and treated with 1 mg/ml isopropyl-thio-β-D-galactoside to induce expression of the cytoplasmic enzyme β-galactosidase prior to conversion to spheroplasts at A₅₅₀ = 0.5. Enzyme levels (Miller, 1972) are determined for pelleted spheroplasts that were treated with protonophores and the corresponding supernatants to confirm that 85% or more of the β-galactosidase remains in the pellet (Larsen et al., 1999).

Dividing the Energy Transduction Cycle into Two Halves

Of the several known siderophores that *E. coli* K12 can use to obtain iron, it synthesizes only enterochelin and its intermediates. TonB does not transduce energy to nonligand-bound transporters (Larsen et al., 1994, 1999). Therefore, in an *aroB* strain, the progression of TonB from an energy-transducing conformation to a discharged conformation is prevented (Larsen et al., 1999). Because of this, the energy transduction cycle can be functionally divided into two stages by use of *aroB* strains. For example, if a conformational change is prevented in an *aroB* strain, then it likely occurs following TonB transduction of energy to OM transporters.
**Ability of TonB Cys Substitutions to Form Dimers**

The use of Cys substitutions to monitor in vivo conformational dynamics and protein–protein relationships has been very powerful in *E. coli* (Falke and Hazelbauer, 2001; Kaback and Wu, 1997). Because most of the TonB system interactions take place in the oxidizing environment of the periplasm, the spontaneous formation of disulfide-linked dimers is a powerful tool for dissection of in vivo interactions. Cys substitutions at TonB carboxy-terminal aromatic amino acids F202C, W213C, Y215C, or F230C spontaneously form disulfide-linked dimers in the CM in vivo (Ghosh and Postle, 2005). These dimers do not form if ExbB/D are absent or if the TonB carries a transmembrane domain mutation in addition to the Cys substitution. Lack of dimer formation could result from failure of an energy-transducing conformation to form or from folding of the TonB carboxy terminus so rapidly that disulfide-linked dimers cannot be trapped. Current data suggest that the latter is most likely the case (Larsen *et al.*, 2007).

In this protocol, the TonB F202C dimers (for example) are expressed from a plasmid-encoded arabinose promoter at chromosomal levels as determined previously. Midexponential-phase cultures are harvested by centrifugation at 4°C and lysed by boiling for 5–10 min in the presence of 50 mM iodoacetamide to ensure that disulfide cross-links detected occurred prior to lysis (Cadieux and Kadner, 1999; Ghosh and Postle, 2005). Samples are electrophoresed on nonreducing SDS-polyacrylamide gels, and TonB dimers are detected by immunoblotting. The dimers appear to exist in three different conformations that appear as bands with three different apparent molecular masses (Fig. 3).

It is unfortunately not possible to directly assay the effect of protonophore DNP or CCCP on this process because the (1) disulfide cross-linked dimers preexist in cells and (2) newly synthesized TonB requires pmf for export across the cytoplasmic membrane (Skare *et al.*, 1989). This precludes experiments where TonB expression is induced in the presence of protonophores.

**Potentially Mechanistically Informative Assays**

The protocols described previously have been adapted and developed using chromosomal levels of all the TonB system components. Some interesting and potentially very useful in vivo assays have been developed where the meaning of results obtained is less clear either because some components are overexpressed or because additional experimental results have raised questions about older experiments. These assays are described briefly so that future readers are made alert to the possibility of eventual refinements that will be more definitively interpretable.
Interaction between TonB and OM Transporter BtuB In Vivo

TonB-gated transporters share a reasonably high degree of similarity. Most notably, at the amino terminus each has a pentapeptide with the consensus D/E T X X V, known as the TonB box. Certain proline substitutions in this region inactivate the transporters completely (Barnard et al., 2001; Heller et al., 1988; Schoffler and Braun, 1989). Second site suppressors of TonB box mutations occur at TonB-Gln160. Direct contact between TonB-Q160 and the TonB box of transporters has been demonstrated in vivo (Cadieux and Kadner, 1999; Ogierman and Braun, 2003). However, in those experiments, both the OM transporter and the

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**Fig. 3.** In vivo formation of disulfide-linked dimers requires pmf mediators ExbB and ExbD and the TonB transmembrane domain. Plasmids encoding TonB and TonB-Cys substitutions were expressed at chromosomal levels in A [KP1344 (ΔtonB::blaM)] or B [KP1440 (ΔtonB::blaM, exbB::Tn10, tolQ(am))]. (C) The plasmid-encoded TonB parent and TonB-Cys substitutions also carry a deletion of Val17 in the transmembrane domain that prevents TonB activity. They are expressed at chromosomal levels in KP1344. An immunoblot of strains resolved on a nonreducing 11% SDS-polyacrylamide gel is shown. Arrows point to positions of three disulfide-linked dimers formed by TonB with Cys substitutions at F202, W213, Y215, and F230. Figure adapted from Ghosh and Postle (2005) and reprinted with permission.
three known cytoplasmic proteins (TonB/ExbB/ExbD) were expressed from plasmids where the ratios of the proteins to one another were unknown and where they were each almost certainly overexpressed relative to any unknown proteins in the system. Because TonB does not transduce energy to nonligand-bound transporters \textit{in vivo} (Larsen \textit{et al}., 1999), results showing that the presence or absence of transport ligand has little effect on disulfide formation are consistent with the idea that overexpression may not accurately reflect normal interactions (Ogierman and Braun, 2003). In another study, TonB interactions with mutant TonB boxes were increased in the presence of ligand, but contrary to expectation, contact with the mutant inactive TonB box still occurred at high levels, although the residues contacted were different (Cadieux and Kadner, 1999). Although not the expected result, it may well correctly reflect what occurs \textit{in vivo}. The problem is that since the system is unbalanced, it is not possible to know with certainty.

\textit{Substrate-Induced Signaling by OM Transporters}

Because TonB does not transduce energy to nonligand-bound OM transporters, there must be a signal to indicate ligand occupancy. Ligand-enhanced disulfide cross-link formation between TonB and the TonB box suggested that, in the case of the BtuB transporter, the TonB box is involved (Cadieux and Kadner, 1999). It was subsequently demonstrated \textit{in vivo} that Cys substitutions at TonB box residues showed up to a \sim 6-fold increase in labeling by the thiol-specific reagent 1-biotinamido-4-[4\prime(maleimidomethyl)cyclohexane-carboxamido]butane in the presence of the ligand compared to its absence. The increase in labeling reflects an increase in periplasmic accessibility for the TonB box; however, the presence and absence of TonB had no effect on extent of labeling (Cadieux \textit{et al}., 2003). These experiments were carried out in the presence of what was almost certainly a \textit{tonB} phenocopy due to highly overexpressed BtuB (on high copy number pUC8 backbone). Thus, while there may be no difference in TonB box accessibility whether or not TonB is present, this question could not be answered with this experimental system.

\textit{In Vivo Shuttling between CM and OM}

As noted earlier, TonB fractionates with both the CM and the OM. This behavior suggested that TonB might shuttle to the OM to transduce stored potential energy to OM transporters (Letain and Postle, 1997). An experimental system was set up where the levels of all TonB system components were at chromosomal levels and the accessibility of the TonB amino terminus to the periplasm could be determined \textit{in vivo} by whether and under
what circumstances it could be labeled with Oregon Green Maleimide (OGM), which is marketed and has been used successfully as a membrane-impermeant reagent. TonB that was capable of associating with the OM was labeled by OGM, whereas TonB that could not associate (due to deletion of the carboxy terminus) remained unlabeled. Both full-length and truncated TonB were situated apparently identically in the CM complex of ExbB/D, as each could cross-link to ExbB and each was conformationally responsive to pmf (assays described earlier). It was concluded that TonB shuttles between CM and OM during energy transduction.

There is potentially a problem with this interpretation. Unpublished results indicate that both cytoplasmically localized TonB lacking its signal sequence and the cytoplasmic protein GroEL can be labeled with OGM, and therefore OGM is not absolutely membrane impermeant in this experimental system. Thus, even in vivo experiments where all the TonB system components are balanced can be subject to potential difficulties in interpretation. The explanation for the original results in light of these new data appears to be that the ExbB/D complex protects the extreme amino terminus of truncated TonB from labeling via the cytoplasmic route (Savenkova and Postle, in preparation).

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