ExbB Cytoplasmic Loop Deletions Cause Immediate, Proton Motive Force-Independent Growth Arrest

Charles M. Bulathsinghala,* Bimal Jana, Kristin R. Baker, Kathleen Postle

Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, Pennsylvania, USA

The *Escherichia coli* TonB system consists of the cytoplasmic membrane proteins TonB, ExbB, and ExbD and multiple outer membrane active transporters for diverse iron siderophores and vitamin B₁₂. The cytoplasmic membrane proteins harvest and transmit the proton motive force (PMF) to outer membrane transporters. This system, which spans the cell envelope, has only one component with a significant cytoplasmic presence, ExbB. Characterization of sequential 10-residue deletions in the ExbB cytoplasmic loop (residues 40 to 129; referred to as $\Delta 10$ proteins) revealed that it was required for all TonB-dependent activities, including interaction between the periplasmic domains of TonB and ExbD. Expression of eight out of nine of the $\Delta 10$ proteins at chromosomal levels led to immediate, but reversible, growth arrest. Arrest was not due to collapse of the PMF and did not require the presence of ExbD or TonB. All $\Delta 10$ proteins that caused growth arrest were dominant for that phenotype. However, several were not dominant for iron transport, indicating that growth arrest was an intrinsic property of the $\Delta 10$ variants, whether or not they could associate with wild-type ExbB proteins. The lack of dominance in iron transport also ruled out trivial explanations for growth arrest, such as high-level induction. Taken together, the data suggest that growth arrest reflected a changed interaction between the ExbB cytoplasmic loop and one or more unknown growth-regulatory proteins. Consistent with that, a large proportion of the ExbB cytoplasmic loop between transmembrane domain 1 (TMD1) and TMD2 is predicted to be disordered, suggesting the need for interaction with one or more cytoplasmic proteins to induce a final structure.

The TonB system of Gram-negative bacteria uses the proton motive force (PMF) of the cytoplasmic membrane (CM) to energize active transport across the outer membrane (OM). The known components of this system are a collection of high-affinity OM TonB-gated transporters and a complex of the CM proteins TonB, ExbB, and ExbD. Current data indicate that TonB mediates transport by direct contact of its periplasmic carboxy terminus with OM transporters while remaining anchored in the CM (1; for recent reviews, see references 2, 3, 4, and 5).

The three known proteins in the CM—TonB, ExbB, and ExbD—appear to form a complex (6–16). TonB is present in the CM as a dimer (17, 18). The TonB/ExbB/ExbD ratio in the cell is 2:14:4 (19, 20), although it is not known if this reflects the ratio of the three proteins in an energy transduction complex.

TonB and ExbD have similar topologies; each is anchored by an uncleaved signal anchor in the CM, with the majority of the protein occupying the periplasm (type II topology) (12, 13, 15, 21). TonB and ExbD can form a formaldehyde cross-link through their periplasmic domains that requires PMF, ExbB, H20 in the TonB transmembrane domain (TMD), and D25 in the ExbD TMD (22). TonB H20 plays a structural role, whereas ExbD D25 mediates conformational changes in the TonB carboxy terminus by modulating its interactions with ExbD (23, 24).

ExbB appears to be the scaffolding on which TonB and ExbD assemble (25). It is the only protein of the three that is stable when expressed by itself, and is required for stabilization of both TonB and ExbD (6, 10, 11, 20, 26, 27). It formaldehyde cross-links *in vivo* with either TonB or ExbD, but a stable ternary complex has yet to be identified *in vivo* or *in vitro* (9, 10, 22, 28). It has three transmembrane domains with an unusual type III topology (N out, C in), with the result that the majority of ExbB occupies the cytoplasm (14) (Fig. 1).

We hypothesize that the cytoplasmic domains of ExbB function in communication between the cytoplasm and the periplasm.

The paralogous region of MotA binds to FliG in the cytoplasm (29). A recent study showed that residues in the extreme cytoplasmic carboxy terminus of ExbB were required for PMF-dependent interactions between TonB and ExbD, suggesting that signal transduction occurs from the cytoplasm to the periplasm (30). To test this hypothesis, we initiated a deletion scanning analysis of the cytoplasmic loop of ExbB between TMD1 and TMD2.

In this study, a 10-residue-deletion scanning mutagenesis indicated that the cytoplasmic loop of ExbB (residues 40 to 129) was essential for TonB system activity. Induction of expression of the majority of ExbB deletion proteins resulted in immediate growth arrest that, surprisingly, was not due to PMF dissipation. Growth arrest was reversible and subsided as the ExbB proteins with 10-residue deletions (referred to as $\Delta 10$ proteins) were proteolytically degraded. Growth arrest was dominant, requiring only chromosomal levels of $\Delta 10$ protein coexpression. In contrast, inactivity in iron transport was not dominant in several cases, indicating that growth arrest by the $\Delta 10$ proteins was intrinsic to these proteins and confirming that they did not cause proton leakage. These results suggested that the ExbB cytoplasmic domains were linked

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Address correspondence to Kathleen Postle, postle@psu.edu.

* Present address: Charles M. Bulathsinghala, Penn State Milton S. Hershey Medical Center, Department of Medicine, Hershey, Pennsylvania, USA.

C.M.B. and B.J. contributed equally to the research.

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FIG 1 Topology of ExbB. Topology of ExbB (14, 25), and revised location of transmembrane domains (41). Numbers on the right indicate predicted boundaries of the three transmembrane domains. The cytoplasm and periplasm are labeled on their respective sides of the cytoplasmic membrane, which is represented by two straight parallel black lines. Features are not drawn to scale.

into a key cell growth pathway that became blocked in the strains with the deletion proteins.

MATERIALS AND METHODS

Strains and plasmids. Strains and plasmids used in this study are listed in Table 1. KP1057 (W3110 *acrA::TnpH*) was constructed by P1_{vir} transduction of *acrA::TnpH* from MPHT37 into W3110. KP1445 (GM1 *exbB::Tn10 recA::cat*) was constructed by P1_{vir} transduction of the *recA::cat* mutation from KP1397 into KP1037 (GM1 *exbB::Tn10*) with selection for resistance to chloramphenicol (34 $\mu\text{g ml}^{-1}$). KP1563 (GM1 $\Delta\text{exbBD}::kan$) was constructed by P1_{vir} transduction of the kanamycin resistance cassette that replaces the entire *exbB-exbD* operon in RA1003 into GM1.

Two sets of plasmids expressing the $\Delta 10$ proteins were created, one with the *exbB-exbD* operon intact and one where the *exbD* gene was absent. To create the first set of deletions, the *exbB-exbD* operon was amplified by PCR and cloned into the *Sma*I site of plasmid pBAD24 to create pKP660. In-frame deletions of 10 *exbB* codons were constructed in pKP660 using extra-long PCR as previously described (11). To create the second set of plasmids, the *exbD* gene from pKP660 was removed by extra-long PCR, resulting in pKP920. Each of the *exbB* $\Delta 10$ mutations was then reconstructed with pKP920 as the template. The plasmid DNA sequences of the entire *exbB-exbD* operon and the isolated *exbB* gene were verified at the Penn State Genomics Core Facility (University Park, PA), and the existence of unintended base changes was ruled out. Primer sequences used in the construction of the *exbB* $\Delta 10$ mutations are available upon request.

Media and culture conditions. LB broth and plates and M9 minimal medium were prepared as previously described (31). For all assays, cultures were grown with aeration by shaking at 37°C in M9 minimal medium supplemented with the following: 0.5% (wt/vol) glycerol (EMD Chemicals; defined amino acids as described previously [32], 80 $\mu\text{g ml}^{-1}$ thiamine, 10 mM MgSO_4 , 0.5 mM CaCl_2 , 1.85 μM FeCl_3) and ampicillin (100 $\mu\text{g ml}^{-1}$). Alternatively, for sucrose density gradient and fractionation assays, samples were supplemented with 0.4% Casamino Acids and 40 $\mu\text{g ml}^{-1}$ tryptophan in lieu of defined amino acids. L-Arabinose was purchased from Sigma. For growth assays, cultures were grown with aeration by shaking at 37°C in LB overnight and subcultured in fresh LB with 100 $\mu\text{g ml}^{-1}$ ampicillin.

[⁵⁵Fe]ferrichrome transport assay. Initial rates of [⁵⁵Fe]ferrichrome transport were determined as previously described with the following modifications (33). Briefly, strains at an A_{550} of 0.4 (measured on a Spectronic 20 spectrophotometer; path length, 1.5 cm) were induced for 60 min with L-arabinose at the final concentrations listed in Table 2. Then, 1.4 A_{550} -ml cells were harvested by centrifugation, and the pellet was suspended in 1.4 ml of M9 minimal medium supplemented as described above but without added iron and with 0.1 mM nitrilotriacetic acid. Samples were separated into three 400- μl aliquots, and the remaining volume

of cells (200 μl) was precipitated with trichloroacetic acid (TCA) to determine steady-state levels of ExbB proteins by immunoblot analysis. Transport was initiated by the addition of [⁵⁵Fe]ferrichrome prepared as described previously and characterized as described previously (33). For dominance assays, proteins were induced for 60 min with 0.01% arabinose prior to harvesting and processed as described above.

Growth experiments and immunoblot analysis. To evaluate the effects of mutant protein expression on cell growth, KP1445 (*exbB-exbD*) carrying plasmids coexpressing $\Delta 10$ proteins and ExbD in KP1445 was grown in LB overnight and subcultured into LB. After 120 min of growth, expression of ExbB and the $\Delta 10$ proteins was induced by addition of 0.1 or 0.01% L-arabinose. At all subsequent time points, samples were diluted 1:10, and the A_{550} was measured in a quartz cuvette with a 1.0-cm path length using a Shimadzu UV 1540 minispectrophotometer. To determine steady-state levels of protein, 0.2 A_{550} -ml cells were removed at subsequent time points and immediately precipitated with equal volumes of 20% TCA on ice. Samples were suspended in 50 μl of 1 $\times$ sample buffer (34), heated at 95°C for 5 min, resolved on 15% polyacrylamide gels, and immunoblotted with anti-ExbB polyclonal antibody. This antibody was produced using purified denatured full-length ExbB excised from an SDS-polyacrylamide gel as the antigen (19, 35). None of the deletions removed an immunodominant epitope, because the $\Delta 10$ proteins were all similarly detectable by the polyclonal antibody when they were synthesized *in vitro* in the relative absence of proteases (see Fig. S1 in the supplemental material).

In vivo formaldehyde cross-linking. Due to the proteolytic instability of most of the $\Delta 10$ proteins, we used a rapid formaldehyde cross-linking procedure to assay their *in vivo* interactions. Subcultured cells were grown to an A_{550} of 0.4 to 0.5 and induced for 30 min with L-arabinose at the concentrations listed in Table 2. Cells were pelleted at room temperature and resuspended in 100 mM sodium phosphate buffer, pH 8.0, and treated with 1% monomeric formaldehyde for 3 min. Cross-linking was quenched with a final concentration of 1% acetic acid. Cell pellets were solubilized at 60°C for 5 min in gel sample buffer, electrophoresed on SDS-polyacrylamide gels (34), and immunoblotted with polyclonal anti-

TABLE 1 Strains and plasmids

Strain or plasmid	Genotype	Reference
Strains		
GM1	<i>ara</i> Δ (<i>pro-lac</i>) <i>thi</i> F' <i>pro lac</i>	57
KP1037	GM1 <i>exbB::Tn10</i>	6
KP1057	W3110 <i>acrA::TnpH</i>	This study
KP1392	GM1 <i>exbB::Tn10 tolQ(Am) recA::cat</i>	20
KP1397	W3110 Δ <i>ana-tonB-trp metE163::Tn10 recA::cat</i>	This study
KP1445	GM1 <i>exbB::Tn10 recA::cat</i>	This study
KP1563	GM1 $\Delta\text{exbBD}::kan$	This study
RA1003	W3110 $\Delta\text{exbBD}::kan$	44
MPHT37	<i>acrA::TnpH</i> at codon 72 in MC4100	58
SH03	W3110 <i>tolC210::Tn10</i>	59
Plasmids		
pBAD24	Cloning vector; expression induced by L-arabinose	40
pKP660	pBAD24 expressing <i>exbBD</i> from the pBAD promoter	22
pKP725	<i>exbB</i> $\Delta 40-49$ <i>exbD</i>	This study
pKP650	<i>exbB</i> $\Delta 50-59$ <i>exbD</i>	This study
pKP651	<i>exbB</i> $\Delta 60-69$ <i>exbD</i>	This study
pKP652	<i>exbB</i> $\Delta 70-79$ <i>exbD</i>	This study
pKP653	<i>exbB</i> $\Delta 80-89$ <i>exbD</i>	This study
pKP654	<i>exbB</i> $\Delta 90-99$ <i>exbD</i>	This study
pKP655	<i>exbB</i> $\Delta 100-109$ <i>exbD</i>	This study
pKP656	<i>exbB</i> $\Delta 110-119$ <i>exbD</i>	This study
pKP657	<i>exbB</i> $\Delta 120-129$ <i>exbD</i>	This study
pKP920	pBAD24 expressing <i>exbB</i> from the pBAD promoter	This study
pKP921	<i>exbB</i> $\Delta 40-49$	This study
pKP922	<i>exbB</i> $\Delta 50-59$	This study
pKP923	<i>exbB</i> $\Delta 60-69$	This study
pKP924	<i>exbB</i> $\Delta 70-79$	This study
pKP925	<i>exbB</i> $\Delta 80-89$	This study
pKP926	<i>exbB</i> $\Delta 90-99$	This study
pKP927	<i>exbB</i> $\Delta 100-109$	This study
pKP928	<i>exbB</i> $\Delta 110-119$	This study
pKP929	<i>exbB</i> $\Delta 120-129$	This study

TABLE 2 Physical properties and phenotypes of ExbB and deletion proteins

ExbB	Deleted residues	% Arabinose ^a	Molecular mass (kDa)		pI ^c
			Calculated	Apparent ^b	
ExbBD		0.00025	26.3	26	7.9
ExbBΔ40–49	IFFSKSVEFF	0.1	25.0	22.5	7.9
ExbBΔ50–59	NQKRRRLKREQ	0.1	25.0	23.5	5.6
ExbBΔ60–69	QLLAEARSLN	0.03	25.2	24	7.9
ExbBΔ70–79	QANDIAADFG	0.001	25.3	25	9.2
ExbBΔ80–89	SKSLSLHLLN	0.1	25.2	26	6.8
ExbBΔ90–99	EAQNELELSE	0.001	25.1	21	9.6
ExbBΔ100–109	GSDDNEGIKE	0.00025	25.2	21.5	9.5
ExbBΔ110–119	RTSFRELRRV	0.1	25.0	25.5	5.9
ExbBΔ120–129	AAVGRQMGRG	0.1	25.3	25	6.3

^a Concentration of L-arabinose required to achieve chromosomal levels of expression.

^b Determined by SDS-polyacrylamide gel electrophoresis.

^c Calculated isoelectric point.

bodies recognizing ExbB or ExbD (19) or with anti-TonB monoclonal antibody 4F1, which recognizes an epitope consisting of TonB residues 120 to 127 (36). For dominance studies, cultures were grown with 0.01% L-arabinose for 60 min prior to cross-linking.

EtBr accumulation assay. To establish the initial correlation between PMF collapse and growth rate, cultures of strain GM1 were grown in M9 minimal medium with 1.85 μM Fe to an A_{550} of 0.4 to 0.5 and treated for 15 min with various concentrations of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) in DMSO (0.0, 7.5, 15.0, 22.5, 30.0, 45.0, 60.0 μM) with shaking at 37°C. After 15 min, 0.4 A_{550} -ml cells were transferred directly to a 1.5-ml disposable cuvette. The final volume in each cuvette was adjusted to 2 ml using M9 growth medium. Ethidium bromide (EtBr) fluorescence was measured on a PerkinElmer LS55 fluorescence spectrometer immediately after addition of EtBr to a final concentration of 6.25 μM at the 520-nm and 590-nm EtBr excitation and emission wavelengths, respectively. The widths of the excitation and emission slits were set at 15 nm. Data were collected continuously for 180 s using the time drive application from the FLWINLAB software. The rate of EtBr accumulation was linear, and the slopes were calculated using linear regression analysis. Rates of EtBr accumulation from at least two independent triplicate assays were graphed as arbitrary fluorescence units (FU)/time. Immediately prior to EtBr addition, aliquots from the cultures were precipitated with TCA, resolved on 13% SDS-polyacrylamide gels, and visualized on immunoblots probed with anti-ExbB antibodies (19).

To determine the effect of various levels of CCCP on cell growth, KP1445 was grown to an A_{550} of 0.5 and treated with the same concentrations of CCCP as stated above. Growth at A_{550} was monitored for 3 h.

To assess the effects of ExbBΔ10 protein expression on EtBr accumulation, KP1445 carrying the relevant plasmids was grown in M9 minimal medium with 1.85 μM Fe. At an A_{550} of 0.4 to 0.5, mutant proteins were induced with L-arabinose for the final 15 or 60 min using 0.01% or 0.1% L-arabinose. To measure EtBr accumulation, 0.8 A_{550} -ml cells were transferred to a disposable cuvette and adjusted to a volume of 2.0 ml, and 6.25 μM EtBr was added. Fluorescence was measured as described above.

RESULTS

The ExbB cytoplasmic region between TMD1 and TMD2 is essential for activity. The function of the ExbB soluble cytoplasmic domain between TMD1 and TMD2 has not been determined (Fig. 1). To rapidly identify nonessential regions, nine 10-codon deletions affecting residues 40 through 129 were created using PCR amplification of plasmid pKP660 carrying both *exbB* and *exbD* expressed from their native operon under expression from the arabinose pBAD promoter. Ten-amino-acid-deletion proteins (referred to as Δ10 proteins) of ExbB were expressed at near-

chromosomal levels by induction with different concentrations of L-arabinose to compensate for their high rates of endogenous proteolytic degradation (Table 2).

Although the calculated masses of the Δ10 proteins varied from 25.0 kDa to 25.3 kDa, immunoblot analysis revealed that their apparent molecular masses varied considerably, from 21 kDa to 25.5 kDa (Table 2). This was apparently due to large differences in calculated pI, which ranged from 5.6 to 9.5. Differential proteolysis was ruled out, because mutant proteins synthesized *in vitro* had masses identical to those of proteins synthesized *in vivo* (Table 2; also, see Fig. S1 in the supplemental material). The effect of pI differences on apparent mass in SDS-polyacrylamide gel electrophoresis has been documented (37). Like wild-type ExbB, all the Δ10 proteins were correctly localized in the CM (see Fig. S2A in the supplemental material). As seen previously in the absence of ExbB (20), the majority of TonB fractionated with the OM in the presence of the mutant proteins (see Fig. S2B in the supplemental material).

The ability of the Δ10 proteins to support TonB-mediated energy transduction was measured by evaluating initial rates of [⁵⁵Fe]ferrichrome transport, which can discriminate within the range of ~20 to 100% TonB activity (38). Except for ExbBΔ100–109, all the deletion proteins were essentially inactive, supporting 0 to 7% of the initial transport rates observed for wild-type ExbB (Fig. 2A). ExbBΔ100–109 retained the ability to support ~25% of the wild-type level. Although chromosomal levels of the Δ10 proteins could be achieved, the steady-state levels of the coexpressed wild-type ExbD protein varied with the ability of the deletion protein to stabilize them and with the amount of L-arabinose used for induction (Fig. 2B; Table 2).

The Δ10 proteins are impaired in formation of ExbB tetramer+X complexes. *In vivo*, a small proportion of wild-type ExbB can be trapped in formaldehyde cross-linked homodimers, with a greater proportion trapped in homotetramer complexes that include ~85 kDa of unknown protein (ExbB tetramer+X complexes) (30). To assess the capacity of the Δ10 proteins to form these complexes, each was expressed at chromosomal levels and then cross-linked with monomeric formaldehyde (Fig. 3). While ExbB-specific bands were observed in the region of the tetramer+X, all were present at much lower levels, and the apparent sizes of the complex varied with the apparent changes in molecular mass of most Δ10 proteins. The exception was ExbBΔ80–89, for which a very low level of

FIG 2 Most of the ExbB proteins with cytoplasmic loop deletions are inactive. Initial rates of iron transport were measured in triplicate assays. (A) Averages are shown as a percentage of the value for KP1392 (*exbB exbD*), in which plasmid-encoded ExbB and ExbD (pExbBD) were expressed from pKP660. Standard deviations are indicated on each bar. (B) Steady-state levels of ExbB (top), ExbD (middle), and TonB (bottom) present at the time of assay. Immediately prior to the assay, samples with equal A_{550} -ml cells were TCA precipitated and immunoblotted with anti-ExbB, anti-ExbD, or anti-TonB antibodies.

tetramer+X complex was observed only on much longer exposures. The formaldehyde cross-linked homodimeric bands, which are not efficiently formed by wild-type ExbB, were also not abundant for most $\Delta 10$ proteins. ExbB $\Delta 90$ –99 and ExbB $\Delta 100$ –109 both formed high levels of presumed homodimers at apparent masses consistent with the behavior of their monomeric forms. Homodimers were detectable only in longer exposure for ExbB $\Delta 40$ –49 and ExbB $\Delta 80$ –89.

Formaldehyde cross-linked ExbB-TonB complexes were absent from most of the strains expressing the $\Delta 10$ proteins. There were, however, low levels of ExbB-TonB complexes detectable from ExbB $\Delta 70$ –79, ExbB $\Delta 90$ –99, ExbB $\Delta 100$ –109, and ExbB $\Delta 120$ –129 with apparent masses that corresponded to the mass differences for the relevant $\Delta 10$ proteins (Fig. 4). The significance of these low levels is established below.

The $\Delta 10$ proteins do not support the PMF-dependent interaction between TonB and ExbD. ExbB is required for TonB and ExbD to form a PMF-dependent formaldehyde cross-link (22). To determine the effect of the $\Delta 10$ proteins on ExbD cross-linking profiles, strains expressing the ExbB deletions at the level of chromosomally encoded ExbB were subjected to formaldehyde treat-

FIG 3 $\Delta 10$ proteins support low levels of ExbB tetramer+X complex. The $\Delta 10$ proteins were expressed at near-chromosomal levels in KP1445 (*exbB exbD*) from plasmids that also expressed ExbD. Equivalent numbers of cells were treated using rapid *in vivo* formaldehyde cross-linking at pH 8.0, resolved on 13% SDS-polyacrylamide gels, and immunoblotted with anti-ExbB antibody. Samples are identified above each lane (WT, GM1). Positions of ExbB monomers, dimers, and tetramer+X complexes are indicated on the right. Molecular mass markers are indicated on the left. The bottom panel shows a short exposure to allow comparison of steady-state monomer levels.

ment. Wild-type ExbD (15.5 kDa) formed three cross-linked complexes observed previously: TonB-ExbD, ExbD-ExbB, and ExbD-ExbD (Fig. 5) (22). Longer development of the immunoblot showed that wild-type ExbD formed homodimers in the presence of all the ExbB deletion proteins (data not shown). The ability to form the ExbB-ExbD complex was limited to ExbB $\Delta 70$ –79, ExbB $\Delta 90$ –99, ExbB $\Delta 100$ –109, and ExbB $\Delta 120$ –129, with the pattern of apparent mass shift in the immunoblot reflecting the differences in apparent masses of the respective $\Delta 10$ proteins (see Fig. 2B for comparison). These $\Delta 10$ proteins were the same ones that could form a low but detectable level of the ExbB-TonB formaldehyde cross-linked complex. None of the $\Delta 10$ proteins formed the PMF-dependent ExbD-TonB complex except ExbB $\Delta 100$ –109, whose complex was detectable upon longer exposure, consistent with its ability to support $\sim 25\%$ iron transport activity.

It is important to remember that the absence of detectable cross-linking does not necessarily mean that the two proteins are no longer in a complex, only that the formaldehyde cross-linkable residues were no longer in the correct apposition for cross-linking. Conformational changes within the complex or complete absence of interaction cannot be distinguished by this means.

Expression of the $\Delta 10$ proteins results in immediate, but reversible, growth arrest. For all but ExbB $\Delta 100$ –109, induction of expression of the $\Delta 10$ proteins with 0.1% or 0.01% L-arabinose resulted in the immediate inhibition of cell growth (Fig. 6). The length of time the bacteria remained in stasis depended upon the amount of L-arabinose used for their induction. All of the deletion proteins except ExbB $\Delta 100$ –109 inhibited growth for more than 540 min in the presence of 0.1% L-arabinose, but when they were induced with 0.01% L-arabinose, the cells recovered and resumed

FIG 4 ExbB Δ 70–79, ExbB Δ 90–99, ExbB Δ 100–109, and ExbB Δ 120–129 cross-link to TonB *in vivo*. The Δ 10 proteins were expressed at near-chromosomal levels in KP1445 (*exbB exbD*) from plasmids that also expressed ExbD. Equivalent numbers of cells were treated using rapid *in vivo* formaldehyde cross-linking at pH 8.0, resolved on 11% SDS-polyacrylamide gels, and immunoblotted with anti-TonB antibody. Sample identities are indicated above each lane (WT, GM1). Positions of TonB monomers and of TonB-Lpp, TonB-ExbD, TonB-ExbB, and TonB-FepA complexes are indicated on the right. Molecular mass markers are indicated on the left. The bottom panel shows a short exposure to allow comparison of steady-state monomer levels.

growth after 30 to 360 min. Immunoblot analysis of ExbB protein expression at various time points after induction revealed that cells resumed growth when steady-state levels of the respective Δ 10 protein declined, presumably as the arabinose was consumed. Consistent with that idea, for cells induced with 0.01% arabinose, treatment with a second dose of 0.01% L-arabinose during the recovery phase induced a second round of mutant protein synthesis and stopped cell growth again (data not shown). Thus, growth arrest was reversible and occurred only when the deletion proteins were present.

Protein was present at each time point during induction with 0.01% arabinose for ExbB Δ 100–109 and ExbB Δ 70–79 (Fig. 6H and E) but substantially reduced or absent for the remaining proteins at 300 min (180 min postinduction) and beyond (Fig. 6B, C, D, F, G, I, and J). Even though ExbB Δ 70–79 was present at all time points, it inhibited growth only up to 180 min postinduction, suggesting that it was the least compromised of the group and must build to a higher level in order to halt cell growth. In contrast to ExbB Δ 70–79, growth arrest was very sensitive to the low levels of ExbB Δ 80–89 that could be expressed (Fig. 6F). Exposures for each panel were comparable, and steady-state levels of the various Δ 10 proteins in most cases could be standardized to a cross-reactive band just below the position of each ExbB monomer where expression was induced with 0.01% L-arabinose.

To determine if ExbD was necessary for growth arrest, plasmids encoding each of the ExbB deletion proteins were reengineered using extra-long PCR to eliminate *exbD*. The newly constructed plasmids (pKP920-929) were then transformed into KP1445 (*GM1 exbB::Tn10 recA::cat*), and growth was assayed in the absence of ExbD as described above. The Tn10 insertion in

FIG 5 ExbB Δ 70–79, ExbB Δ 90–99, ExbB Δ 100–109, and ExbB Δ 120–129 cross-link to ExbD *in vivo*. The Δ 10 proteins were expressed at near-chromosomal levels in KP1445 (*exbB exbD*) from plasmids that also expressed ExbD. Equivalent numbers of cells were treated using rapid *in vivo* formaldehyde cross-linking at pH 8.0, resolved on 13% SDS-polyacrylamide gels, and immunoblotted with anti-ExbD antibody. Samples are identified above each lane (WT, GM1). Positions of ExbD monomers and of TonB-Lpp, TonB-ExbD, TonB-ExbB, and TonB-FepA complexes are indicated on the right. At far right, a longer exposure of the ExbB Δ 100–109 sample demonstrates that a TonB-ExbD complex is detectable, consistent with the 25% iron transport activity this protein supports. Molecular mass markers are indicated on the left. The bottom panel shows a short exposure to allow comparison of steady-state monomer levels, which vary due to different levels of inducer required for chromosomal expression of the Δ 10 proteins.

exbB is polar on *exbD* expression (27). Induction of each of the proteins except ExbB Δ 100–109 still caused immediate growth arrest (data not shown). Similarly, expression of the Δ 10 ExbB proteins in a *tonB*[−] background did not alter the growth arrest phenotype, indicating that growth arrest occurred independently of either ExbD or TonB (data not shown).

Growth arrest caused by the Δ 10 proteins is not due to dissipation of PMF. ExbB and ExbD appear to work in concert to harness the PMF of the CM and energize TonB (24). Protonophore-mediated collapse of PMF leads to immediate growth arrest. Since the ExbB Δ 10 proteins that arrested growth also did not support the PMF-dependent TonB-ExbD formaldehyde cross-link, they may have collapsed the CM PMF. To test that hypothesis, we measured the ability of the Δ 10 proteins to interfere with the PMF-dependent efflux of ethidium bromide (EtBr). EtBr normally undergoes efflux out the cell by the PMF-dependent AcrAB-TolC complex. When PMF is collapsed, EtBr accumulates in the cytoplasm, intercalates with the DNA, and causes an increase in fluorescence of the cells (39). To establish a standard for comparison, we measured the rate of EtBr uptake caused by increasing levels of the protonophore CCCP (carbonyl cyanide *m*-chlorophenyl hydrazine) (Fig. 7A). The concentration of CCCP required to completely stop the growth of parent strain KP1445 was 30 μ M, which corresponded to an EtBr accumulation rate of 0.23 fluorescence units (FU) per second (Fig. 7B and C).

FIG 6 Induction of $\Delta 10$ proteins causes immediate growth arrest. Growth curves for each $\Delta 10$ protein induced at 120 min (arrow) with 0.01 (○) or 0.1% L-arabinose (●) in strain KP1445 (*exbB exbD*) are shown. Immunoblots with anti-ExbB antibody indicate ExbB protein levels at various time points (shown above each lane, in minutes) after induction. Curves for wild-type GM1 (□) and KP1445 (Δ) are the averages from three separate growth experiments and are included in each panel for comparison. All plasmids also carried *exbD*. (A) *ExbB*BD; (B) *ExbB*Δ40–49; (C) *ExbB*Δ50–59; (D) *ExbB*Δ60–69; (E) *ExbB*Δ70–79; (F) *ExbB*Δ80–89; (G) *ExbB*Δ90–99; (H) *ExbB*Δ100–109; (I) *ExbB*Δ110–119; (J) *ExbB*Δ120–129.

FIG 7 Effects of the protonophore CCCP on EtBr accumulation and cell growth. (A) Exponential-phase cells of KP1445 (*exbB exbD*) were treated with increasing concentrations of CCCP for 15 min prior to measurement of the rates of EtBr accumulation, as described in Materials and Methods. (B) The rates of EtBr incorporation at each CCCP concentration are plotted as a bar graph. The values are averages from three individual experiments, with error bars reflecting the standard deviations. (C) Exponential-phase cells of KP1445 were treated with increasing concentrations of CCCP at 150 min (arrow), and a growth curve was determined. Data show a representative result from one of three individual experiments.

To test the effects of the $\Delta 10$ proteins on PMF, their expression was induced with the higher level of 0.1% L-arabinose, as in experiments whose results are shown in Fig. 6, for either 15 or 60 min. The majority of the $\Delta 10$ proteins had no effect on EtBr accumulation at either time of induction (Fig. 8A and B). The ExbB $\Delta 120$ –

FIG 8 Growth arrest caused by $\Delta 10$ proteins is not due to PMF collapse. ExbB $\Delta 10$ proteins were induced for 15 or 60 min in KP1445 (*exbB exbD*) with 0.1% L-arabinose. After induction, cultures were divided and assayed for either EtBr accumulation (A and B, respectively) or steady-state levels of ExbB protein by immunoblotting following TCA precipitation (C). Protein samples from the 15-min induction were also evaluated for steady-state levels of AcrAB and TolC by immunoblotting with appropriate antibodies (D). Rates of EtBr accumulation are averages from three individual experiments. All plasmids also encoded ExbD. Induction times and identities of samples are given above each lane of the immunoblot. Anti-AcrAB and -TolC antibodies are indicated on the right.

FIG 9 The absence of ExbD does not stop proton leakage induced by expression of ExbBΔ50–59 and ExbBΔ120–129. Δ10 proteins were induced with 0.1% L-arabinose from plasmids lacking the *exbD* gene for 15 min, and EtBr accumulation was assayed. ExbBΔ120–129 was also assayed in the presence of the inactive D25N ExbD protein (far right). Data are rates of EtBr accumulation and are averages from three individual experimental results; error bars show standard deviations.

129 protein caused the greatest PMF dissipation, leading to a rate of EtBr accumulation of 0.12 FU/s after 15 min of induction (Fig. 8A). This rate of proton leakage was not sufficient to stop cell growth, which required a proton leakage rate of 0.23 FU/s (Fig. 7). ExbBΔ50–59 caused a smaller decrease in PMF than ExbBΔ120–129, and both deletions caused still smaller decreases in PMF when measured after 60 min of induction (Fig. 8B).

The levels of the Δ10 proteins at the time of assay were determined by immunoblot and compared to normal chromosomal levels (Fig. 8C). Upon 0.1% L-arabinose induction, the wild-type ExbB was highly overexpressed and had little effect on growth (Fig. 6A). In contrast, the steady-state levels of most of the Δ10 proteins reached only near-chromosomal levels. It was clear that these proteins were surprisingly toxic, even at only 15 min of induction. ExbBΔ100–109, the only active deletion protein, could be expressed to levels similar to those of wild-type ExbB and, like wild-type ExbB, was also not growth inhibitory (Fig. 6H).

The rate of EtBr accumulation was unchanged by the absence of ExbD, consistent with the previous result above, where it had no effect on growth arrest (Fig. 9). For ExbBΔ120–129, the EtBr accumulation rate was also unchanged in the presence of ExbD D25N, which cannot respond to PMF (24). The absence of TonB also had no effect on EtBr accumulation rates (data not shown).

Because decreased levels of the AcrAB and TolC proteins could equally account for the increased EtBr accumulation of ExbBΔ50–59 or ExbBΔ120–129, the steady-state levels of AcrAB and TolC were also measured for all the Δ10 proteins after 15 min of induction. Thus, reduced levels of AcrAB, TolC, and TonB were not responsible for EtBr accumulation (Fig. 8D).

Chromosomal levels of Δ10 proteins arrest growth of wild-type cells. All ExbBΔ10 proteins were correctly localized to the CM, and a few (ExbBΔ70–79, ExbBΔ90–99, ExbBΔ100–109, and ExbBΔ120–129) retained the ability to form near-native levels of the ExbB-ExbD formaldehyde-cross-linked complex (Fig. 5; also, see Fig. S2 in the supplemental material). These Δ10 proteins were

candidates to exhibit a dominant phenotype by poisoning complexes they could assemble with. ExbBΔ40–49, ExbBΔ50–59, ExbBΔ80–89, and ExbBΔ110–119 did not form the ExbB-ExbD complex, even at the longest exposures (data not shown).

Due to their proteolytic instability, we were initially concerned that we would not be able to achieve sufficiently high levels of expression that dominance of the Δ10 proteins for growth arrest could be determined. This turned out not to be a problem, since chromosomal levels of Δ10 protein expression proved to be dominant. ExbBΔ40–49, ExbBΔ50–59, ExbBΔ90–99, and ExbBΔ120–129, which have representative effects in the various assays, were chosen to illustrate: ExbBΔ40–49 and ExbBΔ50–59 were unable to form any detectable formaldehyde cross-linked complexes; however, ExbBΔ50–59 caused modest PMF dissipation and ExbBΔ40–49 did not. ExbBΔ90–99 was able to form ExbB homodimers and ExbB-ExbD heterodimers (Fig. 3 and 5); ExbBΔ120–129 supported low-level formation of the ExbB-ExbD and ExbB-TonB complexes (Fig. 4 and 5) and also caused the greatest PMF dissipation, as measured by the EtBr accumulation assay (Fig. 8). Induction of Δ10 protein expression with the smaller amount of L-arabinose from Fig. 6, 0.01%, caused immediate growth arrest in a wild-type strain (Fig. 10A). Induction of wild-type ExbB/D with the same amount of L-arabinose was highly overexpressed yet had little effect on cell growth (Fig. 10). The remaining four Δ10 proteins that could arrest growth were also dominant after addition of 0.01% L-arabinose (data not shown).

At 1 h after induction, while cell growth remained arrested, the steady-state levels of the Δ10 proteins were determined relative to the chromosomally encoded wild-type ExbB in each strain (Fig. 10B). Due to the differences in apparent mass, identification of the mutant proteins and comparison with wild-type ExbB levels was straightforward. ExbBΔ90–99 and ExbBΔ120–129 were both present at levels similar to those of wild-type ExbB, while ExbBΔ40–49 and ExbBΔ50–59 were present at somewhat lower levels. These results, coupled with the inability of ExbBΔ40–49 and ExbBΔ50–59 to form any detectable formaldehyde-cross-linked complexes, indicated that the dominant Δ10 proteins did not exert their effects by competition with wild-type ExbB. Their effects appeared to be due to interference with an unknown pathway that was key for cellular growth.

Several ExbBΔ10 proteins are not dominant for iron transport. As shown above, each of the ExbBΔ10 proteins was defective in supporting iron transport, with ExbBΔ100–109 retaining the most activity at 25%. Their dominance in iron transport assays was tested by measuring their effects on initial rates of iron transport in GM1 (*exbB*⁺) (Fig. 11A). The steady-state levels achieved varied according to the proteolytic stability of each protein and were consistent with the levels of L-arabinose needed to adjust expression to chromosomal levels (Fig. 11B; Table 2). Four of the Δ10 proteins, ExbBΔ70–79, ExbBΔ90–99, ExbBΔ100–109, and ExbBΔ120–129, were the most dominant. The dominant effects of ExbBΔ70–79 and ExbBΔ100–109 in the iron transport assay could be attributed to their higher levels of accumulation (Fig. 11B) and their ability to assemble into complexes with TonB and ExbD (Fig. 4 and 5). The dominant effects of ExbBΔ120–129, which had a steady-state level equal to that of the chromosomally encoded wild type, could be attributed to the fact that it caused a significant decrease in PMF, which is required to energize iron transport. While the dominance due to ExbBΔ90–99 may have been due to its ability to inefficiently assemble into an ExbB-ExbD complex (Fig. 3 and 5), the low steady-state level of this variant

FIG 10 $\Delta 10$ proteins are dominant for growth arrest. (A) Exponential-phase-grown cells of GM1, bearing the plasmid-encoded protein ExbB $\Delta 40-49$, ExbB $\Delta 50-59$, ExbB $\Delta 90-99$, or ExbB $\Delta 120-129$ or vector plasmid pBAD24, were treated with the lower level of L-arabinose (0.01%; arrow), and growth curves were plotted for 180 min. After 1 h of induction (200 min on the abscissa), proteins from 0.2 A₅₅₀-ml cells were precipitated with TCA immediately upon harvest. (B) Steady-state levels of both wild-type ExbB and the $\Delta 10$ proteins were determined by immunoblotting with anti-ExbB antibodies. The positions of wild-type ExbB and the $\Delta 10$ proteins are indicated on the right. Apparent masses of the $\Delta 10$ proteins vary, as shown in Fig. 2B.

suggested that this was not the case. Dominance due to assembly into complexes generally requires higher steady-state levels of the mutant protein than of the wild-type protein. While the source of dominance of ExbB $\Delta 90-99$ was not clear, it nonetheless set a benchmark for how little of a $\Delta 10$ protein was required.

Though inactive, several of the ExbB $\Delta 10$ proteins were not dominant with respect to iron transport, when induced with levels of L-arabinose used for dominant growth arrest. ExbB $\Delta 50-59$, ExbB $\Delta 60-69$, ExbB $\Delta 80-89$, and ExbB $\Delta 110-119$ were unable to decrease initial rates of iron transport below 65 to 70%, confirming the idea that they did not compete with wild-type ExbB and that they did not collapse PMF (or, in the case of ExbB $\Delta 50-59$, did not significantly collapse PMF). All had steady-state levels at or above the level of ExbB $\Delta 90-99$, which was dominant for iron transport.

For the iron transport dominance tests, the ExbB $\Delta 10$ proteins were expressed at the levels that caused growth arrest. Full induction of the pBAD promoter occurs with 2.0% L-arabinose (40). Even the use of 0.1% L-arabinose might be considered a high level of induction and thus potentially responsible for the growth arrest observed. However, because several mutant proteins were non-

FIG 11 Several $\Delta 10$ proteins are not dominant for iron transport. Expression of the $\Delta 10$ proteins in GM1 (wild type for ExbB and ExbD) from plasmids that also expressed ExbD was induced with 0.01% L-arabinose. Due to their different proteolytic instabilities, different proteins were overexpressed to different degrees with respect to chromosomal level of ExbB. (A) Proteins were expressed for 30 min and subjected to measurements of the initial rates of [⁵⁵Fe]ferrichrome transport. Data from multiple triplicate experiments samples were normalized to the value for GM1 (100%). Averaged normalized transport percentages from three individual experiments are shown, with standard deviations. (B) Steady-state levels of ExbB present at the time of assay. Immediately prior to the assay, samples with equal amounts of cells (A₅₅₀-ml) were TCA precipitated and immunoblotted with anti-ExbB antibodies.

dominant, nonspecific growth arrest dominance arising from high-level induction of ExbB $\Delta 10$ proteins from the pBAD promoter was ruled out. Consideration of all the evidence for ExbB $\Delta 60-69$ and ExbB $\Delta 110-119$ showed that these two proteins did not assemble into complexes with either TonB or ExbD. Thus, these two specific ExbB $\Delta 10$ proteins, at least, caused growth arrest by interference in an unknown pathway. Although caveats exist for the interpretations of the other mutant phenotypes, we think it likely that all of the $\Delta 10$ proteins except ExbB $\Delta 100-109$ interfered with the same unknown pathway.

DISCUSSION

The ExbB and ExbD proteins of the TonB system appear to harvest the energy of the PMF and transmit it via TonB to OM transport proteins. They are paralogous to two other PMF-dependent systems in *Escherichia coli*. All three systems have different functions, with the Tol system (paralogues TolQR) being important in outer membrane integrity and cell division and the Mot system (MotAB) being used to energize flagellar rotation.

The greatest amino acid similarity among the *E. coli* paralogues ExbB, TolQ, and MotA occurs in their last two TMDs, regions that could theoretically bind protons and respond to PMF. Recent

studies on ExbB indicate that its three TMDs are involved in scaffolding for assembly of protein complexes and signaling between cytoplasm and periplasm but have no direct role in proton translocation. These studies also suggest that, because of the high degree of residue conservation among last two TMDs in the paralogues TolQ and MotA and the *Vibrio alginolyticus* MotA orthologue PomA, they are also unlikely to participate directly in proton translocation (41).

The majority of ExbB soluble sequences occupy the cytoplasm, where they might interact with cytoplasmic proteins, as has been observed for interaction of the cytoplasmic domain of MotA with FliG (42, 43). There is no similarity among the corresponding soluble cytoplasmic loops or C-terminal cytoplasmic tails of the three *E. coli* paralogues. Thus, it seems likely that cytoplasmic sequences are a means by which the different activities of these family members are manifested or regulated.

Here we show that the cytoplasmic loop region of ExbB between TMD1 and TMD2 appears to connect ExbB, and thus the TonB system, to regulation of cell growth. Deletion scanning analysis of that region showed that eight out of nine 10-residue deletions within that cytoplasmic loop rendered ExbB inactive. This was not particularly surprising given the size of the deletions, although it should be noted that TonB itself is tolerant of mutations and deletions (23, 44, 45). A recent 10-residue-deletion scanning mutagenesis also rendered ExbD inactive for all but the most N-terminal of the deletions but allowed division of the periplasmic domain of ExbD into discrete functional regions based on mechanistically informative rather than simple phenotypic assays (46).

The eight inactive $\Delta 10$ proteins had the unexpected phenotype of invoking immediate growth arrest when induced with 0.01% L-arabinose, a relatively high level of inducer. However, due to their proteolytic instability, the steady-state levels of $\Delta 10$ protein that led to growth arrest were near chromosomal levels. This immediate growth arrest was different from the growth inhibition seen in the MotAB system. High-level overexpression of MotA in the presence of the first 60 residues of MotB fused to 50 residues from the vector pBR322 causes growth inhibition due to proton leakage over a period of hours (47–49). Mutations in the highly conserved Asp32 in the TMD of the modified MotB relieve the growth impairment (50). It is now thought that MotA and MotB together are responsible for proton conduction (51).

In contrast to the MotAB paralogues, growth arrest by the ExbB $\Delta 10$ proteins was not mediated through proton leakage. In the two cases where partial PMF dissipation was observed, it was not sufficient to account for the growth arrest. In addition, growth arrest caused by ExbB $\Delta 10$ proteins occurred independently of ExbD (the paralogue of MotB) or of TonB. Thus, low steady-state levels of the inactive ExbB $\Delta 10$ proteins by themselves were capable of causing immediate growth arrest. Immediate growth arrest was not observed for the $\Delta 10$ ExbD proteins or for TonB mutant proteins (data not shown) and is thus not a general characteristic of TonB system proteins.

Taken together, these results suggest that the immediate growth arrest was due to changed interactions of $\Delta 10$ proteins with a key but unknown protein(s), different from any that interact with soluble domains of MotA. Proteins with deletions within the corresponding MotA domain are inactive and unstable but do not result in growth arrest when overexpressed (52). As long as the $\Delta 10$ proteins were continuously synthesized, cell growth remained arrested. When these proteolytically unstable $\Delta 10$ pro-

FIG 12 The ExbB cytoplasmic loop is predicted to contain a disordered region. The amino acid sequence of *E. coli* ExbB was analyzed by the PONDR program. Amino acid sequences were scanned from the amino to the carboxy terminus for regions of disorder (abscissa), indicated by curves above the ordinate midline. A significant region of disorder (>30 residues) is indicated by the thick horizontal black bar at the midline between residues ~78 and 130.

teins were no longer synthesized, cell growth resumed. Consistent with that hypothesis, growth arrest was dominant. The fact that inactivity in iron transport was not dominant for several $\Delta 10$ proteins following induction with 0.01% L-arabinose (significantly less than the full induction concentration of 0.2%) allowed us to rule out induction as the cause of the dominance.

We speculate that the inactive $\Delta 10$ proteins were unable to release a necessary and cyclic contact with the unknown protein(s) that occurs normally with wild-type ExbB. Consistent with that, a large proportion of the ExbB cytoplasmic loop is predicted to be disordered (Fig. 12), which suggests that it may achieve a final tertiary conformation through induced fit with another protein. The ExbB cytoplasmic loop deletions clearly changed the conformation of ExbB. They rendered ExbB proteolytically unstable, even though wild-type ExbB is the only protein in the energy transduction complex that remains stable in the absence of the other partners (26).

Since PMF collapse was ruled out, what might be the mechanism of the immediate growth arrest? Expression of the $\Delta 10$ ExbB proteins does not induce expression from a σ^E -*lacZ* fusion under conditions where overexpression of OmpC resulted in $\sim 5\times$ induction of expression (data not shown). Thus, growth arrest is not mediated through the σ^E membrane stress pathway. Likewise, growth arrest is not due to depletion of the essential ATP-dependent membrane protease, FtsH, which degrades misfolded proteins. Expression of ExbB $\Delta 60$ -69 in strain AR3291 (W3110 *sflC21* *AftsH3::kan* [53]) still resulted in immediate growth arrest in the absence of FtsH (data not shown). Alternative candidates include inhibition of key cellular processes, such as DNA replication, protein synthesis, cell membrane, and peptidoglycan synthesis. When acidic phospholipids are depleted, DnaA arrests DNA synthesis; however, that depletion requires hours (54). Inhibition of protein synthesis seems unlikely given the very low steady-state levels of the $\Delta 10$ proteins that lead to growth arrest. At chromosomal levels of expression, there would be $\sim 2,500$ copies per cell (19), while estimates for the number of ribosomes in an *E. coli* cell ranges from 68,000 to 72,000 depending on the growth rate (55). Inactivation of the antitoxin in a toxin-antitoxin system, where most effects are reversible, is also possible (56).

Why might the TonB system be connected to an essential cell process? Perhaps it is important to coordinate transport of nutri-

ents across the OM, which translates to expenditure of PMF by the TonB system, with the overall cellular physiology and checkpoint decisions about whether to continue growing and replicating. In any case, identification of the protein(s) involved in growth arrest will increase our understanding of the TonB system.

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REFERENCES

- Gresock MG, Savenkova MI, Larsen RA, Ollis AA, Postle K. 2011. Death of the TonB shuttle hypothesis. *Front. Microbiol.* 2:206.
- Wandersman C, Delepelaire P. 2004. Bacterial iron sources: from siderophores to hemophores. *Annu. Rev. Microbiol.* 58:611–647.
- Braun V. 2006. Energy transfer between biological membranes. *ACS Chem. Biol.* 1:352–354.
- Wiener MC. 2005. TonB-dependent outer membrane transport: going for baroque? *Curr. Opin. Struct. Biol.* 15:394–400.
- Postle K, Larsen RA. 2007. TonB-dependent energy transduction between outer and cytoplasmic membranes. *Biometals* 20:453–465.
- Skare JT, Postle K. 1991. Evidence for a TonB-dependent energy transduction complex in *Escherichia coli*. *Mol. Microbiol.* 5:2883–2890.
- Skare JT, Ahmer BMM, Seachord CL, Darveau RP, Postle K. 1993. Energy transduction between membranes—TonB, a cytoplasmic membrane protein, can be chemically cross-linked *in vivo* to the outer membrane receptor FepA. *J. Biol. Chem.* 268:16302–16308.
- Jaskula JC, Letain TE, Roof SK, Skare JT, Postle K. 1994. Role of the TonB amino terminus in energy transduction between membranes. *J. Bacteriol.* 176:2326–2338.
- Larsen RA, Thomas MT, Wood GE, Postle K. 1994. Partial suppression of an *Escherichia coli* TonB transmembrane domain mutation ($\Delta V17$) by a missense mutation in ExbB. *Mol. Microbiol.* 13:627–640.
- Braun V, Gaisser S, Herrmann C, Kampfenkel K, Killman H, Traub I. 1996. Energy-coupled transport across the outer membrane of *Escherichia coli*: ExbB binds ExbD and TonB *in vitro*, and leucine 132 in the periplasmic region and aspartate 25 in the transmembrane region are important for ExbD activity. *J. Bacteriol.* 178:2836–2845.
- Higgs PI, Myers PS, Postle K. 1998. Interactions in the TonB-dependent energy transduction complex: ExbB and ExbD form homomultimers. *J. Bacteriol.* 180:6031–6038.
- Roof SK, Allard JD, Bertrand KP, Postle K. 1991. Analysis of *Escherichia coli* TonB membrane topology by use of PhoA fusions. *J. Bacteriol.* 173:5554–5557.
- Kampfenkel K, Braun V. 1992. Membrane topology of the *Escherichia coli* ExbD protein. *J. Bacteriol.* 174:5485–5487.
- Kampfenkel K, Braun V. 1993. Topology of the ExbB protein in the cytoplasmic membrane of *Escherichia coli*. *J. Biol. Chem.* 268:6050–6057.
- Hannavy K, Barr GC, Dorman CJ, Adamson J, Mazengera LR, Gallagher MP, Evans JS, Levine BA, Trayer IP, Higgins CF. 1990. TonB protein of *Salmonella typhimurium*. A model for signal transduction between membranes. *J. Mol. Biol.* 216:897–910.
- Plastow GS, Holland IB. 1979. Identification of an *Escherichia coli* inner membrane polypeptide specified by a lambda-tonB transducing phage. *Biochem. Biophys. Res. Commun.* 90:1007–1014.
- Sauter A, Howard SP, Braun V. 2003. *In vivo* evidence for TonB dimerization. *J. Bacteriol.* 185:5747–5754.
- Ghosh J, Postle K. 2005. Disulphide trapping of an *in vivo* energy-dependent conformation of *Escherichia coli* TonB protein. *Mol. Microbiol.* 55:276–288.
- Higgs PI, Larsen RA, Postle K. 2002. Quantitation of known components of the *Escherichia coli* TonB-dependent energy transduction system: TonB, ExbB, ExbD, and FepA. *Mol. Microbiol.* 44:271–281.
- Held KG, Postle K. 2002. ExbB and ExbD do not function independently in TonB-dependent energy transduction. *J. Bacteriol.* 184:5170–5173.
- von Heijne G, Gavel Y. 1988. Topogenic signals in integral membrane proteins. *Eur. J. Biochem.* 174:671–678.
- Ollis AA, Manning M, Held KG, Postle K. 2009. Cytoplasmic membrane protonmotive force energizes periplasmic interactions between ExbD and TonB. *Mol. Microbiol.* 73:466–481.
- Swayne C, Postle K. 2011. Taking the *Escherichia coli* TonB transmembrane domain “offline”? Non-protonatable Asn substitutes fully for TonB His20. *J. Bacteriol.* 193:3693–3701.
- Ollis AA, Postle K. 2012. ExbD mutants define initial stages in TonB energization. *J. Mol. Biol.* 415:237–247.
- Karlsson M, Hannavy K, Higgins CF. 1993. ExbB acts as a chaperone-like protein to stabilize TonB in the cytoplasm. *Mol. Microbiol.* 8:389–396.
- Fischer E, Günter K, Braun V. 1989. Involvement of ExbB and TonB in transport across the outer membrane of *Escherichia coli*: phenotypic complementation of *exb* mutants by overexpressed *tonB* and physical stabilization of TonB by ExbB. *J. Bacteriol.* 171:5127–5134.
- Ahmer BMM, Thomas MG, Larsen RA, Postle K. 1995. Characterization of the *exbBD* operon of *Escherichia coli* and the role of ExbB and ExbD in TonB function and stability. *J. Bacteriol.* 177:4742–4747.
- Pramanik A, Hauf W, Hoffmann J, Cernescu M, Brutschy B, Braun V. 2011. Oligomeric structure of ExbB and ExbB-ExbD isolated from *Escherichia coli* as revealed by LILBID-mass spectrometry. *Biochemistry* 50:8950–8956.
- Lowder BJ, Duyvesteyn MD, Blair DF. 2005. FliG subunit arrangement in the flagellar rotor probed by targeted cross-linking. *J. Bacteriol.* 187:5640–5647.
- Jana B, Manning M, Postle K. 2011. Mutations in the ExbB cytoplasmic carboxy terminus prevent energy-dependent interaction between the TonB and ExbD periplasmic domains. *J. Bacteriol.* 193:5649–5657.
- Miller JH. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Gerhardt P, Murray RGE, Wood WA, Krieg NR (ed). 1994. Methods for general and molecular bacteriology. ASM Press, Washington, DC.
- Larsen RA, Postle K. 2001. Conserved residues Ser(16) and His(20) and their relative positioning are essential for TonB activity, cross-linking of TonB with ExbB, and the ability of TonB to respond to proton motive force. *J. Biol. Chem.* 276:8111–8117.
- Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685.
- Postle K. 2007. TonB system, *in vivo* assays and characterization. *Methods Enzymol.* 422:245–269.
- Larsen RA, Myers PS, Skare JT, Seachord CL, Darveau RP, Postle K. 1996. Identification of TonB homologs in the family *Enterobacteriaceae* and evidence for conservation of TonB-dependent energy transduction complexes. *J. Bacteriol.* 178:1363–1373.
- Armstrong DJ, Roman A. 1992. Mutagenesis of human papillomavirus types 6 and 16 E7 open reading frames alters the electrophoretic mobility of the expressed proteins. *J. Gen. Virol.* 73:1275–1279.
- Larsen RA, Chen GJ, Postle K. 2003. Performance of standard phenotypic assays for TonB activity, as evaluated by varying the level of functional, wild-type TonB. *J. Bacteriol.* 185:4699–4706.
- Takatsuka Y, Nikaido H. 2009. Covalently linked trimer of the AcrB multidrug efflux pump provides support for the functional rotating mechanism. *J. Bacteriol.* 191:1729–1737.
- Guzman LM, Belin D, Carson MJ, Beckwith J. 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P-BAD promoter. *J. Bacteriol.* 177:4121–4130.
- Baker KR, Postle K. 2013. Mutations in *Escherichia coli* ExbB transmembrane domains identify scaffolding and signal transduction functions and exclude participation in a proton pathway. *J. Bacteriol.* 195:2898–2911.
- Paul K, Brunstetter D, Titen S, Blair DF. 2011. A molecular mechanism of direction switching in the flagellar motor of *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A.* 108:17171–17176.
- Morimoto YV, Nakamura S, Hiraoka KD, Namba K, Minamino T. 2013. Distinct roles of highly conserved charged residues at the MotA-FliG interface in bacterial flagellar motor rotation. *J. Bacteriol.* 195:474–481.
- Larsen RA, Deckert GE, Kasteed KA, Devanathan S, Keller KL, Postle

- K. 2007. His20 provides the sole functionally significant side chain in the essential TonB transmembrane domain. *J. Bacteriol.* **189**:2825–2833.
45. Postle K, Kastead KA, Gresock MG, Ghosh J, Swayne CD. 2010. The TonB dimeric crystal structures do not exist *in vivo*. *mBio* **1**:e00307-10. doi:10.1128/mBio.00307-10.
46. Ollis AA, Kumar A, Postle K. 2012. The ExbD periplasmic domain contains distinct functional regions for two stages in TonB energization. *J. Bacteriol.* **194**:3069–3077.
47. Blair DF, Berg HC. 1990. The MotA protein of *E. coli* is a proton-conducting component of the flagellar motor. *Cell* **60**:439–449.
48. Wilson ML, Macnab RM. 1988. Overproduction of the MotA protein of *Escherichia coli* and estimation of its wild-type level. *J. Bacteriol.* **170**:588–597.
49. Stolz B, Berg HC. 1991. Evidence for interactions between MotA and MotB, torque-generating elements of the flagellar motor of *Escherichia coli*. *J. Bacteriol.* **173**:7033–7037.
50. Zhou J, Sharp LL, Tang HL, Lloyd SA, Billings S, Braun TF, Blair DF. 1998. Function of protonatable residues in the flagellar motor of *Escherichia coli*: a critical role for Asp 32 of MotB. *J. Bacteriol.* **180**:2729–2735.
51. Kim EA, Price-Carter M, Carlquist WC, Blair DF. 2008. Membrane segment organization in the stator complex of the flagellar motor: implications for proton flow and proton-induced conformational change. *Biochemistry* **47**:11332–11339.
52. Muramoto K, Macnab RM. 1998. Deletion analysis of MotA and MotB, components of the force-generating unit in the flagellar motor of *Salmonella*. *Mol. Microbiol.* **29**:1191–1202.
53. Qu JN, Makino SI, Adachi H, Koyama Y, Akiyama Y, Ito K, Tomoyasu T, Ogura T, Matsuzawa H. 1996. The *tolZ* gene of *Escherichia coli* is identified as the *ftsH* gene. *J. Bacteriol.* **178**:3457–3461.
54. Fingland N, Flatten I, Downey CD, Fossum-Raunehaug S, Skarstad K, Crooke E. 2012. Depletion of acidic phospholipids influences chromosomal replication in *Escherichia coli*. *MicrobiologyOpen* **1**:450–466.
55. Bremer H, Dennis PP. 1996. Modulation of chemical composition and other parameters of the cell by growth rate, p 1553–1569. In Neidhardt FC (ed), *Escherichia coli* and *Salmonella*: cellular and molecular biology, vol 2. ASM Press, Washington, DC.
56. Hayes F, Van Melder L. 2011. Toxins-antitoxins: diversity, evolution and function. *Crit. Rev. Biochem. Mol. Biol.* **46**:386–408.
57. Sun TP, Webster RE. 1987. Nucleotide sequence of a gene cluster involved in entry of E colicins and single-stranded DNA of infecting filamentous bacteriophages into *Escherichia coli*. *J. Bacteriol.* **169**:2667–2674.
58. Xu J. 1992. The *Escherichia coli* *acrAB* and *acrEF* operons. Washington State University, Pullman, WA.
59. Huntley S. 2002. Interaction between components of the AcrAB-TolC, AcrEF-TolC and AcrD multidrug efflux systems of *Escherichia coli*. Washington State University, Pullman, Washington, WA.