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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

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Ferric siderophores, vitamin B_{12}, and group B colicins are taken up through the outer membranes of *Escherichia coli* cells by an energy-coupled process. Energy from the cytoplasmic membrane is transferred to the outer membrane with the aid of the Ton system, consisting of the proteins TonB, ExbB, and ExbD. In this paper we describe two point mutations which inactivate ExbD. One mutation close to the N-terminal end of ExbD is located in the cytoplasmic membrane, and the other mutation close to the C-terminal end is located in the periplasm. *E. coli* CHO3, carrying a chromosomal *exbD* mutation in which leucine at position 132 was replaced by glutamine, was devoid of all Ton-related activities. A plasmid-encoded ExbD derivative, in which aspartate at position 25, the only charged amino acid in the predicted membrane-spanning region of ExbD, was replaced by asparagine, failed to restore the Ton activities of strain CHO3 and negatively complemented $ExbD^+$ strains, indicating an interaction of this mutated ExbD with wild-type ExbD or with another component. This component was shown to be ExbB. ExbB that was labeled with 6 histidine residues at its C-terminal end and that bound to a nickel-nitrilotriacetic acid agarose column retained ExbD and TonB specifically; both were eluted with the ExbB labeled with 6 histidine residues, demonstrating interaction of ExbB with ExbD and TonB. These data further support the concept that TonB, ExbB, and ExbD form a complex in which the energized conformation of TonB opens the channels in the outer membrane receptor proteins.

Escherichia coli and related gram-negative bacteria take up ferric siderophores, vitamin B_{12}, and group B colicins through their outer membranes by an energy-coupled transport system (8, 20, 35). The energy source is the electrochemical potential across the cytoplasmic membrane (4, 12, 38), which is thought to be transmitted to the outer membrane by the Ton system, composed of the TonB, ExbB, and ExbD proteins anchored in the cytoplasmic membrane (9, 13, 21, 22). TonB reacts with outer membrane receptor proteins (2, 11, 14, 17, 27, 42, 44), to which the substrates to be transported are bound. Upon removal of loops at the cell surface, two receptor proteins, FhuA, and FepA, were converted to open channels through which ferric siderophores nonspecifically diffused without requiring TonB (26, 31, 40). This finding suggested that the receptor proteins form closed channels that are presumably opened by interaction with the Ton system. The same reaction renders cells sensitive to the phages T1 and ϕ80, which infect only via the FhuA receptor-energized $TonB^+$, $ExbB^+$, and $ExbD^+$ cells (12). Recently, it has been shown that induction of the ferric citrate transport system requires the presence of the FecA outer membrane protein and the Ton system, through which ferric citrate acts as an inducer from the cell surface (16, 34). In the latter case, the Ton system displays another function, namely, signal transduction across the outer membrane.

TonB (13, 36, 37) and ExbD (21) are anchored via their N-terminal ends to the cytoplasmic membrane, and the remainders of the proteins are located in the periplasm. ExbB traverses the cytoplasmic membrane three times, starting with its N-terminal end in the periplasm, with most of the protein forming large loops in the cytoplasm (22). Plasmid-encoded ExbB prevents intrinsic proteolytic degradation of plasmid-encoded TonB and ExbD, which suggests binding of ExbB to TonB and ExbD (10). A similar finding has been obtained for chromosomally encoded TonB and ExbB (45). TonB and ExbB interact with the hydrophobic N-terminal transmembrane segments, as shown by the inhibition of TonB degradation by an N-terminal fragment of ExbB (51), by cross-linking experiments that yielded a TonB antibody-reactive 59-kDa protein that was not obtained in an *exbB*::Tn*10* mutant (44), and by weak suppression of a mutant with a point mutation in the region of *tonB*, encoding the transmembrane segment, by a mutant with a point mutation in *exbB*, which encodes the region located in the cytoplasmic membrane (28). *E. coli* contains, in addition to the Ton system, the Tol system, consisting of the proteins TolA, TolQ, and TolR (48, 56). TolA is equivalent to TonB (30), TolQ shows sequence homologies to ExbB (9), and TolR shows sequence homologies to ExbD (9). The transmembrane topology of TolQ (23, 53) is very similar to that of ExbB (22), and the transmembrane topology of TolR (23, 33) is very similar to that of ExbD (21). TolQ and TolR can partially substitute for ExbB and ExbD functionally and vice versa (5, 6), whereas TolA and TonB cannot replace each other (6). However, the replacement of the N-terminal transmembrane segment of TonB with that of TolA resulted in an active TonB that functioned more efficiently with TolQ and/or TolR (not specified in the experiment) than with ExbB and/or ExbD (25). Transmembrane anchors of other proteins fused to TonB yielded inactive TonB (19, 24, 25). The transmembrane

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anchor of TonB that was fused to β-lactamase interfered with wild-type TonB activity (24), indicating that the TonB fragment competes for binding to another component, presumably ExbB.

ExbB and ExbD have been considered to play accessory roles in TonB activity since *exbB* mutants are leaky. The leakiness comes from the partial replacement of ExbB activity by TolQ activity and of ExbD activity by TolR activity (5, 6). Double mutations in *exbB* and *tolQ* showed no Ton-related activity. However, TolQ and TolR support only very weakly growth on ferric siderophores, which is the physiological function of the Ton system. With this in mind, we wanted to know whether ExbD is essential for the functioning of the Ton system. Up to the end of this study, no chromosomal *exbD* mutant was available to examine whether ExbD is essential for Ton-related activities. The mutants used were derived from strain H1388 containing *exbB*::Tn*10* (15), which exerted a strong polar effect on *exbD* expression (6). Very recently, the polar effect of *exbB*::Tn*10* on *exbD* expression was determined (1). Under repressing aerobic conditions, the level of *exbD* expression was 20% of that of *exbB* expression, and under inducing aerobic conditions, *exbD* expression levels were 5% of those of *exbB*. An *exbD*::Tn*phoA* insertion mutant displayed the same phenotype as the *exbB*::Tn*10* mutant (1).

Here we report on the isolation of a spontaneous chromosomal *exbD* mutant that carries a point mutation in the region which the topology model of ExbD localizes in the periplasm (21). We show that ExbD is essential for all Ton-related activities in a *tolR* mutant. We also demonstrate that the transmembrane region of ExbD is important for ExbD activity using mutated *exbD* with asparagine instead of aspartic acid at position 25 [*exbD*(D25N)], encoding ExbD(D25N), which also indicates an important functional role of the negatively charged amino acid at the border of or within the hydrophobic cytoplasmic membrane. We also converted the cytoplasmic region of ExbD containing an accumulation of negatively charged amino acids (9), which is unusual for cytoplasmic transmembrane proteins (18, 54), to a neutral sequence of amino acids and determined the phenotype of the mutated protein. In addition, we show binding of ExbD and TonB to ExbB adsorbed to a nickel-nitrilotriacetic acid (Ni-NTA) resin via 6 histidine residues added to the C-terminal end of ExbB. The results obtained indicate two important functional regions of ExbD, one located close to the C-terminal end in the periplasm and one located close to the N-terminal end in the cytoplasmic membrane. The results also support the concept that TonB, ExbB, and ExbD form a complex, in which all three proteins are important for inducing a conformation in the outer membrane receptor proteins that allows uptake of ferric siderophores, vitamin B_{12}, and group B colicins and infection by the phages T1 and φ80 (12).

MATERIALS AND METHODS

Bacterial strains and plasmids used. The *E. coli* strains and plasmids used in this study are listed in Tables 1 and 2 (see also Fig. 2). Plasmid pKK11 contained the *Sac*I-*Pst*I fragment of pKE61 *exbB exbD* (9) in plasmid pT7-6. Plasmid pHE20 contained the *Eco*RI-*Nsi*I *exbB* fragment of pKE7 (9) in pT7-5 cleaved with *Eco*RI and *Pst*I. pHE30 contained the *Eco*RI-*Cla*I *exbB* fragment (*Cla*I site from vector pT7-5) of pHE20 in pWSK29. pHE31 contained the *Eco*RI-*Xho*I *exbB* fragment of pHE30 in pBCKS+. pCH100 was constructed by cloning the *Ssp*I-*Eco*RI *exbB exbD* fragment of pKK11 in pBCSK+ cleaved with *Hin*dII and *Eco*RI. pCH9 contained the *exbB exbD* fragment of pCH100 in pWSK29 cleaved with *Xho*I and *Eco*RI. pHK702 contained the *Hin*dIII-*Eco*RI fragment of wild-type *exbD* from pCH9 in pSU19. pHE40 contained the *Hin*dIII-*Eco*RI fragment of pKK11 in pBCSK+. pHE41 contained the *Hin*dIII-*Eco*RI fragment of pKK11 in pBCKS+. In pHE42 the *Acy*I-*Eco*RI fragment of wild-type *exbD* of pHE40 was replaced by mutated *exbD*(D25N), encoding ExbD(D25N). In pHE43 and pHE44 the *Hin*dIII-*Eco*RV fragment of wild-type *exbD* of pHE41 was replaced by mutated *exbD* fragments encoding ExbD(H16R) and ExbD(D10N, D11N), respectively. pCH5 contained the mutated *exbD* gene fragment of plasmid pHE42 in pCH100 cleaved with *Hin*dIII and *Hin*dII. pCH6 and -7 contained the mutated *exbD* gene fragments of plasmids pHE43 and -44 in pCH100 cleaved with *Hin*dIII and *Hin*dII. Plasmids pCH10 to -12 contained *exbB exbD* of CH5 to CH7 in the *Xho*I-*Eco*RI site of pWSK29 downstream of the phage T7 gene 10 promoter. In pCH92 and pCH102, 0.14-kb fragments were excised from wild-type *exbD* (pCH9) and from mutated *exbD* (pCH10) (Table 2), respectively, by *Bst*EII. In pCH9485, a 36-bp *Nsi*I fragment of wild-type *exbD* on pHE41 was deleted and cloned with *Hin*dII-*Hin*dIII in pCH9. pCH9481 contained a 36-bp *Nsi*I fragment with the mutations D10N and D11K in reverse orientation in pCH9485 cleaved with *Nsi*I. All constructs were sequenced. In pCH14, DNA sequencing revealed a spontaneous deletion of nucleotides 1335 to 1340 and exchanges of amino acids E-7 with D and N-8 with D. pCH709 was constructed by cloning the *Ssp*I-*Eco*RI fragment of pKK11 into pT7-6 cleaved with *Sma*I and *Eco*RI. For the expression of mutated *exbD* genes, wild-type *exbD* of pCH709 was replaced by the *Bst*XI-*Eco*RI fragments of pCH102, pCH9481, pCH5, and pCH92, resulting in plasmids pCH7102, pCH7481, pCH710, and pCH792, respectively. pCH012/1 to pCH012/4 contained the *Eco*RI-*Hin*dIII *exbD* fragments of CHO3 from four independent PCRs cloned in pBCSK+ cleaved with *Eco*RI-*Hin*dIII. pCH115 and pCH0161 were constructed by cloning the *Eco*RI-*Hin*dIII and the *Eco*RI-*Hin*dII fragments of pCH012/1 in pHE40 and pCH100, respectively.

Mutagenesis of *exbD*. For site-directed mutagenesis, the *Hin*dIII-*Eco*RI fragment of pCH9 was cloned into pBCKS+ (pHE41) and pBCSK+ (pHE40) and amplified by PCR with *Taq* polymerase (Boehringer, Mannheim, Germany). The D25N replacement was introduced with the primer 5′-CAACGTGACGCCGTT TATCAACGTGATG-3′ (the replaced nucleotide is underlined) (pCH10); the ExbD(H16R) replacement was introduced with the primer 5′-CACGTTGATA TCACGCATTTCG-3′ (pCH11); the ExbD(D10N, D11N) replacements were introduced with the primer 5′-CACGTTGATATCATGCATTTCGCCGTTAT TGTTGAGGTTTTCG-3′ (pCH12); and the ExbD(D10N, D11K) replacements were introduced with the primer 5′-CACGTTGATATCATGCATTTCGCCG TTTTGTTGAGG-3′ (pCH9483). Fragments of the amplified DNA were excised by *Eco*RI-*Hin*dIII (1.2 kb), *Eco*RI-*Eco*RV (0.64 kb), and *Hin*cII-*Hin*dIII (0.8 kb) digestion and cloned into plasmid pBCSK+. The resulting plasmids were transformed in *E. coli* 5K and used for sequencing.

Sequencing of the chromosomal *exbD* mutant. The *exbD* gene of *E. coli* CHO3 was isolated by PCR. Since *Taq* polymerase may introduce mutations, four independent PCRs were performed with the primer 5′-GCGTCCTGCATTCAC

TABLE 1. *E. coli* strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
Strains		
AB2847	*aroB thi malT tsx*	14
BL21	F^- *hsd gal*	46
WM1576	K38 HfrC pGP1-2	49
DH5α	F^- *hsdR17* (r_K^- m_K^+) *supE44 thi-1 gyrA relA1 recA1 endA1* Δ(*argF lacZYA*) U169(φ80 Δ*lacZ M15*) λ^-	Stratagene
TPS13	*tolQ ara* Δ(*lac pro*) *thi* F′ *lac pro*	47
HE2	TPS13 *exbB*::Tn*10*	5
H1081	*aroB*	K. Hantke
CHO3	TPS13 *exbD tolQ aroB*	This study
H1388	*exbB*::Tn*10 aroB pro lac malT tsx thi*	15
Plasmids		
pIM750	*exbD*	50
pHK702	*exbD*	This study
pCG752	*tonB*	10
pCG756	*tonB exbD*	10
pKE7	*exbB exbD*	9
pKK11	*exbB exbD*	This study
pWSK29		55
pBCKS+		Stratagene
pBCSK+		Stratagene
pQE61		Diagen
pSU19		32
pGP1-2	Encodes phage T7 RNA polymerase	49
pT7-5	Contains phage T7 gene 10 promoter	49
pT7-3	*blaM* downstream of the gene 10 promoter	49
pT7-7	Like pT7-5 but equipped with an ideal ribosome binding site	49

TABLE 2. Phenotypes of *E. coli* HE2 (*exbB*::Tn*10 tolQ* [*tolR*]) and *E. coli* CHO3 (*exbD tolQ* [*tolR*]) complemented with plasmids carrying mutated *exbD* genes

Plasmid[a]	Amino acid replacement(s) in ExbD on indicated plasmid	Strain	Level of growth inhibition with reagent[b]:			
			ColB	ϕ80	Albo	Ferri
None		HE2	r	r	r	0
None		CHO3	r	r	r	0
pCH9 (*exbB exbD*)	None	HE2	4	4	2	14
pCH9 (*exbB exbD*)	None	CHO3	4	4	2	15
pHE30 (*exbB*)	None	HE2	2	4	r	0
pHE30 (*exbB*)	None	CHO3	r	r	r	0
pHE21 (*exbD*)	None	HE2	r	r	r	0
pHE21 (*exbD*)	None	CHO3	4	4	2	30[c]
pCH115 (*exbD132*)	L132Q	CHO3	r	r	r	0
pCH0161 (*exbB exbD132*)	L132Q	CHO3	r	r	r	0
pCH10 (*exbB exbD25*)	D25N	HE2	r	r	r	0
pCH10 (*exbB exbD25*)	D25N	CHO3	r	r	r	0
pCH102 (*exbB exbD258*)	D25N, Δ81–141	HE2	3	4	r	0
pCH102 (*exbB exbD258*)	D25N, Δ81–141	CHO3	r	r	r	0
pCH92 (*exbB exbD8*)	Δ81–141	HE2	3	4	r	0
pCH92 (*exbB exbD8*)	Δ81–141	CHO3	r	r	0	
pCH9485 (*exbB exbD415*)	Δ4–15	HE2	3	4	0	30[d]
pCH9485 (*exbB exbD415*)	Δ4–15	CHO3	4	4	r	30[c]
pCH14 (*exbB exbD5678*)	Δ5–6, E7D, N8D	HE2	3	4	1	0
pCH14 (*exbB exbD5678*)	Δ5–6, E7D, N8D	CHO3	4	4	0	30[c]
pCH9481 (*exbB exbD81*)	*Nsi*I rev	HE2	r	r	r	0
pCH9481 (*exbB exbD81*)	*Nsi*I rev	CHO3	r	r	r	0
None		AB2847	4	5	2	ND
pCH100 (*exbB exbD*)	None	AB2847	4	5	2	ND
pCH5 (*exbB exbD25*)	D25N	AB2847	2	1	r	ND

[a] The *exbB* and *exbD* genes were cloned in the low-copy-number vector pWSK29 (51), except in the case of pCH5, which is a high-copy-number pBCSK+ derivative.
[b] ColB, colicin B; Albo, albomycin; Ferri, ferrichrome; r, resistance; ND, not determined. A growth inhibition level of 0 for albomycin indicates growth inhibition with an undiluted solution (0.1 mM), and for 0.1 mM ferrichrome it indicates no growth promotion. For albomycin, colicin B, and phage ϕ80, the last dilutions of a 10-fold dilution series that resulted in a clear zone of growth inhibition are listed. For example, a growth inhibition level of 4 indicates that the colicin solution could be diluted 10^4-fold and still inhibit completely growth of the bacteria seeded onto the agar plate. In addition to colicin B, colicins D, M, and 10 were tested and gave the same results obtained with colicin B.
[c] The densities of the growth zones were low, so that ferrichrome, poorly taken up by the cells, diffused over a larger area.
[d] Very weak growth zone.

CAG-3′ from bp 285 to 302 of the published *exbB-exbD* sequence (9) and for the complementary strand primers 5′-GCACGGCGTGTGCGGCGGCGAG-3′ (bp 2051 to 2030) and TGTGAATTCACTCTTACCTG (bp 1982 to 1962). Sequencing was performed by the dideoxy chain termination method (41) with an AutoRead sequencing kit (Pharmacia Biotech, Freiburg, Germany) and an A.L.F. Automatic Sequenator (Pharmacia Biotech).

Construction of ExbB(His)$_6$. The QIAexpress system (Diagen, Hilden, Germany) was used for tagging ExbB at the C-terminal end with 6 histidine residues [ExbB(His)$_6$]. A 750-bp fragment of plasmid pKE61 (*exbB*) (9) was amplified by PCR with *Pfu* polymerase (Stratagene, La Jolla, Calif.) and the primers 5′-GGGGGATCCCATATGGGTAATAATTTAATGCAG-3′ and 5′-GCCATCGGAGATCTTCCTGCGCG-3′. The primers were designed to introduce *Bam*HI, *Nde*I, and *Bgl*II restriction sites. The PCR product was purified by agarose gel electrophoresis, recovered from agarose with a QIAEX kit (Diagen), cleaved with *Bam*HI-*Bgl*II, and cloned into pQE16 (Diagen). *E. coli* DH5α (GIBCO BRL, Eggenstein, Germany) was transformed with the resulting plasmid pQE165 (Fig. 1), which contains *exbB* downstream of the strong promoter of phage T5, which is inducible by isopropyl-β-D-thiogalactopyranoside (IPTG). For high-level expression of ExbB(His)$_6$, the *Eco*RI-*Pvu*II fragment of pQE165 was cloned into plasmid pT7-5 cleaved with *Eco*RI and *Sma*I (pQE166), and from there, the *Nde*I-*Pst*I fragment was cloned into pT7-7 cleaved by *Nde*I and *Pst*I (pQE167) (Fig. 2).

Purification of ExbB(His)$_6$ on Ni-NTA agarose and assays of ExbD and TonB binding to ExbB(His)$_6$. The protocol provided by the supplier of Ni-NTA agarose (Diagen) was employed with the following specifications: *E. coli* BL21 (pQE167) was grown at 37°C in 100 ml of tryptone-yeast extract medium (10) to an optical density at 578 nm of 0.5. IPTG was added to a final concentration of 2 mM, and culturing continued for 3 h. Cells (20-ml portions) were harvested by centrifugation, stored for 20 min at −20°C, thawed, suspended in 1 ml of adsorption buffer (20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole [pH 7.9]), and disrupted three times by French pressure treatment. The cell suspension was centrifuged, and the sediment was suspended in 0.2 ml of ice-cold 0.1 M Tris-HCl–2% octylglucoside, pH 8. After storage on ice for 60 min, the suspension was centrifuged for 30 min at 30,000 × *g*, and the entire supernatant was applied to a column of Ni-NTA agarose prepared as follows: Ni-NTA agarose was washed twice with distilled water and then sedimented by centrifugation at 600 × *g*. The sediment was washed with three times the volume of adsorption buffer, poured (0.5 ml) into a column, and washed once more with adsorption buffer. At this stage, radiolabeled ExbD and TonB were applied for the binding assay. If ExbB was recovered, the column was washed with wash buffer and then with elution buffer, as described below.

Radioactively labeled ExbD and TonB were prepared by growing 10 ml of *E. coli* WM1576 transformed with plasmid pIM750 (*exbD*), pCG752 (*tonB*), or pCG756 (*tonB exbD*) in the presence of [^{35}S]methionine and rifamycin, as described previously (9, 10). The membrane fraction was prepared by disrupting cells with lysozyme-EDTA; the cells were then removed by repeated washing with 0.1 M Tris-HCl, pH 8. The radioactively labeled proteins were solubilized in 0.1 ml of Tris-HCl–2% octylglucoside, pH 8, and applied to the Ni-NTA agarose column loaded with ExbB(His)$_6$. The column was washed with 5 ml of adsorption buffer, 5 ml of wash buffer (13.3 mM Tris-HCl, 0.33 M NaCl, 40 mM imidazole [pH 7.9]), and then with 5 ml of elution buffer (20 mM Tris-HCl, 0.5 M NaCl, 1 M imidazole [pH 7.9]). Samples of 1 ml were collected, and the protein was precipitated with 10% trichloroacetic acid. The sediment was washed with ethanol, dried in vacuo, and solubilized in 40 μl of sample buffer, and 20 μl was applied to a 15% polyacrylamide gel containing 0.1% sodium dodecyl sulfate (SDS) for identification (9, 10). After electrophoresis, proteins were stained with Serva Blue and radiolabeled proteins were identified by autoradiography.

Transport of [$^{55}Fe^{3+}$]ferrichrome. Transport of radiolabeled ferrichrome was essentially determined as described previously (10). Freshly grown colonies on tryptone-yeast extract agar were collected, washed with M9 salt solution supplemented with 0.4% glucose, and suspended in the same medium to a density of 5 × 10^8 cells per ml. NTA was added to a final concentration of 0.25 mM. After a 5-min incubation at 37°C, transport was started by adding 0.1 μM $^{55}Fe^{3+}$ in 0.5 μM deferrichrome (final concentrations in the assay). Samples (0.1 ml) were taken every two minutes from 1 to 15 min, filtered through cellulose nitrate (0.45 μm), and washed twice with 4 ml of 0.1 M LiCl. The filters were dried, and the radioactivity was measured in a liquid scintillation counter.

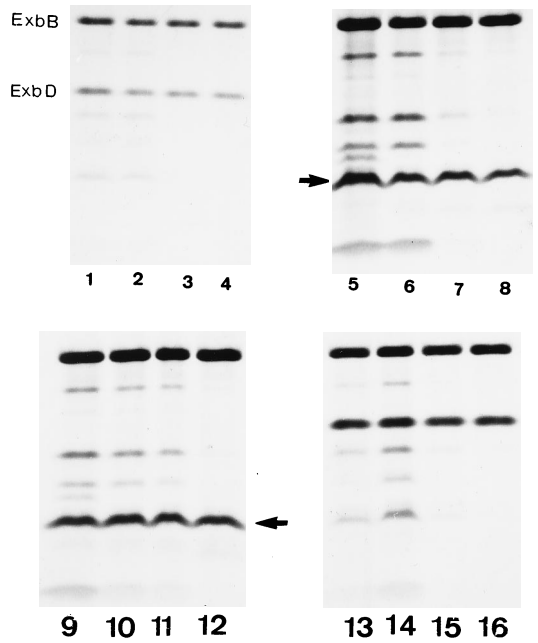

FIG. 1. Stability of pulse-labeled ExbD(D25N) (lanes 1 to 4), ExbD(Δ81–141) (lanes 5 to 8), ExbD(D25N, Δ81–141) (lanes 9 to 12), and wild-type ExbD (lanes 13 to 16) during the chase period after 0 min (lanes 1, 5, 9, and 13), 10 min (lanes 2, 6, 10, and 14), 20 min (lanes 3, 7, 11, and 15), and 50 min (lanes 4, 8, 12, and 16). Wild-type *exbB* and mutated *exbD* were cloned on pT7-6 (described in Materials and Methods) and transcribed in *E. coli* WM1576 by T7 RNA polymerase in the presence of 0.2 mg of rifamycin per ml. Cells were labeled for 5 min with 370 kBq of [^{35}S]methionine and chased for the times indicated with a 200-fold surplus of nonradioactive methionine. The labeled proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (8) on a gel that contained 9.7% acrylamide, and the electrode buffer contained 0.1 M Tris-hydrochloride–0.1 M *N*-Tris(hydroxymethyl)-methylglycine. The proteins were identified by autoradiography, and their electrophoretic mobilities were compared with those of standard proteins (not shown). The arrows indicate the position of the C-terminally truncated ExbD derivatives.

RESULTS

Isolation of a chromosomal *exbD* mutant. We isolated a spontaneous *exbD* mutant of *E. coli* TPS13 carrying a mutation in *tolQ* (47) with a polar effect on *tolR* expression (7). The *tolQ* mutant was chosen to avoid partial complementation of *exbB* and *exbD* by *tolQ* and *tolR* (5, 7). Albomycin and colicin B were simultaneously used for selection of *exbD* mutants to prevent the selection of the more frequently occurring receptor mutants *fhuA* (albomycin resistant) and *fepA* (colicin B resistant). In addition, synthesis of enterochelin was interrupted by introducing an *aroB* mutation to be able to test growth on ferrichrome as the sole iron source. After P1 transduction, with a P1 phage lysate of *E. coli* H1081 containing *aroB* and carrying a Tn*10* insertion close to *aroB*, the lack of enterochelin production was examined on the blue chrome azurol S plates, which turn yellow around colonies secreting an iron chelator (43). The resulting strain, *E. coli* CHO3, was resistant to all group B colicins tested (colicins B, D, M, and 10) and to phages T1 and ϕ80 and could not grow on ferrichrome (Table 2, in which colicins M, D, and 10 and phage T1 are not listed). The Ton$^+$ phenotype was largely restored by transformation with pHE21 *exbD*, but pHE30 *exbB* had no effect on the restoration of this phenotype (Table 2). Transformants of strain CHO3 carrying pCH9 (*exbB exbD*) had fully restored Ton-related activities, which may result from an improved transcription of *exbD* from the promoter upstream of *exbB* (1, 9) and from the stabilization of ExbD by ExbB (10). It is also likely that the stoichiometries of the ExbB and ExbD proteins play a role in activity. The complete inactivity of strain CHO3 *exbD* in all Ton-related functions proves the essential requirement of ExbD in Ton activities.

To identify the mutation in *exbD* of *E. coli* CHO3, the *exbB-exbD* region of isolated chromosomal DNA was amplified by four independent PCRs. The DNA fragments were cloned with *Eco*RI-*Hin*dIII in pBCSK+, resulting in the plasmids pCH012/1 to pCH012/4. The sequencing of all four plasmids revealed only a single mutation, CTG to CAG, resulting in the replacement of leucine at position 132 by glutamine. Plasmids pCH012/1 to pCH012/4 carrying this mutation did not complement *E. coli* CHO3. Replacement of the *Eco*RI-*Hin*dIII fragment of wild-type *exbB-exbD* on pCH100 by the *exbB-exbD*(L132Q) fragment, as well as the *Eco*RI-*Hin*dIII fragment of wild-type *exbD* on pHE40 by the *exbD*(L132Q) fragment, resulted in plasmids pCH0161 and pCH115, respectively, which failed to complement *E. coli* CHO3. This shows that the single-amino-acid replacement L132Q located 10 residues away from the C-terminal end rendered ExbD inactive. The amount and stability of ExbD(L132Q) were similar to those of wild-type ExbD (data not shown).

Isolation and phenotypes of mutants with plasmid-borne *exbD* mutations. Plasmid-borne *exbD* was mutated by PCR with primers with nucleotide replacements at defined sites. Aspartate at position 25 is the only charged amino acid within the predicted transmembrane region of ExbD (21) and may therefore play an important functional role. It was replaced by asparagine, which is about the same size as aspartate; therefore, steric constraints leading to inactivation of ExbD should not occur. ExbD(D25N) encoded on pCH10 was totally inactive in all Ton-related activities measured in strain CHO3 (pCH10) (Table 2). ExbD(D25N) exerted an effect on strain HE2 (*exbB* [*exbD*]) carrying wild-type *exbD* on the chromosome and *exbB exbD25* on pCH10, since it abolished the residual ExbD activity of strain HE2 [Table 2; compare HE2 (pHE30) *exbB* with HE2(pCH10) *exbB exbD25*]. Apparently, overexpressed ExbD(D25N) displaced wild-type ExbD at the site of ExbD activity, which is supposedly in the complex formed by TonB, ExbB, and ExbD (see below). To see whether only residual ExbD activity in strain HE2 was abolished by ExbD(D25N), pCH10 was transformed into *E. coli* AB2847 (*exbB*$^+$ *exbD*$^+$). No negative complementation was observed, suggesting that ExbD(D25N) was unable to abolish wild-type ExbD activity formed in normal amounts (data not shown). This interpretation was examined by cloning *exbD25* into a high-copy-number vector, yielding pCH5. AB2847(pCH5) displayed a strongly reduced sensitivity to colicin B (Table 2), colicins D and M (not shown), ϕ80, and albomycin. Transformants of AB2847 containing wild-type *exbB exbD* (pCH100) or *exbD* on low- or high-copy-number plasmids (data not shown) showed the same level of sensitivity as untransformed AB2847 (Table 2), strengthening the proposal of the specific negative complementation of ExbD(D25N).

Negative complementation of ExbD(D25N) in strain HE2 was abolished by the deletion of the C-terminal end of ExbD (D25N) from residues 81 to 141 (Δ81–141) [Table 2, strain HE2(pCH102) (*exbB exbD258*)]. The proteins encoded by plasmids pCH92 and pCH102 consist of the first 80 amino acids of wild-type *exbD* (pCH92) or of *exbD*(D25N) (pCH102) followed by 2 amino acids (Val and Thr) added by the cloning procedure. The calculated molecular weight of the proteins is 8.98 kDa. To test whether lack of negative complementation by pCH102 was caused by the instability of ExbD(D25N, Δ81–141), *exbD258* was cloned downstream of the phage T7 gene 10 promoter and transcribed specifically by the T7 RNA polymer-

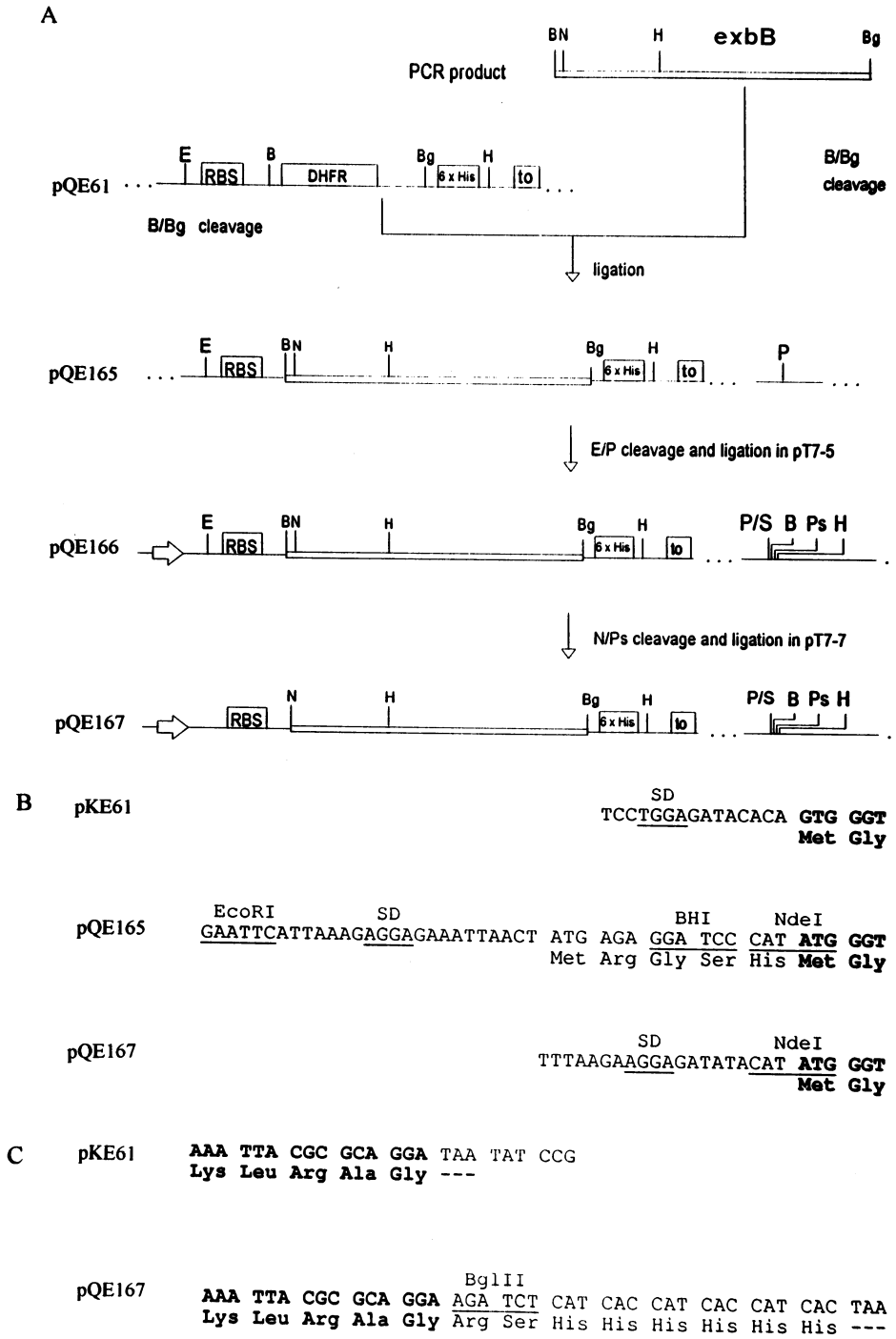

FIG. 2. (A) Construction of the *exbB* gene encoding ExbB(His)$_6$ starting from PCR-amplified *exbB* of plasmid pKE61, which was cloned into pQE61, resulting in pQE165. The *exbB* derivative was cloned into pT7-5 (pQE166) and from there into pT7-7 (pQE167). Restriction sites: B, *Bam*HI; N, *Nde*I; Bg, *Bgl*II; H, *Hin*dIII; E, *Eco*RI; P, *Pvu*II, Ps, *Pst*I; and S, *Sma*I. RBS, synthetic ribosome binding site; DHFR, mouse dihydrofolate reductase; 6 × His, sequence of 6 histidine residues; to, transcription terminator. (B) 5′ nucleotide sequences of *exbB* and derived N-terminal amino acid sequences of ExbB in plasmids pKE61 and pQE165 and in QE167. (C) 3′ nucleotide sequences of *exbB* and C-terminal amino acid sequences of ExbB and ExbB(His)$_6$.

ase. After labeling with [^{35}S]methionine, a surplus of unlabeled methionine was added and the stability of the radiolabeled proteins was examined during the chase period of 50 min (10). The sample with ExbD(D25N, Δ81–141) contained some degradation products which, however, were mainly or exclusively derived from ExbB (Fig. 1, lanes 9 to 12; the truncated ExbD marked by an arrow corresponded to the expected size of 9 kDa). Similar degradation products were observed in the samples of wild-type ExbD truncated after residue 80 [ExbD(Δ81–141)] (Fig. 1, lanes 5 to 8), in ExbD(D25N) (Fig. 1, lanes 1 to 4), and in the samples of wild-type ExbD (Fig. 1, lanes 13 to 16). The amount of ExbD(D25N, Δ81–141) was above the

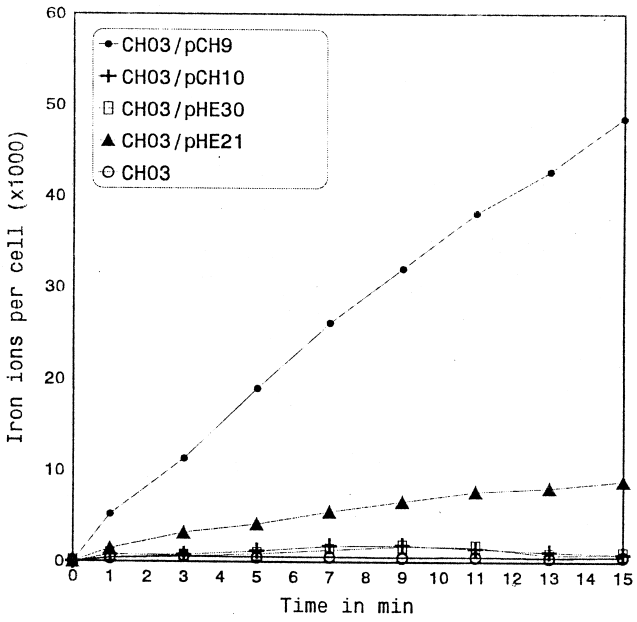

FIG. 3. Transport of [$^{55}Fe^{3+}$]ferrichrome into *E. coli* CHO3 transformed with the plasmids indicated on the figure.

amount of chromosomal wild-type ExbD, so that a shortage of ExbD(D25N, Δ81–141) was not the reason the activity was not present. Since ExbD(D25N, Δ81–141) was virtually not degraded, the mutation presumably did not abolish the physical interaction of ExbD with ExbB, unless the derivative became stable without interacting with ExbB.

Negative complementation of strain HE2 was obtained by plasmid pCH9481, which encoded ExbD containing residues 4 to 15 (the *Nsi*I fragment) in reverse order (*Nsi*I rev). This ExbD derivative was inactive in strain CHO3 and abolished the residual ExbD activity of strain HE2 (Table 2). ExbD(*Nsi*I rev) contained no accumulation of negative charges at the inner side of the cytoplasmic membrane (the sequence reads [from residue 4] His Phe Ala Val Phe Val Glu Val Phe Val Lys Met; compare this with the sequence of wild-type ExbD [His Leu Asn Glu Asn Leu Asp Asp Asn Gly Glu Met]). Excision of the *Nsi*I fragment resulted in an ExbD deletion derivative [ExbD(Δ4–15) encoded on pCH9485] that partially restored the sensitivity of strain CHO3 and somewhat increased the sensitivity of strain HE2 to colicin B, ϕ80, albomycin, and ferrichrome (Table 2).

Another deletion derivative of ExbD carrying additional point mutations in the cytoplasmic region, ExbD(Δ5–6, E7D, N8D) (pCH14), also exhibited residual activity in CHO3 and HE2 (Table 2). The derivatives ExbD(D10N, D11N), ExbD(D10N, D11K), and ExbD(H16R) were fully active in *E. coli* CHO3 and *E. coli* HE2 (data not shown).

Transport of [$^{55}Fe^{3+}$]ferrichrome into *E. coli* CHO3 and *E. coli* CHO3 transformants carrying wild-type and mutant *exbD* genes. To corroborate the data of the growth promotion assays, the transport rate of radioactively labeled iron delivered as a ferrichrome complex was determined. Only pCH9 *exbB exbD* restored fully the transport rate of strain CHO3, while CHO3(pHE21) *exbD* displayed a residual transport (Fig. 3). Either the level of expression of *exbD* was too low because of the lack of a promoter which is in front of *exbB* (1) or the stoichiometry between ExbB and ExbD deviated too strongly from the wild-type stoichometry. *E. coli* CHO3(pCH10) containing *exbB exbD25* was transport negative, as were the other plasmid-encoded *exbD* mutants which rendered CHO3 resistant to the Ton-dependent ligands (only pHE30 *exbB* is shown in Fig. 3). This result agrees with the growth promotion assays (Table 2). The weak growth promotion of some of the mutants cannot be observed in the iron uptake assay, since the growth promotion assay is more sensitive than the transport assay (26).

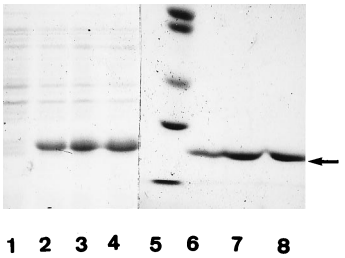

FIG. 4. Synthesis of ExbB$(His)_6$ in *E. coli* BL21(pQE167) grown in tryptone-yeast extract medium containing 0.2 mg of ampicillin per ml. Uninduced ExbB$(His)_6$ (lane 1) and ExbB$(His)_6$ after induction with 2 mM IPTG for 1 h (lane 2), 2 h (lane 3), and 3 h (lane 4) are shown. The synthesized proteins were separated by SDS-PAGE and stained with Serva Blue. ExbB$(His)_6$ was purified on Ni-NTA agarose, showing the fractions (lanes 6 to 8) that contained the protein identified by SDS-PAGE. Lane 5 contains standard proteins of (from bottom to top) 17.8, 29, and 45 kDa. ExbB$(His)_6$ is marked by an arrow.

Isolation of purified ExbB protein. Negative complementation of wild-type ExbD by mutated ExbD suggests the formation of inactive oligomers between wild-type and mutated ExbD or between ExbD and another reaction partner, such as ExbB and/or TonB. ExbB was isolated to examine the binding of ExbD to ExbB and/or TonB. ExbB was chosen since the amounts of radiolabeled ExbB observed on autoradiograms were greater than the amounts of radiolabeled ExbD (10) (Fig. 1). The stronger labeling of ExbB reflects the higher protein content, since ExbB contains 6 and ExbD contains 9 methionine residues.

exbB from plasmid pKE61 was mutated with *Pfu* polymerase and cloned into plasmid pQE61, resulting in an ExbB protein labeled at the C-terminal end with $(His)_6$ (Fig. 2) that was suitable for affinity purification on Ni-NTA resin. Cloning in plasmid pT7-7 resulted in an unaltered N-terminal end of ExbB and the addition of the amino acids Arg and Ser and the $(His)_6$ tail at the C-terminal end of ExbB (Fig. 2B and C). The resulting plasmid, pQE167, contained *exbB* downstream of an ideal ribosome binding site and the strong gene 10 promoter of phage T7. Sequencing of the *exbB* derivative showed that the construct was as expected and revealed the replacements T by A at nucleotide 733 (Leu to Gln), G-1181 by C (Arg to Arg), and G-1287 and C-1288 by C and G (Ala to Arg), as was found previously (24, 28) and which is at variance with the originally published sequence (9). ExbB was expressed in *E. coli* BL21(pQE167) (Fig. 4, lanes 1 to 4) and purified by Ni-NTA agarose chromatography (Fig. 4, lanes 6 to 8). The N-terminal amino acid sequence of the purified protein was determined by Edman degradation. The first step yielded substoichiometric amounts of methionine and glycine, showing that the N-terminal methionine is usually cleaved off but was partially retained in the purified protein because of its overexpression. The remainder of the sequenced region read Asn Asn Leu Met Gln Thr Asp Leu and corresponded to the ExbB sequence derived from the nucleotide sequence (9).

Activity of ExbB$(His)_6$. The activity of ExbB$(His)_6$ encoded on pQE165, pQE166, and pQE167 was tested and compared with the activity of ExbB encoded by pKE61 *exbB exbD* (the wild type) in *E. coli* H1388 (*exbB*::Tn*10*). The transformants

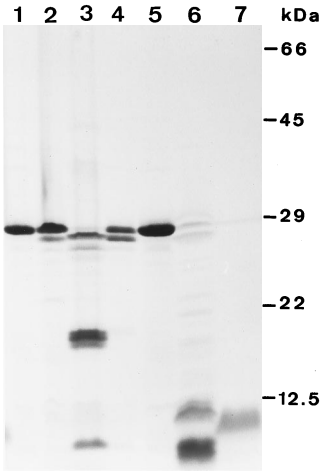

FIG. 5. Determination of the arrangement of ExbB(His)$_6$ in the cytoplasmic membrane of *E. coli* BL21(pQE167) by treating spheroplasts (lanes 1 to 4) and cell lysates (lanes 5 to 7) with 50 μg of trypsin (lanes 2 and 6), 50 μg of proteinase K (lanes 3 and 7), and 100 μg of aminopeptidase K (lane 4) for 30 min at 0°C. Untreated samples (lanes 1 and 5) are also shown. The positions of standard proteins and their molecular masses are given on the right.

carrying pQE165, pQE166, and pQE167 displayed a 10-fold higher level of sensitivity to colicin M than strain H1388 and an unaltered residual sensitivity to albomycin, while the transformants carrying pKE61 were 100-fold more sensitive to colicin M and albomycin. Growth of all transformants was fully supported by ferrichrome, ferric coprogen, and ferric citrate. Interpretation of these results have to take into account the polar effect of the *exbB*::Tn*10* insertion on the expression of *exbD* (1) located downstream of *exbB* (9), as was also observed with strain HE2 transformed with wild-type *exbB* (Table 2). Therefore, strains H1388 and HE2 carrying plasmid pQE165, pQE166, or pQE167 were transformed with pHK702 *exbD* (wild type) to see whether sensitivity to colicins B and M reached the levels of sensitivity of strains H1388 and HE2 transformed with wild-type *exbB* and *exbD*. Indeed, ExbB (His)$_6$ restored the sensitivities of the transformants to the same level as that of wild-type ExbB, demonstrating that the addition of the sequence Arg Ser (His)$_6$ to the C terminus did not affect ExbB activity.

Topology of ExbB(His)$_6$ in the cytoplasmic membrane. To examine whether ExbB(His)$_6$ was properly inserted into the cytoplasmic membrane, spheroplasts and cell lysates were prepared from [^{35}S]methionine-labeled *E. coli* WM1576(pQE167) and treated with trypsin, proteinase K, or aminopeptidase K. This procedure has been employed previously to determine the transmembrane topology of ExbB (22). With spheroplasts, aminopeptidase K truncated a portion of ExbB at the N-terminal end that is exposed to the periplasm (Fig. 5, lane 4). Trypsin treatment resulted in a similar product (Fig. 5, lane 2), since there is a single trypsin cleavage site at the N terminus (lysine residue 24) which may be partially accessible from the periplasmic side, a possibility supported by the much lower yield obtained with trypsin than with aminopeptidase K. The model predicts no other lysine or arginine residue in the periplasm (21). Spheroplasts treated with proteinase K yielded the same ExbB(His)$_6$ degradation products (Fig. 5, lane 3) found previously for ExbB (22). In cell lysates, trypsin and proteinase K degraded most of ExbB(His)$_6$ (Fig. 5, lanes 6 and 7) to the same products as those of the degraded ExbB wild type. These results showed that ExbB(His)$_6$ is inserted into the cytoplasmic membrane, as is wild type ExbB.

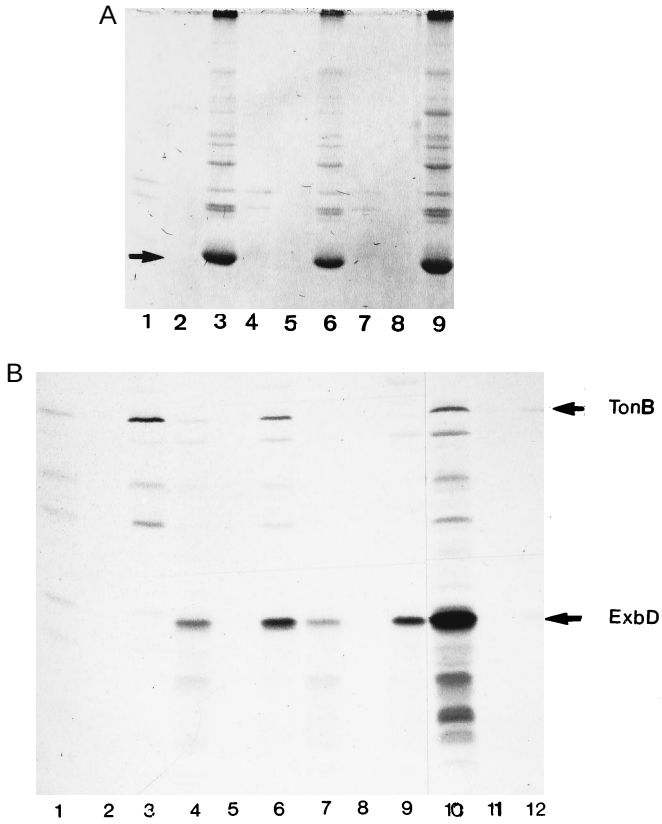

FIG. 6. Interaction of ExbD and TonB with ExbB(His)$_6$ bound to Ni-NTA agarose. (A) Identification of the eluted proteins after SDS-PAGE by staining with Serva Blue. (Only ExbB, marked by an arrow, can be identified by staining. Lanes 10 to 12 are not shown, since no ExbB was applied to the column in this experiment). (B) Identification of the radiolabeled ExbD and TonB proteins after SDS-PAGE by autoradiography. TonB (lanes 1 to 3), ExbD and TonB (lanes 4 to 6), ExbD (lanes 7 to 9), and ExbD and TonB (lanes 10 to 12), the last two proteins chromatographed on Ni-NTA agarose in the absence of ExbB(His)$_6$, were applied to the column, which was washed with adsorption buffer (lanes 1, 4, 7, and 10), wash buffer (lanes 2, 5, 8, and 11), and elution buffer (lanes 3, 6, 9, and 12). Radiolabeled TonB and ExbD were prepared by a procedure identical to that described in Materials and Methods, and the same amounts were applied to the column (final volume, 0.1 ml, prepared from a 10-ml culture).

Interactions among ExbB, ExbD, and TonB. ExbB(His)$_6$ was extracted from the membrane fraction of *E. coli* BL21 (pQE167) with 0.1 M Tris-HCl, pH 8, containing 2% octylglucoside and was then applied to an Ni-NTA agarose column. No ExbB(His)$_6$ was eluted from the column washed with adsorption and wash buffer (Fig. 6A, lanes 1, 2, 4, 5, 7, and 8). ExbD, TonB, and ExbD-TonB were labeled with [^{35}S]methionine, extracted by the same procedure from the membrane fraction of *E. coli* BL21 transformed with pIM750 *exbD*, pCG752 *tonB*, and pCG756 *exbD tonB*, and then applied to the Ni-NTA agarose column loaded with ExbB(His)$_6$. A portion of the radiolabeled proteins along with degradation products or additionally radiolabeled proteins were eluted from the column washed with the adsorption buffer (Fig. 6B, lanes 1, 4, and 7). Subsequent treatment with wash buffer did not elute radioactive proteins (Fig. 6B, lanes 2, 5, and 8). TonB (Fig. 6B, lane 3), ExbD-TonB (lane 6), and ExbD (lane 9) were eluted along with ExbB (Fig. 6A, lanes 3, 6, and 9) with the buffer containing 1 M imidazole that displaced ExbB bound via the His tag to Ni. Only trace amounts of ExbD and TonB were retarded on the Ni-NTA agarose column when no ExbB(His)$_6$ was bound

at the resin (compare Fig. 6B, lane 10 with lanes 11 and 12). In an additional control experiment, the periplasmic FhuD protein of the ferric hydroxamate transport system tagged with $(His)_{10}$ at the N terminus (39) and a cytoplasmic protein of *Neisseria gonorrhoeae* tagged with $(His)_6$ at the C terminus (obtained from J. Maier) were bound to the Ni-NTA agarose, and the experiment was repeated with radiolabeled ExbD and TonB. The results obtained were similar to those shown in Fig. 6B, lanes 10 to 12, demonstrating the lack of binding to both proteins. In another control experiment, β-lactamase, encoded on plasmid pT7-3, was overexpressed and isolated from the periplasm. About 15% of this protein remained on the column and was coeluted with ExbB. The results of these experiments indicated specific binding of ExbD and TonB to ExbB.

DISCUSSION

The Ton system is required for coupling the electrochemical potential of the cytoplasmic membrane to the outer membrane transport system (6, 20, 35). This study shows that ExbD is an essential component of the Ton system, since a chromosomal point mutation in *exbD* abolished all Ton-related activities in CHO3 *exbD tolQ* (*tolR*). Also, residual Ton-related activities displayed by *E. coli* HE1 (*exbB* [*exbD*]) (5, 7) were not observed in *E. coli* CHO3. An increase in the level of TolR activity by transformation with a multicopy *tolR* plasmid did not reconstitute any of the Ton-related activities. This agrees with our previous results showing that *tolQ* and *tolR* had to be on a plasmid to restore Ton-related activities of an *exbB*::Tn*10* mutant with a low expression level of *exbD* (7). Although TolR is active in the Tol system as well as in the Ton system, TolR functions only with TolQ but not with ExbB (7).

Transformation of *E. coli* CHO3 with a multicopy plasmid carrying *tolQ* and *tolR* restored sensitivity to phage φ80, colicins B and M, and albomycin and supported growth on ferrichrome. However, one has to bear in mind that the physiologically relevant function of TolR is in the Tol system and its residual activity in the Ton system is an evolutionary relic (5–7).

Both the L132Q replacement in the periplasmic region of ExbD and the D25N replacement in the transmembrane region abolished ExbD activity. ExbD(L132Q) demonstrates the importance of the periplasmic region for ExbD activity, as has also been found for TonB (7, 20, 26, 27, 42, 44, 52). ExbD(D25N) indicates the functional role of the transmembrane region. The aspartate residue is the only charged amino acid in a hydrophobic region of ExbD that various computer-assisted programs predict to be located within the cytoplasmic membrane (9, 21). Given the uncertainty of such predictions at the border of hydrophobic segments, D-25 could also be located at the inner face of the cytoplasmic membrane. Nevertheless, the inactivity of this mutant agrees with the inactivity of TonB (51), ExbB (28), and TolQ (22, 48, 53) mutated in regions that are predicted to span the cytoplasmic membrane and with the suppression of mutations in the transmembrane segment of TonB by ExbB (28) and of TolQ by TolR (29). These findings indicate that interaction between TonB, ExbB, and ExbD and between TolA, TolQ, and TolR occur within the cytoplasmic membrane. It is likely that ExbB reacts with ExbD, as has been proposed for the TolQ-TolR interaction (29). One may also speculate that the aspartate residue of ExbD forms a salt bridge to the only charged amino acid in the transmembrane region of TonB, histidine 20, whose conversion to arginine strongly reduced TonB activity (51). Both Asp-25 and His-20 are predicted to be located close to the inner side of the cytoplasmic membrane, which makes a functional interaction of the two proteins via these two amino acids physically possible.

ExbB(D25N) abolished the residual ExbD activity of *E. coli* HE2, most likely by displacing the small amounts of wild-type ExbD in strain HE2 by ExbD(D25N). In *E. coli* AB2847, which formed normal amounts of wild-type ExbD, ExbD(D25N) had to be strongly overexpressed for negative complementation. Competition between wild-type and mutant ExbD indicates oligomer formation of ExbD with itself or with another component of the cell. This component is probably ExbB, as shown by this study by the binding of ExbD to $ExbB(His)_6$ and previously by the prevention of ExbD degradation through ExbB (10). In both assays, ExbD did not require TonB for binding to ExbB, which shows a direct and not a TonB-mediated binding of ExbD to ExbB.

Truncation of the C-terminal end of ExbD(D25N) up to residue 80 abolished negative complementation. Since the ExbD(D25N, Δ81–141) protein was present in large amounts, either the deleted sequence contributes in the wild type to the interaction of ExbD with ExbB (and/or TonB) directly, by serving as a binding site, or by being important for the ExbD conformation or ExbD(D25N, Δ81–141) is no longer inserted properly into the cytoplasmic membrane. Because of the negative complementation of wild-type ExbD and the physical stabilization of ExbD (D25N, Δ81–141) by ExbB, it is unlikely that ExbD(D25N, Δ81–141) is not inserted into the cytoplasmic membrane, unless ExbD forms homo-oligomers or hetero-oligomers with ExbB and TonB prior to insertion into the cytoplasmic membrane and the complex with ExbD(D25N, Δ81–141) prevents their insertion into the cytoplasmic membrane. The latter possibility cannot be ruled out, since a cytoplasmic derivative of TonB lacking residues 1 to 32 was accumulated only in the presence of ExbBD (24). It was argued that ExbB functions as a chaperone of TonB in the cytoplasm. We favor the interpretation that the C-terminal periplasmic portion of ExbD participates in the formation of the complex among TonB, ExbB, and ExbD, so that ExbD(D25N) no longer displaces wild-type ExbD when the C-terminal portion is removed. L-132 may be located at a site of functional interaction.

Another ExbD derivative that contained residues 4 to 15 in reverse order also complemented *E. coli* HE2 negatively. Wild-type ExbD contains four negatively charged and no positively charged amino acids at the inner side of the cytoplasmic membrane (9), which is uncommon for proteins spanning the cytoplasmic membrane (18). Such proteins usually contain a surplus of positively charged amino acids at the cytoplasmic side (the positive inside rule [54]). ExbD(*Nsi*I rev) was inactive in *E. coli* CHO3, indicating that the sequence of the N-terminal portion located in the cytoplasm plays a crucial role in ExbD activity. However, deletion of the inverted sequence resulted in an ExbD derivative that was partially active in *E. coli* CHO3 and increased the activity level of strain HE2. This puzzling result suggests that the reverse sequence imposes a conformation on ExbD which is incompatible with its activity. Other mutations in the cytoplasmic region of ExbD did not affect ExbD activity.

The stoichiometry of the presumed complex formed by TonB, ExbB, and ExbD is unknown. The amounts of radiolabeled proteins found in cells are different, in that more ExbB is formed than TonB and more TonB is formed than ExbD (10). The 59-kDa product formed after cross-linking that reacted with antibodies raised against TonB and that was absent in an *exbB*::Tn*10* mutant suggests equimolar amounts of TonB and ExbB in the complex. However, since the yield of cross-

linked proteins was very small (44), the 59-kDa complex might not reflect the in vivo composition of the complex.

The data contained in this paper ascribe an essential function to ExbD and within ExbD to aspartate 25 and leucine 132. It is conceivable that aspartate 25 of ExbD and histidine 20 of TonB are involved in sensing the electrochemical potential of the cytoplasmic membrane, which induces a conformation in the Ton complex that triggers the opening of the channels in the outer membrane receptor proteins. The energized conformation of the receptors not only allows translocation of the ferric siderophores and vitamin B_{12} and uptake of the B-group colicins, it also triggers the release of the DNA from the phages T1 and ϕ80 (12) and it initiates a sequence of events that finally initiates transcription of the *fec* genes involved in ferric citrate transport (16, 34, 57). A transmembrane signaling device similar to the Fec system of *E. coli* has been found in *Pseudomonas putida*. Expression of the *pupB* gene that encodes a receptor protein in the outer membrane required for the uptake of pseudobactin BN8 requires the PupB and the TonB proteins (3).

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