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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

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The *exb* locus in *Escherichia coli* consists of two genes, termed *exbB* and *exbD*. Exb functions are related to TonB function in that most TonB-dependent processes are enhanced by Exb. Like *tonB* mutants, *exb* mutants were resistant to colicin M and albomycin but, in contrast to *tonB* mutants, showed only reduced sensitivity to colicins B and D. Overexpressed *tonB* on the multicopy vector pACYC177 largely restored the sensitivity of *exb* mutants to colicins B, D, and M but only marginally increased sensitivity to albomycin. Suppression of the *btuB451* mutation in the structural gene for the vitamin B_{12} outer membrane receptor protein by a mutation in *tonB* occurred only in an *exb*$^+$ strain. Degradation of the unstable overproduced TonB protein was prevented by overproduced ExbB protein. The ExbB protein also stabilized the ExbD protein. Pulse-chase experiments with radiolabeled ferrichrome revealed release of ferrichrome from *exbB*, *tonB*, and *fhuC* mutants, showing that ferrichrome had not crossed the cytoplasmic membrane. It is concluded that the ExbB and ExbD proteins contribute to the activity of TonB and, like TonB, are involved in receptor-dependent transport processes across the outer membrane.

Transport of iron(III) siderophores and vitamin B_{12} across the outer membrane of *Escherichia coli* (2, 4, 17, 21) stands in contrast to the passive diffusion of most substrates through porins (18). The activity of the receptor proteins for iron(III) siderophore and vitamin B_{12} transport depends on the TonB protein of the cytoplasmic membrane, since these substrates stay at the receptor proteins in *tonB* mutants and in unenergized *tonB*$^+$ cells (3; H. Schöffler and V. Braun, Mol. Gen. Genet., in press). The group B colicins also remain bound to the outer membrane receptors unless *tonB*$^+$ cells are energized (3, 20). Mutations in a single codon of the *tonB* gene suppressed the *btuB451* point mutation in the structural gene of the vitamin B_{12} receptor (14), and mutations in the *fhuA* gene (Schöffler and Braun, in press), which encodes the receptor for ferrichrome, the antibiotic albomycin, colicin M, and the bacteriophages T5, T1, and φ80. Suppression of mutations in receptor genes by *tonB* mutations suggests a direct interaction between the TonB protein and the receptor proteins. The TonB function seems to be involved only in transport processes across the outer membrane.

Exb designates a function of *Escherichia coli* similar to TonB with the difference that TonB-dependent processes absolutely require TonB whereas Exb-dependent functions require Exb to a different extent. There are TonB-dependent functions which do not require Exb, but all Exb-dependent functions rely on TonB. For example, *tonB* mutants are insensitive to colicins B and M and to the antibiotic albomycin and are unable to take up ferrichrome. *exb* mutants have the same properties except that they exhibit reduced sensitivity to colicin B. The *exb* locus has been mapped at min 65 of the *E. coli* linkage map (20). Recently, we cloned (9) and sequenced (8) the *exb* region and identified two genes, termed *exbB* and *exbD*, which are required for complementing *exb* mutants. The *exbB* and *exbD* genes encode proteins with molecular masses of 26.1 and 15.5 kilodaltons (kDa), respectively, which were both preferentially localized in the cytoplasmic membrane (8). Since the ExbB and ExbD proteins affect the same cellular activities as the TonB protein, and the requirement for ExbB and ExbD differs in various TonB-dependent functions, we studied whether overproduction of TonB compensates for the lack of ExbB and ExbD and vice versa. Indeed, the results presented in this paper show that the low sensitivity of *exb* mutants to colicins B, D, and M could be greatly increased by overexpression of *tonB*. In addition, suppression of the *btuB451* mutation by a mutation in *tonB* was enhanced in an *exb*$^+$ background. The ExbB protein prevented degradation of the TonB protein. Radioactive ferrichrome taken up by *exb* mutants was released by a surplus of unlabeled ferrichrome, indicating that ferrichrome is located close to the cell surface in *exb* mutants.

The data are consistent with a model in which the ExbB protein increases the stability, and both proteins enhance the activity, of the TonB protein.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacteria and plasmids used in this study are listed in Table 1. The *exb* mutant W3110-6 was isolated among spontaneous albomycin-resistant derivatives of W3110. P1 cotransduction of *exb*$^+$ with *zge*::Tn*10* restored ferrichrome-iron transport to strain W3110-6 and susceptibility to albomycin and colicins B, D, and M to the level of the parental strain W3110. Strain GU2 was obtained by P1 cotransduction of *exbB*::Tn*10* from H1388 into RK4794. Isolation of plasmids and transformation were carried out by conventional protocols (16). Transformants were selected by plasmid-mediated antibiotic resistance, and plasmid-carried genes were assayed by complementation of chromosomal mutations.

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TABLE 1. *E. coli* strains and plasmids

Strain or plasmid	Relevant genotype	Source or reference
E. coli		
AB2847	*aroB thi malT tsx*	This institute
BR158	As AB2847, but *tonB*	This institute
LF947	*fhuC12 fepA12 recA minB lacY xyl thi mtl*	10
H455	*aroB pro lac malT tsx thi*	This institute
RK4794	*btuB451 metE*	14
GU2	RK4794 *exbB*::Tn*10*	This study
H1388	As H455, but *exbB*::Tn*10*	13
W3110	Wild type	This institute
W3110-6	As W3110, but *exb*	This study
P575	*exb argE his proA thr leu rpsL thi*	A. Pugsley
WM1576	HfrC	22
Plasmids		
pBJM2	pACYC177 carrying *tonB* Km^r	15
pCG8	pBJM2 (Q/L)[a]	14
pCG15	pBJM2 (Q/K)[a]	14
pCG752	pT7-5 carrying *tonB*	This study
pKE7	pUC18 carrying *exbB exbD* Ap^r	
pKE61	pT7-5 carrying *exbB exbD*	K. Eick-Helmerich
pCG754	pT7-5 carrying *tonB exbB exbD*	This study
pCG755	pT7-5 carrying *tonB exbB*	This study
pCG756	pT7-5 carrying *tonB exbD*	This study
pFB5-6	pBR322 carrying *fhuA* Ap^r	10
pFB103-1	pBR322 carrying *fhuA* Tc^r	10
pCF1-V1	pBR322 carrying *fhuACDB* Ap^r	10
pPF3-V1	pBR322 carrying *fhuACDB* Tc^r	10
pT7-5	Phage T7 gene *10* promoter	22
pGP1-2	pACYC177 carrying the T7 RNA polymerase gene	22

[a] Q/L and Q/K, *tonB* mutations that change glutamine (Q) to leucine (L) or to lysine (K) at position 165 of the polypeptide.

Plasmid pCG752 was obtained by cloning the *tonB Hin*dII fragment of pBJM2 (17) into the *Sma*I site of pT7-5 (22). This plasmid carries the *tonB* gene in the orientation of the phage T7 gene *10* promoter, whereas *tonB* was inserted in the reverse orientation in pCG753. Plasmid pKE61 was constructed by cloning the *exbBD Sma*I-*Pst*I fragment of pKE7 (9) into the *Sma*I-*Pst*I sites of pT7-5 (Fig. 1). Plasmid pCG754 contains the *Eco*RI fragment of pKE7 in the *Eco*RI site of pCG752. Plasmid pCG755 contains the *exbB Taq*I fragment of pKE7 in the *Cla*I site of pCG752. Plasmid pCG756 carries the *Xho*II fragment of pKE7 in the *Bam*HI site of pCG752 (Fig. 1).

Media and growth conditions. Bacteria were grown in TY broth (1% tryptone, 0.5% yeast extract, 0.5% sodium chloride) and on TY agar (TY broth containing 1.5% agar). Ampicillin (25 μg/ml), kanamycin (50 μg/ml), and tetracycline (15 μg/ml) were added to select for transformants and to maintain the plasmids. For assays, overnight cultures were diluted 100-fold with TY broth and grown at 37°C (pGP1-2-carrying strains were grown at 30°C) to an A_{578} of 0.5. Exponentially growing cells were collected by centrifugation, suspended in M9 buffer (0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.1% NH_4Cl, 0.05% NaCl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$), and adjusted to the same density to assay [$^{55}Fe^{3+}$]ferrichrome uptake and susceptibility to albomycin and to colicins B, D, and M.

TonB synthesis. *E. coli* W3110 and W3110-6 were transformed with plasmids pCG752 and pCG753 and also with plasmid pGP12 (22), which carries a temperature-inducible T7 RNA polymerase gene. Cells were radioactively labeled by a procedure communicated by Stan Tabor (Harvard Medical School, Boston, Mass.). Briefly, cells (3×10^8) in the logarithmic growth phase were suspended in 1.0 ml of M9 buffer, supplemented with 0.4% glucose, 20 μg of thiamine per ml, and 0.01% each of 18 amino acids (no cysteine and methionine). They were incubated with shaking at 30°C for 45 min. Then, the temperature was shifted to 42°C for 15 min. Rifampin (20 mg/ml in methanol) was added to a final concentration of 0.2 mg/ml. After 15 min at 42°C, the temperature was shifted down to 30°C for 30 min. Cells were pulse-labeled with 0.74 mBq of [^{35}S]methionine for 5 min at 30°C. One sample (0 min, 0.25 ml) was centrifuged for 20 s at $10,000 \times g$, and the sediment was immediately dissolved in 0.02 ml of sample buffer (60 mM Tris hydrochloride [pH 6.8], 0.05% β-mercaptoethanol, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.002% bromphenol blue) by heating for 5 min at 95°C. The rest was incubated with 6.3 mg of methionine for 5, 15, and 60 min, after which 0.25-ml samples were processed as described for the 0-min sample.

Protease accessibility. Cells in the logarithmic growth phase (3×10^8 per ml) were labeled with 0.74 mBq of

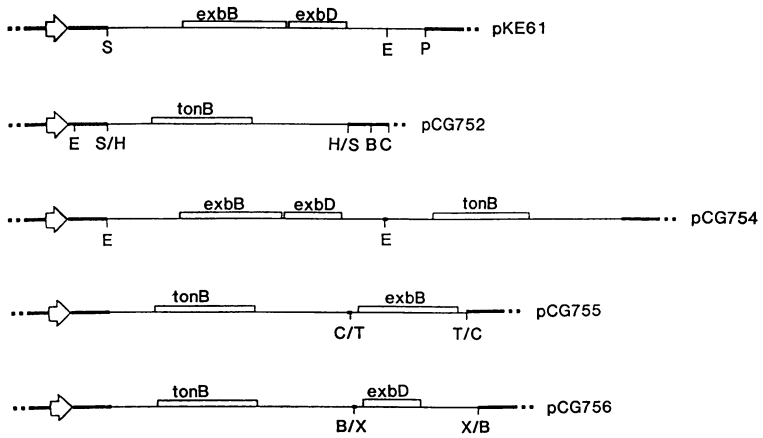

FIG. 1. Plasmids used for expression of the *tonB*, *exbB*, and *exbD* genes alone and in combination. The arrows mark the position and transcription polarity of the T7 promoter. In the plasmids shown, the cloned genes have the same direction of transcription. Only part of the vector DNA is drawn, indicated by the heavy dashed line. E, *Eco*RI; S, *Sma*I; H, *Hin*dII; B, *Bam*HI; C, *Cla*I; T, *Taq*I; X, *Xho*II.

[^{35}S]methionine for 5 min. They were divided in three portions and spun down for 20 s at 10,000 × *g*. One pellet was suspended in 25 μl of ice-cold 0.1 M Tris hydrochloride–5 mM sodium EDTA, pH 8, to which 1 μl of lysozyme (25 mg/ml) was added. The other two portions were suspended in 112 μl of ice-cold 0.2 M Tris hydrochloride–0.5 M sucrose, pH 8, to which was added 12 μl of 5 mM sodium EDTA, pH 8, and 12 μl of lysozyme (6.4 mg/ml). The suspension was mixed, and then 125 μl of ice-cold 0.2 M Tris hydrochloride–0.5 mM sodium EDTA, pH 8, was added. The samples were incubated for 15 min on ice. The sucrose samples received 5 μl of 1 M $MgSO_4$. Trypsin (10 or 25 μg in 1 μl) was added to one sucrose-containing sample (10 μg in 1 μl) and to the sucrose-deficient sample (25 μg) and incubated for 15 min on ice. The spheroplasts of the sucrose samples were pelleted for 5 min at 10,000 × *g*, and the sediments were heated for 5 min at 95°C in 40 μl of sample buffer. The sample with the lysed cells was heated with 15 μl of double-strength sample buffer. Half of each sample was applied to a 15% SDS-polyacrylamide gel. Digestion with proteinase K (10 μg) followed the same procedure. After 20 min on ice, degradation was stopped by addition of 4 μl of 0.1 M phenylmethylsulfonyl fluoride in isopropanol.

[^{55}Fe]ferrichrome transport assay. Overnight cultures were diluted 100-fold into fresh TY medium and grown to a density of 3 × 10^8 cells per ml. Cells were harvested by centrifugation at 0°C and suspended to equal densities in 1 ml of M9 buffer which contained 0.01 mM deferri-ferrichrome and 0.1 mM nitrilotriacetate. After 10 min of incubation at 30°C, transport was started by addition of 10 μl of deferri-ferrichrome (final concentration, 1 μM) which had been loaded with 0.1 μM carrier-free ^{55}Fe(III) by preincubation at 37°C for 30 min in the presence of 30 mM HCl. If required, 1 μM ferrichrome was used to avoid substrate limitation. Throughout incubation the suspension was agitated by magnetic stirring. At intervals, 0.1-ml samples were filtered, washed twice with 5 ml of 0.1 M LiCl, and dried. Incorporated ^{55}Fe(III) was determined by liquid scintillation counting. The counting efficiency was 35%.

Assays of colicin and albomycin susceptibility. Growth inhibition by albomycin and by colicins B, D, and M was measured in the agar diffusion assay. TY plates were seeded with 2 × 10^8 cells (0.2 ml) in 2 ml of top agar (TY broth containing 0.8% agar). Colicins and albomycin were from laboratory stocks. Immediately before the assay, serial dilutions of colicins and albomycin were prepared with phosphate-buffered saline (10 mM sodium phosphate, 0.9% sodium chloride, pH 7). In the case of albomycin, 10 μl of the dilutions was spotted on filter paper disks, which were placed on the seeded TY agar plates. With colicins, 3 μl of the dilutions was spotted directly onto the plates. After 20 h of incubation at 37°C, growth inhibition was recorded.

RESULTS

Increased susceptibility of *exb* mutants to colicins B, D, and M by overexpressed *tonB*. *exb* mutants are largely resistant to colicin (13, 20). In strains H1388 and W3110-6, both *exb* genes are affected, since complementation to Exb wild-type required transformation with plasmids carrying *exbB* and *exbD* (8). Plasmid-encoded *tonB* (pBJM2) restored the susceptibility to colicin M to a large extent in H1388 and to a lesser extent in W3110-6 (Table 2). The sensitivity of the former was only three- to ninefold below the sensitivity obtained when the *exb* mutant was transformed with pKE7 *exbB exbD* (Table 2). Sensitivity to colicin B could also be restored in H1388 and W3110-6 by transformation with pBJM2 (Table 3). Both strains were only three- to ninefold less sensitive than transformants carrying pKE7. Corresponding results were obtained with colicin D. The *exb* mutants were 300- to 1,000-fold less sensitive to colicin D. Transformants of *exb* mutants carrying pKE7 exhibited a 30- to 100-fold-higher susceptibility to colicin D, and transformants containing pBJM2 were 10- to 30-fold more sensitive.

TABLE 2. Restoration of colicin M susceptibility in *exb* mutants by plasmid-coded *tonB*

Strain	Growth inhibition[a] by colicin M at dilution factor:				
	3^2	3^3	3^4	3^5	3^6
H455	+++	+++	+++	++	+
H1388	0	0	0	0	0
H1388(pKE7)	+++	+++	++	+	(+)
H1388(pBJM2)	+++	+++	++	0	0
W3110	+++	+++	+++	+	0
W3110-6	0	0	0	0	0
W3110-6(pKE7)	+++	+++	+++	+	0
W3110-6(pBJM2)	++	++	(+)	0	0

[a] Symbols: +++, clear growth inhibition zone; ++, clear zone with turbid edges; +, turbid; (+), very turbid zone; 0, no growth inhibition.

Failure of plasmid-encoded *tonB* to substitute for deficient chromosomal *exb* in the uptake of albomycin and ferrichrome. Uptake of ferrichrome, the structurally analogous antibiotic albomycin, and colicin M requires the FhuA, TonB, ExbB, and ExbD proteins. Since plasmid-encoded *tonB* reactivated the uptake of colicin M into *exb* mutants, we examined albomycin sensitivity and ferrichrome uptake in *exb* mutants transformed with pBJM2 (*tonB*$^+$). None of the transformed *exb* mutants (W3110-6, H1388, and P575) became albomycin sensitive. Transformation with pKE7 (*exbB exbD*) rendered all three *exb* mutants albomycin sensitive, but not to the extent of the chromosomal *exb*$^+$ strains W3110 and H455 (Table 4; turbid lysis zones in contrast to clear zones).

To see whether the failure of complementation to albomycin susceptibility was due to inefficient uptake, we examined transport of $^{55}Fe^{3+}$-labeled ferrichrome. The amount of ferrichrome found in the pBJM2 transformants was as low as in untransformed H1388, W3110-6, and P575 (data not shown). Transformation with pKE7 restored the ferrichrome transport rates in all three *exbB* strains to nearly those of the H455 and W3100 parent strains, indicating that the chromosomal *exbB* mutation was complemented by pKE7.

Failure of overproduced ExbB and ExbD to substitute for TonB. To examine whether overproduced ExbB and ExbD

TABLE 3. Phenotypic complementation of colicin B susceptibility in *exb* mutants by plasmid-coded *tonB*[a]

Strain	Growth inhibition by colicin B at dilution factor:					
	3^4	3^5	3^6	3^7	3^8	3^9
H455	+++	+++	+++	+++	+++	++
H1388	++	+	(+)	0	0	0
H1388(pKE7)	+++	+++	+++	++	+	+
H1388(pBJM2)	+++	+++	+++	+	(+)	(+)
W3110	+++	+++	+++	++	+	0
W3110-6	+	(+)	(+)	0	0	0
W3110-6(pKE7)	+++	+++	+++	++	+	(+)
W3110-6(pBJM2)	+++	++	++	+	(+)	0

[a] See Table 2, footnote *a*.

TABLE 4. Albomycin susceptibility of *exb* mutants conferred by overproduced components of ferric hydroxamate uptake

Strain	Diameter (mm) of zone[a] at albomycin concn:			
	100 μM	10 μM	1 μM	0.1 μM
W3110	21	16	8	0
W3110-6	0	0	0	0
W3110-6(pKE7)	21	17*	12*	8*
W3110-6(pPF3-V1)	23*	14*	8†	0
W3110-6(pFB103-1)	24†	15†	0	0
H455	23	18	12	8
H1388	0	0	0	0
H1388(pKE7)	27*	22*	17*	12*
H1388(pCF1-V1)	24*	15*	0	0
H1388(pFB5-6)	24†	15†	0	0
P575	0	0	0	0
P575(pKE7)	24	19	15*	12†
P575(pCF1-V1)	22*	12*	0	0
P575(pFB5-6)	22*	12†	0	0

[a] Symbols indicate turbid (*) and very turbid (†) inhibition zones.

would substitute for TonB, ferrichrome uptake and susceptibility to albomycin and to colicins B, D, and M were determined with several pKE7 transformants of the *tonB* mutant BR158. Although pKE7-encoded ExbB was overproduced (9) far above pBJM2-encoded TonB (data not shown), all transformants tested remained resistant to the colicins and to albomycin and failed to transport ferrichrome.

Susceptibility of *exb* mutants to albomycin and increased uptake of ferrichrome conferred by overexpressed *fhu* genes. We examined whether overexpression of *fhu* genes compensated the *exb* defect. As shown in Table 4, overexpression of the entire *fhu* operon in transformants with plasmids pPF3-V1 and pCF1-V1 and overexpression of *fhuA* in transformants with plasmids pFB5-6 and pFB103-1 rendered the *exb* mutants W3110-6, H1388, and P575 slightly albomycin sensitive. The growth inhibition zones were turbid on plates seeded with transformants carrying the entire *fhu* operon and very turbid with *fhuA* transformants. Apparently, albomycin was taken up to such an extent that growth was retarded, but most of the cells were not killed. Ferrichrome uptake rates support these findings. Transformants of strains H1388 and W3110-6 carrying pCF1-V1 showed a twofold increase in ferrichrome uptake, which, however, was not sufficient to support growth on nutrient broth-dipyridyl plates containing ferrichrome as the sole iron source. The increase in the ferrichrome uptake rate of H455 and W3110 transformed with pCF1-V1 was much higher (ninefold), which illustrates the marginal compensation of the *exbB* defect by the overproduced FhuA, −B, −C, and −D proteins. pFB5-6 transformants of the *exbB* mutants exhibited no increase in ferrichrome uptake.

Sensitivity to colicin M varied in that undiluted colicin M (titer, 10^5) gave rise to a much smaller zone of growth inhibition on plates seeded with H1388(pFB5-6) than on plates seeded with H1388, whereas W3110-6(pFB5-6) was slightly sensitive (small clear inhibition zone), in contrast to W3110-6, which was resistant.

Release of ferrichrome from transport mutants. Cells in the logarithmic growth phase were incubated for 8 min with radioactive [$^{55}Fe^{3+}$]ferrichrome and subsequently with a 10-fold excess of nonradioactive ferrichrome. The strains used carried the *fhuA* gene on pFB103-1 to provide more ferrichrome-binding sites (10, 15). The wild-type strain AB2847(pFB103-1) transported ferrichrome at a constant rate, starting from a fourfold higher initial level (Fig. 2, right panel) than AB2847 without pFB103-1 (not shown). Addition of nonradioactive ferrichrome (marked by an arrow) terminated the uptake, but no radioactive ferrichrome was released from the cells. Most of the radioactive ferrichrome was chased from the *tonB* derivative by nonradioactive ferrichrome (Fig. 2; note the different scale). Similarly, [^{55}Fe]ferrichrome was released from the *exb* mutant, but a small amount remained cell bound (Fig. 2), corresponding to the residual transport activity of *exb* mutants. However, this amount of transported ferrichrome was insufficient to provide enough iron for cellular growth (about 2,000 ions remain cell bound, in contrast to 20,000 in AB2847). *fhuC* mutants, which encode a mutant transport protein of the cytoplasmic membrane (6, 7, 10; W. Köster and V. Braun, Mol. Gen. Genet., in press), also released [^{55}Fe]ferrichrome upon chase (Fig. 2). This suggests that in all three mutant types, most (*exbB*) or all (*tonB* and *fhuC*) of the ferrichrome had not crossed the cytoplasmic membrane.

ExbB enhances suppression of the *btuB451* mutation by *tonB* mutations. *btuB451* mutants bind but do not transport vitamin B_{12} (14, 17). Two types of *tonB* mutations, both affecting residue 165 of the TonB protein, restored growth of the vitamin B_{12}-dependent *metE* mutant RK4794 (*btuB451*) on a vitamin B_{12}-containing minimal medium (14). RK4794 derivatives containing *tonB* mutant plasmids with a glutamine-to-leucine (Q/L) or a glutamine-to-lysine (Q/K) exchange grew equally well. However, the *exb* mutant GU2 differed in growth. Transformants containing the *tonB* plasmid pCG8 (Q/L) grew on 5 nM vitamin B_{12}, whereas those with pCG15 (Q/K) showed a very low growth rate with the same amount of vitamin B_{12}. Very small colonies appeared after several days of incubation, in contrast to the *tonB* (Q/L) transformants, which formed normal-sized colonies after overnight growth. A transformant of GU2(pCG15) also carrying pKE7 exhibited the same growth properties as RK4794(pCG15), indicating that the growth defect of GU2 was related to the *exb* mutation.

ExbB stabilizes the TonB protein. TonB is functionally (1) and physically (19) unstable. To examine whether ExbB and ExbD prevented degradation of TonB, pulse-chase experiments were performed in chromosomal *exb*$^+$ and *exb* mutant cells. Since the amount of TonB protein expressed by chromosomal *tonB* was too low to be detected by autoradiography, the *tonB* gene was cloned downstream of the strong phage T7 gene *10* promoter and transcribed by the T7 RNA polymerase while the cellular polymerase was suppressed with rifampin. After temperature induction of the T7 polymerase, *E. coli* W3110 and W3110-6 *exb* were labeled for 5 min with [^{35}S]methionine and then chased with 50 mM methionine for 5, 10, and 20 min. Both cell types contained only one labeled protein, which decreased in amount during the chase period (Fig. 3, *exb*$^+$ in lanes 1 to 4; *exb* mutant in lanes 5 to 8). The molecular mass of the TonB protein, estimated from the electrophoretic mobility relative to standard proteins, was 38 kDa, which corresponds to previously determined values (19) but is higher than the value calculated from the nucleotide sequence (19). A similar result was obtained when protein synthesis after pulse-labeling was inhibited with chloramphenicol (0.1 g/ml). The rate of disappearance of the TonB protein was indistinguishable in chromosomal *exb*$^+$ and *exb* cells. When these experiments were performed with a plasmid containing the same DNA fragment in reverse orientation in pT7-5, the TonB protein was not labeled, but instead the adjacent P14 protein, whose

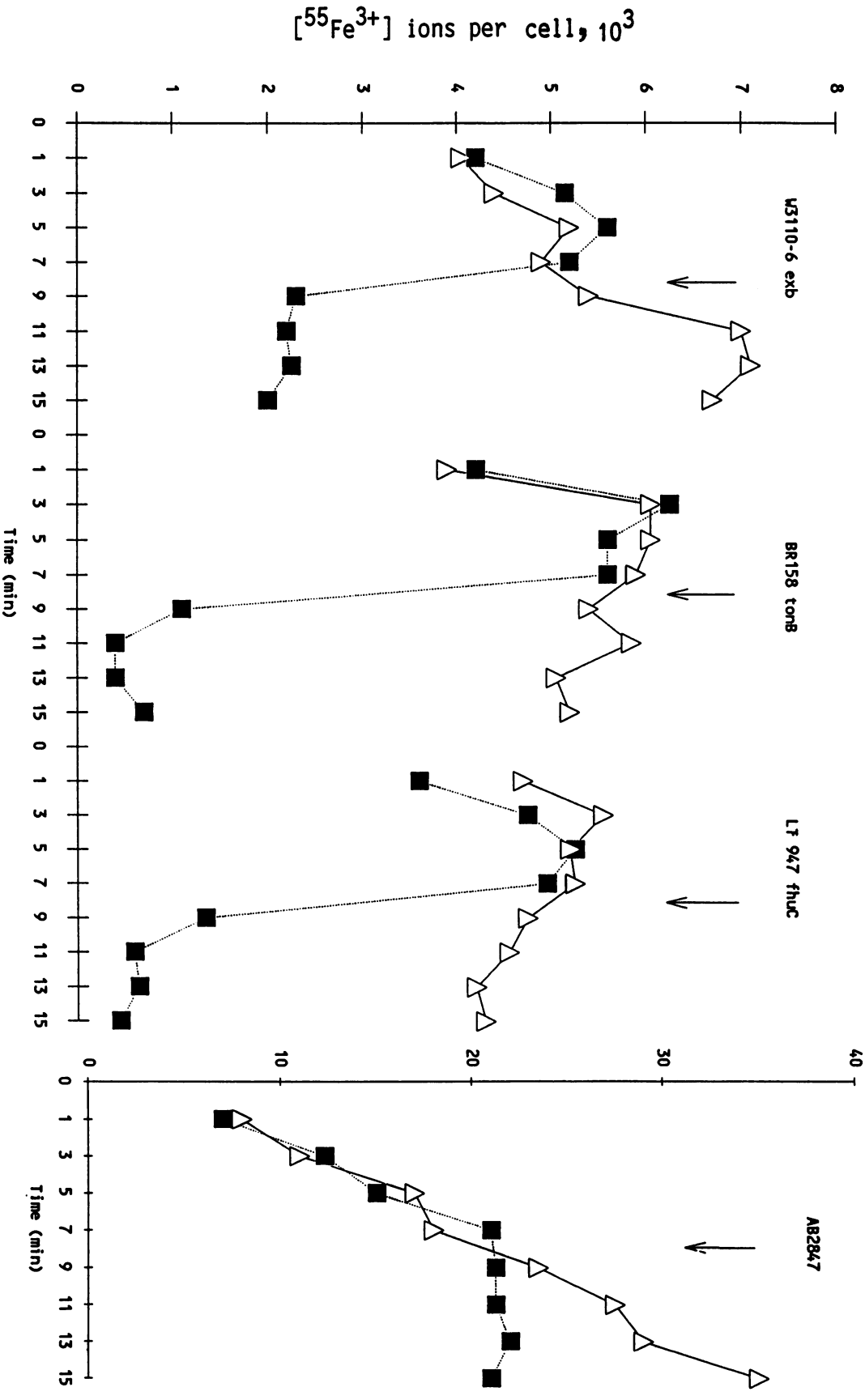

FIG. 2. Release of [$^{55}Fe^{3+}$]ferrichrome from transport mutants by addition of surplus unlabeled ferrichrome. Cells were incubated for 8 min with 0.1 μM [$^{55}Fe^{3+}$]ferrichrome, after which 1 μM unlabeled ferrichrome was added (arrow, solid symbols). No unlabeled ferrichrome was added to the control samples (open symbols). The strains used and their relevant properties are indicated on the figure. Note the different scales used for presentation of the transport rates of the wild-type strain AB2847 versus the transport rates of the mutants.

FIG. 3. Pulse-chase experiment demonstrating instability of the TonB protein. *tonB* was expressed under control of the phage T7 promoter. Cells were labeled with radioactive methionine for 5 min. Samples were withdrawn immediately after addition of nonradioactive methionine and 5, 10, and 20 min thereafter [strain W3110 (pGP1-2, pCG752), lanes 1 to 4, respectively; strain W3110-6(pGP1-2, pCG752), lanes 5 to 8, respectively].

structural gene is transcribed opposite to *tonB* (19), was very strongly labeled. During the chase period, the amount of P14 protein remained constant (data not shown).

To examine whether the amount of chromosomally encoded ExbB protein was insufficient to prevent degradation of the overproduced TonB, we cloned *tonB* together with *exbB*, *exbD*, or both *exbB* and *exbD* downstream of the gene *10* promoter of pT7-5 (Fig. 1). Two proteins were [^{35}S] methionine-labeled in *E. coli* WM1576 transformed with plasmid pKE61 (*exbB exbD*) (Fig. 4, lane 1). The molecular masses of these proteins, deduced from their electrophoretic mobilities relative to standard proteins, were 26 kDa for ExbB and 16.8 kDa for ExbD, which corresponds to the sizes of the ExbB and ExbD proteins estimated previously by gel electrophoresis (9) and are close to the values calculated from the *exbB* and *exbD* nucleotide sequences (26.1 and 15.5 kDa, respectively) (8). The amounts of the ExbB

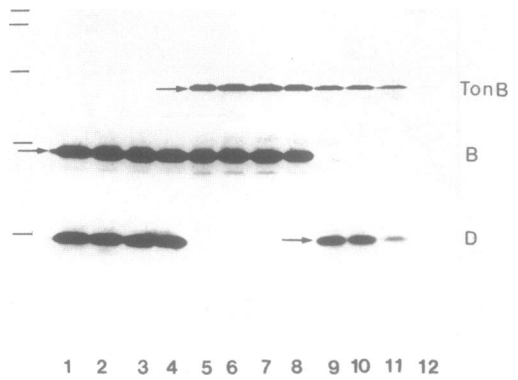

FIG. 4. Stabilization of TonB and ExbD by ExbB. Strain WM1576(pGP1-2) was transformed with pKE61 *exbB exbD* (lanes 1 to 4), pCG755 *tonB exbB* (lanes 5 to 8), and pCG756 *tonB exbD* (lanes 9 to 12). After temperature induction of the T7 RNA polymerase, cells were labeled for 5 min with radioactive methionine and then chased for 0, 5, 15, and 60 min with unlabeled methionine (first to last lane in each group, respectively). The proteins of the chase samples were separated on a 15% polyacrylamide gel in the presence of SDS. The arrows indicate the TonB, ExbB (B), and ExbD (D) proteins. The positions of the standard proteins human transferrin (80 kDa), bovine serum albumin (67 kDa) ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and myoglobin (18 kDa) used for estimation of the molecular masses of TonB, ExbB, and ExbD are marked in this order from top to bottom on the left side of the autoradiograph.

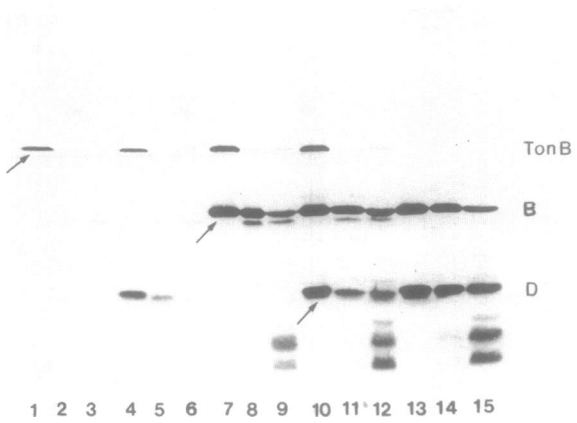

FIG. 5. Degradation of the TonB protein by trypsin. Cells of WM1576(pGP1-2) carrying pCG752 (lanes 1 to 3), pCG756 (lanes 4 to 6), pCG755 (lanes 7 to 9), pCG754 (lanes 10 to 12), or pKE61 (lanes 13 to 15) were labeled with [^{35}S]methionine for 5 min. Untreated spheroplasts (lanes 1, 4, 7, 10, and 13), trypsin-treated spheroplasts (lanes 2, 5, 8, 11, and 14), and trypsin-treated cell lysates (lanes 3, 6, 9, 12, and 15) were loaded onto a polyacrylamide gel, and the proteins were separated by electrophoresis in the presence of SDS. The TonB, ExbB (B), and ExbD (D) proteins are marked by arrows.

and ExbD proteins stayed constant during the chase periods of 5, 15, and 60 min (Fig. 4, lanes 2 to 4). In WM1576 (pCG755 [*tonB exbB*]), two major proteins were labeled, and the amount of TonB, like ExbB, did not decrease during the chase (Fig. 4, compare lane 5 with lanes 6 to 8). ExbB apparently prevented degradation of TonB. The TonB stability conferred by ExbB was unaltered when ExbD was also expressed by WM1576(pCG754). In contrast, ExbD alone failed to stabilize TonB, and in the absence of ExbB the amount of ExbD decreased during the chase (Fig. 4, lanes 9 to 12).

TonB is degraded by proteinase K when cells are converted to spheroplasts (19). To examine whether ExbB prevents proteolysis of TonB, we used trypsin, which has a higher cleavage specificity than proteinase K. Trypsin degraded TonB in spheroplasts and in disrupted cells (Fig. 5, lanes 1 to 3). Overexpression of ExbD (lanes 4 to 6), ExbB (lanes 7 to 9), or ExbB and ExbD (lanes 10 to 12) did not protect overexpressed TonB from trypsin cleavage, although faint TonB bands were still seen. Repetition of this experiment with proteinase K in place of trypsin yielded the same results. Protection of TonB by ExbB and ExbD was not observed (data not shown). However, ExbB markedly delayed proteolysis of ExbD by trypsin (Fig. 5, compare lanes 5 and 6 with 11 and 12 and with 14 and 15) and by proteinase K. We suggest that protection of ExbD by ExbB in the presence and absence of added proteases indicates physical interaction between the two proteins.

DISCUSSION

Overproduced TonB increased the susceptibility of *exb*-deficient mutants to the group B colicins B, D, and M. Particularly striking was the restoration of colicin M sensitivity to near wild-type levels in H1388. Strain W3110-6 remained less sensitive to colicin M than H1388 following amplification of *tonB*, which may reflect the known difference in the mutations at the *exb* locus (8). These results indicate that ExbB and ExbD were not essential for either

the uptake of colicin M or for TonB activity, since amplification of the *tonB* copy number sufficed to reestablish colicin M susceptibility. However, ExbB and ExbD enhanced the efficiency of the TonB-dependent uptake of group B colicins considerably. In the absence of ExbB, the TonB activity expressed by the chromosomal gene was high enough to render both *exb* mutants sensitive to the highly active colicin B and D preparations, but was not sufficient to confer sensitivity to the less-active colicin M preparation used in these assays.

In ferrichrome and albomycin uptake, deficiency of ExbB and ExbD could not be bypassed by amplification of TonB. Only double transformants carrying pBJM2 and pKE7 efficiently took up ferrichrome and albomycin. Overproduced FhuA receptor rendered *exb* mutants slightly albomycin sensitive. Apparently, traces of the elevated amounts of albomycin bound to the amplified receptor were taken up into the cells even when the TonB activity was low. The lack of efficient bypass of the *exb* mutation by overexpressed *tonB* for ferrichrome and albomycin uptake may reflect the requirement for a higher number of active TonB molecules. In contrast to the few molecules of colicins that are sufficient to kill a cell, albomycin has to be transported at a rate of 50 molecules per min per cell to inhibit bacterial growth (unpublished results). The notion of different levels of TonB activity required for various TonB-dependent processes is supported by the ExbB and ExbD independence of phages T1 and φ80 infection, for which only 1 particle per cell is required. In support of this, the number of plaques formed by phages T1 and φ80 also remained unaltered in certain point mutants in the TonB box of the *fhuA* receptor gene, which showed strongly reduced sensitivities to colicin M and resistance to albomycin unless suppressed by certain mutations in the *tonB* gene (Schöffler and Braun, in press). It was concluded that the residual coupling between the altered FhuA protein and wild-type TonB was sufficient for phage infection but inadequate for colicin M and albomycin uptake (Schöffler and Braun, in press). The mutations in *tonB* apparently improved the coupling to a level that colicin M and albomycin sensitivity was recovered. The same argument applies to the requirement for *exbBD* for suppression of the *btuB451* mutation by a *tonB* mutation. The *btuB451 exb* double mutant GU2 could only grow on 5 nM vitamin B_{12} when suppressed by a *tonB* mutant plasmid encoding a TonB protein carrying a glutamine-to-leucine (Q/L) replacement at residue 165 (14) (residue 165 may actually be residue 160 in the TonB polypeptide, since translation apparently initiates at the sixth codon of the open reading frame [19]). The *tonB* mutant plasmid (glutamine-to-lysine [Q/K] replacement) only suppressed the *btuB451* mutation in the *exbBD*$^+$ wild-type parent strain. We assume that the latter suppression was too low due to a weak TonB-BtuB interaction, which was increased by ExbB and ExbD to a level that enough vitamin B_{12} was transported to support growth.

We obtained two lines of evidence for ExbB and ExbD involvement in transport across the outer membrane. ^{55}Fe-labeled ferrichrome could be released by a surplus of unlabeled ferrichrome from *exb* mutants which expressed increased amounts of the FhuA receptor protein from a *fhuA* multicopy plasmid. These cells bound five times more ferrichrome than cells expressing normal amounts of chromosomally encoded FhuA. Very little ferrichrome could be chased from the latter when they carried an *exb* mutation (data not shown). Apparently, ferrichrome was mainly released from the cell surface receptor, which means that it stayed there in *exb* mutants. Previously, we have shown that the apparent K_m of ferrichrome binding to the receptor was 10 times higher than to the subsequent transport components (23), so that in the chase experiments most of the ferrichrome was in fact bound to the receptor of the transport mutants. In addition, the requirement for the ExbB and ExbD functions in the uptake of colicin M, like the requirement for TonB and FhuA (3), could be bypassed by osmotic shock treatment, which renders the outer membrane temporarily permeable for larger molecules. Therefore, we propose that ExbB and ExbD, like TonB, participate in the transport of group B colicins and of iron siderophores across the outer membrane. The simplest assumption regarding its function is that it enhances TonB activity. According to this model, ExbB and ExbD increase the portion of active TonB. Prevention of TonB degradation by Exb (K. Postle, personal communication), which, as shown in this paper, is exerted by ExbB, could explain an ExbB- and ExbD- mediated increase in TonB activity. However, ExbB would only delay TonB inactivation, since cessation of TonB synthesis from a chromosomal *tonB* gene in an *exb*$^+$ strain led to a decline of vitamin B_{12} transport activity of more than 75% within one generation and to 25% colicin D resistance among the cell population within 20 min (1).

ExbB and ExbD are only active in concert with TonB. If the amount of active TonB required for the various TonB-dependent processes differs, ExbB would be involved to a different extent, which has indeed been observed for ferrichrome, ferri-enterochelin, ferri-citrate, and vitamin B_{12} uptake and sensitivity to the group B colicins and to phages T1 and φ80. ExbB increases the sensitivity of cells to very different compounds. The common denominator of all these processes is the cell surface receptor and TonB-dependent uptake across the outer membrane. However, the TonB-dependent uptake routes may be similar in principle but differ in detail. For example, the TonB box assumed to be involved in the interaction of the TonB protein with the receptors occurs close to the N-terminus of the receptor proteins and of the colicins, and mutations in either one impair colicin uptake (Schöffler and Braun, in press; J. Mende, personal communication).

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LITERATURE CITED

1. **Bassford, P. J., C. A. Schnaitman, and R. J. Kadner.** 1977. Functional stability of the *bfe* and *tonB* gene products in *Escherichia coli*. J. Bacteriol. **130**:750–758.
2. **Braun, V.** 1985. The iron transport systems of *Escherichia coli*, p. 617–652. *In* A. N. Martonosi (ed.), The enzymes of biological membranes, vol. 3. Plenum Publishing Corp., New York.
3. **Braun, V., S. Frenz, K. Hantke, and K. Schaller.** 1980. Penetration of colicin M into cells of *Escherichia coli*. J. Bacteriol. **142**:162–168.
4. **Braun, V., K. Hantke, K. Eick-Helmerich, W. Köster, U. Pressler, M. Sauer, S. Schäffer, H. Schöffler, H. Staudenmaier, and L. Zimmermann.** 1987. Iron transport systems in *Escherichia coli*, p. 35–51. *In* G. Winkelmann, D. van der Helm, and J. B. Neilands (ed.), Iron transport in microbes, plants and animals. Verlag Chemie, Weinheim.
5. **Braun, V., K. Schaller, and H. Wolff.** 1973. A common receptor protein for phage T5 and colicin M in the outer membrane of

Escherichia coli B. Biochim. Biophys. Acta **328**:87–97.
6. **Burkhardt, R., and V. Braun.** 1987. Nucleotide sequence of the *fhuC* and *fhuD* genes involved in iron(III) hydroxamate transport: domains in *fhuC* homologous to ATP-binding proteins. Mol. Gen. Genet. **209**:49–55.
7. **Coulton, J. W., P. Mason, and D. A. Allatt.** 1987. *fhuC* and *fhuD* genes for iron(III)-ferrichrome transport into *Escherichia coli* K-12. J. Bacteriol. **169**:3848–3849.
8. **Eick-Helmerich, K., and V. Braun.** 1989. Import of biopolymers into *Escherichia coli*: nucleotide sequences of the *exbB* and *exbD* genes are homologous to those of the *tolQ* and *tolR* genes, respectively. J. Bacteriol. **171**:5117–5126.
9. **Eick-Helmerich, K., K. Hantke, and V. Braun.** 1987. Cloning and expression of the *exbB* gene of *Escherichia coli* K-12. Mol. Gen. Genet. **206**:246–251.
10. **Fecker, L., and V. Braun.** 1983. Cloning and expression of the *fhu* genes involved in iron(III)-hydroxamate uptake by *Escherichia coli*. J. Bacteriol. **156**:1301–1314.
11. **Hancock, R. E. W., K. Hantke, and V. Braun.** 1976. Iron transport in *Escherichia coli* K-12: involvement of the colicin B receptor and of a citrate-inducible protein. J. Bacteriol. **127**: 1370–1375.
12. **Hantke, K.** 1981. Regulation of ferric iron transport in *Escherichia coli* K-12: isolation of a constitutive mutant. Mol. Gen. Genet. **182**:288–292.
13. **Hantke, K., and L. Zimmermann.** 1981. The importance of the *exbB* gene for vitamin B_{12} and ferric iron transport. FEMS Microbiol. Lett. **12**:31–35.
14. **Heller, K. J., R. J. Kadner, and K. Günter.** 1988. Suppression of the *btuB451* mutation by mutations in the *tonB* gene suggests a direct interaction between TonB and TonB-dependent receptor proteins in the outer membrane of *Escherichia coli*. Gene **64**:147–153.
15. **Hoffmann, H., E. Fischer, H. Kraut, and V. Braun.** 1986. Preparation of the FhuA (TonA) receptor protein from cell envelopes of an overproducing strain of *Escherichia coli* K-12. J. Bacteriol. **166**:404–411.
16. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1981. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Mann, B. J., C. D. Holroyd, C. Bradbeer, and R. J. Kadner.** 1985. Reduced activity of TonB-dependent functions in strains of *Escherichia coli*. FEMS Microbiol. Lett. **33**:255–260.
18. **Nikaido, H., and T. Nakae.** 1979. The outer membrane of gram-negative bacteria. Adv. Microb. Physiol. **21**:163–250.
19. **Postle, K., and J. T. Skare.** 1988. *Escherichia coli* TonB protein is exported from the cytoplasm without proteolytic cleavage of its amino terminus. J. Biol. Chem. **263**:11000–11007.
20. **Pugsley, A. P., and P. Reeves.** 1978. Characterization of group B colicin-resistant mutants of *Escherichia coli* K-12: colicin resistance and the role of enterochelin. J. Bacteriol. **127**:218–228.
21. **Reynolds, P. R., G. P. Mottur, and C. Bradbeer.** 1980. Transport of vitamin B_{12} in *Escherichia coli*. Some observations on the roles of the gene products of *btuC* and *tonB*. J. Biol. Chem. **255**:4313–4319.
22. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82**:1074–1078.
23. **Wookey, P., S. Hussein, and V. Braun.** 1981. Functions in outer and in inner membranes of *Escherichia coli* for ferrichrome transport. J. Bacteriol. **146**:1158–1161.