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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

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***Escherichia coli* with mutations in the *exb* region are impaired in outer membrane receptor-dependent uptake processes. They are resistant to the antibiotic albomycin and exhibit reduced sensitivity to group B colicins. A 2.2-kilobase-pair DNA fragment of the *exb* locus was sequenced. It contained two open reading frames, designated *exbB* and *exbD*, which encoded polypeptides of 244 and 141 amino acids, respectively. Both proteins were found in the cytoplasmic membrane. They showed strong homologies to the TolQ and TolR proteins, respectively, which are involved in uptake of group A colicins and infection by filamentous bacteriophages. *exbB* and *exbD* were required to complement *exb* mutations. Osmotic shock treatment rendered *exb* mutants sensitive to colicin M, which was taken as evidence that the ExbB and ExbD proteins are involved in transport processes across the outer membrane. It is concluded that the *exb*- and *tol*-dependent systems originate from a common uptake system for biopolymers.**

The *exb* locus of *Escherichia coli* is of particular interest because it is involved in the uptake of certain compounds which are too large to be taken up by the usual transport systems. The term *exb* originally defined mutations which conferred insensitivity to colicin B (13, 14). *exb* mutants hyperexcreted enterochelin (14), which competed with the uptake of colicin B through binding to the same cell surface receptor (15, 24, 33, 34). *exb* mutants that do not secrete enterochelin showed reduced sensitivity to colicin B and the other colicins of group B (13). Hyperexcretion of enterochelin was caused by iron starvation, which derepresses enterochelin synthesis. *exbB* mutants exhibit a reduced uptake rate of Fe^{3+} via enterochelin (17, 33) and no transport via ferrichrome (17).

Previously, we cloned a 2.2-kilobase (kb) DNA fragment into plasmid pACYC184, which, when transformed into various *exb* mutants, restored growth on ferrichrome as the sole iron source (11). Two proteins with molecular masses of 26 kilodaltons (kDa) and 17.8 kDa were synthesized in an in vitro transcription-translation system programmed by the recombinant plasmid DNA (11). From preliminary deletion analyses, we concluded that the gene encoding the 26-kDa protein was sufficient to complement *exb* mutants.

In this paper we describe the nucleotide sequence of the 2.2-kb DNA fragment of plasmid pKE7. It contains two open reading frames encoding proteins of 26.1 and 15.5 kDa. The entire DNA fragment was required to complement *exb* mutants. We maintain the former designation *exbB* for the structural gene of the 26.1-kDa protein and term the gene for the 15.5-kDa protein *exbD*. The term *exbC* was previously used for poorly defined mutants (7, 34). The amino acid sequences deduced from the nucleotide sequences of *exbB* and *exbD* are strongly homologous to the amino acid sequences deduced for the *tolQ* and *tolR* gene products, respectively (44). The latter are involved in the uptake of colicins A, E1, E2, E3, and K, which belong to the group A colicins, and DNA of infecting filamentous bacteriophages f1, fd, and M13 (44). It seems that the different uptake routes of the group A and group B colicins evolved from a common system for transport of biopolymers into *E. coli* cells.

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MATERIALS AND METHODS

Bacterial strains and plasmids. All strains are derivatives of *E. coli* K-12 and are listed in Table 1. They were grown in TY medium, containing (in grams per liter) Bacto-tryptone (Difco Laboratories), 8; yeast extract, 5; and NaCl, 5 (pH 7), or in NB medium (nutrient broth, 8; NaCl, 5; pH 7). For the complementation analysis, 0.2 mM 2,2'-dipyridyl and 3 μM ferrichrome were added to the NB medium. Only exb^+ wild-type strains were able to grow on the latter medium. The *fhuA* mutant W3110-1 was isolated by selecting bacteriophage T5-resistant derivatives. *tonB* mutants were selected as being simultaneously resistant to albomycin and colicin B. Ampicillin (30 μg/ml), chloramphenicol (40 μg/ml), neomycin (50 μg/ml), and tetracycline (15 μg/ml) were used to maintain the plasmids.

Growth inhibition assays. Growth inhibition by colicins was determined by spotting 10 μl of 10-fold dilutions of a colicin stock solution with dilution titers of 10^4 to 10^5 onto TY agar plates seeded with 10^8 cells of the strain to be tested. Sensitivity to albomycin was tested by placing filter paper disks impregnated with 10 μl of 0.1 mM albomycin onto TY agar plates seeded with 10^8 cells of the test strain. Colicins B and M were from previously isolated preparations (28, 31), and colicin D was isolated from an *E. coli* strain carrying plasmid pColD-CA23 (45).

Recombinant DNA techniques. Plasmids were isolated as described by Birnboim (3). Purification of DNA, cleavage with restriction enzymes, ligation, and agarose gel electrophoresis were performed as summarized by Maniatis et al. (23). DNA fragments were recovered from agarose gels as described by Silhavy et al. (39).

Construction of plasmids. Plasmids pKH3, pKE7, pKE41, and pKE42 were described previously (11). Plasmid pKE65 contained the *Eco*RI fragment of pKE7 cloned into the *Eco*RI site of pACYC184. Plasmid pKE70 was constructed by cloning the *Eco*RI-*Nsi*I fragment of pKE7 into the *Eco*RI-*Pst*I site of pDS6 (43). Plasmid pKE69 contained the *Ban*I

TABLE 1. *E. coli* strains

Strain	Relevant genotype	Source or reference
AB2847	*aroB thi mal tsx*	15
H455	As AB2847, but *pro lac*	17
H1388	As H455, but *exbB*::Tn*10*	17
EH72	As H455, but *exbB*::Tn*10*	This study
W3110	Wild type	
W3110-1	As W3110, but *fhuA*	This study
W3110-2	As W3110, but *tonB*	This study
W3110-6	As W3110, but *exb*	E. Fischer
W3110-63	As W3110-6, but *tonB*	I. Mayer
P575	*exb*	34
GUC41	*exb fhuA metC thr leu*	13
DS410	*minA lacY xyl mtl rpsL thi*	9
JM101	Δ(*lac-pro*) Δ*thi supE* (F′ *traD36 proAB*) *lacI*^{q}ZM15	49

fragment of pKE7 in the *Hinc*II site of pLG339 (42). Plasmids pKE67 and -68 were constructed by inserting the *Hinc*II fragment of pUC4-K (47) into an *Eco*RV site of pKE7 (see Fig. 5).

Determination of nucleotide sequence. Plasmid pKE7 contains a 2.2-kb *Sau*3A fragment of the *E. coli* K-12 chromosome cloned into the *Bam*HI site of pUC18 (49). Defined DNA fragments of pKE7, obtained from single and double digests with endonucleases with known restriction sites (*Sau*3A, *Hpa*II, *Hae*III, and *Taq*I), were cloned into the appropriate sites of phage M13mp18 and M13mp19 DNA (25, 49) for sequencing by the enzymatic dideoxy-chain termination method (35). The phages were grown with *E. coli* JM101 as the host. [α-^{35}S]dATP was used for labeling, and dGTP was replaced by 7-deaza-dGTP for sequencing G+C-rich regions which showed band compressions. Both DNA strands were completely sequenced across all restriction sites used for the isolation of templates.

DNA hybridization. DNA hybridization (40) was carried out with the nonradioactive DNA labeling and detection kit (catalog no. 1093657) of Boehringer, Mannheim, Federal Republic of Germany. DNA was labeled by random primed incorporation of digoxigenin-labeled deoxyuridine triphosphate. For Southern blotting, PALL A membranes (Pall, Dreieich, Federal Republic of Germany) were used.

Translation assays. Proteins encoded by plasmids or by DNA fragments of plasmids were identified either by using the in vitro transcription-translation kit of Amersham-Buchler (Braunschweig, Federal Republic of Germany) or in minicells. The latter were prepared from the minicell-producing strain DS410 (10) which had been transformed by the plasmids. Minicells were isolated by three cycles of sucrose density gradient centrifugation and labeled with [^{35}S]methionine. To 10^{10} minicells treated for 1 h at 37°C with 4 mM cycloserine, 1 ml of M9 salt solution containing 10% methionine assay medium (Difco) and 600 KBq of [^{35}S]methionine was added. After incubation for 1 h at 37°C, the minicells were collected by centrifugation, suspended in 50 μl of sample buffer, and heated for 5 min at 100°C, and the extracts were subjected to polyacrylamide gel electrophoresis with 15% acrylamide and 0.27% *N,N′*-methylene bisacrylamide or 18% acrylamide and 0.32% *N,N′*-methylene bisacrylamide by the method of Lugtenberg et al. (22). Polyacrylamide gels were stained with Coomassie blue, dried, and autoradiographed.

Fractionation of cells. Bacteria grown in 50 ml of medium to a density of 5×10^8 cells per ml were collected by centrifugation and suspended in 0.5 ml of ice-cold 0.2 M Tris hydrochloride, pH 8. Then, 1 ml of 0.2 M Tris hydrochloride, pH 8, 1 M sucrose, and 0.1 ml of lysozyme (2 mg/ml) were added. The suspension was frozen at −70°C and thawed twice before 0.1 ml of 10 mM sodium EDTA, pH 8, was added. Incubation continued for 15 min at 20°C. Cytoplasm and membranes were separated by centrifugation at 10,000 × *g* for 10 min. The sediment was suspended in 3.2 ml of distilled water containing 66.6 μl of 1 M $MgCl_2$ and 0.1 mg of DNase. After shaking for 10 min, the suspension was centrifuged for 1 h at 34,000 × *g*. The total membrane in the sediment was suspended in 0.2 ml of extraction buffer (2% Triton X-100, 10 mM $MgCl_2$, 50 mM Tris hydrochloride, 0.05 mg of DNAse [pH 8]) (37). After 30 min at 20°C, the suspension was centrifuged at 10,000 × *g* for 10 min. The sediment contained the outer membrane, and the supernatant solution contained the components of the cytoplasmic membrane. Proteins were precipitated by addition of 0.2 ml of chloroform, followed by 0.48 ml of methanol. After thorough mixing, the suspension was centrifuged for 5 min at 10,000 × *g*. The sediment consisted mainly of cytoplasmic membrane proteins.

Radioactively labeled periplasmic proteins of minicells were isolated by the chloroform procedure (1). Labeled cells from a 3-ml culture were suspended in 50 μl of TY medium to which 20 μl of chloroform was added. After 15 min of incubation at 20°C, 0.2 ml of Tris hydrochloride (pH 8) was added, and the suspension was centrifuged for 20 min at 6,000 × *g*. Proteins in the supernatant fraction were precipitated with 0.25 ml of ethanol at −20°C. They were dissolved in sample buffer and subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

Osmotic shock treatment in the presence of colicin M. The procedure described previously (4) was modified slightly to increase the percentage of surviving cells of the rather shock-sensitive W3110 strains when osmotic shock treated in the absence of colicin. *E. coli* W3110 and its *fhuA*, *tonB*, and *exb* derivatives were grown in 20 ml of TY medium at 37°C with shaking to a density of about 5×10^8 cells per ml. Cells were pelleted at 4°C by centrifugation for 5 min at 6,000 × *g*. They were washed twice in 1 ml of 10 mM Tris hydrochloride–30 mM $CaCl_2$, pH 7, finally suspended in 0.5 ml of 33 mM Tris hydrochloride (pH 7), and adjusted with the same buffer to an A_{578} of 0.6. Sucrose (40% in 0.1 ml of Tris buffer) was added to two 0.1-ml samples, and Tris buffer alone was added to two 0.1-ml samples. After incubation for 5 min at 20°C, the samples were centrifuged for 15 min at 1,500 × *g* at 4°C. The supernatants were carefully removed, and colicin M (10 μl; dilution titer, 10^6) was added to one sucrose-treated and one untreated sample and rinsed into the tubes with 0.1 ml of ice-cold 0.5 mM $MgCl_2$. The other two samples received only the $MgCl_2$ solution. The samples were thoroughly mixed and then kept for 15 min on ice. Appropriate dilutions were plated on TY agar.

Data analysis. Nucleotide sequence data were analyzed with the IBI Pustell DNA program, version 4.0 (International Biotechnologies, Inc., New Haven, Conn.). Amino acid sequences were compared among various proteins with the FASTA.DOC program (Pearson, University of Virginia, Charlottesville, Va.) by using the GenBank release 40.0 (BBN Laboratories Inc., Cambridge, Mass., 1986). An extended Chou-Fasman analysis of the ExbB and ExbD proteins predicting regions of α helix, β turn, and pleated-sheet structures and hydrophilic, hydrophobic, and flexible segments was done with the HYCON program (W. Tröger,

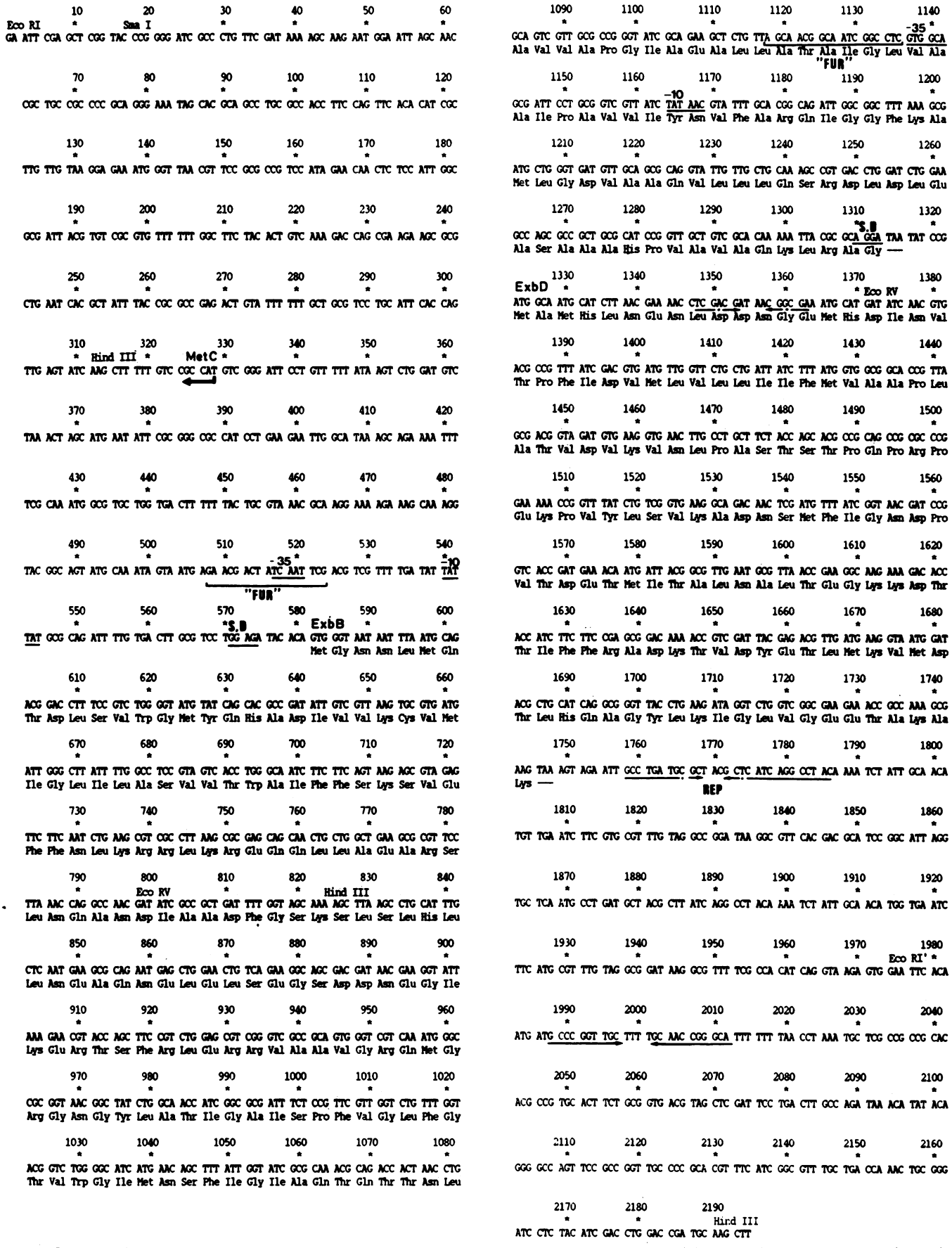

FIG. 1. Nucleotide sequences of exbB and exbD and deduced amino acid sequences. Potential −10 and −35 promoter regions, the Shine-Dalgarno sequence (S.D.), and the consensus sequence of iron-repressible genes (Fur) are marked. The sequence includes part of *metC*, which is transcribed in the opposite direction (marked by an arrow) from *exbB* and *exbD*. Potential stem-and-loop structures which could serve as transcription termination sites and a repetitive extragenic palindromic sequence (REP) are indicated by arrows.

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RESULTS AND DISCUSSION

Nucleotide sequence of the *exbBD* region. The *Sma*I-*Pst*I fragment of pKE7 (11) was cleaved with the endonucleases *Sau*3A, *Hpa*II, *Taq*I, and *Hae*III, and the resulting fragments were cloned into phages M13mp18 and M13mp19 for sequencing by the enzymatic dideoxy-chain termination method. Both strands were completely sequenced. They contained two open reading frames of 732 and 423 base pairs (bp) on one strand, designated *exbB* and *exbD*, respectively, (Fig. 1). Potential −10 and −35 promoter regions were located upstream of *exbB* and *exbD* (Fig. 1) with homology values of 47 and 40%, respectively, to the consensus sequence as calculated by the method of Mulligan et al. (26).

A consensus sequence in the promoter region of iron-regulated genes, which reads GATAATGATAATCAT TATC (5, 9, 32) and serves as a binding site for the iron-loaded Fur repressor (16), was also present in the −35 regions preceding the *exbB* and *exbD* genes (Fig. 1). Previously, iron regulation has been shown in cells carrying operon fusions between phage Mu dl (Ap *lac*) and *exb* [they were designated *exbB*::Mu dl(Ap *lac*) because the existence of *exbD* was unknown]. Upon addition of 2,2′-dipyridyl to the growth medium to reduce the available iron, β-galactosidase activity was increased from 75 to 170 U under aerobic conditions and from 15 to 200 U under anaerobic conditions (17). In addition, increased amounts of the ExbB protein were found in cells carrying plasmid pKE7 after growth in dipyridyl-containing medium (11).

Potential ribosome-binding sites were present in front of both open reading frames (Fig. 1). A double-stranded RNA stem-and-loop structure which could serve as a transcription termination site could be formed from bp 1344 to 1368 (free energy, −6.2 kcal [46]), but a stronger site (−16.2 kcal) was located 9 bp and a third potential terminator (−35.6 kcal) was located 238 bp downstream of the *exbD* translation termination codon. The latter stem and loop was followed by 5 U nucleotides, which is typical of rho-independent transcription termination (30). An extra repetitive palindromic sequence (REP) occurred downstream of *exbD* which showed similarity to the REP consensus sequence GCCT GATGCGACGCT-(0–5 bp)-ACGTCTTATCAGGCCTAC (2, 12, 18, 41). Such structures have been implicated in stabilization of translationally active upstream mRNA. Additional open reading frames on the *exb* strand were not larger than 180 bp. The complementary strand contained 329 nucleotides of the 5′ *metC* sequence and additional open reading frames of 322 and 243 bp whose G+C content, however, differed markedly from the average *E. coli* G+C content. The G+C content of the *exb* region was 51%, and the codon usage corresponded to that of moderately expressed *E. coli* genes. *exb* mutants are frequently methionine auxotrophs, for example, *E. coli* P575 and GUC41, but the *exb* mutants we constructed were *metC*$^+$ (Table 1).

Amino acid sequences of the *exbB* and *exbD* genes. The amino acid sequences deduced from the nucleotide sequences are presented in Fig. 1. The sequences predict an ExbB protein consisting of 244 amino acids and an ExbD protein of 141 amino acids, with calculated molecular masses of 26,135 and 15,525 daltons, respectively. The predicted molecular mass of ExbB agrees with the molecular mass of 26 kDa estimated previously from electrophoretic mobility on SDS-polyacrylamide gels (11), whereas the deduced molecular mass for ExbD was somewhat larger (17.8 kDa). The gel composition previously used was not optimal for molecular mass determination of proteins of such a small size. The N-terminal sequences of both proteins did not correspond to signal sequences of exported proteins. The ExbB protein consisted of 9.8% positively and 8.5% negatively charged amino acids; the net charge was +3; 44.6% of the amino acids were unpolar and 28.6% were polar. The corresponding values for ExbD were 10.6% and 14.7%; −5; and 47.8% and 26.2%. The amino acid composition was not unusual except that the ExbD protein contained no cysteine and no tryptophan residues (Table 2).

TABLE 2. Amino acid composition of the ExbB and ExbD proteins

Amino acid	No. of residues[a] per polypeptide	
	ExbB	ExbD
Phenylalanine	11	5
Leucine	29	14
Isoleucine	16	8
Methionine	7	9
Valine	21	13
Serine	15	4
Proline	4	8
Threonine	9	14
Alanine	34	12
Tyrosine	3	3
Histidine	3	3
Glutamine	13	2
Asparagine	12	8
Lysine	8	10
Aspartic acid	9	12
Glutamic acid	12	8
Tryptophan	3	0
Arginine	13	2
Glycine	21	6
Cysteine	1	0

[a] Totals: 244 for ExbB and 141 for ExbD.

A hydropathy plot of the ExbB and ExbD proteins made by the method of Kyte and Doolittle (20) revealed two extensive hydrophobic segments for ExbB and one such domain for the ExbD protein (Fig. 2). The mean hydrophobicity along the entire sequence was calculated to be +0.3 for the ExbB protein and −0.02 for the ExbD protein. Proteins associated with the cytoplasmic membrane display values between −0.27 and −0.05, and integral cytoplasmic membrane proteins have values between +0.66 and +0.97 (17a, 39). The corresponding values for the periplasmic arabinose-(−0.25) and penicillin-(−0.22) binding proteins and for the outer membrane porins (OmpF (−.0.48) and LamB (−0.62) are more negative (19, 48).

Analysis of the ExbB protein by the rules of Chou and Fasman predicted an α-helical conformation for the segment comprising amino acid residues 50 to 100, interrupted by a β-turn at residue 80. The hydropathy of this region was low, and it was high in the regions from residues 20 to 45, 135 to 160, and 165 to 200, where the secondary-structure predictions were ambiguous. The C-terminal end, starting at residue 206, may be α helical. For the ExbD protein, the program indicated α-helical structures between residues 1 to 12, 28 to 40, 86 to 96, and possibly between 102 to 141.

Comparison of the ExbB and ExbD amino acid sequences with those of other proteins. A computer-assisted search for homologies between the ExbB and ExbD proteins (21) and procaryotic proteins in the GenBank and FASTA.DOC

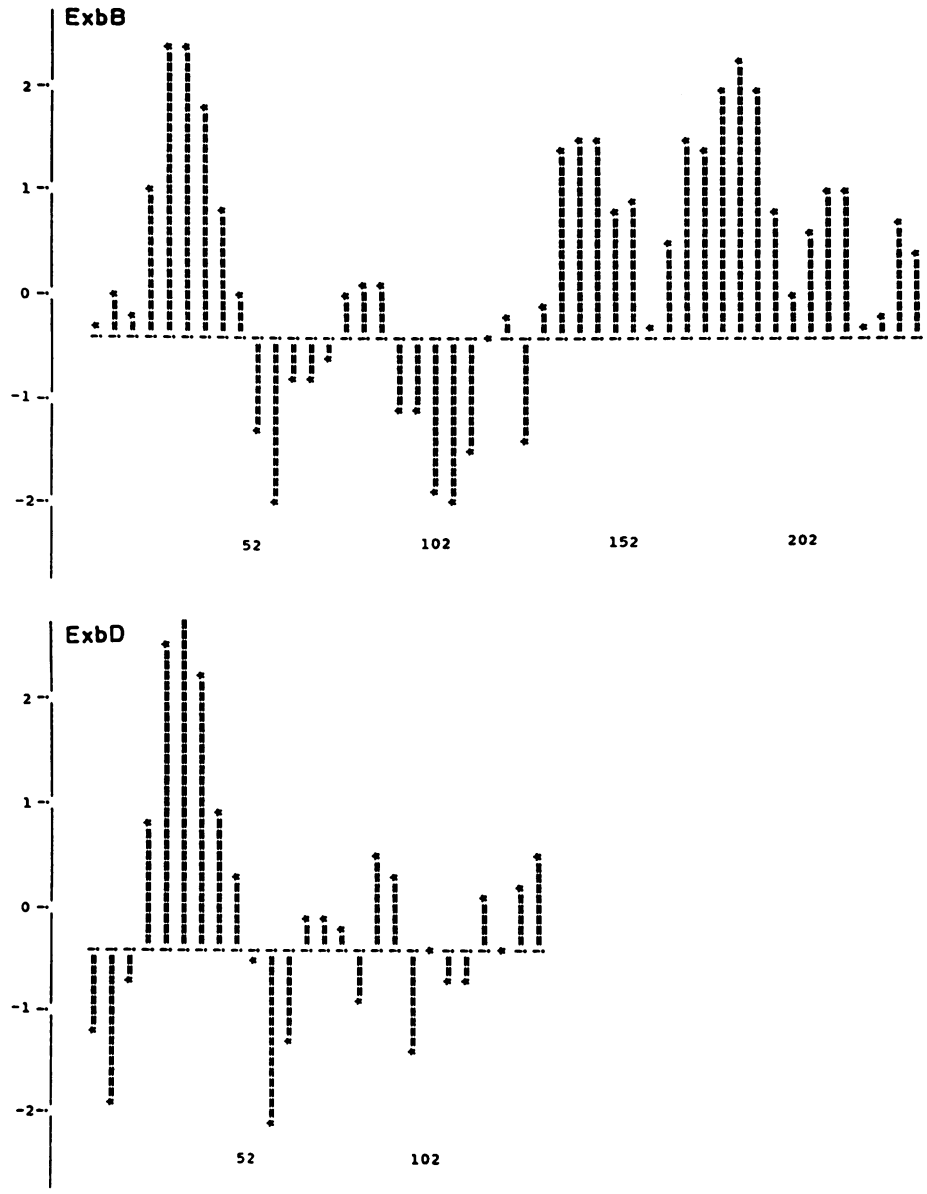

FIG. 2. Mean hydrophobicity averaged over nine neighboring amino acids (IBI Pustell Program) along the ExbB and ExbD proteins. One bar represents the values of five amino acids.

program revealed striking similarities to two very interesting proteins. The amino acid sequence of ExbB is similar to that of TolQ, and the sequence of ExbD is similar to that of TolR (Fig. 3). Alignment of ExbB with TolQ resulted in the highest homology when only two sequence gaps were introduced into ExbB comprising 1 and 2 amino acids. Then, 26.3% of the amino acids were identical and 79.1% of the other amino acids exhibited similar physicochemical properties (conservative amino acid replacements). The optimal alignment of ExbD with TolR required the introduction of three sequence gaps of 2 and 3 amino acids into ExbD, which resulted in 25% identical and 70% similar amino acids. Mutations in the *tolQ* and *tolR* genes render cells tolerant to colicins of group A and do not allow uptake of DNA from filamentous phage after they have adsorbed to the tip of the F pilus (44). *exbB* and *exbD* mutants are tolerant to the group B colicins, to which colicins B, D, G, H, Ia, Ib, M, and S1 belong (7). The mutants are insensitive to high concentrations of colicin M (16) and less tolerant to colicins B and D (17; this paper). The uptake of the group B colicins is completely dependent on activity of the *tonB* gene product, in contrast to the group A colicins, which do not require TonB (8). The ExbB and ExbD proteins and the TolQ and TolR proteins take part in two independent uptake systems for substances which have in common the requirement for receptor proteins at the cell surface. The nucleotide sequence homology between *exbB* and *tolQ* is 51.2%; between *exbD* and *tolR* it is 49.7%. The sequence homologies suggest a common evolutionary origin. Presumably a single system once catalyzed the uptake of substances too large to diffuse through the water-filled pores of the outer membrane. This is still the function of the two uptake routes after their divergence from the original system.

Restriction analysis of the chromosomal *exb* region in wild-type and mutant strains. Chromosomal DNA fragments were isolated from the parent *exb*$^+$ strains AB2847, H1443, and

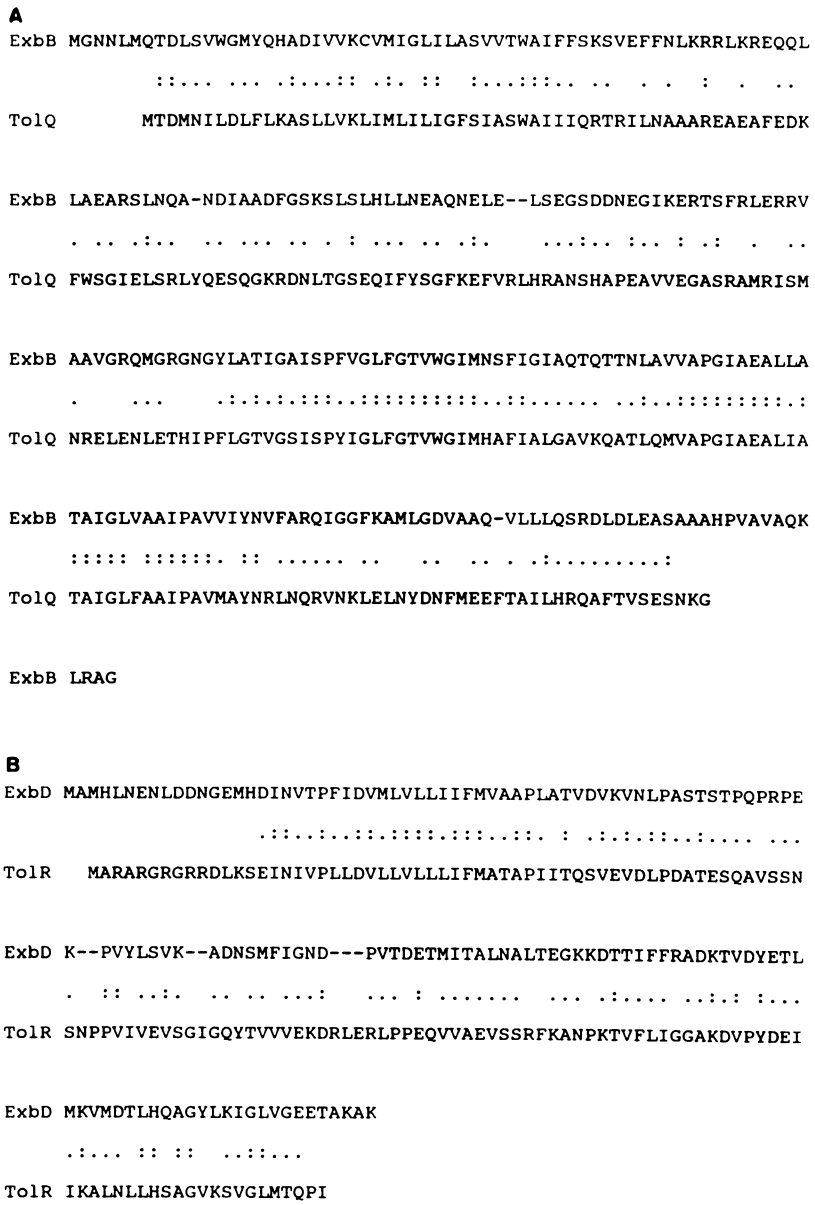

FIG. 3. Comparison of the amino acid sequences (one-letter symbols) of the ExbB protein and the TolQ protein (A) and of the ExbD protein and the TolR protein (B). Identical amino acids are marked by double dots, and amino acids with similar chemical properties are marked by single dots. The dashes mark artificial sequence gaps introduced to improve the homologies between the proteins.

W3110. They were hybridized with the labeled DNA fragments listed in Fig. 4, and their sizes were determined by agarose gel electrophoresis. With the data obtained, it was examined whether the sequenced DNA was a genuine fragment of the chromosome and whether the *exb* regions of the wild-type strains differed. In addition, the *exb* mutants used for complementation studies were characterized. Chromosomal fragments obtained with any of the restriction endonucleases and with *Hin*dIII-*Eco*RI double digests yielded identical hybridization patterns for all three wild-type strains, which corresponded to the pattern expected for fragments excised from the sequenced DNA (data not shown). The corresponding restriction analysis of the *exb* mutant H1388 revealed the location of the Tn*10* transposon in the middle of the 0.5-kb *Hin*dIII fragment so that it is either upstream of or within *exbB*. The independently isolated *exb* mutants ZI55 and H1822 yielded DNA fragments of identical size, so that Mu dl (Ap *lac*) must have inserted at similar sites in both mutants. The transposon was localized close to the 0.5-kb *Hin*dIII fragment within a 5.6-kb *Hin*dIII fragment so that it was inserted in the *exbB* gene. The analysis of the spontaneous *exb* mutant W3110-6 indicated a complex DNA rearrangement at the *exb* locus, and it remained unclear whether one or both *exb* genes were affected.

Contribution of the ExbB and ExbD proteins to the Exb phenotype. We examined the sensitivity of cells to colicins B, D, and M and to albomycin, since previous experiments have shown that *exb* mutants are resistant to albomycin and colicin M and partially resistant to the other colicins (17). We determined the sensitivity of the exb^+ strains and *exb* mutants described above and of transformants carrying *exbB*

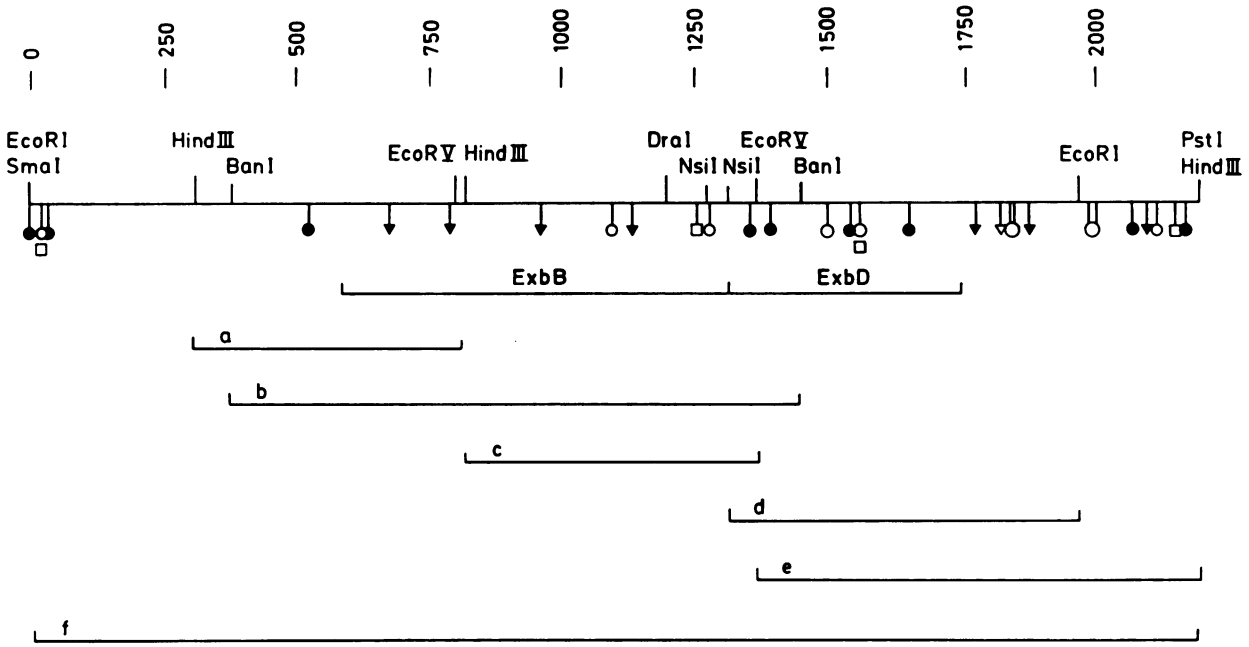

FIG. 4. Hybridization fragments a to f used to analyze the chromosomal *exb* region of wild-type and *exb* mutant strains. The DNA fragment corresponds to the sequenced region shown in Fig. 1. The symbols indicate restriction sites for the endonucleases *Hae*II (▽), *Hpa*II (○), *Sau*3A (□), and *Taq*I (●).

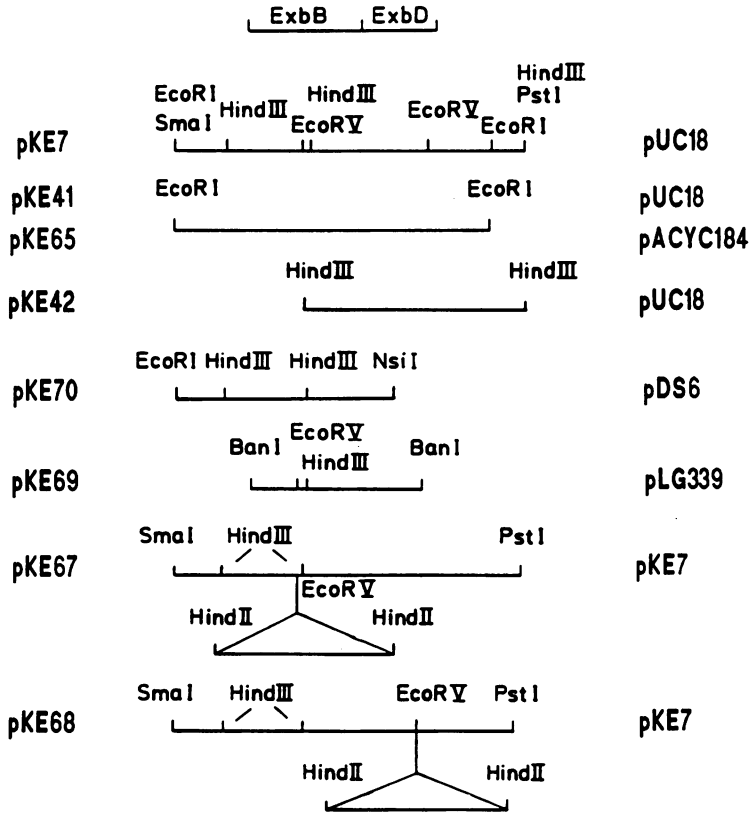

FIG. 5. DNA fragments used to complement *exb* mutants. The vector DNA is not shown. The triangles indicate kanamycin resistance cassettes inserted into *exbB* and *exbD*. Numbers to the left designate plasmids and those to the right indicate the vectors used for cloning.

TABLE 3. Sensitivity of *exb* mutants and *exbB* and *exbD* transformants to colicins and albomycin

Strain	Colicin sensitivity (dilution factor)[a]			Albomycin sensitivity[b]
	B	D	M	
AB2847	2	4	5	+
H455	2	4	4	+
H1388	2	3	1	−
H1388(pKH3)	4	4	4	+
H1388(pKE7)	5	4	5	+
H1388(pKE41)	4	4	5	+
H1388(pKE65)	4	4	5	+
H1388(pKE70)	3	4	3	(+)
H1388(pKE69)	2	3	1	−
H1388(pKE67)	2	3	1	−
H1388(pKE42)	2	3	1	−
EH72	2	3	1	−
EH72(pKE65)	4	4	4	+
W3110	2	4	4	+
W3110-6	0	0	0	−
W3110-6(pKH3)	3	4	3	+
W3110-6(pKE7)	3	4	3	+
W3110-6(pKE65)	3	4	3	+
W3110-6(pKE70)	3	3	3	(+)
W3110-6(pKE69)	0	0	0	−
W3110-6(pKE68)	0	0	0	−
W3110-6(pKE42)	0	0	0	−
P575	2	0	0	−
P575(pKE65)	3	4	2	+
GUC41[c]	1	0		
GUC41(pKH3)	4	5		
GUC41(pKE41)	4	5		
GUC41(pKE42)	0	0		

[a] The numbers indicate the factors by which the colicin stock solutions could be diluted and still yield a clear zone of growth inhibition; for example, 2 means a 10^2-fold dilution.

[b] Albomycin was not titrated; +, sensitive; (+), turbid zone of growth inhibition; −, insensitive.

[c] GUC41 *fhuA* was colicin M and albomycin resistant.

or *exbD* or both on plasmids (Fig. 5). In addition, we compared the *aroB* mutant AB2847 and its *exb* derivatives devoid of enterochelin synthesis with the enterochelin-synthesizing strain W3110 and its *exb* mutant to determine the contribution of enterochelin competition for the common FepA receptor in colicin B and D sensitivity. Dilution titers over 4 to 5 log units were used to examine sensitivity.

E. coli AB2847 was sensitive to all colicins and to albomycin (Table 3). Strain AB2847 showed reduced sensitivity to colicin B which was unchanged in the *exb* mutant H1388. However, the pKE7 transformant of H1388 was 1,000 times more sensitive and the pKE41 and pKE65 (Fig. 5) transformants were 100 times more sensitive than AB2847. Increased copy number of the *exbB* and *exbD* genes strongly enhanced sensitivity to colicin B. Sensitivity to colicins D and M was already high in the wild type and was restored to the wild-type level in the H1388 transformants. The *exb* derivative H1388 was resistant to all agents used. H1388 transformed with plasmid pKE7 (*exbBD*) or pKE65 (*exbBD*) was fully sensitive to albomycin and the colicins. H1388 became partially sensitive when transformed with pKE70 (*exbB*). Colicin concentrations 1 or 2 log units higher had to be applied to obtain a clear zone of growth inhibition, and albomycin at the highest concentration used caused only a turbid zone of growth inhibition on nutrient agar plates. No complementation of increased colicin and albomycin sensitivity was obtained with plasmid pKE69, which differs from pKE70 in the lack of a potential stem-and-loop structure characteristic for a transcription terminator in the vector DNA downstream from the *exbB* gene. We assume that in contrast to pKE70, no stable mRNA is formed from pKE69. Transformants of H1388 containing pKE42 (*exbD*) or pKE67 (*exbD*, with a kanamycin resistance cassette in *exbB*) were insensitive to albomycin and to the colicins. For restoring full sensitivity, the *exb* mutation in H1388 had to be complemented with both *exb* genes. The Tn*10* transposon in H1388 apparently affects both genes, either by inhibiting transcription when located in the promoter region upstream of *exbB* or by a polar effect on *exbD* expression when located within *exbB*.

Results qualitatively similar to those with strain H1388 were obtained with the *exb* mutant W3110-6. Strain W3110-6, in contrast to H1388, was completely resistant to colicins B and D. Transformants carrying *exbB* together with *exbD* on plasmids were 10 times more sensitive to colicin B than, as sensitive to colicin D as, and 10 times less sensitive to colicin M than the wild type. W3110-6 transformed with pKE70 exhibited the same phenotypic pattern as if the DNA rearrangement in the *exb* locus of this mutant had mainly affected the *exbB* gene. Plasmids pKE69 and pKE67 were unable to complement the *exb* mutation. Strain W3110 and its derivatives were able to synthesize enterochelin, which increased the insensitivity of the *exb* mutant significantly. Conversion to an *aroB* mutation (W3110-7) increased sensitivity to colicins B and D 100-fold (data not shown). The enterochelin-synthesizing *exb* mutant GUC41 was also nearly resistant to colicins B and D (Table 3). Transformants with *exbBD* plasmids became fully sensitive, while no complementation was achieved with pKE42 (Table 3).

Bypass of the Exb functions by osmotic shock. Colicin M inhibits murein biosynthesis (37) by interfering with polyisoprenylphosphate regeneration (17a). Colicin M binds to the FhuA outer membrane receptor and is subsequently transported across the outer membrane in a *tonB* and energy-dependent process (4). We have previously shown that the requirement for the outer membrane FhuA receptor and for the TonB function can be bypassed by osmotic shock treatment (4). Now, we examined whether the same procedure renders *exb* mutants sensitive to colicin M. The number of surviving cells was determined after osmotic shock treatment in the presence and absence of colicin M. The results obtained with W3110-6 were compared with those for a *tonB*, a *tonB exb*, and a *fhuA* mutant of strain W3110. Strain W3110 and its derivatives were rather sensitive to the shock treatment alone, so that the percentage of surviving cells ranged between 9 and 14%, in contrast to AB2847 used previously (4), of which 22 to 62% survived. The parent strain was sensitive to colicin M without osmotic shock treatment (Table 4, 0.1% survivors), whereas the *exb*, *tonB*, and *fhuA* mutants remained largely unaffected (Table 4, 90% survivors). The percentage of surviving wild-type cells decreased further after combined treatment with colicin M and osmotic shock due to the additional killing caused by the osmotic shock (0.02% survivors). The *exbB*, *exbB tonB*, and *fhuA* mutants exposed to colicin M during osmotic shock treatment were killed (0.2 to 0.5% survivors). The percentage of survivors in the presence of colicin M decreased on average to about 4% of the shock value without colicin M. No significant difference in colicin M sensitivity was observed among the shock-treated mutants. The Exb defect seemed to be bypassed as effectively as the lack of the outer

TABLE 4. Sensitization of *exb* mutants to colicin M by osmotic shock treatment

Strain	Genotype	Surviving cells (% of initial input)		
		Colicin M, + shock	Colicin M, no shock	Shock alone
W3110	Wild type	0.02	0.1	11
W3110-6	*exbB*	0.5	92	13
W3110-63	*exbB tonB*	0.4	91	10
W3110-2	*tonB*	0.3	89	9
W3110-1	*fhuA*	0.2	90	14

membrane receptor activity in the *fhuA* mutant and the lack of uptake across the outer membrane in *tonB* mutants. Since osmotic shock treatment renders the outer membrane permeable, we propose that the ExbB and ExbD proteins participate in TonB-dependent outer membrane transport processes.

Subcellular localization of the ExbB and ExbD proteins. The hydrophobic nature of the ExbB and ExbD proteins (Fig. 2) and their apparent function in uptake processes led us to determine the location of both proteins within *E. coli* cells. The minicell-producing strain DS410 was transformed with plasmid pKH3, and minicells were isolated and labeled with [^{35}S]methionine. Then, minicells were disrupted with EDTA-lysozyme, followed by repeated freezing and thawing, the membrane fraction was isolated, and the cytoplasmic membrane was preferentially solubilized in Triton X-100–$MgCl_2$. The periplasmic fraction was isolated separately by the chloroform procedure. The proteins were separated by SDS-polyacrylamide gel electrophoresis and determined by autoradiography. The bulk of both proteins were in the cytoplasmic membrane fraction (Fig. 6, lane 2). The outer membrane (lane 1) and the periplasmic (lane 3) fractions contained trace amounts of the ExbB and ExbD (barely visible) proteins. No ExbB or ExbD and only small amounts of the vector chloramphenicol transacetylase were found in the cytoplasmic fraction (lane 4). Outer and cytoplasmic membranes of minicells are difficult to separate. In addition, strong overexpression of proteins disturbs fractionation. For these reasons, the ExbB protein and the chloramphenicol transacetylase were contained in several fractions. However, most of ExbB and all of ExbD were found in the cytoplasmic membrane fraction. Moreover, we found both proteins predominantly in the cytoplasmic membrane of vegetative cells in which *exbB* and *exbD* were transcribed by the phage T7 polymerase (data not shown). Localization of the ExbB and ExbD proteins in the cytoplasmic membrane does not contradict their assumed participation in transport processes across the outer membrane. The TonB protein was also localized in the cytoplasmic membrane (29), so that all three proteins which act together on the same transport processes are in the same compartment.

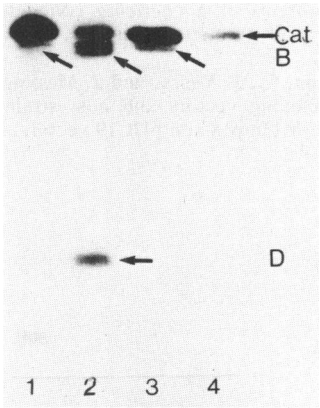

FIG. 6. Localization of the ExbB and ExbD proteins in subcellular fractions. Minicells isolated from strain DS410 containing plasmid pKH3 were labeled with [^{35}S]methionine and separated into outer membrane (lane 1), cytoplasmic membrane (lane 2), periplasmic space (lane 3), and cytoplasm (lane 4). The proteins were separated by SDS-polyacrylamide gel electrophoresis. The figure shows the autoradiograph. B, ExbB; D, ExbD protein, Cat, chloramphenicol transacetylase. The arrows point to the ExbB and ExbD proteins.

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