Topography of the ExbB Protein in the Cytoplasmic Membrane of Escherichia coli*

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The ExbB protein together with the ExbD and TonB proteins is involved in energy-coupled transport across the outer membrane of Escherichia coli. To understand this unusual process it is required to determine the subcellular location of ExbB and its transmembrane arrangement. Using ExbB-β-lactamase fusion proteins as reporters for a periplasmic versus a cytoplasmic location of the fusion sites, and accessibility of ExbB in spheroplasts and cell lysates to aminopeptidase K, trypsin, and proteinase K, we arrived at a model of ExbB topology in the cytoplasmic membrane. Starting with the N terminus in the periplasm ExbB contains three transmembrane segments (residues 16-39, 128-155, 162-184) a small periplasmic loop and two large portions in the cytoplasm. Two of the 18 fusion proteins studied, ExbB34-β-lactamase and ExbB41-β-lactamase, conferred a high ampicillin resistance. Protease experiments revealed a high respectively low percentage of the molecules in a reverse transmembrane orientation. Both proteins were lacking positive charges at the inner side of the cytoplasmic membrane which determine the orientation of transmembrane segments.

Uptake of substrates through the outer membrane of Escherichia coli occurs either by diffusion, facilitated diffusion, or by energy-coupled transport. The latter process is particularly remarkable since no energy source is known to be present in the outer membrane. Involved in transport through the outer membrane are receptor proteins which are exposed to the cell surface and to which bind the substrates to be transported. These substrates are iron complexes, ferric siderophores, through which the otherwise insoluble Fe3+ is taken up via specific transport systems (1). The ferric siderophores are too large to diffuse through the outer membrane pores formed by the porins (2). In addition, an outer membrane receptor is part of the transport system for vitamin B12 (3). Furthermore, certain bacterial protein toxins, the group B colicins, and infection by phages T1 and φ80 require receptor proteins. These ligands bind to the receptors but are not translocated across the outer membrane unless cells express three proteins, termed TonB, ExbB, and ExbD. While several receptors are present to which the ligands bind specifically, the TonB, ExbB, and ExbD proteins are required together for the uptake of all these ligands (1).

The TonB protein is degraded by cellular proteases (4). Degradation of plasmid-encoded TonB protein was prevented by plasmid encoded ExbB, which also stabilized ExbD (5). These results led us to propose a protein complex composed of TonB, ExbB, and ExbD in which TonB and ExbD bind to ExbB (TonB-ExbB-ExbD). This conclusion was supported by experiments in which chromosomally encoded TonB was stabilized by chromosomally encoded ExbB (6).

TonB (7) and ExbD (8) were found to be anchored in the cytoplasmic membrane by their N-terminal hydrophobic sequences while the remainder of the proteins extended into the periplasmic space located between the outer and the cytoplasmic membrane. Thus, both proteins seem to be well suited for an interaction with the outer membrane receptors. Such an interaction was demonstrated in that tonB point mutations suppressed point mutations in the FhuA (9), BtuB (10), and Cir (11) receptor proteins and in colicin B (12). The mutations of the receptors and colicin B were located in a region close to the N-terminal end, termed TonB box (13), sharing a similar amino acid sequence among all TonB-dependent receptors and colicins, suggesting this region to be involved in the interaction between receptors and colicins with TonB. In addition, wild-type FhuA protein stabilized the TonB protein, and the extent of stabilization correlated with the extent TonB mutants restored the activity of FhuA TonB box mutants (14). We infer that outer membrane receptors might form gated pores which upon interaction with TonB are opened. In this view TonB assumes two conformations, an energized one and an unenergized one of which the energized conformation induces pore opening. In this way energy from the cytoplasmic membrane is transmitted to the outer membrane receptors and explains how an energy-consuming transport occurs in the outer membrane where no energy source resides.

Since TonB activity depends on ExbB and ExbD it is essential for an understanding of the regulation of outer membrane receptor activity to unravel the structure of the proposed TonB-ExbB-ExbD complex. An important aspect is the transmembrane arrangement of ExbB which has not been studied yet. In this report we investigated the membrane topology of ExbB by two experimental approaches. We undertook proteolysis experiments using spheroplasts and cell lysates to ascertain which regions of ExbB are exposed at the periplasm and which are exposed at the inner side of the cytoplasmic membrane. In addition, we constructed fusion proteins between ExbB and the mature TEM β-lactamase lacking its own signal sequence (15). The β-lactamase is a periplasmic enzyme and renders ampicillin resistance only when it is located in the periplasm since it has to degrade the antibiotic before it inhibits the periplasmic penicillin-binding proteins involved in murein (peptidoglycan) biosynthesis.

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ExbB-β-lactamase fusions confer ampicillin resistance when the ExbB portion translocates β-lactamase through the cytoplasmic membrane. If insertion of the ExbB portion into the cytoplasmic membrane occurs undisturbed by the fused β-lactamase, the latter appears in the periplasm when the ExbB segment at the fusion site is naturally located in the periplasm. The results we obtained with ExbB indicate that the ExbB polypeptide passes three times through the cytoplasmic membrane with the N terminus in the periplasm and the C terminus in the cytoplasm. Only two small segments were localized in the periplasm while the preponderant portion of the protein was in the cytoplasm.

MATERIALS AND METHODS

Bacteria and Plasmids—Escherichia coli 5K (badR lacY rpsL thi thr ser fluA) (16) was used for the selection of ExbB-β-lactamase fusion proteins. E. coli BL21 (F' hsdS29 gal d) was used for radio labeling of proteins (17). E. coli JM101 (F' lac pro) superp tran36 prod B lac ZM15 was employed for DNA single strand preparation (18).

Plasmid pKB61 contains the genes exbb or exbb downstream of the gene 10 promoter of phage T7 (5). Plasmid pJS633 is a β-lactamase fusion vector (15) carrying the TEM β-lactamase gene blaM and tetracycline and kanamycin resistance genes. Plasmid pKK2 carries the phoU promoter of pKK2 by a 3′ lac gene of E. coli exbb that exbb is under the control of the tandem promoters tet and gene 10 (8). Plasmid pTT-3 contains the blaM gene under the control of the gene 10 promoter of phage T7 (19). Plasmid pMLB1034 (20) was used to construct exbb-lacZ fusions. Plasmid pTT-exbb is a pTT-5 derivative which originally carried a chromosomal EcoRI fragment containing exbb of which exbb was deleted in the third fragment by exonuclease III.

Construction of Exbb-β-lactamase Fusions—Plasmid pKK2 was cleaved with XhoI at codon 82 of exbb and incubated for various periods of time with Bal31 exonuclease. Bal31 was heat inactivated, the derivatives were blunt ended with the Klenow fragment of deoxyribonuclease I in the presence of deoxyribonucleotides, cleaved with PvuII upstream of xhoI, and religated. E. coli JM101 was transformed with pKK2 which carried exbb gene.

Transformants were selected with kanamycin (75 mg/liter) and subsequently with ampicillin (200 mg/liter) on TY agar plates containing 8 g of Bacto tryptone (Difco Laboratories), 5 g of yeast extract, and 5 g of NaCl/liter. Ampicillin-resistant colonies were streaked on TY agar plates supplemented with increasing concentrations of ampicillin (5, 25, 50, 100, 200, 400, and 800 mg/liter). Sensitivity of single colonies to 5 mg/liter ampicillin was indicative of a cytoplasmic resistance to 50 mg/liter of a periplasmic location of β-lactamase.

Single strand DNA of plasmids containing in frame exbb-β-lactamase fusions was isolated from E. coli JM101 infected with M13KO7 helper phage (Pharmacia Biochemicals). DNA was sequenced with the dideoxy chain termination method (21) using the primer 5'-dCTCTGGTCAACCAACTGTA-3' which was complementary to codons 15–21 of mature β-lactamase.

Construction of In-frame exbb-β-lactamase Gene Fusion—A 2.7-kilobase XhoI fragment of pKK2 was cloned into the BamHI site of plasmid pMLB1034. Transformants of E. coli SK were selected on TY agar plates containing 50 mg/liter of ampicillin. The transcription polarity of the insert was examined by restriction analysis.

Radiolabeling of Proteins—Genes exbb and exbb were transcribed under the control of the gene 10 promoter of phage T7 by the T7 RNA polymerase which was chromosomally encoded in E. coli BL21 (27) under control of the lacI repressor. Cells (2 ml) in the logarithmic growth phase were collected by centrifugation at an absorbance of 0.4 and then suspended in 0.8 ml of a medium containing 0.6% NaHP04, 0.3% KH2PO4, 0.1% NH4Cl, 0.05% NaCl, 1 mM MgSO4, 0.1 mM CaCl2, 1 mM sodium citrate, 0.4% glucose, 20 μg/liter thiamine, and 0.01% methionine assay medium (Difco Laboratories). T7 RNA polymerase synthesis was induced by adding 1 μM isopropyl-β-d-thiogalactoside. After shacking the culture for 40 min at 37 °C, 20 μl of rifamycin (20 mg/ml methanol) was added to inhibit the E. coli RNA polymerase. Cells were shaken for an additional 30 min after which 170 Kβd [35S]methionine were added to label cells for 15 min at room temperature. Cells were sedimented by centrifugation, suspended in 40 μl of sample buffer and, and heated for 5 minutes in a boiling water bath. Ten μl were applied to a 10% polyacrylamide gel (3% stacking gel, 15% running gel) for electrophoresis in the presence of 0.1% sodium dodecyl sulfate (SDS-PAGE).

The dried gels were autoradiographed using X-Omat S100 films of Kodak.

Subcellular Fractionation—Cells radiolabeled as described above were suspended in 0.4 ml of ice-cold 0.2 M Tris-HCl, 0.5 M sucrose, pH 8, EDTA (50 μl, 5 mM, pH 8), lysosome (50 μl, 6.4 mg/ml), and 0.5 ml of 0.2 M Tris-HCl, 0.5 mM EDTA, pH 8, were added and the suspension kept on ice. Spheroplast formation and stability was controlled by light microscopy showing that more than 90% of the cells converted after 15 min to spheroplasts which remained stable during the course of the experiments. Spheroplasts were collected by centrifugation for 30 min at 15,600 × g and 4 °C. The proteins in the supernatant fraction containing the periplasm were precipitated by adding 0.5 ml of 30% trichloroacetic acid. The sediment was dissolved in 40 μl of sample buffer and subjected to SDS-PAGE as described above.

The proteins in the supernatant fraction containing the periplasm were precipitated by adding 0.5 ml of 30% trichloroacetic acid. The sediment was dissolved in 40 μl of sample buffer by heating for 5 min and then subjected to SDS-PAGE as described above.

Proteolysis of Spheroplasts and Cell Lysates with Trypsin, Proteinase K, and Aminopeptidase M—Cells (2 ml) were labeled with [35S]methionine as described above. They were converted to spheroplasts in half the volume (0.5 ml) used above. Cell lysates were prepared by incubating cells for 15 min in 30 μl of ice-cold 0.1 M Tris-HCl, 0.5 mM EDTA, pH 8, and 1 μl lysosome (25 mg/ml). Various amounts of trypsin (trypsin and proteinase K, 1–4 μl of 50 μg/ml solution) and 50 μl of aminopeptidase M (2 mg/ml of 50% glycerol) were added and incubated for 20 min or 1 h on ice. Spheroplasts and cell lysates were prepared in separate reaction tubes. Proteolysis of spheroplasts with trypsin was terminated with a 5-fold surplus of soy bean trypsin inhibitor (50 mg/ml), with proteinase K by adding 2 μM (final concentration) of freshly prepared 0.1 M phenylmethylsulfonyl fluoride in isopropyl alcohol. The spheroplasts were collected by centrifugation, suspended in 40 μl of preheated (95 °C) sample buffer, and boiled for 5 min. Proteolysis of cell lysates was terminated by adding 40 μl of preheated sample buffer and further boiled for 5 min. A quantity of the volume of the probes in the sample buffer was used for SDS-PAGE.

RESULTS

Construction of Exbb-β-lactamase (Bio) Fusion Proteins—Cells survive ampicillin treatment when they express fusion proteins which translocate the β-lactamase moiety through the cytoplasmic membrane into the periplasm. Cells producing cytoplasmic β-lactamase lyse and release the enzyme which can protect nearby sensitive cells from being killed by cleaving ampicillin before it enters sensitive cells. This procedure selects for high cell densities recombinant in-frame Exbb-β-lactamase fusions which can be recognized by the growth zones around colonies on nutrient agar plates. However, the fusion proteins retaining β-lactamase in the cytoplasm cannot grow as individual cells on ampicillin plates.

For the construction of Exbb-β-lactamase fusions, we started from plasmid pKB61 which carries the exbb exbb genes in tandem as they are contained on the E. coli chromosome (5). The exbb exbb genes were excised with PuluI-BamHI and cloned into the EcoRV-BamHI site of plasmid

The abbreviation used is: PAGE, polyacrylamide gel electrophoresis.
pJB8633 placing exbB exbD downstream of the tandem tetracycline (tet) promoter and the gene 10 promoter of phage T7. The resulting plasmid pKK2 was cleaved with BseEII at codon 82 of exbD and digested for various periods of time with Bal31 exonuclease. The derivatives were blunt-ended, cleaved with PvuII upstream of blaM (β-lactamase gene), religated, and transformed into E. coli 5K. Randomly distributed in-frame exbB′-blaM fusions were obtained in 10–30% of the transformants. Eighteen gene fusions were sequenced and found to contain fusion sites at residues 6, 12, 22, 34, 41, 49, 65, 67, 74, 75, 100, 107, 111, 130, 153, 154, 164, and 186 of the ExbB protein.

Since the derivatives were located downstream of the phage T7 promoter they could be highly and specifically expressed by the T7 RNA polymerase while transcription of chromosomal genes by the E. coli RNA polymerase was inhibited by rifamycin. The proteins were labeled with [35S]methionine, separated by SDS-PAGE, and the gel autoradiographed (Fig. 1). The ExbB (26.6 kDa) and ExbD protein (15.7 kDa) encoded by the original plasmid pKE61 (contains β-lactamase gene) also under T7 control (lane 1) and plasmid pKK2 (lane 2) had disappeared in the transformants carrying the fusion plasmids (lanes 3–15). Instead, larger proteins with apparent molecular masses ranging from 52.5 to 31.3 kDa were formed by the transformants. The molecular weights calculated from the sequences corresponded to the molecular weights estimated from the electrophoretic mobility of the proteins. The larger fusions migrated slightly slower than estimated, and the shorter fusions migrated as expected. Plasmid pKK2 and its derivatives also expressed the 29-kDa Tn903 kanamycin inhibitor (22 kDa), and cytochrome c (12.5 kDa). The proteins were applied to SDS-PAGE, and the gel autoradiographed (Fig. 1).

For determination of the ExbB membrane topology using the fusion proteins, their subcellular location had to be determined. After radiolabeling cells were converted to spheroplasts and the released periplasm separated from the spheroplasts by centrifugation. The spheroplasts in the sediment were lysed and the membranes sedimented by centrifugation. The four examples shown in Fig. 2 localized the fusion proteins (marked by arrows) exclusively in the membrane fraction. No fusion proteins were observed in the trichloroacetic acid precipitate of the periplasm and the cytoplasm. The authentic ExbB and ExbD proteins were also in the membrane fraction (Fig. 2, lane 14), and the vector-encoded proteins were in the periplasm (lane 13) and cytoplasm (lane 15).

For further information see legend to Fig. 1.
Seperal attempts to obtain $\beta$-lactamase fusions at the C-terminal end of ExbB, using Bal31 digests that started from the BstEI1 site of codon 82 of exbD, resulted either in $\beta$-lactamase fusions to exbD or to exbB upstream of site 185. Fusions to residue 185 were obtained four times. Therefore, we tried to fuse blaM to codon 205 of exbB (DraI site after fill-in) of plasmid pKK2. The few transformants obtained contained plasmids with deletions at codon 243 (BglI site after fill-in) of plasmid pKK2. The few transformants obtained contained plasmids with deletions and duplications, and none was ampicillin-resistant. We then attempted to create a $\beta$-galactosidase fusion in codon 225 of ExbB. The 2.7-kilobase XhoII fragment of pKK2 was cloned into the BamHI site of the $\beta$-galactosidase fusion vector pMLB 1034. Of the 120 transformants examined only recombinant plasmids containing the XhoII insert in opposite orientation to lacZ were obtained. The failure to obtain at the C-terminal end of ExbB in-frame $\beta$-lactamase and $\beta$-galactosidase fusions suggests that such fusion proteins are detrimental to the cells.

Topology Mapping by Proteolysis—To collect additional data for construction of a transmembrane model of ExbB, we determined susceptibility of ExbB to added proteases. Proteases gain access to the periplasmic side of the cytoplasmic membrane in spheroplasts in which the outer membrane is permeable for proteins. In lysed cells both sides of the membrane are accessible. E. coli BL21 transformed with plasmid pT7-exbB was labeled with [35S]methionine. Increasing concentrations of trypsin were added and incubation continued for 20 min on ice. For further details see legend to Fig. 1.

Further degradation was apparently prevented since the highest enzyme concentration and the longest time used (Fig. 6, lane 2) resulted in a sharp band indicating a largely homogeneous degradation product. Under these conditions a substantial fraction of ExbB still remained undegraded. As a control endoproteolytic activity of aminopeptidase K was tested in spheroplasts and cell lysates of E. coli BL21 pT7-3 bla expressing periplasmic $\beta$-lactamase. No endoproteolytic cleavage of $\beta$-lactamase was observed (data not shown) so that ExbB was indeed degraded at the N-terminal end. Furthermore, it was previously shown that aminopeptidase K of the same source used in this study displays exclusively an N-terminal exoprotease activity (22).

Further cleavage experiments were performed with protease K which is much less specific than trypsin. Three discrete bands with molecular masses of approximately 26, 16.6, and 8.7 kDa were obtained in spheroplasts, which were not further degraded by increasing the concentration of protease K (Fig. 5, lanes 2–4). The 8.7-kDa fragment was weaker labeled (lanes 1–4) and cell lysates (lanes 5–8). E. coli BL21 transformed with pT7-exbB was induced with isoprityl-1-thio-$\beta$-d-galactopyranoside and labeled with [35S]methionine. Increasing concentrations of trypsin (lanes 2, 50 $\mu$g; lanes 3 and 6, 100 $\mu$g; lanes 4 and 7, 200 $\mu$g; lane 8, 400 $\mu$g) were added and incubation continued for 20 min on ice. For further details see legend to Fig. 1.
molecules were inserted into the cytoplasmic membrane and that the peptide bonds in the periplasmic loop were difficult to cleave, presumably because the loop is small and sterically poorly accessible for the protease.

**Two Membrane Orientations of ExbB41-β-lactamase**—The unexpectedly high ampicillin resistance of cells expressing ExbB41-Bla prompted an investigation of the topology of this protein with proteinase K. Spheroplasts and cell lysates were incubated for 1 h with 0.4 mg of proteinase K. For comparison ExbB164-Bla conferring ampicillin resistance, ExbB65-Bla (no ampicillin resistance), and a previously characterized ExbD134-Bla fusion located in the periplasm (8) were included in this study. Interestingly, the ExbD134-Bla fusion was nearly fully active in conferring sensitivity to colicins B and M.2

ExbD134-Bla and ExbB164-Bla were degraded in spheroplasts by proteinase K (Fig. 6, lanes 4 and 8). Exbb which is coexpressed with the ExbD134-Bla plasmid (marked by an arrow) was degraded to the 16.6- and 8.7-kDa fragments. ExbB65-Bla was resistant to proteinase K except the N-terminal region which was removed (lane 12). This protein was degraded in the cell lysate (lane 14) indicating protection through the membrane in spheroplasts. These data are all consistent with the model. In contrast, ExbB41-Bla was not entirely degraded by proteinase K to small products as expected from the ampicillin resistance but mainly showed the N terminally truncated product (lane 16). However, comparison with ExbB65-Bla (lane 12) revealed a substantial amount of additional degradation products (23, 15.5, and 10 kDa) demonstrating that a fraction of the protein was exposed to the periplasm and was degraded in this orientation. This fraction is sufficient to confer the degree of ampicillin resistance observed with cells expressing ExbB41-Bla.

To further investigate the transmembrane orientation of ExbB41-Bla, spheroplasts were treated with aminopeptidase K. ExbB34-Bla was included in this experiment since it also conferred high ampicillin resistance. For comparison, ExbB65-Bla and ExbB49-Bla were treated with aminopeptidase and the products obtained were separated by SDS-PAGE. A portion of the ExbB65-Bla (Fig. 7, lane 1 untreated, lane 2 treated with aminopeptidase), of ExbB49-Bla (lanes 3 and 4), and of ExbB41-Bla (lanes 5 and 6) was converted to

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2 K. Kampfenkel and V. Braun, unpublished results.
FIG. 7. Degradation of ExbB65-Bla (lane 2), ExbB49-Bla (lane 4), ExbB41-Bla (lane 6), ExbB34-Bla (lane 8), and ExbB, ExbD (lane 10) with 0.1 mg of aminopeptidase K for 1 h on ice. The untreated samples were applied to lanes 1, 3, 5, 7, and 9. The proteins were labeled and separated by SDS-PAGE as described in Fig. 1.

...a slightly smaller product, as was ExbB (lane 10), whereas no truncated product was observed with ExbB34-Bla (lane 8). Apparently, ExbB34-Bla was mainly inserted in an inverse orientation in the cytoplasmic membrane. This conclusion was supported by the strong degradation of ExbB34-Bla in spheroplasts by proteinase K (data not shown) demonstrating that most of the hybrid molecules were exposed in the periplasm to proteinase K. ExbD was not attacked by aminopeptidase K (lane 9) as expected from the location of the N terminus in the cytoplasm. The result obtained with ExbD shows that the spheroplast preparations used in these experiments contained no substantial amounts of lysed cells and proves that the aminopeptidase was free of other protease activities.

Comparison of the Hydropathy Profile between the ExbB-TolQ and the ExbD-TolR Proteins—TolQ and TolR display about 25% sequence identity and 75% similarity (amino acids with similar physicochemical properties) with ExbB and ExbD, respectively (23). TolQ could partially replace the function of ExbB, and TolR the function of ExbD (24). Therefore, it was of interest to compare the hydropathy profile of TolQ and TolR with that of ExbB and ExbD by applying the same computer-assisted prediction method (25). The distribution of hydrophilic and hydrophobic amino acids along the polypeptide chains was indeed very similar between ExbB-TolQ and ExbD-TolR (Fig. 8) which supports their structural and functional relatedness. It is likely that TolQ shows a transmembrane arrangement as determined for ExbB in this paper, and TolR a transmembrane topology as ExbD (8). In fact, TolQ (21.9 kDa) like ExbB was resistant to trypsin in spheroplasts and was degraded with proteinase K to three products of 21.1, 15.7, and 9 kDa (data not shown). These data are consistent with a transmembrane arrangement as proposed for ExbB. The model predicts a somewhat shorter N-terminal segment of TolQ in the periplasm, and also a shorter C-terminal end in the cytoplasm than ExbB contains.

FIG. 8. Prediction of transmembrane helices employing the method of Rao and Argos (25). The transmembrane helices are indicated by cross-bars extending in ExbD (A) approximately from residues 23 to 42, in TolR (B) from residues 21 to 40, in ExbB (C) from residues 16 to 39, 128 to 155, and 162 to 194, in TolQ (D) from residues 9 to 36, 127 to 159, and 162 to 191.

DISCUSSION

Knowledge of the membrane topology of TonB, ExbB, and ExbD is important for the understanding of the presumed energy transduction from the cytoplasmic to the outer membrane mediated by the TonB-ExbB-ExbD complex. TonB and ExbD display a very similar arrangement in that they are anchored via their N-terminal ends in the cytoplasmic mem-
brane, and the remainder of the polypeptides extend into the periplasm. According to the results presented in this paper, the transmembrane arrangement of ExbB is entirely different. The model of ExbB predicts three transmembrane segments, two short sequences in the periplasm, and two large portions in the cytoplasm. To arrive at this model, a combination of various methods, construction and characterization of fusion proteins, degradation with proteases, and computer-assisted prediction methods had to be applied. The enzyme assay and the protease digestion experiments were reliable. β-Lactamase activity was measured in viable cells in which synthesis of ExbB and its fusion derivatives was not particularly high since T7 RNA polymerase synthesis was not induced. We found no indication that the fusion proteins harmed cells. Growth rates were as in the untransformed cells, and viable cell counts corresponded to the absorbance of the cell culture. The yield and stability of the spheroplasts was the same as with transformants carrying exbB or exbD on the same plasmids. Induction of T7 RNA polymerase synthesis increased the concentration of ExbB and the ExbB fusion proteins which were largely degraded with trypsin and completely with protease K in cell lysates suggesting that they formed no inclusion bodies.

β-Lactamase activity of all ExbB-β-lactamase hybrid proteins except two (ExbB34-Bla and ExbB41-Bla) clearly indicated the location of the fusion sites as predicted by computer analysis (25) and by applying the "positive inside rule" (26). The latter proposes that transmembrane proteins contain a surplus of positive charges at the inside of the cytoplasmic membrane. Fusions at sites 6, 12, and 22 contain an ExbB portion which is probably too small to act as a topological signal. The N-terminal amino acid sequence is also hydrophilic so that it does not serve as a signal sequence for β-lactamase export. No β-lactamase fusion proteins were obtained in the C-terminal portion of ExbB. The site nearest to the C terminus that could be fused to β-lactamase was residue 185 (total length of ExbB comprises 244 residues). Neither random fusion techniques nor attempts to create fusions at defined sites resulted in ExbB-β-lactamase hybrid proteins. In addition, no ExbB-β-galactosidase fusion protein was obtained in this region which excludes the possibility that the subcellular location of the protein to which ExbB was fused (β-lactamase periplasmic, β-galactosidase cytoplasmic) caused the failure. Rather the lack of C-terminal fusions suggests that cells do not tolerate such artificial ExbB variants. There are other cases where no β-lactamase fusions to the C-terminal portion of proteins were obtained such as the HlyB and HlyD proteins of the export apparatus of the E. coli hemolysin (27) and the OppB and OppD membrane proteins of the Salmonella typhimurium peptide transport system (28). Furthermore, the ExbB34-Bla and ExbB41-Bla fusions conferred ampicillin resistance, indicating a periplasmic location of β-lactamase, which conflicted with the ampicillin resistance pattern of the other fusions and with the computer-assisted prediction of transmembrane helices (Fig. 8C) and the model derived thereof (Fig. 3). According to the model, residue 34 is located within the cytoplasmic membrane, and residue 41 faces the cytoplasm. The HlyB protein provides another example of a membrane internal residue which translocates β-lactamase to the periplasm (27). Similar observations have been made for fusion proteins between the periplasmic alkaline phosphatase (PhoA) and N-terminal portions of MalF (maltose transport) (29), leader peptidase (30), and the tetracycline resistance protein of pBR322 (31) where, in contrast to the proposed membrane topology models, PhoA was exported to the periplasm.

Independent evidence for the transmembrane arrangement of ExbB was obtained by protease digestions using spheroplasts and cell lysates. ExbB in spheroplasts was degraded at the N-terminal end with aminopeptidase K to a defined product of about the size (26 kDa, Fig. 5, lane 10) predicted by the model if the remainder of the protein was protected from further digestion by the cytoplasmic membrane. A fragment of the same size was obtained with protease K which gave rise to two additional products with molecular masses of 16.6 and 8.7 kDa (Fig. 5, lanes 2–4). These products were expected if protease K degraded the N-terminal end (26-kDa product) as did aminopeptidase K, and in addition cleaved in the periplasm loop between the second and third transmembrane segment (16.6- and 8.7-kDa products). The results obtained with cell lysates, in which both sides of the cytoplasmic membrane became accessible to the added proteases, were entirely consistent with those gained with spheroplasts. Tryptsin and protease K degraded ExbB in cell lysates (Fig. 4, lanes 6–8; Fig. 5, lanes 6–8) whereas ExbB was trypsin resistant in spheroplasts (Fig. 4, lanes 2–4). All arginine and lysine residues were localized in the cytoplasm except lysine 24 which resides in the first transmembrane segment (Fig. 3), and for this reason was sterically protected against trypsin attack. These data are consistent with the model (Fig. 3) which contains three transmembrane segments extending from residues 16–39, 128–155, and 162–194 (Fig. 8C), a large cytoplasmic loop, a short periplasmic loop, and the C terminus in the cytoplasm. Protease K degraded ExbB (Fig. 5, lanes 6–8) and the ExbB-Bla fusions in lysates (Fig. 6, lanes 10, 14, and 18) to small products consistent with the localization of most of the protein in the cytoplasm. Accordingly, all ExbB-Bla fusions which were assigned to the cytoplasm by the protease experiments conferred no ampicillin resistance. The two fusions in the periplasmic loops rendered cells ampicillin-resistant. The exceptions were the fusions ExbB34-Bla and ExbB41-Bla which according to the model were cytoplasmic but conferred ampicillin resistance. The protease digestion experiments performed with ExbB41-Bla explained this apparent contradiction. Protease K yielded in spheroplasts a major degradation product slightly smaller than the fusion protein (Fig. 6, lane 16) consistent with an orientation as expected by the model. In addition, aminopeptidase K degraded part of ExbB41-Bla (Fig. 7, lane 6). However, there was a fraction which was degraded further by protease K (Fig. 6, lane 16, stronger bands of degradation products as compared with ExbB65-Bla, lane 12). Apparently, a fraction of ExbB41-Bla was inserted in the cytoplasmic membrane with the opposite orientation. An even larger proportion of ExbB34-Bla showed the reverse ExbB orientation since aminopeptidase K yielded no degradation product (Fig. 7, lane 8), and protease K cleaved most of the protein to small peptides. The hybrid proteins ExbB34-Bla and ExbB41-Bla do not contain a positive charge at the inner face of the cytoplasmic membrane (Fig. 3) which would orient the transmembrane segment. In summary, combination of computer-assisted prediction of the membrane topology, the enzymatic activities of β-lactamase fusion proteins, and the accessibility of ExbB and the ExbB-Bla hybrid proteins to aminopeptidase K, protease K, and trypsin resulted in a consistent model of the transmembrane arrangement of ExbB.

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