Insertion of the Polytopic Membrane Protein Lactose Permease Occurs by Multiple Mechanisms

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Received October 28, 1994; Revised Manuscript Received January 4, 1995⊗

ABSTRACT: The lactose permease of *Escherichia coli* has 12 transmembrane hydrophobic domains in probable α-helical conformation connected by hydrophilic loops. Previous studies [Consler, T. G., Persson, B., et al. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90,* 6934−6938] demonstrate that a peptide fragment (the XB domain) containing a factor Xa protease site immediately upstream of a biotin acceptor domain can be engineered into the permease, thereby allowing rapid purification to a high state of purity. Here we describe the use of the XB domain to probe topology and insertion. Cells expressing permease with the XB domain at the N terminus, at the C terminus, or in loop 6 or 10 on the cytoplasmic face of the membrane catalyze active transport, although only the chimeras with the XB domain at the C terminus or in loop 6 are biotinylated. In contrast, chimeras with the XB domain in periplasmic loop 3 or 7 are inactive, but strikingly, both constructs are biotinylated. Furthermore, the XB domain in all the constructs, particularly in the loop 3 and loop 7 chimeras, is accessible from the cytoplasmic face of the membrane, as evidenced by factor Xa proteolysis or avidin binding studies with spheroplasts and disrupted membrane preparations. Finally, alkaline phosphatase fusions one loop downstream from each periplasmic XB domain exhibit high phosphatase activity. Thus, the presence of the XB domain in a periplasmic loop apparently blocks translocation of a discrete segment of the permease consisting of the loop and the two adjoining helices without altering insertion of the remainder of the protein. The results provide a strong indication that XB domain insertion cannot be used to study the topology of polytopic membrane proteins. On the other hand, the approach yields unique and important information regarding insertion, and it seems likely that certain regions of lactose permease may be inserted as helical hairpins. The findings are discussed in the context of other observations indicating that different regions of the permease may be inserted by different mechanisms.

Lactose (lac)[1] permease of *Escherichia coli* is a hydrophobic, polytopic membrane protein that catalyzes the coupled stoichiometric translocation of β-galactosides and H^+ (reviewed in Kaback, 1983, 1989, 1992; Poolman & Konings, 1993). The permease is encoded by the *lacY* gene which has been cloned (Teather et al., 1978) and sequenced (Büchel et al., 1980). Moreover, the *lacY* gene product has been solubilized from the membrane, purified to homogeneity, reconstituted into proteoliposomes, and shown to be solely responsible for β-galactoside transport (Newman et al., 1981; Viitanen et al., 1986) as a monomer (see Sahin-Tóth et al., 1994). Based on circular dichroism and hydropathy analysis, a secondary-structure model was proposed (Foster et al., 1983) consisting of 12 hydrophobic segments in α-helical conformation that traverse the membrane in zig-zag fashion connected by hydrophilic domains (loops) with both the N and C termini on the cytoplasmic face (Figure 1). Evidence favoring general features of the model and showing that the C terminus, as well as loops 4 and 6, are on the cytoplasmic face of the membrane has been obtained from a variety of experimental approaches (see Kaback, 1983, 1989, 1992). Moreover, analysis of a large number of lac permease−alkaline phosphatase (*lacY-phoA*) fusions has provided unequivocal support for the topological predictions of the 12-helix model (Calamia & Manoil, 1990). Recently, the combined use of site-directed mutagenesis and site-directed fluorescence labeling has led to a description of helix packing in the C-terminal half of the permease (Jung et al., 1993; Kaback et al., 1993).

In a previous communication, Consler et al. (1993) described lac permease chimeras containing a peptide fragment (the XB domain) with a factor Xa protease site (Ile-Glu-Gly-Arg) at the N terminus of the biotin acceptor domain from the *Klebsiella pneumoniae* oxaloacetate decarboxylase (Cronan, 1990). Two chimeras were made that are fully active with respect to lactose transport, one with the XB domain in the middle cytoplasmic loop (loop 6) and one with the XB domain at the C terminus. It was also demonstrated that both constructs are biotinylated *in vivo* and can be solubilized from the membrane and rapidly purified in a functional state by monovalent avidin chromatography. In these experiments, the XB domain was used to study topology of lac permease and its insertion into the membrane.

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⊗ Abstract published in *Advance ACS Abstracts,* March 1, 1995.

[1] Abbreviations: lac, lactose; XB domain, factor Xa protease site immediately preceding a biotin acceptor domain; RE, restriction enzyme; mAb, monoclonal antibody; PA-HRP, protein A-conjugated horseradish peroxidase; ECL, enhanced chemiluminescence; avidin-HRP, avidin-conjugated horseradish peroxidase; IPTG, isopropyl 1-thio-β-D-galactopyranoside; PCR, polymerase chain reaction; PVDF, poly(vinylidene difluoride); XP, 5-bromo-4-chloro-3-indolyl phosphate; KP_i, potassium phosphate; EDTA, ethylenediaminetetraacetate; $NaDodSO_4$, sodium dodecyl sulfate.

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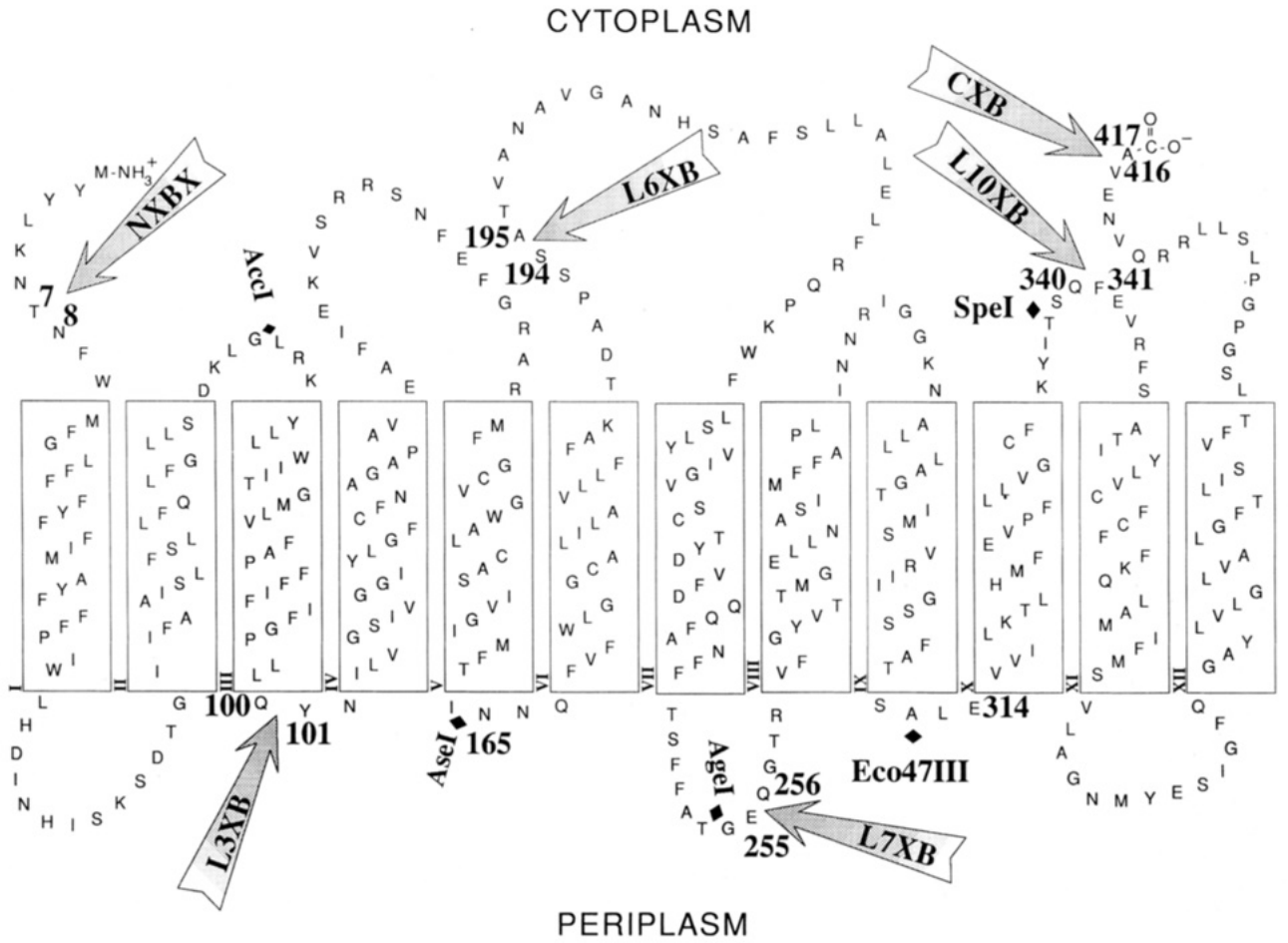

FIGURE 1: Secondary-structure model of *E. coli* lac permease showing six chimeric constructs and the insertion sites of the XB domain. The single-letter amino acid code is used, and the 12 putative transmembrane helices are shown in boxes. The model is based on the hydropathy plot of the primary amino acid sequence of the permease (Foster et al., 1983) and recent findings (King et al., 1991; Zen et al., 1994) indicating that Asp237 and Asp240 are within transmembrane domain VII. The arrows indicate XB domain insertion sites, and the nearby amino acid residues are numbered. The RE sites used for construction of the mutants are also shown.

The results demonstrate that XB domain insertion is not a viable approach for determining the topology of polytopic membrane proteins. However, data are presented indicating that the approach can provide interesting information concerning the mechanism of insertion of the permease. When considered in conjunction with other observations, the findings indicate that insertion of lac permease is a complicated process that utilizes different mechanisms for different regions of the protein.

MATERIALS AND METHODS

Materials. Oligodeoxynucleotides were synthesized on an Applied Biosystems 391 DNA synthesizer and used without further purification. Restriction endonucleases (REs) and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). Sequenase was purchased from United States Biochemicals (Cleveland, OH). Site-directed rabbit polyclonal antiserum against a dodecapeptide corresponding to the C terminus of lac permease (Carrasco et al., 1984) was prepared by BabCo (Richmond, CA). Rabbit antibody to bacterial alkaline phosphatase was purchased from 5 Prime → 3 Prime (Boulder, CO). Protein A-conjugated horseradish peroxidase (PA-HRP), enhanced chemiluminescence (ECL) detection kits, and [1-^{14}C]lactose were obtained from Amersham (Arlington Heights, IL). [^{125}I]Avidin and [^{35}S]-adenosine 5′-(α-thiotriphosphate) were from New England Nuclear (Boston, MA). Avidin-conjugated horseradish peroxidase (avidin-HRP) was purchased from Pierce (Rockford, IL). Isopropyl 1-thio-β-D-galactopyranoside (IPTG), calf intestinal alkaline phosphatase, factor Xa protease, *Thermus aquaticus* (Taq) DNA polymerase, and associated polymerase chain reaction (PCR) reagents were from Boehringer Mannheim (Indianapolis, IN). GeneClean glassmilk DNA purification kits for removing DNA from agarose were obtained from Bio101 (La Jolla, CA), and Magic Miniprep kits for preparing plasmid DNA were from Promega (Madison, WI). Poly(vinylidene difluoride) (PVDF) membranes were from Schleicher and Schuell (Keene, NH), and 0.7 μM glass microfiber filters (GF/F) were from Whatman (Maidstone, England). All other materials were reagent grade and were obtained from commercial sources.

Bacterial Strains. The following *Escherichia coli* K-12 strains were used: T184 [*lacI*$^+$*O*$^+$*Z*$^-$*Y*$^-$(A), *rpsL, met, thr, recA, hsdM, hsaR*/F′ *lacI*^{q}*O*$^+$*Z*$^{\Delta 118}$(Y$^+$A$^+$)] (Teather et al., 1980), HB101 [*hsdS20 (r*$^-$*B,m*$^-$*B), recA13, ara-14, proA2, lacY1, galK2, rpsL20(Sm*^{r}*), xyl-5, mtl-1, supE-44,* λ$^-$/F$^-$] (Boyer & Roulland-Dussoix, 1969), and CC181 [F128 *lac*^{q}/Δ(*ara,leu*)*7697* Δ*lacX74 phoA*Δ*20 galE galK thi rpsE rpoB argE*(am) *recA1 lacY328*(am)] (Calamia & Manoil, 1990).

Construction of Chimeras. The construction of plasmids encoding lac permease with an XB domain at the C terminus (pLacY/CXB) or in loop 6 (pLacY/L6XB) have been described (Consler et al., 1993). The general procedure used to construct pLacY/NXBX, pLacY/L3XB, pLacY/L7XB, or pLacY/L10XB was to introduce a unique *Sal*I site into the *lacY* gene by PCR mutagenesis, followed by insertion of the

Table 1: DNA Sequences of Oligodeoxynucleotides Used for Constructing Chimeric *lacY* Genes[a]

Construct		Oligonucleotide sequence
LacYNSalI	→	5'-TATTTAAAAAACGTCGACGGATGTTC-3'
	←	5'-GAACATCCGTCGACGTTTTTTAAATA-3'
LacYL3SalI	→	5'-CCACTGTTAGTCGACAACATTTTA-3'
	←	5'-TAAAATGTTGTCGACTAACAGTGGC-3'
LacYL7SalI	→	5'-TTCTTTGCTACCGGTGTCGACGGTACGCGGGTA-3'
LacYL10SalI	→	5'-TTTAAATATATTACTAGTGTCGACGAAGTGCGT-3'
XBX domain	→	5'-CGTCAGGTGAATGAAGTCGACATCGAGGGGCGGAC GGCGGCTGCT-3'
	←	5'-CCTTTTCCGTCGACCCGCCCCTCGATGGTGTCGCCGA CCGC-3'
LacYL3XB-N165-phoA	→	5'-AAACTCGGTCTACGCAAATACCTGC-3'
	←	5'-TTTCCCGCTAGCATTATTGATGGTGAACATGATGCC-3'

[a] Sequences of mutagenic primers are presented in the 5′ → 3′ direction. The *Sal*I restriction site is shown in boldface type. The symbols → or ← for each primer denote sense and antisense, respectively.

300-bp *Sal*I fragment encoding the factor Xa protease site and an XB domain excised from pLacY/L6XB. Table 1 shows the sequences of the oligonucleotides used for mutagenesis and the direction (sense or antisense) of the oligonucleotides relative to the open reading frame of *lacY*. The positions of the XB domains in the six chimeric permease constructs are shown in Figure 1.

For constructing plasmids pLacY/L7XB or pLacY/L10XB, sense primers were designed to place a *Sal*I restriction site immediately after the *Age*I site in the DNA encoding loop 7 or after the *Spe*I site in the DNA encoding loop 10, respectively. An antisense oligonucleotide for PCR mutagenesis was designed to anneal at least 200 base pairs away from the mutagenic oligonucleotide and to allow for digestion with a second restriction endonuclease. The PCR product was digested with *Age*I or *Spe*I and with the second RE so as to leave cohesive ends, thereby ensuring unidirectional insertion into the expression vector pTC/cassette *lacY* (K. H. Zen and H. R. Kaback, unpublished data) which was also digested with *Age*I or *Spe*I and the second RE. As a precautionary measure, twice-digested pTC/cassette *lacY* DNA was treated with alkaline phosphatase to reduce regeneration of wild-type *lacY*. Screening of the ligation products for a new *Sal*I site identified pLacY/L7*Sal*I and pLacY/L10*Sal*I. The 300-bp XB domain obtained from *Sal*I digestion of pLacY/L6XB was then ligated with pLacY/L7*Sal*I or pLacY/L10*Sal*I, which had been digested with *Sal*I and treated with alkaline phosphatase. Screening for the insert in the correct orientation yielded plasmids pLacY/L7XB and pLacY/L10XB. In both constructs, the XB domain is inserted in frame with *lacY*.

Permease with the biotin acceptor domain at the N terminus (NXBX permease) or in loop 3 (L3XB permease) was constructed by two-stage PCR mutagenesis. Sense and antisense primers (Table 1) were designed for two-stage PCR mutagenesis to introduce a unique *Sal*I site by changing codons 7 and 8 (T7V/N8D) or codons 100 and 101 (Q100V/Y101D) of *lacY*, yielding plasmids pLacY/N*Sal*I and pLacY/L3*Sal*I. The plasmid pLacY/L3*Sal*I was then digested with *Sal*I, treated with alkaline phosphatase, ligated with the 300-bp XB domain fragment obtained from *Sal*I digestion of pLacY/L6XB. Ligation products were screened by restriction fragment analysis to verify the orientation of fragment insertion, yielding pLacY/L3XB. For creating the XBX domain, sense and antisense primers (Table 1) were designed for PCR to introduce an additional factor Xa protease site at the C terminus of the XB domain by using plasmid pLacY/L6XB as a template. The second factor Xa protease site was introduced so that a significant change in electrophoretic mobility would be evidenced after cleavage with factor Xa protease. Both ends of the PCR product encoding the XBX domain were trimmed with *Sal*I and then ligated with the vector pLacY/N*Sal*I, which had been digested with the same RE and treated with alkaline phosphatase. The plasmid pLacY/NXBX was obtained by screening the orientation of the XBX domain insertion.

To construct pLacY/L3XB-N165-*phoA*, plasmid pT7-5/*lacY-phoA* (kindly provided by M. L. Ujwal) in which an *Nhe*I site is at the fusion junction between the *lacY* and the mature *phoA* coding sequence was used as a vector. Primers (Table 1) were designed to amplify the *Acc*I/*Ase*I fragment of L3XB permease (see Figure 1) and create an *Nhe*I site in the DNA encoding loop 5 of permease. The ends of the PCR product were trimmed with *Acc*I and *Nhe*I and ligated with pT7-5/*lacY-phoA* which had been digested with the same two REs and treated with alkaline phosphatase. The ligation mixture was transformed into competent *E. coli* CC181. Ampicillin-resistant colonies were isolated, and plasmid pLacY/L3XB-N165-*phoA* was characterized by restriction analysis. For constructing pLacY/L7XB-E314-*phoA*, pLacY/L7XB was digested with *Age*I and *Eco47*III, and the fragment containing the L7XB domain was ligated with the vector pT7-5/lacY-E314-*phoA* (kindly provided by M. L. Ujwal) which had been digested with the same two REs and treated with alkaline phosphatase. The ligation mixture was used to transform *E. coli* CC181, and plasmid pLacY/L7XB-E314-*phoA* was purified and characterized.

DNA Sequencing. DNA fragments from PCR amplification that were ligated into *lacY* gene, as well as the ligation junctions, were verified by sequencing double-stranded DNA

(Hattori & Sakaki, 1986) using dideoxynucleotide termination (Sanger et al., 1977; Sanger & Coulson, 1978).

Colony Phenotype. Ligation mixtures or plasmids were transformed into the cryptic strain *E. coli* HB101 (*lacZ*$^+$*Y*$^-$), and as a qualitative assay for lac permease activity, transformants were plated on MacConkey (Difco) indicator medium containing 10 μg/mL streptomycin, 100 μg/mL ampicillin, and 25 mM lactose. Colonies expressing active lac permease appear red on MacConkey indicator plate.

For qualitative assay of alkaline phosphatase activity, *E. coli* CC181 cells (*phoA*$^-$) transformed with given plasmids or ligation mixtures were plated on agar containing Luria broth, IPTG (0.4 mM), 5-bromo-4-chloro-3-indolyl phosphate (XP; 40 μg/mL), spectinomycin (100 μg/mL), and ampicillin (100 μg/mL). Colonies that express active alkaline phosphatase appear blue on XP indicator plate. Selected colonies were grown overnight in Luria broth, and plasmid DNA was isolated by Magic Minipreps (Promega) for restriction digestion and sequencing.

Lactose Transport. Lactose transport was measured in *E. coli* T184 (*lacZ*$^-$*Y*$^-$) harboring a given plasmid. Cells from overnight cultures were diluted 20-fold in fresh Luria broth containing 10 μg/mL streptomycin and 100 μg/mL ampicillin and grown aerobically to an OD_{420} of 0.5−0.75 (2−3 h). IPTG was added to a final concentration of 0.2 mM, and the cells were grown for an additional 1 h. Cells were harvested by centrifugation, washed in 100 mM potassium phosphate (KP_i; pH 7.5)/10 mM magnesium sulfate, concentrated by centrifugation, diluted to an OD_{420} of 10.0 in the same solution (ca. 0.7 mg of protein/mL), and kept on ice until use. Aliquots (50 μL) of the cell suspension were used for each time point, and reactions were carried out at room temperature in plastic tubes. Assays were initiated by the addition of [1-^{14}C]lactose (10 mCi/mmol) to a final concentration of 0.4 mM and stopped at given times by the addition of 3.0 mL of 100 mM KP_i (pH 5.5)/100 mM LiCl, followed by rapid filtration through glass fiber filters (Whatman GF/F). The filters were washed once with stop solution, and radioactivity trapped on the filters was determined by liquid scintillation spectrometry using Cytoscint (ICN Biomedicals). Nonspecific adsorption of radioactivity was determined by adding stop solution to the suspension prior to addition of radioactive lactose, followed by rapid filtration and washing as described.

Preparation of Spheroplasts. Spheroplasts were prepared essentially as described (Herzlinger et al., 1984). Freshly grown cells were centrifuged (ca. 1 g wet weight), resuspended in 15 mL of 10 mM Tris-HCl (pH 8.0)/0.75 M sucrose, and incubated at 20 °C for 7 min. Lysozyme was added to a final concentration of 0.1 mg/mL, and incubation was continued for 5 min. The suspension was then diluted with 30 mL of 1.5 mM ethylenediaminetetraacetate (EDTA; potassium salt) containing 0.1 mg/mL lysozyme, incubated at 20 °C for an additional 45 min, and examined by phase-contrast microscopy for spheroplast formation. The spheroplasts were harvested by centrifugation ($10000g_{max}$) for 15 min and washed once in 100 mM KP_i (pH 7.0) containing 0.5 M sucrose.

Preparation of Disrupted Membranes. Freshly grown cells were centrifuged, washed twice in 50 mM Tris-HCl (pH 8.0)/100 mM NaCl/1 mM EDTA, resuspended in ice-cold osmotic shock buffer [25 mM Tris-HCl (pH 8.0)/45% sucrose/1 mM EDTA], incubated on ice for 20 min, harvested by centrifugation, resuspended in cold distilled water, and allowed to stand 10 min on ice before adding 0.1 mg/mL lysozyme. After incubation for 20 min, the cell suspensions were clarified by a brief period of sonication (ca. 15 s). Unlysed cells were removed by low-speed centrifugation, and cell membranes were harvested by ultracentrifugation at $150000g_{max}$ for 45 min in a Beckman Optima TL ultracentrifuge.

Western Blotting. Membrane fractions were subjected to sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide electrophoresis using 12% and 5% polyacrylamide, respectively, in the running and stacking gels (Newman et al., 1981). Proteins were electroblotted to PVDF membranes and probed with rabbit polyclonal antibody directed again a dodecapeptide corresponding to the C terminus of the permease (Carrasco et al., 1984) or monoclonal antibody (mAb) 4A10R which is also directed partially against the C terminus of the permease (Carrasco et al., 1982; Herzlinger et al., 1985). Immune complexes were detected by PA-HRP with ECL (Zen et al., 1994). Alternatively, avidin-HRP with ECL substrates was used to detect biotinylated proteins (Consler et al., 1993).

Avidin Binding. Spheroplasts or disrupted membrane preparations from equal amounts of cell culture were resuspended in the same volume of blocking buffer [100 mM KP_i (pH 7.0)/0.5 M sucrose/5% bovine serum albumin]. Aliquots (0.5 mL) were withdrawn, mixed with 1 μL of [^{125}I]-avidin (124 μCi/μg), and incubated at 25 °C for 30 min. The samples were then washed twice by centrifugation, and bound radioactivity was measured by liquid scintillation spectrometry using Cytoscint (ICN Biomedicals).

Alkaline Phosphatase Assays. Alkaline phosphatase activity of *E. coli* CC181 harboring a given plasmid was assayed by measuring the rate of hydrolysis of *p*-nitrophenyl phosphate by permeabilized cells as described (Miller, 1972). Cells growing exponentially in Luria broth at 37 °C were exposed to 1 mM IPTG for 60 min prior to assay. Portions (0.1 mL) of cell culture were mixed with 0.9 mL 1 M Tris-HCl (pH 8.0), 25 μL of 0.1% $NaDodSO_4$, and 25 μL of chloroform. Reactions were started by the addition of 0.2 mL of *p*-nitrophenyl phosphate. After centrifugation in a microcentrifuge, the absorbance of the supernatant was measured at 420 nm. A unit of activity is defined as 1 μmol of substrate hydrolyzed/min/OD_{600} of cells.

Protein Determination. Protein was assayed as described (Peterson, 1977) with bovine serum albumin as standard.

RESULTS

Construction of Lac Permease-XB Domain Chimeras. In a previous study (Consler et al., 1993), permease chimeras were constructed with a factor Xa protease site and the biotin acceptor domain (XB) from the oxaloacetate carboxylase α subunit of *K. pneumoniae* inserted into cytoplasmic domain loop 6 (L6XB permease) or at the C terminus (CXB permease). In order to test whether or not the domain can be used to probe the topology of lac permease, four additional chimeras were constructed with the XB domain inserted at either the N terminus (NXBX permease) or into loop 10 (L10XB permease) on the cytoplasmic face of the membrane or into periplasmic loop 3 (L3XB permease) or loop 7 (L7XB permease) (Figure 1). The general strategy used to make the chimeras was to create a unique *SalI* restriction site in *lacY* by PCR mutagenesis and then insert the XB domain with *SalI* at both ends. All constructs were verified by RE analysis and sequencing through the entire DNA fragment from PCR amplification, as well as the ligation junctions,

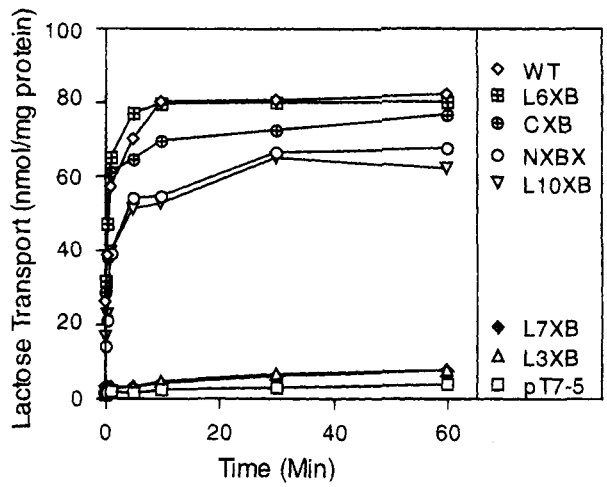

FIGURE 2: Lactose transport. *E. coli* T184 cells harboring pT7-5 (plasmid without *lacY*), pLacY/NXBX, pLacY/L3XB, pLacY/L6XB, pLacY/L7XB, pLacY/L10XB, pLacY/CXB, or pLacY (plasmid encoding wild-type permease) were grown to midlogarithmic phase and induced with IPTG. Aliquots (50 μL) of washed cells resuspended in 50 mM KP_i (pH 7.5)/10 mM magnesium sulfate were assayed for [1-^{14}C]lactose (10 mCi/mmol) uptake at a final concentration of 0.4 mM as described in Materials and Methods.

in double-stranded DNA as described in Materials and Methods.

Colony Phenotype. The ability of the constructs to translocate lactose "downhill" was estimated by transforming *E. coli* HB101 (*lacZ*$^+$*Y*$^-$) harboring a plasmid encoding a given chimera and growing the transformants on MacConkey indicator plates containing 25 mM lactose. HB101 is a "cryptic" strain that produces active β-galactosidase but carries a defective *lacY* gene. Cells expressing functional lac permease allow access of external lactose to cytosolic β-galactosidase, and the subsequent metabolism of the monosaccharides causes acidification which makes the colonies appear red. In contrast, cells unable to transport lactose appear as white colonies. *E. coli* HB101 expressing NXBX or L10XB permease, like L6XB or CXB (Consler et al., 1993), grow as dark red colonies indistinguishable from cells expressing wild-type permease. On the other hand, cells expressing L3XB or L7XB permease grow as white colonies indistinguishable from cells transformed with plasmid containing no *lacY* insert.

Lactose Transport. Time courses of lactose transport by *E. coli* T184 (*lacZ*$^-$*Y*$^-$) expressing each chimeric permease, the negative control (pT7-5 with no insert) and the positive control (pT7-5 encoding wild-type lac permease), are shown in Figure 2. As demonstrated previously (Consler et al., 1993), insertion of the XB domain into loop 6 or at the C terminus of the permease has no significant effect on activity. Moreover, cells expressing NXBX or L10XB permease transport lactose 70−80% as well as cells harboring wild-type permease. On the other hand, as judged by comparison to cells transformed with pT7-5 with no *lacY* gene, cells expressing L3XB or L7XB permease transport the disaccharide to a negligible extent.

Expression and Biotinylation. Expression and biotinylation of each chimera were evaluated by immunoblot analyses using either anti-C-terminal polyclonal antibody or mAb 4A10R followed by PA-HRP, as well as Western blot analyses using avidin-HRP. As demonstrated previously (Consler et al., 1993), spheroplasts or membranes containing L6XB or CXB permease react with both mAb 4A10R and avidin-HRP, demonstrating that the constructs are synthesized, inserted into the membrane, and biotinylated *in vivo* (Figure 3). Although membranes containing NXBX or L10XB permease react with anti-C-terminal polyclonal antibody (Figure 3A,D), avidin blots are completely negative, indicating that neither chimera is biotinylated *in vivo*. Strikingly, although L3XB permease is inactive, the chimera is not only expressed and inserted into the membrane in a stable form, but is biotinylated (Figure 3B). Although data are not shown, expression and biotinylation of L7XB permease which is also inactive are similar to L3XB permease.

Membrane Topology. The membrane topology of the XB domain was studied by factor Xa protease digestion of each chimera in spheroplasts where only the periplasmic surface is exposed (Herzlinger et al., 1984) or sonicated membranes which are scrambled with respect to sidedness (Short et al., 1975; Owen & Kaback, 1978, 1979a,b). When spheroplasts containing L3XB, L6XB, or CXB permease are exposed to factor Xa protease, minimal cleavage is observed (see Figure 3B,C,F), and similar results were obtained with spheroplasts expressing each of the other chimeras. In marked contrast, the factor Xa site in each of these chimeras and NXBX permease in addition (Figure 3A) is highly susceptible to factor Xa protease cleavage in disrupted membrane preparations (data for L7XB permease are not shown). The results indicate that the factor Xa protease site immediately prior to the XB domain in each construct is accessible exclusively from the cytoplasmic face of the membrane. The results with NXBX, L6XB, L10XB, or CXB permease, where the XB domain is inserted into cytoplasmic domains, are consistent with the topology of the permease as determined primarily by *lacY-phoA* fusions (Calamia & Manoil, 1990) and also immunologically (Seckler et al., 1983, 1986; Carrasco et al., 1984; Danho et al., 1985). However, the findings obtained with L3XB or L7XB permease, where the XB domain is inserted into periplasmic loops (see Calamia & Manoil, 1990), indicate that the domain remains on the cytoplasmic face. The findings imply that the XB domain blocks translocation of periplasmic loops 3 or 7, thereby distorting permease structure and abolishing activity.

Measurements of [^{125}I]avidin binding to spheroplasts or sonicated membranes containing L3XB, L7XB, CXB, or wild-type permease confirm the observations with factor Xa protease (Figure 4). For wild-type permease which does not contain an XB domain, spheroplasts bind avidin to the same small extent as sonicated membranes. However, like CXB permease, sonicated membranes containing L3XB or L7XB permease clearly bind significantly more avidin than spheroplasts. The findings indicate strongly that the XB domain in the L3XB and L7XB chimeras remains on the cytoplasmic face of the membrane.

Alkaline Phosphatase Fusions with L3XB and L7XB Permeases. Since insertion of an XB domain into either periplasmic loop 3 or loop 7 appears to block translocation of these loops, it is important to determine whether the succeeding transmembrane domains are assembled with the same or different topology from wild-type lac permease. To address this question, two *phoA* fusions were constructed (L3XB-N165-phoA and L7XB-E314-phoA) that contain alkaline phosphatase devoid of the signal sequence fused to periplasmic loops 5 or 9 of L3XB or L7XB permease, respectively (Figure 1). *E. coli* CC181 harboring pLacY/L3XB-N165-phoA or pLacY/L7XB-E314-phoA grows as

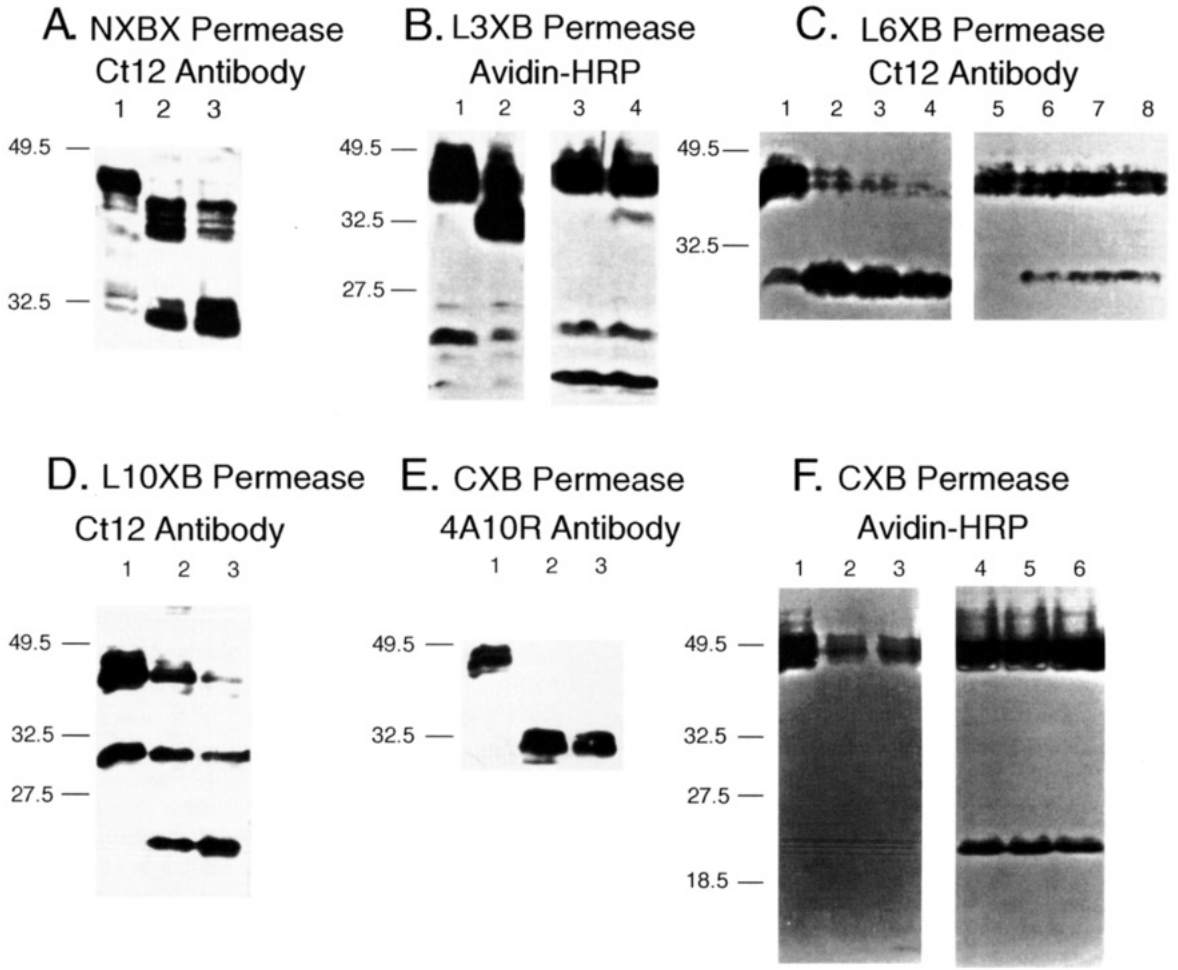

FIGURE 3: Surface topology of lac permease/XB chimeras. Spheroplasts and disrupted membrane vesicles were prepared as described in Materials and Methods from *E. coli* T184 expressing NXBX (*A*), L3XB (B), L6XB (C), L10XB (D), or CXB (E, F) permease. Aliquots of each spheroplast or disrupted membrane preparation were digested with factor Xa protease (2 μg of protease/mg of membrane protein) for a given period of time at 25 °C. Samples were then subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis and electroblotted onto PVDF membranes, followed by treatment with avidin-HRP or with mAb 4A10R or anti-C-terminal polyclonal antibody, PA-HRP, and ECL reagents as indicated in each panel. (A) Disrupted membranes containing NXBX permease digested with factor Xa protease for 0, 60, and 120 min (lanes 1, 2 and 3, respectively). (B) Disrupted membranes (lanes 1 and 2) or spheroplasts (lanes 3 and 4) containing L3XB permease digested with factor Xa protease for 0 (lanes 1 and 3) or 60 min (lanes 2 and 4). (C) Disrupted membranes (lanes 1−4) or spheroplasts (lanes 5−8) containing L6XB permease digested with factor Xa protease for 0 (lanes 1 and 5), 30 (lanes 2 and 6), 60 (lanes 3 and 7), or 120 min (lanes 4 and 8). (D) Disrupted membranes containing L10XB permease digested with factor Xa protease for 0, 30, and 60 min (lanes 1, 2, and 3, respectively). (E) Disrupted membranes containing CXB permease digested with factor Xa protease for 0, 30, and 60 min (lanes 1, 2, and 3, respectively). (F) Disrupted membranes (lanes 1−3) or spheroplasts (lanes 4−6) containing CXB permease digested with factor Xa protease for 0 (lanes 1 and 4), 30 (lanes 2 and 5), or 60 min (lanes 3 and 6). Although the band corresponding to CXB permease (ca. 49 kDa) decreases in intensity, the fragment corresponding to XB in lanes 2 and 3 is not seen due to its small size. Note that biotin carboxyl carrier protein (BCCP) band (22 kDa) is the only endogenous biotinylated cytosolic protein present when spheroplasts were probed with avidin-HRP and that the band corresponding to BCCP is hardly observed in disrupted membrane preparations.

blue colonies on indicator plates and also exhibits high alkaline phosphatase activity (Figure 5A), suggesting that the transmembrane domains following the dislocated XB domains in both chimeras are probably inserted into the membrane with the same or similar topology as wild-type lac permease (see Calamia & Manoil, 1990).

The presence of L3XB-N165-phoA and L7XB-E314-phoA proteins in the membrane was demonstrated by Western blot analysis with either anti-alkaline phosphatase antibody followed by PA-HRP (Figure 5B) or avidin-HRP (Figure 5C). Membranes containing wild-type lac permease with alkaline phosphatase fused at Glu314 (positive control for the immunoblot and negative control for the avidin blot), L3XB-N165-phoA permease, or L7XB-E314-phoA permease react with antibody against alkaline phosphatase (Figure 5B, lanes 1−3), indicating that the fusion proteins are inserted into the membrane. As expected, moreover, only L3XB-N165-phoA and LacYL7XB-E314-phoA react with avidin-HRP, demonstrating that the tripartite chimeras are not only inserted into the membrane but also biotinylated.

DISCUSSION

Consler et al. (1993) demonstrated previously that lac permease with an XB domain inserted into loop 6 or at the C terminus, two sites on the cytoplasmic face of the membrane (Seckler et al., 1983, 1986; Carrasco et al., 1984; Danho et al., 1985; Calamia & Manoil, 1990), catalyzes active transport and is biotinylated *in vivo*. Moreover, as expected, the factor Xa protease site at the N terminus of each XB domain is accessible from the cytoplasmic surface of the membrane. In addition, Reed and Cronan (1991) have shown that a fusion protein containing pro-β-lactamase with a biotin acceptor domain on the C terminus is processed and secreted into the periplasmic space. The findings suggest that construction of chimeras with an XB domain inserted into hydrophilic domains might represent a powerful ap-

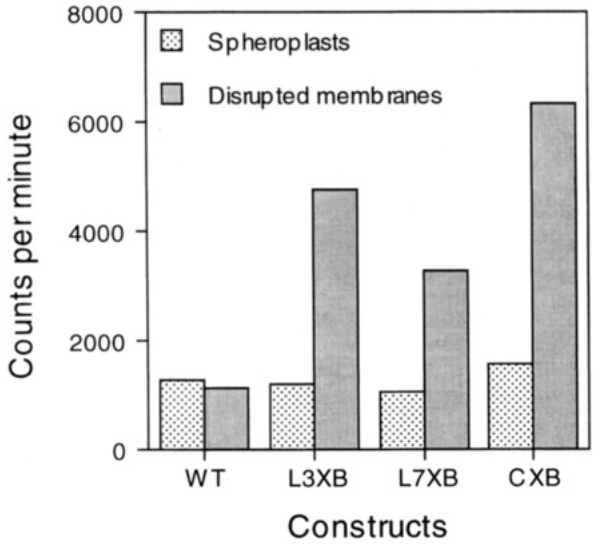

FIGURE 4: [^{125}I]Avidin binding to biotinylated lac permease/XB chimeras. Spheroplasts or disrupted membranes were prepared, and [^{125}I]avidin binding was assayed as described in Materials and Methods.

proach for studying the topology of polytopic membrane proteins.

In this report, we describe additional constructs in which the XB domain is inserted into other hydrophilic domains in lac permease, namely, at the N terminus and into loop 10 which are also cytoplasmic, as well as into loops 3 and 7 which are periplasmic. The rationale is that an XB domain on the cytoplasmic face should be biotinylated because biotin holoenzyme synthetase is present in the cytosol only, while an XB domain on the periplasmic face might not be biotinylated because it is accessible to the cytosol for a limiting period of time. However, the results demonstrate that the hypothesis is invalid. As opposed to L6XB or CXB permease, when an XB domain is inserted at the N terminus or into loop 10 which are also cytoplasmic (Calamia & Manoil, 1990), the chimeras are not biotinylated, although they also exhibit high transport activity. Biotinylation is a post-translational modification that requires a properly folded structure (Reed & Cronan, 1991). Thus, one possibility for the absence of biotinylation with NXBX or L10XB permease is that the XB domain at the N terminus or in loop 10 interacts with other regions of permease, and the interaction prevents proper folding of the biotin acceptor domain. Alternatively, the domain may fold properly but become buried in the tertiary structure of the folded permease. In any case, constructs with an XB domain inserted into periplasmic loop 3 or 7 are biotinylated but inactive. Clearly, therefore, the biotinylation pattern observed is inconsistent with the topology of lac permease as determined from *lacY-phoA* fusions (Calamia & Manoil, 1990) or by immunological criteria (Seckler et al., 1983, 1986; Carrasco et al., 1984; Danho et al., 1985). Therefore, the most straightforward conclusion is that the approach cannot be used to study the topology of lac permease.

On the other hand, insertion of the XB domain into periplasmic loops of the permease has revealed unique and surprising results that have important implications for the mechanism of insertion of this class of membrane proteins. Insertion of the XB domain into loops 3 and 7 which are clearly periplasmic in wild-type permease (Calamia & Manoil, 1990) appears to block translocation of the loops and in all likelihood the two adjoining helices (Figure 6).

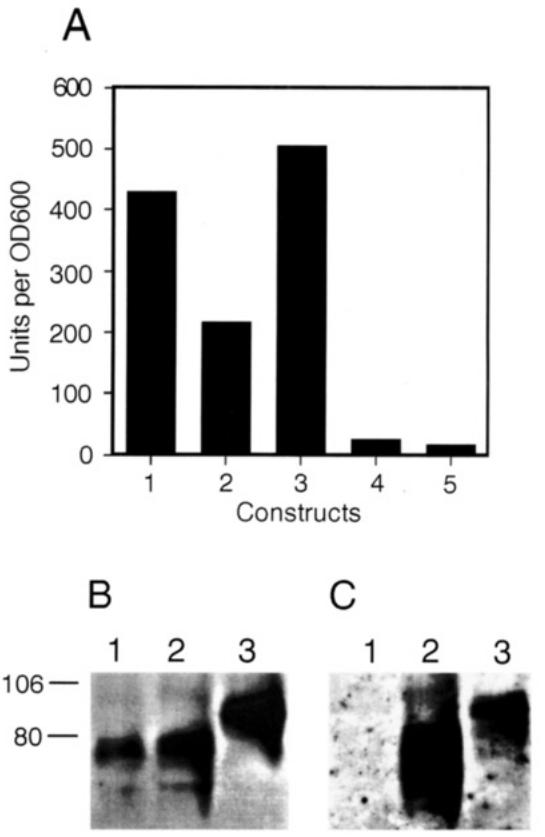

FIGURE 5: Permease/XB/alkaline phosphatase tripartite chimeras. (A) Alkaline phosphatase activity of phoA fusions. Samples 1, 2, 3, 4, and 5 are prepared from *E. coli* CC181 harboring pLacY/E314-phoA, pLacY/L3XB-N165-phoA, pLacY/L7XB-E314-phoA, pLacY/A417-phoA or pT7-5, respectively. Measurements were carried out as described in Materials and Methods. (B) Immunoblots of membranes from *E. coli* CC181 expressing chimera E314-phoA (lane 1), L3XB-N165-phoA (lane 2), or L7XB-E314-phoA (lane 3). Membrane fractions were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis and electroblotted onto PVDF membranes, treated with anti-alkaline phosphatase antibody, followed by PA-HRP and ECL reagents as described in Materials and Methods. (C) Avidin blots of membranes from *E. coli* CC181 expressing lacY/E314-phoA (lane 1), lacY/L3XB-N165-phoA (lane 2), or lacY/L7XB-E314-phoA (lane 3). Electroblotted PVDF membranes were treated with avidin-HRP and subsequently with ECL reagents as described in Materials and Methods.

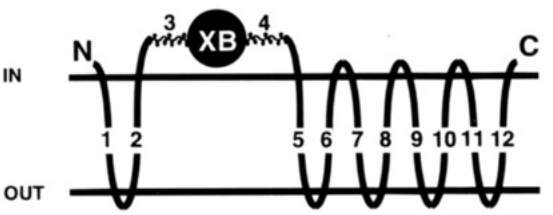

FIGURE 6: Postulated secondary-structure model for lac permease with an XB domain in periplasmic loop 3 (L3XB). As indicated, helix III, loop 3, and helix IV are depicted to be on the cytoplasmic surface of the membrane as the result of insertion of the XB domain into loop 3.

This conclusion is consistent with four lines of evidence: (i) factor Xa protease digestion of L3XB or L7XB permease is observed with disrupted membranes, but minimally with spheroplasts; (ii) significantly more [^{125}I]avidin binding is observed with disrupted membranes relative to spheroplasts containing L3XB or L7XB permease; (iii) the permease is inactivated, presumably as a result of a structural alteration [note that insertion of up to six contiguous His residues into these loops (McKenna et al., 1992) or three factor Xa

protease sites in tandem into loop 7 (Sahin-Tóth et al., 1995) has only a small effect on activity]; and (iv) tripartite chimeras with alkaline phosphatase fusions one periplasmic loop downstream from XB domains in either loop 3 or 7 exhibit high alkaline phosphatase activity. Thus, the first transmembrane domain following the aberrant XB insertions appears to be translocated normally, a finding consistent with observations of Calamia and Manoil (1992) which demonstrate that each of the odd membrane-spanning domains of the permease acts as an independent export signal for alkaline phosphatase. The ability of the XB domain to block translocation of loops 3 or 7 may be due to the run of Ala-Pro repeats at the N terminus of the biotin acceptor domain (Schwarz et al., 1988) or alternatively to the "lollipop" structure of the domain (J. E. Cronan, personal communication), neither of which favors translocation through membranes. In any case, the results are consistent with the notion that helices III and IV and helices VII and VIII in the permease may insert into the membrane as helical hairpins (Von Heijne & Blomberg, 1979; Engleman & Steitz, 1981).

In marked contrast, recent experiments (Zen et al., 1994) indicate that the first two helices in lac permease probably do not insert as a helical hairpin. Thus, nonoverlapping, contiguous polypeptides corresponding to the first transmembrane domain (N_1) and the remaining eleven transmembrane domains (C_{11}) form a relatively stable, functional complex. Taken together with the observations presented here which are consistent with the notion that helices III and IV, as well as helices VII and VIII, insert as helical hairpins, it seems reasonable to conclude that insertion of polytopic membrane proteins like lac permease is a complex process that may involve different mechanisms for different regions of the protein. In this regard, it is also important that disruption of the interaction between Asp237 (helix VII) and Lys358 (helix XI) causes the permease to be inserted into the membrane much less efficiently, thereby raising the possibility that the C-terminal half of lac permease may be inserted post-translationally (Dunten et al., 1993). Finally, the first 22 amino acid residues in the permease, which represent the N-terminal hydrophilic domain and the first half of putative helix I, are not important for activity, but enhance the efficiency of insertion into the membrane (Bibi et al., 1992).

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