The role of helix VIII in the lactose permease of *Escherichia coli*: I. Cys-scanning mutagenesis

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Abstract

Using a functional lactose permease mutant devoid of Cys residues (C-less permease), each amino acid residue in transmembrane domain VIII and flanking hydrophilic loops (from Gln 256 to Lys 289) was replaced individually with Cys. Of the 34 single-Cys mutants, 26 accumulate lactose to >70% of the steady state observed with C-less permease, and an additional 7 mutants (Gly 262 → Cys, Gly 268 → Cys, Asn 272 → Cys, Pro 280 → Cys, Asn 284 → Cys, Gly 287 → Cys, and Gly 288 → Cys) exhibit lower but significant levels of accumulation (30–50% of C-less). As expected (Ujwal ML, Sahin-Tóth M, Persson B, Kaback HR, 1994, *Mol Membr Biol* 1:9–16), Cys replacement for Glu 269 abolishes lactose transport. Immunoblot analysis reveals that the mutants are inserted into the membrane at concentrations comparable to C-less permease, with the exceptions of mutants Pro 280 → Cys, Gly 287 → Cys, and Lys 289 → Cys, which are expressed at reduced levels. The transport activity of the mutants is inhibited by N-ethylmaleimide (NEM) in a highly specific manner. Most of the mutants are insensitive, but Cys replacements render the permease sensitive to inactivation by NEM at positions that cluster in manner indicating that they are on one face of an α -helix (Gly 262 → Cys, Val 264 → Cys, Thr 265 → Cys, Gly 268 → Cys, Asn 272 → Cys, Ala 273 → Cys, Met 276 → Cys, Phe 277 → Cys, and Ala 279 → Cys). The results indicate that transmembrane domain VIII is in α -helical conformation and demonstrate that, although only a single residue in this region of the permease is essential for activity (Glu 269), one face of the helix plays an important role in the transport mechanism. More direct evidence for the latter conclusion is provided in the companion paper (Frillingos S, Kaback HR, 1997, *Protein Sci* 6:438–443) by using site-directed sulfhydryl modification of the Cys-replacement mutants in situ.

Keywords: active transport; bioenergetics; Cys modification; Cys replacement

The lactose permease of *Escherichia coli*, which catalyzes the coupled stoichiometric translocation of β -galactosides and H^+ as a monomer, is an integral membrane protein with 12-transmembrane domains that are most probably in α -helical conformation (reviewed in Kaback et al., 1994; Kaback, 1996). Site-directed mutagenesis of wild-type permease and Cys-scanning mutagenesis of a functional mutant devoid of Cys residues (C-less permease) have provided important insights into the structure and mechanism of the permease. Although as few as four amino acid residues [Glu 269 (helix VIII), Arg 302 (helix IX), His 322 (helix X), and Glu 325 (helix X)] appear to be critically involved in the symport mechanism, the activity of various active Cys-replacement mutants

is altered by alkylation, and these mutants appear in clusters, suggesting that helical interfaces within the permease are important for turnover. Moreover, site-directed fluorescence spectroscopy (Jung et al., 1993, 1994a, 1994c; Wu et al., 1994, 1995a) and in situ site-directed sulfhydryl modification (Frillingos & Kaback, 1996b) show that the reactivity of individually placed Cys residues in a number of transmembrane domains is altered as a result of ligand binding or imposition of a H^+ electrochemical gradient ($\Delta\mu_{H^+}$). Therefore, it appears likely that permease turnover involves widespread conformational changes with few essential side-chain–substrate (H^+ and sugar) or side-chain–side-chain interactions.

Evidence has also accumulated that the C-terminal half of the permease plays a more direct role in the transport mechanism than the N-terminal half. Importantly, with essentially all of the residues in the permease mutated (see Kaback et al., 1994; Kaback, 1996), the four residues found to be irreplaceable with regard to active transport are charged and cluster in transmembrane domains VIII, IX, and X (see Fig. 1). In addition, two pairs of interacting Asp and Lys residues that are not critical for activity are also located in the C-terminal half. Furthermore, the epitope for a monoclonal antibody that uncouples lactose from H^+ translocation has been localized

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Abbreviations: lac, lactose; C-less permease, functional lactose permease devoid of Cys residues; $\Delta\mu_{H^+}$, the H^+ electrochemical gradient across the membrane; NEM, N-ethylmaleimide; IPTG, isopropyl 1-thio- β -D-galactopyranoside; KP_i , potassium phosphate; PMS, phenazine methosulfate; TDG, β -D-galactopyranosyl 1-thio- β -D-galactopyranoside.

Fig. 1. Secondary structure model of *E. coli* lac permease. The one-letter amino acid code is used and putative transmembrane helices are shown in boxes. The four essential amino acid residues in helices VIII (Glu 269), IX (Arg 302), and X (His 322 and Glu 325) are shown as enlarged bold letters and the binding-site residue Cys 148 (helix V) is outlined. The shaded area highlights the region of the permease subjected to Cys-scanning mutagenesis. Also indicated are the restriction endonuclease sites used for construction of the mutants.

to the periplasmic loop between transmembrane domains VII and VIII (Sun et al., 1996b). Finally, downhill transport activity and ligand binding have been shown to be retained in the C-terminal half of the permease (Bibi et al., 1991; Wu et al., 1996a). On the other hand, the N-terminal 22 amino acid residues can be deleted from the permease without abolishing active transport (Bibi et al., 1992), and no essential residues have been found in the N-terminal half of the permease, although Cys 148 (transmembrane domain V) interacts weakly and hydrophobically with the galactosyl moiety of the substrate (Jung et al., 1994b; Wu & Kaback, 1994).

Use of second-site suppressor analysis, site-directed mutagenesis, and site-directed excimer fluorescence has led to a model describing the packing of transmembrane domains VII–XI in the C-terminal half of the permease (see Kaback et al., 1994; Kaback, 1996). The model has been confirmed and extended by engineering divalent metal-binding sites (*bis*- or *tris*-His residues) within the transmembrane domains of the permease (Jung et al., 1995b; He et al., 1995a, 1995b) and by use of site-directed chemical cleavage (Wu et al., 1995b), site-directed spin labeling, and thiol crosslinking (Wu et al., 1996b), which demonstrate that putative helix V, which contains Cys 148, is close to helices VII and VIII. In addition, independent evidence for the close proximity of transmembrane domains VIII–XI has been obtained recently by demonstrating that the last two cytoplasmic loops in the permease comprise a discontinuous epitope for monoclonal antibody 4B11 (Sun et al., 1996a).

Consistent with the predictions of the model, Cys-scanning mutagenesis of transmembrane domains V (Weitzman & Kaback, 1995), VI (Frillingos & Kaback, 1996a), VII (Frillingos et al., 1994), IX, X (Sahin-Tóth & Kaback, 1993), XI (Dunten et al., 1993), and XII (He et al., 1996) reveals that residues where a Cys substitution renders the permease sensitive to inactivation by NEM cluster at interfaces between transmembrane helices thought to comprise part of the pathway for substrate and/or H^+ . In the

present study, we focus on transmembrane domain VIII, which appears to play a central role in the transport mechanism because it is close to residues crucial for lactose-coupled H^+ translocation and to residues involved in substrate binding (Cys 148 and Met 145 in helix V; Wu & Kaback, 1994).

Saturation and site-directed mutagenesis of transmembrane domain VIII have shown that (1) there is a highly mutable stripe of residues on the face of the putative helix opposite to Glu 269 that is proposed to be in contact with the hydrophobic phase of the membrane (Hinkle et al., 1990), and (2) Glu 269 is obligatory for active lactose transport (Ujwal et al., 1994; Franco & Brooker, 1994). The systematic Cys-scanning mutagenesis study presented here demonstrates that only one residue (Glu 269) in transmembrane domain VIII and flanking hydrophilic loops is obligatory for active lactose transport. Furthermore, Glu 269 and nine single-Cys mutants that are sensitive to inactivation by NEM cluster in a fashion indicating that transmembrane domain VIII is an α -helix. The importance of these residues in the transport mechanism is examined further in the companion paper (Frillingos & Kaback, 1997) using *in situ* site-directed sulphydryl modification.

Results

Colony morphology

As a preliminary, qualitative assay of transport activity, each mutant was transformed into *E. coli* HB101, and colonies were grown on MacConkey indicator plates containing 25 mM lactose. HB101 (*lacZ*⁺*Y*[−]) expresses active β -galactosidase but carries a defective *lacY* gene. Cells expressing functional lac permease allow access of external lactose to cytosolic β -galactosidase, and subsequent metabolism of the sugar leads to acidification and the appearance of red colonies. Cells expressing inactive mutants form white colonies, whereas mutants with low activity grow as red

colonies with a white halo. Indicator plates report "downhill" translocation of lactose and give no indication as to whether the cells catalyze lactose accumulation. Of the 34 Cys-replacement mutants described, 32 grow as red colonies indistinguishable from cells expressing C-less permease, P280C¹ grows as red colonies with a white halo, and E269C yields white colonies. Therefore, judging from indicator plates, all of the single-Cys mutants except E269C translocate lactose downhill. Notably, cells expressing permease with Gln, Cys, or His at position 269 placed in the background of wild-type lac permease also grow as white colonies, whereas cells expressing E269D permease grow as red colonies with a white halo (Ujwal et al., 1994; Franco & Brooker, 1994).

Active transport

E. coli T184 (*lacZ*⁻*Y*⁻) was used to test the ability of the mutants to catalyze active lactose transport. The majority of the 34 mutants transport lactose at highly significant rates (Fig. 2A): 22 mutants exhibit rates that are between 70% and 100% or more of C-less permease, 8 mutants (Q256C, T258C, G262C, T265C, G268C, N272C, N284C, and G288C) transport at intermediate rates (35–60% of C-less), 3 mutants (P280C, G287C, and K289C) transport at very low rates (10–20% of C-less), and E269C is inactive. Steady-state levels of lactose accumulation for the great majority of mutants also approximate those of C-less permease (Fig. 2B); steady states of 70–100% or more of C-less are achieved by 26 mutants. G262C, G268C, N272C, P280C, N284C, G287C, and G288C exhibit intermediate levels of accumulation (30–50% of C-less), whereas E269C is unable to accumulate the sugar, as expected (Sahin-Tóth et al., 1992; Ujwal et al., 1994).

Time courses of lactose transport by mutants with low activity are presented in Figure 3 (see Jung et al., 1995a for mutants G287C and G288C). Mutants G262C, G268C, and N272C transport the sugar to 30–40% of the level achieved by C-less permease, whereas P280C transports at a very low initial rate, but reaches a significant level of accumulation in 1 h (40% of C-less). Clearly, however, E269C permease displays negligible activity. Importantly, permease with Gln, Cys, or His at position 269 is completely inactive when transferred into the wild-type background, although E269D permease catalyzes TDG accumulation with a markedly elevated H⁺/TDG stoichiometry relative to wild-type permease (Ujwal et al., 1994; Franco & Brooker, 1994). E269D permease also catalyzes galactose transport to an extent similar to that observed with TDG (data are not shown).

Expression of permease mutants

Western blot analyses of membrane fractions prepared from *E. coli* T184 expressing individual Cys-replacement mutants demonstrates that all of the mutants are present in the membrane at levels comparable to C-less permease with the exception of P280C, G287C, or K289C permease (Fig. 4). Mutants P280C, G287C, and K289C are expressed at reduced levels, consistent with the finding that these mutants exhibit low rates of transport but significant steady-state levels of accumulation (Figs. 2, 3).

¹Site-directed mutants are designated as follows: the one-letter amino acid code is used followed by a number indicating the position of the residue in the wild-type lac permease and a second letter denoting the amino acid replacement at this position.

Fig. 2. Active lactose transport by *E. coli* T184 expressing individual Cys-replacement mutants or C-less permease. Cells were grown at 37°C, and aliquots of cell suspensions (50 μ L containing approximately 35 μ g of protein) in 100 mM KP_i (pH 7.5)/10 mM MgSO₄ were assayed as described in Materials and methods. The single-letter amino acid code along the horizontal axis denotes the original residues replaced with Cys in increasing order from Gln 256 to Lys 289. **A:** Rates of lactose transport measured at 1 min. The rate for C-less permease averaged 81 nmol/min/mg protein. Results are expressed as a percentage of this value. Although not shown (see Fig. 3), T184 cells harboring pT7-5 (vector with no *lacY* gene) transported at a rate of 3.0 nmol/min/mg protein (i.e., 3.7% of C-less). **B:** Steady-state levels of lactose accumulation. Results are expressed as a percentage of the C-less value, which was 185 nmol lactose/mg protein, on average. Although not shown (see Fig. 3), T184 cells harboring pT7-5 accumulated 11 nmol lactose/mg protein in 1 h (i.e., 5.9% of C-less).

Sensitivity of single-Cys mutants to NEM

The effect of NEM, a membrane-permeable sulfhydryl reagent, on the initial rate of lactose transport for each mutant is shown in Figure 5. Although the activity of the majority of the Cys-replacement mutants is not altered significantly and one mutant (T258C) exhibits a twofold increase (compare Figs. 2, 5), ten

Fig. 3. Time courses of lactose transport by single-Cys mutants with low or intermediate activity. *E. coli* T184 transformed with plasmid pT7-5 (vector with no *lacY* gene), pT7-5 encoding C-less permease, or pT7-5 encoding given Cys-replacement mutants were grown and assayed as described in Materials and methods.

mutants are inhibited by more than 75%: G262C, V264C, T265C, G268C, N272C, A273C, M276C, F277C, A279C, and I283C. When viewed on a helical wheel plot, these mutants cluster on one face of helix VIII (Fig. 6). Similar observations were made when active transport of galactose was tested (data not shown). Thus, each one of the ten NEM-sensitive mutants catalyzes galactose accumulation at rates and to levels comparable to those observed for lactose

(when expressed as a percentage of C-less activity; see Fig. 2), and treatment with NEM inhibits galactose transport to the same extent as lactose transport (data not shown).

Discussion

The results presented in this paper extend a series of Cys-scanning mutagenesis studies of lac permease that have led to two major conclusions. First, remarkably few residues are irreplaceable with respect to activity. Second, the transport activity of certain active

Fig. 4. Western blots of membranes containing C-less lac permease or Cys-replacement mutants. Membranes were prepared from IPTG-induced cultures of T184 harboring given plasmids, as described in Materials and methods. Samples containing approximately 50 μ g of membrane protein were subjected to SDS-PAGE, electroblotted, and the blots were incubated with anti-C-terminal lac permease antibody, followed by incubation with horseradish peroxidase-conjugated protein A. The blots were developed with chemiluminescent substrate and exposed to film for 1 min. Membranes prepared from cells harboring pT7-5 with no *lacY* gene exhibited no immunoreactive material. Arrow at the left indicates the migration position of the marker protein carbonic anhydrase (32.5 kDa).

Fig. 5. Effect of NEM on active lactose transport by *E. coli* T184 harboring plasmids encoding single-Cys mutants. Cells were incubated with 2 mM NEM (final concentration) at room temperature for 10 min, the reaction was quenched by addition of 10 mM dithiothreitol (final concentration), and cells were assayed for initial rates of lactose uptake in the presence of 20 mM potassium ascorbate and 0.2 mM PMS (Konings et al., 1971). Rates are presented as percentages of the rate measured in the absence of NEM. Values were not determined (N.D.) for mutants E269C, P280C, G287C, and K289C, which display very low initial rates of transport (<20% of C-less).

Fig. 6. Helical wheel plot of residues in putative helix VIII (from Gly 262 to Ala 279) viewed from the periplasmic surface of the membrane. Lines radiating from the center are proportional to the transport activity of each mutant retained after incubation with NEM (see Fig. 5). Positions highly sensitive to NEM treatment (>75% inhibition of initial rate) are shown as enlarged bold letters, and the essential residue Glu 269 is outlined. Arrows indicate positions where more than three different amino acid replacements were found in a library of functional mutants created by saturation mutagenesis in this region (Hinkle et al., 1990; see Fig. 8).

Cys-replacement mutants is compromised by alkylation, and these mutants appear in clusters in a manner suggesting that helical surfaces within the permease are important for the conformational changes that occur during turnover (see Kaback et al., 1994; Kaback, 1996). With regard to transmembrane domain VIII, only one residue (i.e., Glu 269) is obligatory for active transport, whereas the activity of a set of ten Cys-replacement mutants is strongly inhibited by NEM. The NEM-sensitive positions cluster in a manner indicating that they are located on one face of an α -helix that also contains Glu 269. Moreover, the face comprises part of an interface between helices V, VII, VIII, X, and XI that may line a pathway for substrate translocation (Fig. 7). In the companion paper (Frillingos & Kaback, 1997), further evidence for this argument is presented.

With respect to active transport, the findings confirm and extend data obtained from saturation mutagenesis (Hinkle et al., 1990) and site-directed mutagenesis of Gly residues (Jung et al., 1995a), indicating that the majority of residues in helix VIII are not critical for activity. Thus, apart from Glu 269, which appears to be irreplaceable with respect to binding and/or coupling (Ujwal et al., 1994; Franco & Brooker, 1994), no other residue in helix VIII or the flanking hydrophilic domains is essential (Fig. 2). The low activity observed with mutants G262C and G268C (Fig. 3) and their sensitivity to alkylation (Fig. 5) is consistent with other findings (Brooker et al., 1989; Hinkle et al., 1990; Jung et al., 1995a), indicating that bulkier side chain residues cannot be tolerated at these positions.

As observed previously with mutants Y228C (helix VII; Frillingos et al., 1994) and Y350C (helix XI; Dunten et al., 1993), mutants P280C, G287C, and K289C exhibit reduced levels of expression in the membrane paralleled by low initial rates of transport, but significant levels of lactose accumulation. The findings

are consistent with previous observations demonstrating that replacement of Pro 280 with Ala or Gly (Consler et al., 1991) or Gly 287 with Cys (Jung et al., 1995a) results in functional permease and confirm the conclusion that Pro 280 and Gly 287 are not essential for turnover. In addition, the results presented for mutants G287C and K289C (Figs. 2, 4) are in general agreement with insertional mutagenesis (McKenna et al., 1992) and site-directed mutagenesis studies (Jessen-Marshall et al., 1995; Jung et al., 1995a) showing that insertions or certain point mutations within the conserved sequence motif R/K-X-G-[X]-R/K in loop II/III or VIII/IX (Henderson, 1990; Baldwin, 1993) may cause inhibition.

In an initial screen for accessibility or reactivity of Cys residues at each position from Gln 256 to Lys 289, the effect of NEM on lactose transport was studied in the active mutants. Although most of the 34 mutants are relatively insensitive, 10 mutants are inhibited by more than 75% by the alkylating reagent. Possibly the other Cys residues are inaccessible to NEM and do not react. However, NEM is membrane-permeant and a number of single-Cys permease mutants located within transmembrane domains and disposed toward the inner surface of the membrane are inhibited readily (Sahin-Tóth et al., 1992; Dunten et al., 1993; Sahin-Tóth & Kaback, 1993; Frillingos et al., 1994; Weitzman & Kaback, 1995; Frillingos & Kaback, 1996a) and react with [^{14} C]NEM, as demonstrated *in situ* (Frillingos & Kaback, 1996b; Frillingos & Kaback, 1997). In this respect, it is important that the NEM-sensitive positions described here are essentially continuous on a helical wheel plot (Fig. 6) and comprise a face that is in close proximity to Cys 148, a residue that makes direct contact with substrate, as well as the residues found to be essential for coupling between lactose and H^+ translocation in helices IX and X (i.e., Arg 302,

Fig. 7. Helical wheel model of helices V and VII-XI in lac permease as described by (Wu et al., 1996b). Residues shown as bold capital letters in helices V, VII, X, XI, and VIII represent positions where a Cys-replacement mutant is significantly inactivated by treatment with NEM (>60% inhibition of initial rate). Also shown are the interactions between Asp 237 and Lys 358 and between Asp 240 and Lys 319 (open capital letters) and the four irreplaceable residues (Glu 269, Arg 302, His 322, and Glu 325) (shadowed open capitals) (see He et al., 1995a; Wu et al., 1995b, in addition).

His 322, and Glu 325; Figs. 6, 7). In addition, Glu 269, which is irreplaceable with respect to active transport, is on the same face of helix VIII. As expected, Glu 269 and the NEM-sensitive positions are highly conserved among homologous H^+ -coupled sugar transporters (Marger & Saier 1993; Lee et al., 1994) and represent positions where few or no replacements are found in a library of active permease mutants created by saturation mutagenesis (Hinkle et al., 1990; see Fig. 8). Furthermore, the highly mutable stripe of residues proposed to be in contact with the membrane phospholipids is on the face opposite to that of NEM-sensitive positions identified here (Figs. 6, 8). Therefore, taken as a whole, the observations imply that one face of helix VIII plays an important role in the transport mechanism. More direct evidence for this conclusion is provided in the companion paper (Frillingos & Kaback, 1997).

Materials and methods

Bacterial strains and plasmids included *E. coli* HB101 [*hsdS20* (r_B^- , m_B^-), *recA13*, *ara-14*, *proA2*, *lacY1*, *galK2*, *rpsL20* (Sm^r), *xyl-5*, *mtl-1*, *supE44*, *l⁻IF⁻*] (Boyer & Roulland-Dussoix, 1969) was used to propagate the plasmids described and for detection of lac permease activity on MacConkey-agar indicator plates (Difco Laboratories [Detroit, Michigan]) containing 25 mM lactose. *E. coli* T184 [*lacI⁺O⁺Z⁻Y⁻*(A), *rspL*, *met⁻*, *thr⁻*, *recA*, *hsdM*, *hsdR/F'*, *lacI^qO⁺Z^{D118}* (Y^+A^+)] (Teather et al., 1980) harboring plasmid pT7-5/*lacY* with given mutations was used for expression of lac permease from the *lacZ* promoter/operator. A cassette *lacY* gene (EMBL-X56095) devoid of Cys codons (C-less permease; van Iwaarden et al., 1991) containing the *lacZ* promoter/operator was used for all *lacY* gene manipulations.

[1- ^{14} C]Lactose, [U- ^{14} C]galactose, and [α - ^{35} S]dATP were purchased from Amersham (Arlington Heights, Illinois). Deoxyoligonucleotides were synthesized on an Applied Biosystems 391 DNA synthesizer. 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, California). Restriction endonucleases and T4 DNA ligase were from New England Biolabs (Beverly, Massachusetts). Taq DNA polymerase was from Promega Corporation (Madison, Wisconsin). Sequenase was from United States Biochemical (Cleveland, Ohio).

				262	264	265		268	269		272	273		276	277				
	R	V	F	G	Y	V	T	M	G	<u>E</u>	L	N	A	S	I	M	F	F	
1	P	I	Y		S		M	S	L	a		F	D	g	L	T	L	Y	Y
	c	A	I		C		s	m	G			i		W	M	i	V	L	
	h	G	I		h			a	s					F	s			C	
	l	L			q				v					a	f			S	
															v			i	
2	e				f				g										c
3	k	a			f		a		a				i	c					c

Fig. 8. Sequence of residues Arg 259–Phe 278 in *E. coli* lac permease. Top line shows the native sequence. Lines numbered 1 show a set of mutations at each position that yield active permease (from Hinkle et al., 1990; upper-case letters indicate single mutations, lower-case letters indicate multiple mutations). Bottom lines numbered 2 and 3 show residues that are found in the *Klebsiella pneumoniae* lac permease (McMorrow et al., 1988) and in the *E. coli* raffinose permease (Aslanidis et al., 1989), respectively. The irreplaceable residue Glu 269 is outlined and positions where a Cys-replacement mutant is sensitive to inactivation by NEM (Fig. 5) are numbered and shown in boxes.

All other materials were reagent grade and obtained from commercial sources.

Oligonucleotide-directed site-specific mutagenesis

Cys-replacement mutants were constructed either by a two-stage PCR method (PCR overlap-extension; Ho et al., 1989) or by one-step PCR using pT7-5/*lacY*/cassette encoding C-less permease as template. For mutants in helix VIII (from V260C to S274C, except T266C, M267C, and G268C), a unique *Nco*I restriction site was introduced as a silent mutation in codon 266 (CCATGG) to facilitate construction and verification. The PCR products were digested with appropriate restriction endonucleases and ligated to similarly treated pT7-5/*lacY*/C-less vector (see Fig. 1 for location of sites). Mutations were verified by sequencing the length of the subcloned double-stranded DNA through the ligation junctions, using dideoxynucleotide termination (Sanger et al., 1977) after alkaline denaturation (Hattori & Sakaki, 1986).

Growth of bacteria

E. coli HB101 (Z^+Y^-) or T184 (Z^-Y^-) transformed with each plasmid described was grown aerobically at 37°C in Luria-Bertani medium containing streptomycin (10 μ g/mL) and ampicillin (100 μ g/mL). HB101 cultures were used for preparation of plasmid DNA. Fully grown cultures of T184 were diluted ten-fold and allowed to grow for another 2 h before induction with 0.5 mM IPTG. After further growth for 2 h at 37°C, cells were harvested and used for transport assays or preparation of membranes.

Active transport

Cells were washed with 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ and adjusted to an optical density of 10.0 at 420 nm (approximately 0.7 mg protein/mL). Transport of [1- ^{14} C]lactose (2.5 mCi/mmol; final concentration 0.4 mM) was assayed by rapid filtration, as described (Consler et al., 1991). Transport of [U- ^{14} C]galactose (5 mCi/mmol) at a final concentration of 1 mM was assayed as described (Jung et al., 1994c).

Membrane preparation

Crude membrane fractions from *E. coli* T184 were prepared by osmotic lysis and sonication as described (Frillingos et al., 1994).

Immunologic analyses

Membrane fractions were subjected to 12% SDS-PAGE, as described (Newman et al., 1981). Proteins were electroblotted to polyvinylidene difluoride membranes (Immobilon-PVDF; Millipore) and probed with a site-directed polyclonal antibody against the C terminus of lac permease (Carrasco et al., 1984).

Protein determinations

Protein was assayed in the presence of SDS as described (Peterson, 1977).

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