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The Conserved Motif in Hydrophilic Loop 2/3 and Loop 8/9 of the Lactose Permease of *Escherichia coli*. Analysis of Suppressor Mutations

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Abstract. The major facilitator superfamily (MFS) of transport proteins, which includes the lactose permease of *Escherichia coli,* contains a conserved motif G-X-X-X-D/E-R/K-X-G-R/K-R/K in the loops that connect transmembrane segments 2 and 3, and transmembrane segments 8 and 9. In three previous studies (Jessen-Marshall, A.E., & Brooker, R.J. 1996. *J. Biol. Chem.* **271:**1400–1404; Jessen-Marshall, A.E., Parker, N., & Brooker, R.J. 1997. *J. Bacteriol.* **179:**2616–2622; and Pazdernik, N., Cain, S.M., & Brooker, R.J. 1997. *J. Biol. Chem*. **272:**26110–26116), suppressor mutations at twenty different sites were identified which restore function to mutant permeases that have deleterious mutations in the conserved loop 2/3 or loop 8/9 motif. In the current study, several of these second-site suppressor mutations have been separated from the original mutation in the conserved motif. The loop 2/3 suppressors were then coupled to a loop 8/9 mutation (P280L) and the loop 8/9 suppressors were coupled to a loop 2/3 mutation (i.e., G64S) to determine if the suppressors could restore function only to a loop 2/3 mutation, a loop 8/9 mutation, or both.

The single parent mutations changing the first position in loop 2/3 (i.e., G64S) and loop 8/9 (i.e., P280L) had less than 4% lactose transport activity. Interestingly, most of the suppressors were very inhibitory when separated from the parent mutation. Two suppressors, A50T and G370V, restored substantial transport activity when individually coupled to the mutation in loop 2/3 and also when coupled to the corresponding mutation in loop 8/9. In other words, these suppressors could alleviate a defect imposed by mutations in either half of the permease. From a kinetic analysis, these suppressors were shown to exert their effects by increasing the V_{max} values for lactose transport compared with the single G64S and P280L strains. These results are discussed within the context of our model in which the two halves of the lactose permease interact at a rotationally symmetrical interface, and that lactose transport is mediated by conformational changes at the interface.

Key words: Lactose permease — Symporter — Conserved motif — Sugar transporter

Introduction

Integral membrane proteins, known as cation/substrate cotransporters or symporters, provide an uptake mechanism for a variety of solutes necessary for cellular function [7, 24]. These transported solutes include amino acids, metabolic intermediates, inorganic ions, and a variety of sugars. The lactose permease of *Escherichia coli* has been an extensively studied symporter that has provided a model system for the study of structure and function in transport proteins [33]. The lactose permease is a cytoplasmic integral membrane protein that couples the transport of H^+ and lactose [35, 36]. The cloning and sequencing of the *lacY* gene has shown that the permease contains 417 amino acids and has a predicted molecular weight of 46,504 [3, 31]. Hydropathy plots, alkaline phosphatase fusions, and other genetic studies support a secondary model consisting of twelve transmembrane domains that cross the membrane as α-helices [4, 5, 8, 18]. A recent analysis of proteins that are evolutionarily related to the lactose permease has produced a tertiary model in which the two halves of the protein form a rotationally symmetrical dimer composed of 6 transmembrane domains each [11].

The lactose permease is a member of a large superfamily of transporters termed the major facilitator super-

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family (MFS) that includes symporters, uniporters, and antiporters [12–14, 22, 26]. A conserved motif is found in the members of this superfamily. It is the decapeptide, G-X-X-X-D/E-R/K-X-G-R/K-R/K, which is located in the loop that connects TMS-2 to TMS-3 and is repeated in the TMS-8 to TMS-9 loop [12, 26]. In the loop 8/9 motif of the lactose permease, the motif is not as conserved; the first position is proline, the fifth position is asparagine, and the ninth position is asparagine. The functional importance of amino acid residues within this motif has been extensively investigated in the lactose permease and tetracycline antiporter [15, 28, 39–42]. While some differences between the motif are noted between these two proteins, it has been found that the first position glycine and fifth position aspartate are critical for transport activity in the loop 2–3 motif [15, 41]. Individually, the basic residues within the lactose permease are not critical for transport function, but may play a role in protein insertion and/or stability [15, 28].

One approach that can yield useful information concerning the functional role of particular amino acid residues within proteins is an intragenic suppressor analysis. In this strategy, the amino acid of interest is mutated to a nonfunctional residue and suppressor mutations are isolated that restore protein function. These can be first site revertants in which the nonfunctional residue is replaced by a functional residue, or they can be second-site suppressor mutations in which an amino acid substitution somewhere else in the protein can act to restore functionality. This type of approach has been previously conducted on the lactose permease and the tetracycline antiporter, starting with parental strains that had deleterious mutations in the loop 2/3 or loop 8/9 motif. In the case of the lactose permease, suppressor mutations were found at twenty different locations [16, 17, 27]. According to our tertiary model, most of the suppressors clustered in three regions that were located at the interface between the two halves of the permease.

Our tertiary model for the permease is based on a rotationally symmetrical structure [1]. We hypothesize that conformational changes associated with lactose transport occur at the interface [16]. Evidence for such a model could be provided by an analysis of suppressor mutations. If our model is correct, suppressors which restore activity to a loop 2/3 defect might also be able to restore activity to a loop 8/9 defect and *vice versa*. Such a result would require that the loop 2/3 and loop 8/9 mutations disrupt permease structure in a rotationally symmetrical way (e.g., a twisting around the rotational axis of symmetry) and that the suppressors act in a compensatory manner to alleviate such a change in structure. The goal of the current study is to analyze the effects of the suppressor mutations when separated from the parent mutation and to determine if any of the suppressors can complement both loop 2/3 and loop 8/9 defects, thereby providing support for our model.

Table 1. Bacterial strains and plasmids

Strain	Relevant genotype (chromosome/F/plasmid)	Reference
TI84	*lacI*$^+$ *lacO*$^+$ *lacZ*$^-$ *lacY*$^-$/*lacI*^{Q} *lacO*$^+$ *lacZ*^{U118a} (*lacY*$^+$)/−	32
HS4006/F′I^{Q}Z$^+$Y$^-$	Δ(*lac-pro*)Δ*malB101*/*lacI*^{Q} *lacO*$^+$ *lacZ*$^+$ *lacY*$^-$/−	1
pTE18 (plasmid)	−/−/Δ(*lacI*) *lacO*$^+$ Δ(*lacZ*) *lacY*$^+$ Δ(*lacA*) Amp^R Tet^R	32
pACYC-LacY[b]	−/−/Δ(*lacI*) *lacO*$^+$ Δ(*lacZ*) *lacY*$^+$ Δ(*lacA*) Tet^R	10

[a] *LacZ*^{U118} is a polar nonsense mutation which results in a *Lac Z*$^-$ *Lac Y*$^-$ phenotype [32].

[b] pACYC-LacY was constructed by cloning the 2300 bp EcoRI fragment from pTE18 which carries the wild-type *lacY* gene into the EcoRI site of pACYC184. The *lacY* gene and the tetracycline resistance gene are in the opposite transcriptional direction.

Materials and Methods

Reagents

Lactose (O-β-D-galactopyranosyl-[1,4]-α-D-glucopyranose), melibiose, (O-α-D-galactopyranosyl-[1,6]-α-D-glucopyranose) and maltose (4-O-α-D-glucopyranosyl-D-glucose) were purchased from Sigma. [^{14}C]-lactose was purchased from Amersham. The remaining reagents were analytical grade.

Bacterial Strains and Methods

The bacterial strains and plasmids are described in Table 1. Plasmid DNA was isolated using PERFECTprep Plasmid DNA kit obtained from 5 prime- 3 prime, Inc., and introduced into the appropriate bacterial strain by the $CaCl_2$ transformation procedure of Mandel and Higa [21] or the RbCl transformation procedure [25].

Stock cultures of cells were grown in YT media [23] supplemented with tetracycline (0.01 mg/ml). For transport assays, cells were grown to midlog phase in YT media containing tetracycline (0.005 mg/ml) and 0.25 mM isopropylthiogalactoside (IPTG) to induce synthesis of the lactose permease.

Sugar Transport Assays

For downhill transport assays, midlog phase cells were washed in phosphate buffer, pH 7.0, containing 60 mM K_2HPO_4 and 40 mM KH_2PO_4 and resuspended in the same buffer to a density of approximately 0.5 mg protein per ml. Cells were then equilibrated at 30°C and radioactive sugar (final concentration = 0.1 mM) was added. At the appropriate time intervals, 0.2-ml aliquots of cells were withdrawn and filtered over a membrane filter (pore size = 0.45 μm). The external buffer was then washed away with 5–10 ml of phosphate buffer, pH 7.0, by rapid vacuum filtration. Intracellular radioactivity was deter-

mined by liquid scintillation counting of the filtered cells. For the kinetic experiments, the same procedure for downhill transport was followed except that several different final sugar concentrations were used to evaluate the K_m and V_{max} values.

Calculations

To determine the K_m and V_{max} values for lactose transport, the data were plotted as 1/V *vs.* 1/[S] (a Lineweaver-Burke double reciprocal plot, 30). The *x*- and *y*-intercepts were based on a best-fit regression line plotted through the known *x*-values and *y*-values. For the wild type and each mutant, the uptake rates at each lactose concentration were plotted, and were found to be linear for 60 sec at low lactose concentrations (0.1–0.5 mM) and for a minimum of 30 seconds at higher lactose concentrations. The data used to calculate the K_m and V_{max} values in this study were always within the linear range. Likewise, a visual examination of the Lineweaver-Burke plots indicated linearity. The V_{max} values reported in this study were not adjusted for levels of expression described in Table 2. This adjustment was not done for two reasons. First, the level of expression had a much higher standard error compared to the standard error found in the kinetic calculations, and it was felt an adjustment based on expression levels may be misleading. Second, it has been discovered in other studies that expression levels, which are determined after the isolation of membrane vesicles, do not always reflect the true levels of expression in whole cells [28]. This is because some mutant proteins appear to be rapidly degraded during the membrane isolation procedure.

With regard to their kinetic parameters, some mutants showed rather high standard deviations (>10%). A visual inspection of the raw data suggested that this is related to inaccuracies associated with lactose diffusion. At high lactose concentrations, the background (non-permease mediated) level of lactose uptake is higher, and it becomes difficult to accurately measure the uptake rate if the K_m value is high and/or the V_{max} value is low.

Membrane Isolation and Immunoblot Analysis

For Western blot analysis, HS4006/F′Z^+Y^- cells containing the appropriate plasmid were grown as described for the sugar transport assays. 10 ml of late log cells were pelleted by low speed centrifugation, quick frozen in liquid N_2, and thawed at room temperature. Cells were resuspended in 800 μl of MTPBS (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4). Cells were quick frozen in liquid N_2 and thawed twice. Cells were then sonicated three times for 20 secs each. The membranes were harvested by centrifugation and resuspended in 100 μl of MTPBS. A total of 100 μg of membrane protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Proteins were electroblotted onto nitrocellulose and then probed using an antibody that recognizes the carboxyl-terminus of the lactose permease (graciously provided by Dr. Tom Wilson of Harvard University). The amount of lactose permease was then determined by laser densitometry.

Construction of Mutant Strains/DNA Sequencing

The single mutant strains, G64S and P280L, and the double mutant strains, G64S/F29S, G64S/A50T, G64S/C234F, G64S/Q241L, G64S/F261V, G64S/V367E, P280L/G46C, P280L/L216Q, P280L/C333G, P280L/F354C, and P280L/G370V were originally described in references [16, 17, 27]. To make single mutants that only contained the suppressor mutation, the plasmid DNAs from double mutant strains were digested with a restriction enzyme that had a unique site between the relevant codons and then separated by gel electrophoresis. The appropriate fragments were then isolated from agarose slices using QIAquick Extraction Kit (Qiagen, Chatsworth, CA). The fragment containing the suppressor mutation was then ligated to a fragment containing the rest of the wild-type *lacY* gene and transformed into strain T184 (Table 1). Similarly, to make the reciprocal mutant constructs, the fragment containing the suppressor mutation was ligated to a fragment carrying the G64S or P280L mutation. In a few cases, the DNA fragments used in these ligations were generated by PCR. After the appropriate clones were made, the mutations were confirmed by DNA sequencing using the method of Kraft et al. [19]. The entire *lacY* gene was sequenced to confirm that no other mutations had occurred, and a minimum of two independent mutations (of each type) were saved for further study.

Abbreviations

IPTG, isopropylthiogalactoside; TMS, transmembrane segment.

Results

Construction of Single Suppressor Strains and Their Coupling with Mutations in Loop 2/3 or Loop 8/9

As mentioned, previous studies have identified twenty second-site suppressor mutations that restore substantial or full transport activity to parental strains that had a loop 2/3 or loop 8/9 mutation. These were isolated as spontaneous mutations on MacConkey plates [16, 17, 27]. The locations of these suppressor mutations in the secondary structure of the lactose permease are shown in Fig. 1. Most of the suppressors clustered in one of three common regions that included codons 45–50, 234–241, and 366–370 which are located in periplasmic loop 1/2, TMS-7, and periplasmic loop 11/12, respectively.

In the current study, we have further characterized the following strains: G64S/A50T, G64S/C234F, G64S/T266I, G64S/V367E, P280L/G46C, P280L/L216Q, P280L/C333G, P280L/F354C, and P280L/G370V. In Tables 2–4, these are the strains in which the suppressor is coupled with its original parent mutation. Molecular genetic techniques were used to separate the suppressor mutation from the original parent mutation in loop 2/3 (i.e., G64S) or loop 8/9 (i.e., P280L) in order to analyze the effects of the suppressor mutation alone. We also coupled each of these suppressor mutations with a mutation in the conserved motif in the other half of the permease. For example, if the original suppressor strain was G64S/V367E, the V367E mutation was separated from the G64S mutation and then coupled to a P280L mutation. The P280L/V367E strain is described as a suppressor with a reciprocal parent mutation. In the experiments of Figs. 2, 3, and Table 5, we also characterized a few additional suppressor alone strains: F49L, Q241L, F261V, and A369P.

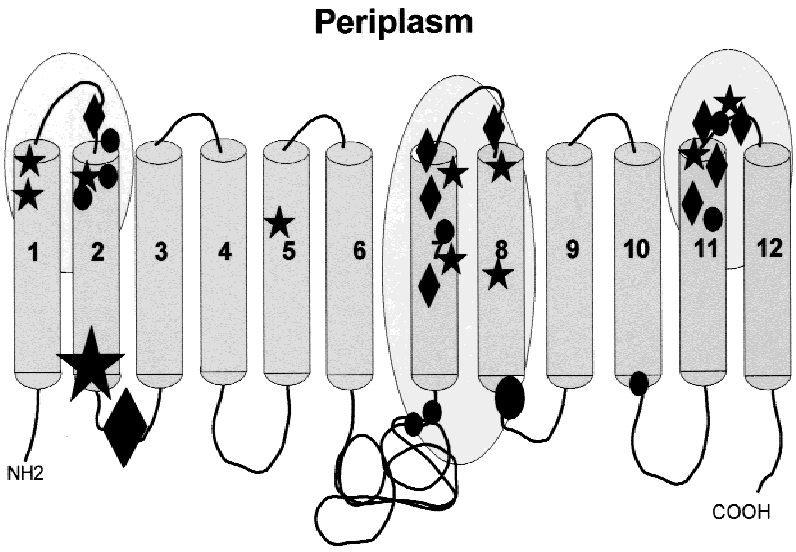

Fig. 1. Locations of Suppressor Mutations that Restore Function to Loop 2/3 or Loop 8/9 Mutations. The location of the first site mutation is shown with a large star, diamond, or oval. Second site suppressors isolated from these strains are designated with smaller stars, diamonds, and ovals. Three regions are circled where the suppressors tended to cluster.

Table 2. Protein expression levels in wild-type and mutant strains[a]

Strain	% Wild-type level[a]
pACYC-LacY	100
pG64S	118 ± 33
p280L	92 ± 5

Suppressor mutation	% of Wild-type level		
	Suppressor alone	Suppressor with original parent[b]	Suppressor with reciprocal parent
pG46C	100 ± 1	70 ± 11	0
pA50T	70 ± 4	50 ± 11	50 ± 11
pL216Q	27 ± 6	93 ± 16	0
pC234F	47 ± 1	77 ± 4	0
pT266I	103 ± 14	77 ± 7	0
pC333G	100 ± 24	27 ± 2	0
pF354C	46 ± 5	25 ± 8	0
pV367E	87 ± 2	86 ± 12	0
pG370V	131 ± 31	199 ± 35	87 ± 8

[a] Protein levels were determined as described under Materials and Methods and expressed as the percentage of the wild-type levels.
[b] A loop 2/3 mutation (G64S) was the original parent of the following suppressor strains: pF29S, pA50T, pC234F, pQ241L, pF261V, and pV367E. A loop 8/9 mutation (P280L) was the original parent of the following suppressor strains: pG46C, pA50T, pL216Q, pC333G, pF354C, and pG370V.

Table 3. Phenotypes of lactose MacConkey plates of wild-type and mutant strains[a]

Strain	Phenotype on 1.0% lactose MacConkey
pACYC-LacY	Red
pG64S	White
p280L	White

Suppressor	Phenotype of 1% lactose MacConkey plates[a]		
	Suppressor alone	Suppressor with original parent	Suppressor with reciprocal parent
pG46C	Red	Red	White
pA50T[b]	Red	Red	Red
pL216Q	White	Red	Pink
pC234F	Red	Red	White
pT266I	Red	Red	White
pC333G	Red	Red	White
pF354C	Red	Red	White
pV367E	Red	Red	White
pG370V	Red	Red	Red

[a] A red phenotype indicates substantial uptake and fermentation of the sugar, a white phenotype indicates negligible transport and fermentation, and a pink phenotype indicates a very low level of uptake and fermentation.
[b] This suppressor mutation was originally isolated as a spontaneous suppressor for both loop 2/3 and loop 8/9 mutant parent strains.

For those suppressors that were coupled to both parent mutations, Table 2 shows the expression levels of the suppressors alone, when they were coupled with the original parent mutation, and when they were coupled with a reciprocal parent mutation. Though some variability is seen in expression levels, most of the suppressor alone strains and the suppressors with their original parents were expressed at substantial levels. However, when the suppressors were coupled with the reciprocal parent, the expression levels of most strains were undetectable. The only two exceptions were the A50T and G370V strains. In these cases, the suppressor with its reciprocal parent was expressed at substantial levels.

Phenotype on MacConkey Plates

To explore initially the effects of the suppressor mutations alone, when coupled with the original parent mu-

Table 4. Phenotypes on melibiose MacConkey plates of wild-type and mutant strains[a]

Strain	Phenotype on 1.0% Melibiose MacConkey
pACYC-LacY	Red
pG64S	White
p280L	Pink

Suppressor	Phenotype on 1% Melibiose MacConkey Plates		
	Suppressor alone	Suppressor with original parent	Suppressor with reciprocal parent
pF29S	Red	Red	Pink
pG46C	Red	Red	Pink
pA50T	Red	Red	Red
pL216Q	Red	Red	White
pC234F	Red	Red	White
pT266I	Red	Red	White
pC333G	Red	Red	Pink
pF354C	Red	Red	Pink
pV367E	Red	Red	Pink
pG370V	Red	Red	Red

tation, and when coupled with the reciprocal parent mutation, their phenotype on sugar MacConkey plates was used as a crude measure of transport activity. As expected, the wild-type strain is red on both lactose MacConkey and melibiose MacConkey plates because it is able to transport effectively and ferment these two sugars. (Note: melibiose is an α-galactoside that is also transported via the lactose permease). The parent strains, G64S and P280L, are severely defective in the transport of either sugar, as evidenced by their white or pink phenotypes on both lactose MacConkey and melibiose MacConkey plates.

All of the suppressors were red when coupled with their original parent strain. This was expected since the suppressors were identified by their red phenotype on melibiose MacConkey plates [16, 17, 27]. Likewise, most of the suppressor mutations alone were also red. To exhibit a red phenotype on MacConkey plates, a mutation usually must exhibit a transport rate that is above 5% of the wild-type transport activity. These results indicate that most of the suppressor-alone strains have at least a low level of lactose transport. An exception was the L216Q mutation which has a white phenotype on lactose MacConkey plates.

Most of the suppressors did not significantly complement a reciprocal parent mutation since they had white or pink phenotypes on MacConkey plates. This was expected based on their low levels of expression described in Table 2. However, two of them, A50T and G370V, which had substantial levels of expression, were also very effective at complementing a defect in the reciprocal parent construct. These results indicate that the A50T and G370V mutations can suppress a defect caused by a loop 2/3 mutation and also suppress a defect caused by a loop 8/9 mutation. It is particularly interesting to note that the A50T suppressor is in the first half of the permease, while G370V suppressor is in the second half.

In vitro Galactoside Transport

To explore further the effects of the suppressor mutations, lactose transport was measured via [^{14}C]-lactose transport assays. To conduct a downhill lactose transport assay, plasmids containing the wild-type or mutant *lacY* genes were transformed into a *lacZ*$^+$ *Escherichia coli* strain (HS4006/F′I^{Q}Z$^+$Y$^-$) which is β-galactosidase positive. When lactose enters the cell, it is rapidly metabolized so that the external lactose concentration is always higher than the internal concentration [29]. Therefore, this in vitro assay measures lactose transport as it moves from a higher to lower concentration, or "downhill".

Figure 2 shows the results of a downhill lactose transport assay involving the wild-type strain, the two original parent mutant strains (pG64S and pP280L), and several suppressor-alone strains. Most of the suppressor-alone strains had relatively low levels of downhill transport. Since previous studies have already shown that the suppressors coupled with the original parent mutation have high levels of transport, these results indicate that the suppressors alter permease structure in a compensatory manner. The first site mutation may disrupt the structure of the permease and the suppressors then counteract this structural change when paired with the disruptive parent mutation in loop 2/3 or loop 8/9. However, when the parent mutation is removed, the suppressor mutation alters permease structure in a way that is inhibitory, presumably because the parent mutation is not being compensated.

Another important aspect of lactose-H$^+$ cotransport is the ability to accumulate sugars against a concentration gradient. To accomplish secondary active transport, the uptake of sugar must be coupled to the uptake of H$^+$ ions so that the proton electrochemical gradient can provide the driving force for the accumulation of sugar [24, 35, 36]. In Fig. 3, the wild-type, parent strains, and suppressor-alone strains were assayed for their ability to accumulate lactose against a gradient. As seen in the downhill assays, most of the suppressor-alone strains were defective in uphill accumulation. These results are also consistent with the idea that the suppressor alters permease structure in a way that compensates for a structural change caused by the parent mutation in loop 2/3 or loop 8/9.

To gain a greater understanding of the nature of the

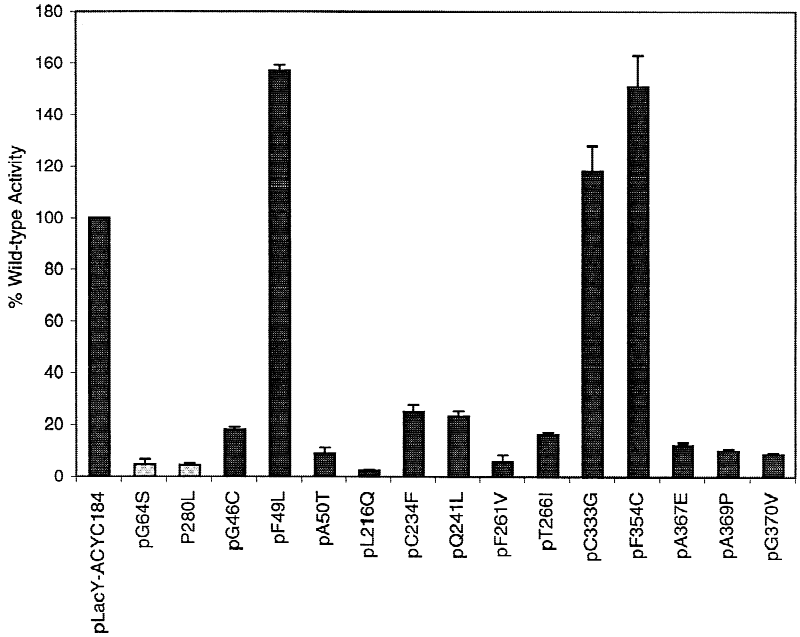

Fig. 2. Downhill lactose transport in the wild-type and single suppressor mutant strains. [^{14}C]-Lactose uptake was measured as described in the Materials and Methods in strain HS4006/F′I^{Q}Z^{+}Y carrying the wild-type or designated mutant plasmids.

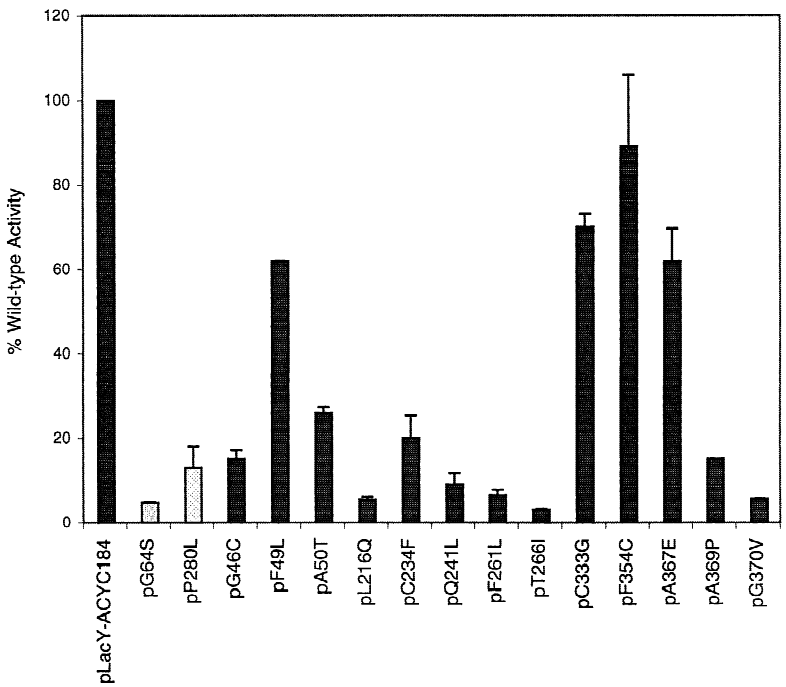

Fig. 3. Uphill lactose transport in the wild-type strain and single suppressor mutant strains. [^{14}C]-Lactose uptake was measured as described in the Materials and Methods in strain T184 carrying the wild-type or designated mutant plasmids.

effects of the suppressor mutations on permease function, several of the suppressor-alone strains were subjected to a kinetic analysis. As shown in Table 5, the suppressor-alone strains had effects on both the K_m and V_{max} values for transport, although the V_{max} effects tended to be quantitatively greater. A few mutations in the second half of the permease (F261V, V367E, and G370V) had moderately elevated K_m values. Previous studies have suggested that some of the sugar binding residues may be in the second half of the permease and the suppressor mutations may be affecting this region [1, 2, 6, 9]. Even so, it should pointed out that the observed

Table 5. Kinetic analysis of several suppressor-alone strains[a]

Strain	K_m ± SE (mM)	V_{max} ± SE (nmol lactose/ min.mg protein)	V_{max}/K_m	Reference
Wild-type	0.5 ± 0.1	197.0 ± 37.6	394.0	22
pG46C	1.1 ± 0.1	18.1 ± 0.4	16.5	This study
pA50T	1.1 ± 0.2	15.2 ± 4.9	13.8	22
pL216Q	1.8 ± 0.2	9.3 ± 1.2	5.2	This study
pQ241L	0.5 ± 0.1	20.9 ± 0.5	41.8	This study
pF261V	2.3 ± 0.3	8.5 ± 1.4	3.7	This study
pC333G	0.7 ± 0.2	208.0 ± 16.0	297.1	This study
pF354C	0.5 ± 0.1	441.2 ± 12.0	882.4	This study
pV367E	2.4 ± 0.7	92.0 ± 15.1	38.3	This study
G370V	2.4 ± 0.4	50.7 ± 14.7	21.1	22

[a] Lactose uptake and kinetic values were determined as described under Materials and Methods.

K_m and V_{max} values may reflect not only sugar binding and translocation, but also cation binding and translocation.

Many of the suppressors show V_{max} values that are less than 10% of wild type (G46C, A50T, L216Q, and F261V) and all but two of them (C333G and F354C) are inhibitory with regard to V_{max} values. The two strains, which do not inhibit the V_{max} value (C333G and F354C), both involve amino acid substitutions with smaller side chain volumes than the wild-type residue. By comparison, most of the other strains (except for L216Q and F261V) involve amino acid substitutions that are larger than the wild-type residue. Overall, the results of Table 5 indicate that most of the suppressors have a negative effect on the velocity of lactose transport. These results are consistent with the idea that most of the suppressor-alone strains affect permease structure in a way that inhibits conformational changes required for H^+ and lactose transport.

As mentioned earlier, when the suppressors were coupled with the reciprocal parent, most of them failed to express detectable levels of lactose permease in the membrane (refer back to Table 2). However, two suppressors, A50T and G370V, were expressed when coupled with either parent mutation. To determine the effects of these suppressors, a kinetic analysis was also conducted (*see* Table 6). As shown previously, the parent mutations primarily affected the velocity of lactose transport rather than the affinity of the sugar as judged by the apparent K_m. The wild-type strain exhibited an apparent K_m for lactose of 0.5 mM with a V_{max} of 197 nmol/min.mg of protein. The G64S and P280L strains showed K_m values of 0.7 mM and 0.1 mM, respectively. The V_{max} values were severely depressed. Both the G64S and P280L strains had V_{max} values for lactose transport that were 5% or less than that of the wild-type strain. The A50T and G370V suppressors were able to restore high levels of lactose transport due to an enhancement in the rate of lactose transport. The suppressors had V_{max} values that ranged from approximately 33% to 100% of wild-type levels. Taken together, these results indicate that the suppressor is altering conformation in a compensatory manner and permitting conformational changes that are inhibited in the parent strain.

Discussion

The results of the current study have shown that second site mutations, which suppress loop 2/3 and loop 8/9 first site mutations, appear to act in a compensatory manner. In other words, the first site mutation disrupts the structure of the permease and the suppressors counteract this structural change when paired with the disruptive parent mutation in loop 2/3 or loop 8/9. However, when the parent mutation is removed, the suppressor mutation usually alters permease structure in a way that is inhibitory, presumably because the parent mutation is not being compensated. It was also observed that two strains, which did not inhibit the V_{max} value (C333G and F354C), both involve amino acid substitutions with smaller side chain volumes than the wild-type residue. By comparison, most of the other strains, which were inhibitory for the velocity of lactose transport, involved amino acid substitutions that are larger than the wild-type residue. These results indicate that most of the suppressor alone strains affect permease structure in a way that inhibits conformational changes required for lactose transport and that bulky substitutions are more likely to be inhibitory.

Several tertiary models for the lactose permease have been proposed based on a variety of data including bifunctional crosslinking, the locations of putative salt bridges, and bioinformatics [11, 16, 34, 37, 38]. Although the precise arrangement of helices is not likely to be accurate until the permease has been crystallized, a unique aspect of our tertiary model is the proposition that the two halves of the lactose permease are rotationally symmetrical [11, 16]. In the current study, two different suppressor mutations, A50T and G370V, can complement a defect in the loop 2/3 motif and also complement the corresponding defect in the loop 8/9 motif. According to our tertiary model, these suppressors are located at the TMS-2/TMS-11 interface between the two halves of the permease (*see* Fig. 4). We propose that the loop 2/3 and loop 8/9 mutations alter the structure at the interface in a way that disrupts conformational changes required for lactose transport. This hypothesis is consistent with the observation that the loop 2/3 and loop 8/9 mutations inhibit the V_{max} values for lactose transport. For a rotationally symmetrical structure, a loop 2/3 and a loop 8/9 mutation could have a similar net effect on the global

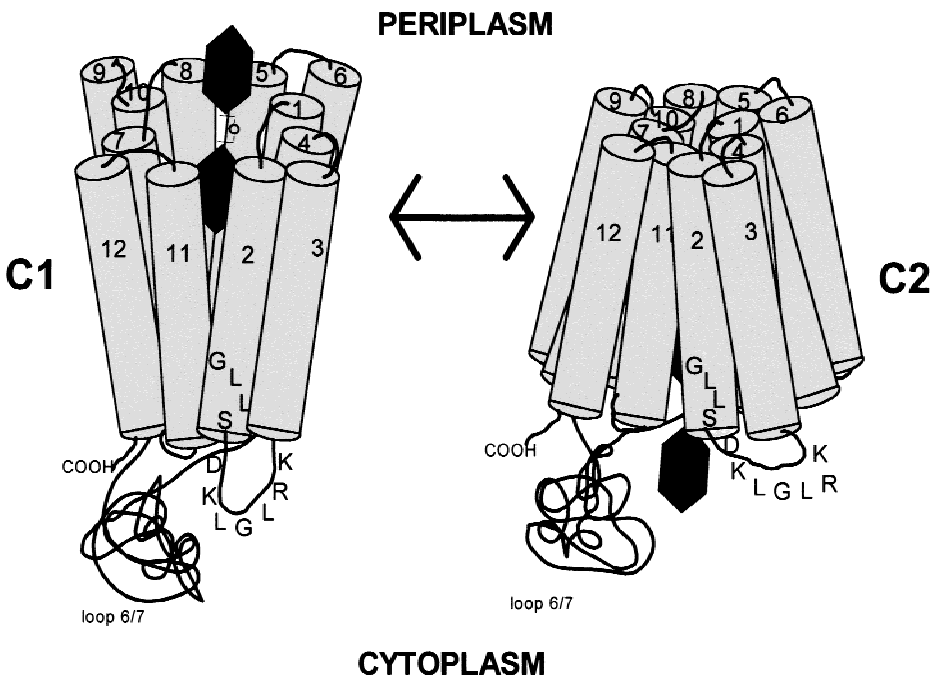

Fig. 4. Putative conformational changes in the lactose permease. The three dimensional arrangement of transmembrane domains is described in Ref. 11 and this mechanism of conformational change was proposed in Ref. 16.

Table 6. Kinetic analysis of lactose uptake in wild-type, parent, and double mutant strains[a]

Strain	K_m ± SE (mM)	V_{max} ± SE (nmol lactose/ min.mg protein)	V_{max}/K_m	Reference
Wild-type	0.5 ± 0.1	197.0 ± 37.6	394.0	22
G64S	0.7 ± 0.3	10.1 ± 3.1	14.4	13
P280L	0.1 ± 0.1	6.1 ± 3.1	61.0	22
A50T/G64S	0.4 ± 0.1	198.0 ± 3.0	495.0	This study
A50T/P280L	0.9 ± 0.4	66.2 ± 23.6	73.6	22
G370V/P280L	1.1 ± 0.2	199.9 ± 34.7	181.7	22
G370V/G64S	0.5 ± 0.1	87.0 ± 8.0	174.0	This study

[a] Lactose uptake and kinetic values were determined as described under Materials and Methods.

conformation of the permease if the mutation affects the relative orientations of the two halves of the permease (*also see* Reference 27). If so, a suppressor with a compensatory effect at the interface could correct a defect imposed by a loop 2/3 mutation and also correct a defect imposed by a loop 8/9 mutation. With regard to the A50T and G370V mutations, the results obtained in this study are adequately explained by such a rotationally symmetrical model.

It is not yet clear why most of the suppressors have negligible levels of expression when coupled with the reciprocal parent (*see* Table 2). It seems unlikely that these double mutants have a defect in protein insertion since the parent mutations and suppressor mutations rarely involve changes in charged amino acid residues. (The only exception is the V367E mutation). Instead, it would seem more probable that the combination of suppressor and reciprocal parent mutation produces a permease that is highly susceptible to protein degradation.

A symmetrical model for structure and function is quite reasonable, and perhaps expected, based on the nature of the *lacY* gene sequence and the sequences found in the MFS [26]. As originally proposed by Maiden et al. [20] and discussed thoroughly in a recent review [26], the genes that encode the lactose permease and other members of the MFS have arisen by a gene duplication/fusion event in which a primordial gene encoding six transmembrane segments duplicated and fused to encode a membrane protein with twelve transmembrane segments. Based on their evolutionary origins, the two halves of the proteins would be expected to have very similar folding patterns and to interact with each other at an interface. The results of our study can be explained by such a structural arrangement. Furthermore, the kinetic analyses of the mutants suggest that the interface is a critical region that is involved with conformational changes associated with lactose transport. In particular, the effect of the parent mutations is to lower the V_{max} for lactose uptake while the suppressors act to restore maximal transport velocity.

Finally, the results of the current study can be discussed with regard to the role of the conserved motif in loop 2/3 and loop 8/9. The conservation of the decapeptide motif, G-X-X-X-D/E-R/K-X-G-R/K-R/K, among a large group of solute transporters suggests that it may play a general role in transport. The loop 2/3 motif begins within the cytoplasmic portion of TMS-2 and includes the hydrophilic loop to TMS-3; the loop 8/9 motif begins within the cytoplasmic portion of TMS-8 and includes the hydrophilic loop to TMS-9. According to our tertiary model, TMS-2 and TMS-8 are at the interface between the two halves of the permease. Based on this location and the preponderance of charged amino acid residues in the conserved motif, we propose that its function is to precisely position the two halves of the permease within the plane of the lipid bilayer. This would maintain the interface between the two halves of the permease in order to perform conformational changes associated with lactose transport.

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