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Mutants of the Lactose Carrier of *Escherichia coli* Which Show Altered Sugar Recognition Plus a Severe Defect in Sugar Accumulation

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Abstract. Lactose and melibiose are actively accumulated by the wild-type *Escherichia coli* lactose carrier, which is an integral membrane protein energized by the proton motive force. Mutants of the *E. coli* lactose carrier were isolated by their ability to grow on minimal plates with succinate plus IPTG in the presence of the toxic lactose analog β-thio-*o*-nitrophenylgalactoside (TONPG). TONPG-resistant mutants were streaked on melibiose MacConkey indicator plates, and red clones were picked. These melibiose positive mutants were then streaked on lactose MacConkey plates, and white clones were picked. Transport assays indicated that the mutants had altered sugar recognition and a defect in sugar accumulation. The mutants had a poor apparent K_m for both lactose and melibiose in transport. One mutant had almost no ability to take up lactose, but melibiose downhill transport was 58% (V_{max}) of normal. All of the mutants accumulated methyl-α-D-galactopyranoside (TMG) to only 8% or less of normal, and two failed to accumulate. Immunoblot analysis of the mutant lactose carrier proteins indicated that loss of sugar transport activity was not due to loss of expression in the membrane. Nucleotide sequencing of the *lac*Y gene from the mutants revealed changes in the following amino acids of the lactose carrier: M23I, W151L, G257D, A295D and G377V. Two of the mutants (G257D and G377V) are novel in that they represent the first amino acids in periplasmic loops to be implicated with changes in sugar recognition. We conclude that the amino acids M23, W151, G257, A295 and G377 of the *E. coli* lactose carrier play either a direct or an indirect role in sugar recognition and accumulation.

Key words: Lactose — Melibiose — Transport — Recognition — Mutant — Accumulation — Permease — Sugar — Symport

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Introduction

The lactose carrier of *Escherichia coli* is a proton-sugar symporter driven by the electrochemical proton gradient (Mitchell, 1963; Poolman & Konings, 1993; Krämer, 1994; Maloney, 1994). The gene encoding the lactose carrier, *lac*Y, a component of the *lac* operon (Müller-Hill, 1996), has been cloned (Teather et al., 1978), its nucleotide sequence determined (Büchel et al., 1980) and its gene product purified to homogeneity (Newman et al., 1981). Based on a variety of data, the lactose carrier is thought to be an integral membrane protein with 12 transmembrane α-helices and with the N- and C-termini on the cytoplasmic side of the inner membrane of *E. coli* (Calamia & Manoil, 1980; Foster et al., 1983; Frillingos & Kaback, 1996; Jung et al., 1994; Kaback et al., 1993; Kaback et al., 1994; Kaback & Wu, 1997; Ujwal et al., 1995; Varela & Wilson, 1996; Vogel et al., 1985). Although little is known concerning the 3-dimensional structure of the lactose carrier, some useful structural information was obtained by studies of salt bridges between various charged amino acids (Kaback & Wu, 1997; King et al., 1991; Lee et al., 1992; Lee et al., 1993; Lee et al., 1995). The lactose carrier is the most extensively characterized secondary active transporter (Kaback & Wu, 1997; Varela & Wilson, 1996), making it a good model for the study of solute transport systems in bacteria.

Early work by Shuman and Beckwith (1979) of sugar selection and recognition in the lactose carrier of *E. coli* resulted in the isolation of a mutant with significant maltose transport activity. Later, additional mutants that transported maltose were isolated. These mutants had

amino acid substitutions at residues Ala-177 and Tyr-236 (Brooker & Wilson, 1985). Studies of sucrose- or arabinose-positive mutants also revealed mutations affecting Ala-177 in the lactose carrier (King & Wilson, 1990; Goswitz & Brooker, 1993). Selection and sequencing of mutants in minimal media containing maltose and cellobiose (an inhibitor of maltose transport) identified mutations resulting at residues Tyr-236, Ser-306 and Ala-289 (Collins et al., 1989). Additional sugar recognition mutants had alterations at Thr-266, Ile-303, Lys-319 and His-322 (Eelkema et al., 1991; Franco et al., 1989; Markgraf et al., 1985). Other investigations have implicated Cys-148, Cys-154, Asp-237, Asp-240, Glu-269, Arg-302, Glu-325 and Lys-358 in the mechanism of sugar transport (Brooker, 1991; Eelkema et al., 1991; Franco et al., 1989; Kaback & Wu, 1997; Markgraf et al., 1985; Olsen & Brooker, 1989; Ujwal et al., 1995). Recent experiments designed to study mutants that transport melibiose better than lactose revealed changes in residues Tyr-26, Phe-27, Phe-29, Asp-240, Leu-321 and His-322 (Varela et al., 1997).

In an attempt to further our understanding the sugar recognition site in the lactose carrier we have isolated additional mutants, determined the sequences of their *lac*Y genes and characterized their transport. All of the mutants had a poor apparent K_m for lactose and melibiose. In addition, all of the mutants had a very severe defect in accumulation of the lactose analogue TMG.

Materials and Methods

Reagents

Bacteriological media were from Difco. Lactose, melibiose, TMG, methyl-α-D-galactopyranoside (α-MG), isopropyl-β-D-thio-galactopyranoside (IPTG), β-thio-*o*-nitrophenyl-β-D-galactopyranoside (TONPG), *o*-nitrophenyl-β-D-galactopyranoside (ONPG) and (3-[N-morpholino] propanesulfonic acid) buffer (MOPS) were purchased from Sigma. Materials for PCR-based DNA amplification and DNA cycle sequencing were from Perkin-Elmer. Manipulation of DNA was performed using kits from New England Biolabs, Qiagen and Pharmacia Biotech. Radioactively labeled [^{14}C]-lactose was purchased from Amersham. Radioactively labeled [^{14}C]-TMG was from DuPont New England Nuclear. Radioactively labeled [^{3}H]-melibiose was the generous gift of Prof. Gérhard Le Blanc (Départment de Biologie Cellulaire du CEA, Villefranche-sur-mer, France). Radiolabeled sugars were purified by descending paper chromatography (Whatman No. 3MM paper) using a 3:1 propanol:water mixture.

Bacterial Strains

All strains are derivatives of *E. coli* K-12. The relevant genotypes are shown in Table 1. Growth of all DP90 cultures was at 37°C in LB broth or on LB agar in the presence of nalidixic acid (chromosomally encoded nalidixic acid resistance gene) at 20 μg/ml. Growth of DW2 cultures was performed at 37°C in LB broth containing 20 μg/ml streptomycin (chromosomally encoded streptomycin resistance gene).

Isolation of Mutants

Bacterial DP90/F′ *lac* mutants were isolated by incubation at 37°C on minimal plates containing 0.2% succinate, 3 mM TONPG and 0.5 mM IPTG. Colonies that appeared on these plates were used to inoculate 1% melibiose MacConkey indicator plates containing 0.5 mM IPTG. These plates were then incubated overnight at 37°C. Next, red colonies were used to inoculate 1% lactose MacConkey plates which were then incubated overnight at 37°C. Bacterial mutants that formed white colonies were chosen for further study (Table 2).

To determine whether the phenotype was due to a mutation on the F′ factor we transferred this F′ factor to *E. coli* DW2 (Δ*lac,* Δ*mel*B) by mating and examined the resulting phenotype of DW2/F′ *lac* transconjugant. In all cases, the original fermentation phenotypes were observed.

Amplification and Sequencing of Mutants

The *lac*Y gene of the selected bacterial mutants was present on an F′ factor. Cells containing the *lac*Y gene on the F′ factor were grown overnight in LB broth at 37°C to saturation. Genomic DNA was prepared from the saturated cells according to the method of Wilson (1988). Using primers prepared by the Harvard Medical School Oligonucleotide Synthesis Facility and specific for the flanking regions of the *lac*Y gene, the DNA encoding the lactose carrier protein was amplified by PCR as described previously (Varela et al., 1997). The amplified DNA was isolated and purified with a QIAquick PCR purification kit from Qiagen. The amplified and purified *lac*Y mutant DNA was subjected to complete nucleotide sequencing by the cycle sequencing method (Perkin-Elmer) (Sanger et al., 1977).

Sugar Transport and Kinetic Analyses

E. coli DP90 cells, harboring the F′ factor and containing the mutant *lac*Y DNA (Table 1), were grown overnight to saturation in LB broth in the presence of nalidixic acid while shaking at 37°C. At this temperature, the normal chromosomal melibiose carrier (MelB) is inactive. At all times the transport values of the DP90Δ*lac*Y*mel*B$^+$ control strain were subtracted from all transport data. Overnight grown cells were used to inoculate fresh LB broth containing the appropriate inducer, depending on the sugar used in the transport assay, and were grown at 37°C to mid-log phase. The cells were then harvested by centrifugation at 5000 rpm in a Sorvall SS-34 rotor at 4°C. The cells were washed 2–3 times with an equal volume of 100 mM MOPS buffer (pH 7) containing 0.5 mM $MgSO_4$ and 1 mM DTT. The washed cells were resuspended in the same buffer to a concentration of 0.45 mg of protein/ml and placed on ice. After equilibration at room temperature or 37°C for 20 min, the transport assays were initiated by the addition of radioactive sugar. After incubation, 0.2 ml samples were removed, filtered through a 0.65 μm pore size nitrocellulose filter and washed with MOP buffer containing 0.5 mM $HgCl_2$. The filters were dissolved in 4 ml Liquiscint (National Diagnostics) containing 10% water. The radioactivity was counted using a liquid scintillation counter.

Kinetic analyses were conducted for downhill transport of sugars as previously described (Varela et al., 1997). Briefly, initial transport rates for lactose and melibiose were determined with various sugar concentrations after cells were incubated in the presence of IPTG or α-MG, respectively, and 0.2 ml of cell suspension were removed for filtering and counting. Apparent K_m and V_{max} values were determined by the Lineweaver-Burk double reciprocal plot.

Table 1. Bacterial strains and genotypes used

Strain	Genotype (chromosome/F′ factor)	Reference or source
DW2	*lac*I$^+$ Δ(*ZY*) *melA*$^+$Δ*B rspL*/	Wilson & Wilson, 1987
DP90-Y$^+$	Δ*lac-pro Nal*^{r}/*F′ lacI*^{Q}Z$^+$Y$^+$ *proAB*	Hobson et al., 1977
DP90-ΔY	Δ*lac-pro Nal*^{r}/*F′ lacI*^{Q}Z$^+$ ΔY *proAB*	Varela et al., 1997
DP90-M23I	Δ*lac-pro Nal*^{r}/*F′ lacI*^{Q}Z$^+$ Y(Met 23 → Ile) *proAB*	This work
DP90-W151L	Δ*lac-pro Nal*^{r}/*F′ lacI*^{Q}Z$^+$ *Y*(Trp-151 → Leu) *proAB*	This work
DP90-G257D	Δ*lac-pro Nal*^{r}/*F′ lacI*^{Q}Z$^+$ Y(Gly-257 → Asp) *proAB*	This work
DP90-A295D	Δ*lac-pro Nal*^{r}/*F′ lacI*^{Q}Z$^+$ Y(Ala-295 → Asp) *proAB*	This work
DP90-G377V	Δ*lac-pro Nal*^{r}/*F′ lacI*^{Q}Z$^+$ Y(Gly-377 → Val) *proAB*	This work

Immunochemical Assay of the Lactose Carrier

The amount of normal and mutant lactose carrier protein expressed in the membrane was measured by the immunoblot method of Lolkema et al. (1988). A polyclonal antibody directed against the C-terminal decapeptide of the lactose carrier was used for detection.

Results

Isolation and Sequencing of Bacterial Mutants

E. coli cells containing an F′ factor (Table 1) encoding the *lac* operon genes were plated onto agar plates with minimal medium containing succinate as the sole carbon source plus a toxic analog of lactose (TONPG), which is a hydrophobic substrate for the wild-type lactose carrier (Müller-Hill et al., 1968). There is cotransport of TONPG and a proton into the cell. This is followed by diffusion of the hydrophobic molecule out of the cell and reaccumulation. The continuous rapid transport of the sugar depletes the cell of its proton motive force. This results in reduced ATP synthesis and failure of the cell to grow. A mutation in the lactose carrier that reduces proton cotransport or reduces the rate of TONPG uptake will permit the cell to grow on these plates. Thus, TONPG-resistant mutants are defective in sugar recognition or energy coupling with protons or both (Müller-Hill et al., 1968). Colonies were picked which were fermentation positive (red) on melibiose MacConkey indicator plates containing IPTG and fermentation negative (white) on lactose MacConkey plates without IPTG (Table 2). The F′ factor DNA from each mutant was isolated. The *lac*Y gene of each mutant was amplified by PCR and completely sequenced (Sanger et al., 1977).

Nucleotide sequencing revealed single point mutations in the *lac*Y gene of each of the TONPG-resistant mutants isolated. The following changes in deduced amino acid sequences were found: M23I, W151L, G257D, A295D and G377V. Two of the TONPG-resistant mutants had the same mutation (G257D). The M23I mutation is in helix I (Fig. 1). The W151L mutation is in helix V. A295D is in helix IX. The G257D mutation is located in the periplasmic loop between helices VII and VIII of the carrier. G277V is in the periplasmic loop between helices XI and XII. These latter two mutants are novel in that they represent the first amino acids in periplasmic loops to be implicated with changes in sugar recognition.

Table 2. Fermentation by lactose carrier mutants

Strain	Colony phenotypes[a]	
	Lactose MacConkey	Melibiose MacConkey
Y$^+$	Red	Red
ΔY	White	White
M23I	White	Red
W151L	White	Red
G257D	White	Red
A295D	White	Red Center
G377V	White	Red Center

[a] Fermentation studies were conducted using MacConkey agar plates containing either 30 mM lactose or 30 mM melibiose plus 1 mM IPTG and incubated overnight at 37°C.

Downhill ONPG Transport in Mutants

Since the mutants isolated here were selected for growth in TONPG, one would expect that the transport of the structurally similar sugar, ONPG, would be affected. Measurement of 2 mM ONPG entry was performed. This sugar is hydrolyzed within the cell by β-galactosidase to galactose and *o*-nitrophenol, which is yellow in color. The results, shown in Table 3, indicate that ONPG entry was significantly reduced for all of the mutants.

Downhill Lactose Transport

When *E. coli* cells containing the *lac* operon system are grown in the presence of the inducer IPTG, β-galactosi-

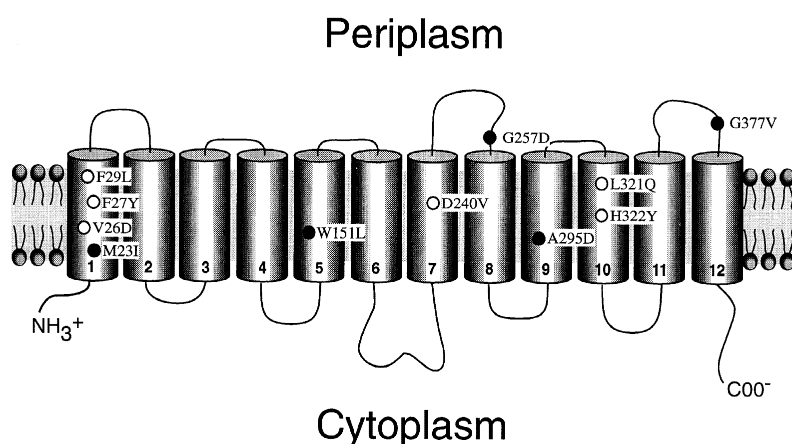

Fig. 1. A two-dimensional model of the lactose carrier of *E. coli* showing the locations of point mutations. The locations of the individual changes in amino acid sequence of the lactose carrier are shown in this 2-dimensional model. The first one-letter code and the number represent the wild-type amino acid along the polypeptide chain of the lactose carrier. The second one-letter code represents the amino acid alterations encoded in the *lac*Y gene of the mutants isolated in this study (black circles) and of a previous study (white circles) in which the sugar transport phenotypes were similar. That is, the transport of melibiose was better than the transport of lactose, and melibiose accumulation was defective (Varela et al., 1997). For a comprehensive illustration of lactose carrier mutants that affect lactose accumulation, please refer to Fig. 1 of Varela and Wilson (1996).

Table 3. Downhill transport of ONPG (2 mM) and lactose (0.4 mM)

Strain	% of normal	
	ONPG	Lactose
Y^+	100	100
ΔY	0	0
M23I	12	6
W151L	25	3
G257D	9	10
A295D	15	3
G377V	34	14

Cells were grown in LB in the presence of 1 mM IPTG (to induce the lactose carrier and β-galactosidase).

dase and the lactose carrier are produced. Lactose that enters the cell is hydrolyzed to glucose and galactose by β-galactosidase, and the hexoses are metabolized. Thus, the concentration of lactose inside the cell remains low. Consequently, the entry of lactose under these conditions is thermodynamically downhill, and accumulation against a concentration gradient (uphill) does not occur. As shown in Table 3, lactose (0.4 mM) downhill transport was severely defective. In the mutants M23I, W151L, G257D and A295D, lactose entry was 10% or less of normal, whereas the G377V mutant had 14% of the normal transport rate.

The study of the kinetics of lactose transport (Table 4) indicated that the apparent K_m was elevated in all cases, the apparent V_{max} was from 8 to 65% of normal. In one case, (W151L) the rate of transport was so low that kinetics could not be performed. The V_{max}/K_m ratio for lactose downhill transport was reduced for each of the lactose carrier mutants. Thus, all of these mutations cause a severe defect in lactose transport.

Downhill Melibiose Transport

When "downhill" transport of melibiose was desired, α-galactosidase was induced with α-MG added to the growth medium (as well as IPTG to induce the carrier). If the cells are grown at 37 to 42°C, the normal melibiose carrier (MelB) shows no transport activity, as it is temperature sensitive. Thus, cells grown at 37°C with IPTG and α-MG show melibiose transport by the lactose carrier.

The kinetics of downhill melibiose transport was studied (Table 4). The apparent K_m for the sugar was reduced in every case. The apparent V_{max} for melibiose transport was between 35 and 58% of normal. For each mutant, the V_{max}/K_m ratio of melibiose downhill transport was reduced.

Uphill Transport (Accumulation) of Sugars

The nonmetabolizable galactoside TMG was used to test for accumulation. A severe defect in accumulation by the mutants was observed (Table 5). Two of the mutants, W151L and A295D, completely failed to accumulate this sugar. The other three mutants accumulated to only 5 to 8% of the normal level.

When accumulation of melibiose was to be studied, the lactose carrier was induced with IPTG, which induces the *lac* operon but not the melibiose operon. Thus, α-galactosidase was not induced, and the transported melibiose accumulated as the free sugar within the cell. Melibiose accumulation by the mutants was also severely defective. Two mutants (W151L and A295D) had 0 and 2% of normal accumulation, respectively. Two (G257D and G377V) gave 11% of the normal accumulation, and M23I had 23% of normal accumulation.

Table 4. Kinetics of lactose and melibiose downhill transport

Strain	Lactose[a]			Melibiose[b]		
	K_m (mM)[c]	V_{max} (nmoles/mg Protein)[c]	V_{max}/K_m	K_m (mM)[c]	V_{max} (nmoles/mg Protein)[c]	V_{max}/K_m
Y^+	1.1	116	105.5	0.7	38	54.3
M23I	10	76	7.6	1.9	13.5	7.1
W151L[d]	—	—	—	8.8	21.9	2.5
G257D	10.8	35	3.2	2.5	10.9	4.4
A295D	2.3	10	4.3	5.2	10.9	2.1
G377V	2.3	43	18.7	2.9	18.3	6.3

[a] To measure the kinetics of lactose transport, cells were grown in the presence of 1 mM IPTG (to induce the carrier).
[b] To measure the kinetics of melibiose transport, cells were grown with 1 mM IPTG (to induce the carrier) and 1 mM α-MG (to induce α-galactosidase).
[c] Values here should be referred to as 'apparent' since the lactose carrier has two substrates (proton and sugar).
[d] The transport rate was too low to obtain reliable kinetic data.

Table 5. Accumulation of sugars by mutants of the lactose carrier

Strain	% Normal	
	TMG Accumulation	Melibiose Accumulation
Y^+	100	100
ΔY	0	0
M23I	8	23
W151L	0	0
G257D	5	11
A295D	0	2
G377V	6	11

Cells were grown in LB plus 1 mM IPTG to induce the lactose carrier. To measure TMG accumulation, cells were exposed to 0.1 mM [^{14}C]-TMG for 10 min. The normal control accumulated TMG 30-fold. Data are the average of 3 independent experiments. Melibiose accumulation was measured in the presence of 0.2 mM [^{3}H]-melibiose at 25°C after 10 min. Data are the average of 3 independent experiments.

Table 6. Immunochemical assay of lactose carrier mutants

Strains	Percent of Wild-type
Y^+	100
M23I	164
W151L	58
G257	140
A295D	44
G377V	30

Immunological Assay for Normal and Mutant Lactose Carrier Protein

The amount of carrier protein was measured using the immunoblot assay (Lolkema et al., 1988). The results are shown in Table 6. Expression of the carrier protein was only somewhat reduced for the W151L, A295D and G377D mutants. The M23I and G257 mutants were expressed at greater than normal amounts. Thus, the loss of transport activity in the mutants is not due to diminished expression or stability of the carrier in the membrane.

Discussion

There were striking alterations in sugar recognition in each of these mutants. The affinity and V_{max} for lactose and melibiose were reduced. It is quite striking that all of the mutants were white on lactose MacConkey indicator plates and red (or red center) on melibiose MacConkey plates. It is interesting to note that three of the mutants (M23I, G257D and G377D) which were white on lactose MacConkey plates had a moderately good apparent V_{max} (35 to 67% of normal) for downhill lactose transport. The white phenotype on the lactose MacConkey plates is probably due to the failure of the carrier to accumulate lactose, which is required for full induction of β-galactosidase.

The wild-type lactose carrier transports melibiose and lactose at similar rates (Hobson et al., 1977; Olsen & Brooker, 1989). One category of mutants had a more severe defect for lactose than for melibiose. The W151L mutant, for example, had almost no lactose uptake but 58% of the normal apparent V_{max} for melibiose (Table 4). The A295D mutant took up lactose at 9% of the normal rate and melibiose at 29% of the normal rate (Table 4). Previously, we found mutants with similar properties although the locations of the amino acid replacements in the lactose carrier were different from those found here. In this earlier work (Fig. 1), the amino acids implicated in having the above transport phenotype included Tyr-

26, Phe-27, Phe-29, Asp-240, Leu-321 and His-322 (Varela et al., 1997).

Accumulation of sugars against a concentration gradient was severely defective in all of these mutants. The accumulation of TMG was particularly defective. In two cases (W151L and A295D) TMG accumulation was zero, and the remaining three mutants had 8% or less of normal accumulation. Since the accumulation of melibiose was also defective, the mechanism of coupling proton uptake to sugar uptake must be defective.

With regard to the location of the residues targeted by mutations, three are located in transmembrane α-helices and two are in periplasmic loops (Fig. 1). The two found in loops are the first mutations with alterations in sugar recognition to be found in periplasmic loops. Gly-257 is located in the loop between helices VII and VIII of the lactose carrier. Jessen-Marshall and Brooker (1996) found that the second-site revertant Gly-257 → Asp restored lactose transport activity that was lost by the Asp-68 → Ser mutation. The Asp-68 → Ser/Gly-257 → Asp revertant completely restored normal lactose accumulation, and downhill transport of lactose was 40% of normal. The loss of a negative charge in the cytoplasmic loop between helices II and III was thus compensated for by the gain of a new negative charge in the periplasmic loop between helices VII and VIII. The second periplasmic loop residue (Gly-377) targeted by a mutation affecting sugar recognition was present in the loop between helices XI and XII of the lactose carrier.

With respect to degree of conservation in the LacY family of transporters of the residues implicated in this study, M23, W151 and A295 are found in LacY of *Klebsiella pneumoniae* and in RafB of *E. coli,* according to the amino acid sequence alignments of Griffith and Samson (1998). However, these residues, all of which were found in transmembrane spanning helices, were absent in the other members of the LacY family: CscB (sucrose carrier), NugP (nucleoside carrier) and XapB (xanthosine carrier) of *E. coli.* In addition, the residues implicated in the present study which were found in periplasmic loops, G257 and G377, were conserved in all sugar-proton symporters (LacY of *K. pneumoniae,* RafB, CscB) of the LacY family but not in the nucleoside-proton symporters (NugP and XapB).

In summary, the active transport (accumulation) of sugar was severely defective in all mutants affecting amino acids M23, W151, G257, A295 and G377 of the lactose carrier of *E. coli.* In addition, mutations occurring at W151 and A295 demonstrated more severe defects in the transport of lactose than of melibiose, suggesting separate sugar binding sites for these sugars, consistent with previous lactose carrier mutants (Varela et al., 1997). Lastly, we demonstrate here that residues G257 and G377 in periplasmic loops between helices VII and VIII and XI and XII, respectively, whether directly or indirectly, affect sugar recognition and accumulation in the lactose carrier.

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