JOURNAL OF BACTERIOLOGY, June 2003, p. 3672–3677 Vol. 185, No. 12
0021-9193/03/$08.00+0 DOI: 10.1128/JB.185.12.3672–3677.2003

Altered Substrate Selection of the Melibiose Transporter (MelY) of *Enterobacter cloacae* Involving Point Mutations in Leu-88, Leu-91, and Ala-182 That Confer Enhanced Maltose Transport

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We isolated mutants of *Escherichia coli* HS4006 containing the melibiose-H^+ symporter (MelY) from *Enterobacter cloacae* that had enhanced fermentation on 1% maltose MacConkey plates. DNA sequencing revealed three site classes of mutations: L-88-P, L-91-P, and A-182-P. The mutants L-88-P and L-91-P had 3.6- and 5.1-fold greater maltose uptake than the wild type and enhanced apparent affinities for maltose. Energy-coupled transport was defective for melibiose accumulation, but detectable maltose accumulation for the mutants indicated that active transport is dependent upon the substrate transported through the carrier. We conclude that the residues Leu-88, Leu-91 (transmembrane segment 3 [TMS-3]), and Ala-182 (TMS-6) of MelY mediate sugar selection. These data represent the first MelY mutations that confer changes in sugar selection.

The melibiose transporter of *Enterobacter cloacae* (MelY) is a secondary active transporter and catalyzes the symport (co-transport) of the α-galactoside sugar melibiose and cation (22, 23). The gene encoding the melibiose transporter, *melY*, has been cloned, sequenced (22), and found to reside in an inducible operon in the bacterial genome (23). The deduced amino acid sequence of the *melY* gene has shown a highly hydrophobic protein of 425 residues and 12 predicted transmembrane segments (TMS) (22). Phylogenetic analyses show that the MelY transporter is a member of oligosaccharide H^+ symporter family 5 and shares highly conserved amino acid sequence motifs with members of the major facilitator superfamily (MFS) (23, 24, 27, 34). MelY is distinct from MelB (27) but shows high sequence identity (22, 23) with the lactose carrier of *Escherichia coli* (LacY) (7), the raffinose transporter of *E. coli* (RafB) (1), the sucrose permease of *E. coli* (CscB) (2, 26), the lactose carrier of *Citrobacter freundii* ($LacY_{Cf}$) (18), and the lactose carrier of *Klebsiella pneumoniae* ($LacY_{Kp}$) (20).

Based on multiple sequence and two-dimensional structural analyses of members of the MFS specific for a diverse array of substrates, it has been predicted that the three-dimensional structures of these seemingly distinct transporters are similar (10, 11, 24, 32, 33). This suggests that MFS transporters function by a common transport mechanism in which substrate specificities are determined by subtle differences in sequence. The homology shared between LacY and MelY (7, 22, 27, 34) and the differences in substrate selection profiles between them provide a unique opportunity for a comparative study.

Shuman and Beckwith first isolated mutants of the *E. coli* lactose carrier that transport maltose (30). The lactose carrier of *E. coli* is a well-studied and important model system for the study of secondary active transport, and it consists of an integral membrane protein of 417 amino acids with 12 transmembrane α-helices residing in the cytoplasmic membrane (15, 16, 34). Although the problem of substrate selection has been well documented in LacY (5, 6, 17, 21, 29; reviewed in reference 34), it is unknown whether any of its homologs dictate substrate selection by similar mechanisms. Sugar selection mutations in TMS-3 have thus far not been found in LacY or any of its homologs.

Here we report the sequencing and characterization of MelY mutations that had enhanced maltose uptake. We implicate novel amino acid residues Leu-88 and Leu-91 within TMS-3 of MelY and Ala-182 within TMS-6 in the transport of maltose, indicating a role for these residues in the determination of sugar selection.

Isolation of mutants. Table 1 shows the bacterial strains and plasmids used in this study. All strains are derivatives of *E. coli* K-12 in order to provide the appropriate genetic background strains for mutant isolation and functional characterization.

E. coli strain HS4006 (Δ*malB101* Δ*melAB* Δ*lac*), a strain that expresses invertase, was transformed by the $CaCl_2$ method with plasmid pNOEC73 (kindly provided as a generous gift from Tomofusa Tsuchiya, Okayama University), which harbors a 1.1-kbp *Bam*HI insert fragment containing the *melY* gene from *Enterobacter cloacae* in plasmid vector pBR322 (3). *Escherichia coli* HS4006/pNOEC73 cells were grown overnight (37°C), plated onto 1% maltose MacConkey plates (100 μg of ampillicin per ml) and incubated overnight at 37°C. Under these conditions, colonies on these plates were white, indicating very little maltose fermentation by *E. coli* HS4006/pNOEC73 (Table 2). However, after several days, red colonies appeared. These maltose fermentation-positive mutants (total, 30) were isolated and restreaked onto 1% maltose MacConkey plates (100 μg of ampillicin per ml) and incubated overnight at 37°C. Ampillicin-resistant clones (indicating the presence of plasmid) that maintained the maltose fermentation-positive phenotype (total, 21) were saved for preparation of plasmid DNA. Upon transformation of competent *E. coli* HS4006 cells with plasmids from the original mutants, those transformants that exhibited transfer of the enhanced maltose fermentation-pos-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype	Reference or origin
Strains		
DW1	*lacI*$^+$Δ(*ZY*) *mel*Δ(*AB*) *rspL*	36
DW2	*lacI*$^+$Δ(*ZY*) *melA*$^+$Δ*B rspL*	36
HS2053	F$^-$ Δ(*lac*)*U169 rpsL relA flbB thi rpoB* (Rif^r) Δ(*malB*)*101 malPQ*::Tn*5*	H. A. Shuman
HS4006	F$^-$ Δ(*lac-pro*)*X111* Δ*malB101 rpsL rpoB*	30
Plasmids		
pBR322	Amp^r Tet(C)^r	3
pNOEC73	*melY*$^+$ Amp^r Tet(C)^s	23
pNOEC73-L88P	*melY*(L-88→P) Amp^r Tet(C)^s	This work
pNOEC73-L91P	*melY*(L-91→P) Amp^r Tet(C)^s	This work
pNOEC73-A182P	*melY*(A-182→P) Amp^r Tet(C)^s	This work

itive phenotype (total, 10), suggesting enhanced maltose transport properties, were selected for archival, DNA sequencing, and transport studies. Table 2 shows the phenotypes for the fermentation of sugars by these transformants.

Sequencing of mutants. Maltose fermentation-positive *E. coli* HS4006/pNOEC73 cells grown to saturation overnight at 37°C were subjected to plasmid DNA preparation by using a kit from Qiagen. The nucleotide sequence of the *melY* gene on plasmid pNOEC73 DNA was determined for each mutant by automated sequencing at the Center for Biotechnology and Genomics at Texas Tech University by using primers complementary to the published sequence of the *melY* gene (22); synthesis was done by the Center for Biotechnology and Genomics.

Sugar uptake assays. Sugar uptake was measured as previously reported (29, 31). For the in vivo uptake measurements of melibiose, *E. coli* strain DW2 cells were transformed with wild-type or mutant pNOEC73 DNA and selected for ampillicin resistance. Transformants were grown to saturation in Luria-Bertani broth medium containing ampillicin (100 μg/ml) and diluted 100-fold in the same medium containing 1 mM α-methyl-galactopyranoside (α-MG) to induce the *melA* gene on the bacterial chromosome. *E. coli* DW2 cells harboring plasmid pBR322 (having no *melY* gene insert) were used as a control. For the in vivo maltose uptake assays, *E. coli* HS4006 cells, containing either wild-type or mutant pNOEC73 plasmid DNA, were grown to saturation overnight (37°C) in Luria-Bertani broth containing ampicillin (100 μg/ml) and diluted 100-fold in the same medium. *E. coli* HS4006 cells containing plasmid pBR322 were used as controls.

TABLE 2. Fermentation by melibiose transporter mutants

Cell	Colony phenotype[a]	
	Melibiose MacConkey	Maltose MacConkey
MelY$^+$	Red	White
ΔMelY	White	White
L-88-P	Red center	Red
L-91-P	Red	Red center
A-182-P	Red	Red center

[a] Fermentation studies were conducted by using MacConkey agar plates containing either 30 mM melibiose or maltose and incubated overnight at 37°C. For melibiose and maltose fermentation, *E. coli* strains DW2 and HS4006 containing the appropriate plasmids were used, respectively.

Cells at the mid-log phase of growth were washed twice with 100 mM morpholinepropanesulfonic acid (MOPS) buffer containing 0.5 mM $MgSO_4$ and 1 mM dithiothreitol. The washed cells were resuspended in the same buffer at a concentration of 0.45 mg of protein/ml and placed on ice. After equilibration at room temperature for 20 min, transport assays were initiated by the addition of either [^{3}H]melibiose (final concentration, 0.5 mM) or [^{14}C]maltose (final concentration, 0.4 mM). After incubation at room temperature for 1, 6, and 12 min, 0.2-ml samples were removed, filtered through a 0.45-μm-pore-size nitrocellulose filter and washed with MOPS buffer containing 0.5 mM $HgCl_2$. The filters were dissolved in 4 ml of scintillation fluid containing 10% water. The amount of radioactivity was determined by counting in a liquid scintillation counter. All transport data were subtracted from control cells.

Sugar accumulation assays. The in vivo accumulation of melibiose and maltose by cells harboring wild-type MelY or its spontaneous mutant derivatives was investigated using *E. coli* DW1 cells (36) and *E. coli* HS2053 cells (a generous gift from Howard Shuman, Columbia University), respectively. *E. coli* DW1/pBR322 or HS2053/pBR322 cells were used as controls, and all transport data from controls were subtracted from the data for the wild type and all mutants.

Sugar transport kinetic measurements. For kinetic analyses of sugar uptake, initial rates (linear) of transport for melibiose (using 1 mM α-MG for induction of α-galactosidase) or maltose (invertase expression is constitutive) were determined after incubation at various concentrations of radioactive sugar (0.1, 0.2, 0.33, 0.5, 2.0, and 5 mM), 50-μl samples were removed either after 15 or 45 s, filtered, and counted as described above. The Lineweaver-Burk and Eadie-Hofstee plots were used to determine apparent K_m and V_{max} values.

Nature of maltose-positive mutations in MelY. Plasmid DNAs from cultures of *E. coli* HS4006 harboring wild-type or mutant pNOEC73 plasmids were isolated, and the *melY* genes were sequenced. The wild-type *melY* sequence was identical to the published sequence (22). In contrast, four of the mutants showed that the codon at position Leu-88 was changed to a Pro; five mutants had Leu-91 replaced with Pro; and one mutant had Ala-182 changed to Pro. In LacY, these positions correspond to Met-83, Met-86, and Ala-177, respectively (7, 22). According to the published hydropathy analysis of MelY (22, 23) and the present topological models of LacY (9, 16, 34), Leu-88 and Leu-91 are predicted to reside in the middle of TMS-3 of MelY (Fig. 1). Ala-182 of MelY is predicted to lie within TMS-6.

Maltose and melibiose uptake by mutants. When HS4006 cells are plated onto maltose MacConkey plates, they can cleave a maltose (an α-glucoside) molecule that has entered the cell into two glucose molecules, which are then rapidly metabolized. The consequence is that the concentration of maltose inside the cell remains low compared to the concentration in the periplasm. Thus, the uptake of maltose into the cytoplasm would occur down its concentration gradient, i.e., thermodynamically "downhill." As shown in Table 3, the nonlinear transport rates of maltose uptake for the L-88-P and L-91-P MelY mutants were 3.6- and 5-fold greater than for wild-type MelY, whereas the A-182-P MelY mutant was only 1.6-fold greater than wild-type MelY.

By using *E. coli* strain DW2 (Table 1), melibiose uptake by

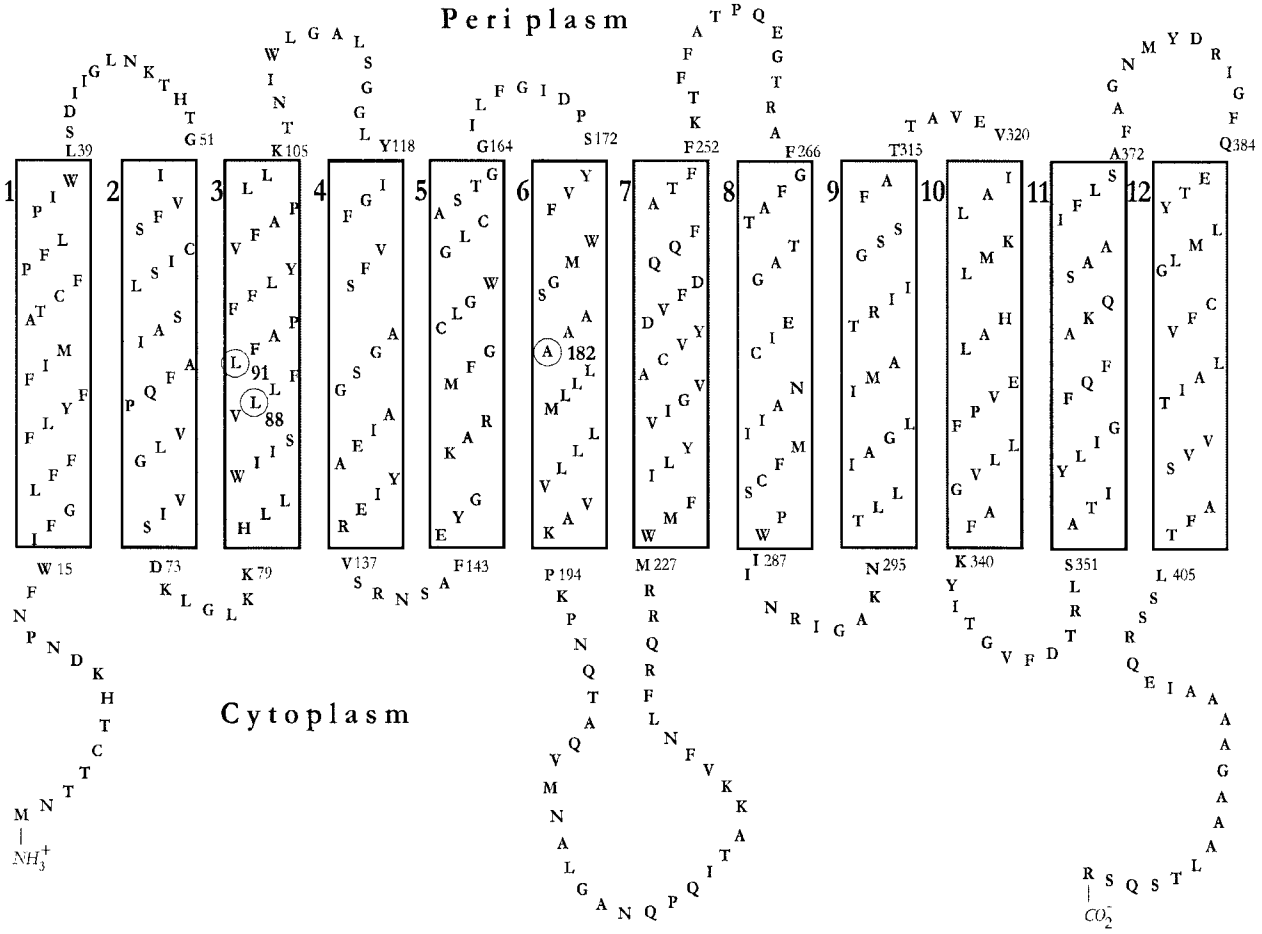

FIG. 1. Structure of the melibiose transporter of *Enterobacter cloacae*. The amino acid sequence and the two-dimensional model are based on nucleotide sequence and hydropathy analyses, respectively (22, 23). The affected residues implicated in the transport of the sugar maltose by MelY are encircled and include Leu-88, Leu-91, and Ala-182; these residues correspond to Met-83, Met-86, and Ala-177 of the lactose carrier, respectively (7, 34). Leu-88 and Leu-91 reside in the middle of TMS-3, and Ala-182 is in the center of TMS-6.

MelY and its mutant derivatives was studied. Such cells, when grown in the presence of the *melA* inducer α-MG, produce α-galactosidase and rapidly metabolize melibiose such that the concentration in the cytoplasm remains low. Thus, the uptake of melibiose is thermodynamically downhill, and accumulation ("uphill") against a concentration gradient does not take place. The mutants had melibiose uptake levels between 68 and 93% compared to that of the wild-type MelY (Table 3).

TABLE 3. Melibiose and maltose uptake by MelY mutants[a]

Cell	% of normal	
	Melibiose	Maltose[b]
$MelY^+$	100	100
ΔMelY	0	0
L-88-P	68 ± 17	361 ± 97
L-91-P	93 ± 16	507 ± 121
A-182-P	91 ± 23	163 ± 91

[a] Melibiose uptake (0.5 mM) was measured after 15 and 45 s of incubation. $MelY^+$ cells accumulated melibiose at a rate of 233 ± 42 cpm, from which a $MelY^-$ cell's background uptake of 55 ± 9 cpm was subtracted. Maltose uptake (0.4 mM) was measured at 1, 6, and 12 min after incubation. $MelY^+$ cells accumulated maltose at a rate of 76 ± 8 cpm, from which a $MelY^-$ cell's background uptake of 26 ± 3 cpm was subtracted. The wild-type MelY showed transport of 1.01 nmol of melibiose/mg of protein and 0.4 nmol of maltose/mg of protein. Shown are the averages ± standard errors of the means (SEM) (*n* ranged from 6 to 9).

[b] For the *t* test, $P \leq 0.05$ compared to the wild-type MelY.

Sugar accumulation by mutants. Because the *malPQ* operon is inactivated by transposition mutagenesis, and the *malB* region is deleted in *E. coli* strain HS2053 (Table 1), the cells cannot metabolize or transport maltose. Therefore, we used the HS2053 cell as the host strain to study maltose accumulation against a concentration gradient by MelY and its mutant derivatives. Appreciable maltose accumulation activity was observed in HS2053 cells containing MelY with L-88-P, L-91-P, or A-182-P mutations but was not observed in cells harboring wild-type MelY or negative control containing only plasmid pBR322 (Table 4). Thus, when maltose is the substrate, the energy-coupling mechanism is somewhat functional in MelY, involving proline replacements for residues Leu-88, Leu-91, and Ala-182.

Melibiose accumulation was measured in DW1 cells (lacking α-galactosidase) containing either MelY or its mutant derivatives, and all transport data from DW1/pBR322 control cells

TABLE 4. Accumulation of melibiose and maltose[a]

Cell	Ratio (in/out)	
	Melibiose	Maltose
MelY$^+$	5.53 ± 0.49	0.31 ± 0.01
L-88-P	0.19 ± 0.01	2.62 ± 0.37
L-91-P	1.02 ± 0.15	3.47 ± 0.63
A-182-P	1.27 ± 0.10	4.55 ± 0.24

[a] Melibiose (0.5 mM) and maltose (0.4 mM) accumulation activities were measured at 25°C after 10 min of incubation. MelY$^+$ cells accumulated melibiose at a rate of 615 ± 55 cpm, from which a MelY$^-$ cell's background uptake of 64 ± 17 cpm was subtracted. MelY$^+$ cells accumulated maltose at a rate of 44 ± 5 cpm, from which a MelY$^-$ cell's background uptake of 32 ± 4 cpm was subtracted. Shown are the averages ± SEM (n ranged from 6 to 9). For the t test, $P \leq 0.05$ for mutant transport data when compared to the normal MelY.

were subtracted. Melibiose accumulation activity in the mutants was not detectable (Table 4), indicating that the energy-coupling properties of all of the MelY mutants were defective for melibiose.

Kinetics of maltose uptake in mutants. An analysis of maltose uptake kinetics (initial, linear rates) was performed for MelY and its mutant derivatives (Table 5). The three mutants showed decreases in both apparent K_m and V_{max} values. Cells containing wild-type MelY showed a relatively high apparent K_m value (14 mM) for maltose transport. In contrast, the L-88-P, L-91-P, and A-182-P mutants showed enhanced apparent K_m values for maltose ranging between 4- and 42-fold difference. The L-88-P and L-91-P mutants showed little or no changes in the apparent V_{max}/K_m ratios for maltose, while A-182-P showed a significant increase in the ratio. Although the Lineweaver-Burk plots were more linear than the Eadie-Hofstee plots (data not shown), the K_m and V_{max} values were similar in both cases.

Concluding remarks. We present the first comparative study of substrate selection alteration between LacY, an important model transport system, and one of its homologs, MelY, a melibiose transporter (22, 23) that has a different substrate selection profile. The data presented here indicated that point mutations in residues located in helices three and six of MelY (Fig. 1) showed alterations in substrate selection. Cysteine-scanning mutagenesis (8), alanine insertion scanning mutagenesis (4), deletion mutagenesis (25), and β-galactosidase insertional mutagenesis (19) of helix three in LacY showed loss of transport activity but not loss of expression levels or stability, suggesting a functional role for residues in helix three (4, 8, 19, 25). In our study, the Pro-88 and Pro-91 mutations in MelY are three residues apart and reside within TMS-3 (Fig. 1). We show that the L-88-P and L-91-P variants of MelY have greater rates of maltose uptake than wild-type MelY (Table 3), and the L-88-P, L-91-P, and A-182-P mutations show enhanced apparent K_m values (Table 5) indicating either increased apparent affinities for maltose or rate-limiting catabolism of the sugar. Cells containing MelY with L-88-P, L-91-L, and A-182-P mutations showed transport of melibiose (Table 3) that was not significantly different from the wild type, suggesting no significant changes in expression levels or kinetic properties, although these were not directly measured in our study. Taken together, our data suggest that Leu-88 and Leu-91 in the wild-type MelY transporter reside on the same face of TMS-3.

TABLE 5. Kinetics of maltose uptake

Cell	Maltose		
	K_m (mM)[a]	V_{max} (nmol/mg protein/min)[a]	V_{max}/K_m
MelY$^+$	14 ± 0.41	121 ± 3.53	8.6 ± 0.25
L-88-P	3.2 ± 0.05	26.2 ± 0.35	8.2 ± 0.11
L-91-P	0.77 ± 0.05	10.5 ± 0.66	13.6 ± 0.87
A-182-P	0.33 ± 0.01	94 ± 1.2	284 ± 5.1

[a] Values here should be referred to as "apparent" since the melibiose-H$^+$ symporter has two substrates (proton and sugar). Initial, linear rates of transport were measured. Shown are the averages ± SEM (n = 9). For the t test, $P \leq 0.05$ for mutant K_m and V_{max} data compared to the normal MelY.

There are two ways to interpret the transport data for the mutants containing altered residues at Leu-88 and Leu-91 in MelY. The first explanation is that TMS-3 participates in channel formation, where the Leu-88 and Leu-91 side chains project into the channel and interact hydrophobically with melibiose but not with maltose, whereas in the Pro-88 and Pro-91 side chains, these mutants of MelY produce a structure such that a direct interaction with maltose is facilitated in the channel. If indeed a direct role for sugar binding is taking place for residues in helix three in LacY, then a modification of the structural model (9) should be made, such that helix three is moved towards the center of the transporter where the aqueous channel is located. Our data that implicate the two Leu residues in MelY are consistent with the idea that the sugar-binding site is hydrophobic (28).

Second, an indirect involvement for residues in TMS-3 is possible. Two structural models of LacY show helix three to be distantly located within the carrier and in proximity to helices two and four (9, 15, 16, 37), suggesting an indirect role for substrate binding and transport by residues of helix three. Furthermore, the three-dimensional structure of OxlT, an oxalate/formate transporter from *Oxalobacter formigenes*, indicates that helix three does not form the substrate translocation pathway (12). If indeed an indirect role for helix three is the case, then in the normal LacY transporter, helix three probably influences the direct involvement of helices two and four with substrate.

It is noteworthy that only Pro substitutions were observed in our study, although the evolutionary reason for this is unclear. Pro probably introduces a slight kink in TMS-3 (33) (which would not be as profoundly kinked as Pro in helices in aqueous environments) that in turn affects the structures of TMS-2 and TMS-4 such that their direct interactions with maltose are enhanced. This view for the roles of residues in TMS-3 of MelY is consistent with the idea that conformational changes (6, 13, 14, 35, 37) occur in the loop between helices two and three of LacY (9). It is striking that although Pro is largely absent in most of the transmembrane segments, it is notably present in TMS-1 and TMS-3 of MelY (Fig. 1). Whether either of these functional roles (direct versus indirect) is possible for TMS-3 in MelY or helix three of LacY awaits elucidation of crystal structures or further conformational studies. Lastly, one cannot rule out the possibility that there is more than one substrate-binding site in MelY, as has been suggested for LacY (37).

King and Wilson (17) observed that substitution of a valine at position 177 in LacY showed enhanced transport of sucrose.

Likewise, substitution of Val, Ile, Leu, Phe, or Pro for Ala-177 in LacY showed transport of arabinose, and substitution of Val or Thr for Ala-177 in LacY showed transport of maltose (17). An alanine is conserved in helix six (Ala-182 in MelY and Ala-177 in LacY) in five of six members of the oligosaccharide H^+ symporter subfamily five of the MFS (the exception is CscB, which has a glycine) (2, 23, 27, 34). Taken together with our mutational and transport data, these data predict that in MelY, comparable amino acid substitutions would show similar substrate specificities during transport. In any event, because an identical amino acid substitution (Ala→Pro) in the same position (Ala-182 in MelY and Ala-177 in LacY) shows maltose transport within the two homologous transporters, this suggests that the structures of the maltose-binding sites, the mechanisms of sugar selection, and the overall three-dimensional structures of LacY and MelY are similar. Thus, this study provides functional evidence for the conservation of an important alanine residue that mediates substrate selection in homologous transporters with different substrates.

In the MelY substrate selection mutants, melibiose accumulation was defective (Table 4), implying that the energy-coupling properties for melibiose were lost. This further implies that, in the mutants, substrate accumulation and energy-coupling mechanisms for melibiose are independent; this is consistent with observations in several LacY sugar selection mutants, which also show a drastic reduction in the accumulation of lactose (6, 17). It is thus suggested that in the MelY mutants studied here, the mechanism responsible for melibiose binding is probably subject to the various proposed energy-coupling mechanisms of LacY (9, 14, 24) and, by extension, wild-type MelY. However, cells containing MelY mutants showed slight accumulation of maltose (Table 4). This suggests that Leu-88, Leu-91, and Ala-182 are probably involved in the energy-coupling mechanism for maltose accumulation and that accumulation activity may be dependent on the type of sugar transported through the carrier.

We thank Jeff Griffith, Tomofusa Tsuchiya, and Thomas Wilson for critical review of the manuscript and Robert Brooker for helpful discussions. We are indebted to Howard Shuman for *E. coli* strain HS2053 and Tomofusa Tsuchiya for plasmid pNOEC73. We gratefully acknowledge Celsa Gallegos, Shea Madrid, and Erin Hald for expert technical assistance.

The work described in this paper was made possible by NIH grants RR-16480 and GM-56626, plus NSF grant MCB-9812362 and IRGs from ENMU.

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