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Amino Acids that Confer Transport of Raffinose and Maltose Sugars in the Raffinose Permease (RafB) of *Escherichia coli* as Implicated by Spontaneous Mutations at Val-35, Ser-138, Ser-139, Gly-389 and Ile-391

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Abstract In order to identify amino acid residues in the *Escherichia coli* raffinose-H^+ permease (RafB) that play a role in sugar selection and transport, we first incubated *E. coli* HS4006 containing plasmid pRU600 (expresses inducible raffinose permease and α-galactosidase) on maltose MacConkey indicator plates overnight. Initially, all colonies were white, indicating no fermentation of maltose. Upon further incubation, 100 mutants appeared red. pRU600 DNA was prepared from 55 mutants. Five mutants transferred the phenotype for fermentation of maltose (red). Plasmid DNA from five maltose-positive phenotype transformants was prepared and sequenced, revealing three distinct types of mutations. Two mutants exhibited Val-35→Ala (MT1); one mutant had Ile-391→Ser (MT2); and two mutants had Ser-138→Asp, Ser-139→Leu and Gly-389→Ala (MT3). Transport studies of [^{3}H]-maltose showed that cells harboring MT1, MT2 and MT3 had greater uptake ($P \leq 0.05$) than cells harboring wild-type RafB. However, [^{14}C]-raffinose uptake was reduced in all mutant cells ($P \leq 0.05$) with MT1, MT2 and MT3 mutants compared to cells harboring wild-type RafB. Kinetic analysis showed enhanced apparent K_m values for maltose and reduced V_{max}/K_m ratios for raffinose compared to wild-type values. The apparent K_i value of maltose for RafB indicates a competitive relationship between maltose and raffinose. Maltose "uphill" accumulation was greater for mutants ($P \leq 0.05$) than for cells with wild-type RafB. Thus, we implicate residues in RafB that are responsible for raffinose transport and suggest that the substituted residues in RafB dictate structures that enhance transport of maltose.

Keywords Amino acid · Bacteria · Maltose · Mutation · Permease · Raffinose · Secondary active transport · Sugar · Symport

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Introduction

Secondary active transport of the sugar raffinose across the cytoplasmic membrane of *Escherichia coli* is mediated by the raffinose permease (RafB) (Schmid & Schmitt, 1976; Aslanidis et al., 1989; Titgemeyer et al., 1994), which is a member of the oligosaccharide:H^+ symporter (OHS) family 5 of the major facilitator superfamily (MFS) of transporters (Saier et al., 1999). Other homologous members of the OHS family include the sucrose permease (CscB) of *E. coli* (Bockmann et al., 1992; Sahin-Toth et al., 1995), the melibiose permease (MelY) of *Enterobacter cloacae* (Okazaki et al., 1997a, 1997b) and the lactose permeases (LacY) of *Citrobacter freundii*, *Klebsiella pneumoniae* and *E. coli* (Buchel et al., 1980; McMorrow et al., 1988; Lee et al., 1994; Varela & Wilson 1996; Abramson et al., 2004a). Because of shared similarities in their amino acid sequences, predicted two-dimensional structures and phylogenetic relationships, the three-dimensional structures of the transporters within the OHS family are believed to be similar (Griffith et al., 1992; Maloney, 1994). Such structural similarities predict a common mechanism for sugar transport in which differences in substrate specificities are dictated by subtle differences in sequence and structure as these transporters have structurally distinctive sugar substrates (Henderson et al., 1984; Griffith et al., 1992; Hirai et al., 2003).

The only member of the OHS family of transporters for which a molecular structure has been solved is the lactose permease of *E. coli* (Abramson et al., 2003). Studies of mutants have been useful for the study of sugar selection profiles in MelY (Shinnick et al., 2003) and in LacY (Shuman & Beckwith, 1979; Brooker et al., 1985; Brooker & Wilson, 1985a, 1985b; King & Wilson, 1990a, 1990b; Brooker, 1991; Olsen et al., 1993; Varela et al., 1997; Shinnick & Varela, 2002). Primarily, transmembrane-associated amino acid residues have been implicated in the selection, recognition and transport of substrates across the membrane. In particular, when altered by spontaneous mutation, the residues Ala-177, Tyr-236 and Thr-266 in LacY (Shuman & Beckwith, 1979; Brooker et al., 1985; Markgraf et al., 1985) and Leu-88, Leu-91 and Ala-182 in MelY confer enhanced transport of maltose (Shinnick et al., 2003). Additional mutations in LacY that result in the enhancement of transport for other distinct sugars have been found and documented (Brooker et al., 1985; Brooker & Wilson 1985a, 1985b; Collins et al., 1989; Franco et al., 1989; Olsen & Brooker, 1989; Brooker, 1990, 1991; King & Wilson 1990a, 1990b; Eelkema et al., 1991; Gram & Brooker, 1992; Goswitz & Brooker, 1993; Olsen et al., 1993; Varela & Wilson, 1996; Varela et al., 1997, 2000; Shinnick & Varela, 2002). Computational and biophysical analyses have been useful in the elucidation of sugar binding properties and of the conformational changes that occur during lactose transport across the membrane (Park & Lee, 2005; Kasho et al., 2006; Vadyvaloo et al., 2006; Yin et al., 2006; Jensen et al., 2007; Klauda & Brooks, 2007). In addition, studies of maltoporin (LamB) have been tremendously helpful in elucidating the molecular basis of maltose transport across the membrane via outer membrane porin channels (Boos & Shuman, 1998). Little or no such substrate selection or physiological information exists, however, for the transport of maltose in other members of the OHS family. Thus, a comparative study of substrate selection would be useful in these sugar transporters, especially since the crystal structure LacY is available (Abramson et al., 2004a, 2004b; Kasho et al., 2006; Mirza et al., 2006; Yin et al., 2006; Jensen et al., 2007). Here, we provide the first mutational and physiological evidence for the existence of amino acid residues that play a functional role in the selection and transport of raffinose and maltose sugars in the RafB permease of *E. coli*.

Methods

Bacterial Strains and Plasmids

E. coli HS4006 (Δ*malB101* Δ*melAB* Δ*lac*), a gift from Dr. Howard A. Shuman (Columbia University, New York, NY), was used as a background strain as it is deleted for its maltose transport system but can inductively express the maltose invertase enzyme (Shuman & Beckwith, 1979; Brooker et al., 1985). Plasmid pRU600, containing an intact Raf operon (*rafR, rafA, rafB, rafD*) cloned into the plasmid vector pACYC184 (Chang & Cohen, 1978), was a gift from Dr. Rudiger Schmidt (Regensburg University, Regensburg, Germany) (Schmid & Schmitt, 1976). The HS4006 bacterial strain was transformed with pRU600 plasmid using the $CaCl_2$ method as described previously (Shinnick et al., 2003) and incubated on 1% maltose, 1% lactose or 1% raffinose MacConkey agar plates containing 30 μg/ml chloramphenicol for 24 h at 37°C for analysis of transformant fermentation phenotypes.

Isolation of Mutants

HS4006 cells harboring plasmid pRU600 were plated onto MacConkey agar plates containing 1% maltose and 30 μg/ml chloramphenicol and incubated at 37°C, 30°C or 25°C for approximately 1 week. Initial colonies (approximately 24–48 h incubation) had white colony phenotypes on the indicator plates. However, after 1–2 further weeks of incubation at 37°C, 30°C or 25°C, colonies appeared that displayed a fermentation-positive phenotype (red color). These mutants were picked, restreaked onto fresh 1% maltose MacConkey agar containing 30 μg/ml chloramphenicol and incubated overnight at 37°C. Mutant clones that retained the maltose-positive fermentation phenotype on these plates were picked, used to inoculate Luria-Bertani (LB) broth containing 30 μg/ml chloramphenicol, incubated overnight at 37°C with shaking and archived at –80°C in 25% glycerol. From the mutant cells that were grown overnight, the putatively mutated pRU600 DNA plasmids were prepared using the miniprep protocol from Qiagen (Chatsworth, CA) and used to transform new HS4006 host cells, which were then incubated at 37°C on 1% maltose MacConkey agar plates containing 30 μg/ml chloramphenicol. The transformation step ensured selection of mutations on plasmid DNA and not in host genomic DNA. After transformation, plasmids were harvested, in the same manner as described above, from transformant cells that were positive for fermentation of maltose. The nucleotide sequences of the *rafB* genes on the mutated plasmids were determined by GeneMed Synthesis using an ABI (Foster City, San Francisco, CA) automated sequencer and primers complementary to F1 (5′-GGTGTTGTTTAGAGATGCAGA-3′) and F2 (5′-CGTGAACTCCACGTTGAGATGTCA-3′) promoter elements flanking the *rafB* insert (Aslanidis et al., 1989).

Sugar Transport Assays

"Downhill" sugar transport assays of [^{3}H]-maltose and [^{14}C]-raffinose were performed as previously described (Varela et al., 1997, 2000; Shinnick & Varela 2002; Shinnick et al., 2003). Briefly, *E. coli* cells of the HS4006 strain containing wild-type or mutated pRU600 plasmids (isolated as described above) were used to inoculate LB broth and incubated overnight at 37°C. Each refresher culture, containing 30 μg/ml chloramphenicol and 30 mM raffinose (inducer of *rafB* expression), was seeded with approximately 0.5 ml of the overnight cultures. The cells were then grown to mid-log phase of growth (OD_{600} approximately 0.4). The cells were washed twice with 100 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS, pH 7.0) by centrifugation at 5,000 rpm in a Beckman (Fullerton, CA) JA-120 rotor for 5 min and then resuspended such that 0.45 mg of protein were present in each sample. The transport reactions were initiated by addition of [^{3}H]-maltose or [^{14}C]-raffinose to a final sugar concentration of 0.4 mM. Samples (0.2 ml) were removed after 10 min, filtered and immediately washed with 100 mM MOPS (pH 7.0) containing 0.5 mM $HgCl_2$, which prevents sugar efflux and traps transported sugar within the cell (King & Wilson, 1990a, 1990b). The filters were dissolved in LiquiScint™ (National Diagnostics, Manville, NJ) containing 10% water. All samples were counted using a Beckman LS-6500 scintillation counter. For our downhill sugar transport studies, we subtracted all values from control cells harboring plasmid vector pACYC184 from all transport data by cells with wild-type and mutated RafB.

Kinetic analyses were conducted for downhill transport of sugars as previously described (Varela et al., 1997). Initial transport rates for [^{3}H]-maltose or [^{14}C]-raffinose were determined with various sugar concentrations (0.1, 0.2, 0.4 and 2.0 mM) after cells were incubated for 15 s and 45 s in the presence of raffinose. Samples of cell suspension (0.2 ml) were removed for filtering through 0.65-μm-pore size nitrocellulose filters and counting in a Beckman LS-6500 liquid scintillation counter. Values from cells with vector plasmid pACYC184 (control) were subtracted from the data. Apparent K_m and V_{max} values were determined using a Lineweaver-Burk double reciprocal plot.

The apparent K_i value of maltose for wild-type RafB was determined with [^{14}C]-raffinose and *E. coli* HS4006 containing plasmid pRU600. Briefly, HS4006/pRU600 cells were used to inoculate LB broth containing chloramphenicol 30 μg/ml overnight at 37°C. These cells (0.5 ml) were then used to seed a subculture at the mid-log phase of growth (OD_{600} approximately 0.4–0.6) in LB broth containing 30 μg/ml chloramphenicol and 30 mM raffinose as inducer. The cells were washed and resuspended twice using 100 mM MOPS (pH 7.0) containing 0.5 mM $MgSO_4$ by centrifugation at 5,000 rpm in a Beckman JA-120 rotor for 10 min. The washed cells were resuspended in the same buffer to a concentration of 0.17 mg protein/ml. The inhibition reactions were initiated by addition of [^{14}C]-raffinose to final sugar concentrations of 0.1, 0.2, 0.4 and 1.0 mM. After equilibration at room temperature (25°C) for 20 min, maltose was added to the mixture at final concentrations of 0.1, 0.2, 0.4 and 2.0 mM. After 15- and 45-s incubation in the presence of unlabeled maltose, 250-μl samples were removed, filtered through a 0.65-μm-pore size filter and washed with 3 ml 100 mM MOPS (pH 7.0) buffer containing 0.5 mM $HgCl_2$. The filters were dissolved in 4 ml LiquiScint containing 10% water. The radioactivity of each sample was measured using a Beckman LS-6500 liquid scintillation counter.

Maltose "uphill" accumulation studies were performed as previously described (Shinnick et al., 2003). Briefly, mutated plasmids were used to transform *E. coli* HS2053 cells, which lack both maltose metabolizing and transporter systems (provided by H. A. Shuman, Columbia University, New York, NY). *E. coli* HS2053 cells harboring mutated pRU600 plasmids were grown overnight to saturation in 50 ml of LB broth containing 30 μg/ml chloramphenicol with shaking at 37°C. Cells grown overnight were used to inoculate fresh LB broth containing 30 μg/ml chloramphenicol and raffinose as an inducer of the RafB transporter and grown at 37°C with shaking to mid-log phase (ΔOD = 0.3–0.6). The cells were then harvested by centrifugation at 5,000 rpm at 4°C. The pellets were drained and the cells resuspended in 5 ml of 100 mM MOPS buffer (pH 7) containing 0.5 mM $MgSO_4$. The washed cells were resuspended in the same buffer to a concentration of 0.45 mg protein/ml and placed on ice. After the cells and radioactive sugar had equilibrated to room temperature (25°C) for 20 min, transport assays were initiated by addition of 50 μl of radioactive [^{3}H]-maltose to the HS2053 cells. After 10-min incubation in the presence of [^{3}H]-maltose, 50-μl samples were removed, filtered through a 0.65-μm-pore size filter and washed with 3 ml 100 mM MOPS buffer (pH 7.0) containing 0.5 mM $HgCl_2$. The filters were dissolved in 4 ml LiquiScint containing 10% water. The radioactivity of each sample was measured using a Beckman LS-6500 liquid scintillation counter. We subtracted values from cells with control plasmid (pACYC184) from transport data obtained from cells with wild-type or mutated RafB.

Results

Isolation of Maltose Fermenting Mutants

We incubated *E. coli* HS4006 cells containing plasmid pRU600 on maltose MacConkey indicator plates (Schmid

Table 1 Fermentation of sugars by cells with wild-type or mutated RafB permeases

Cell	Colony phenotypes		
	Maltose MacConkey (initial[a])	Maltose MacConkey (final[b])	Raffinose MacConkey
RafB$^+$	White	White	Red
ΔRafB	White	White	White
MT-1	—	Red	Pink
MT-2	—	Red	Pink center
MT-3	—	Red	White

The MT-1 and MT-3 mutants originally arose on the indicator plates after incubation at 37°C, whereas the MT-2 mutants arose after incubation at 30°C

[a] Colony phenotype before mutant isolation

[b] Colony phenotype subsequent to isolation and archival of mutants

& Schmitt, 1976; Shuman & Beckwith, 1979). After 1–2 days of incubation, colony phenotypes were white, suggesting no maltose uptake and indicating no fermentation of maltose. Continued incubation of these indicator plates over the course of several weeks, however, resulted in the appearance of red colonies, implying maltose uptake and metabolism (*see* Table 1). One hundred red mutants were colony-purified by serial streak dilution onto fresh maltose MacConkey agar plates, incubating these plates overnight and using colonies that maintained the red phenotype to inoculate LB broth, which were cultured and archived. Approximately 55 of the original 100 mutants picked retained a red color phenotype upon restreaking on the new MacConkey plates. Plasmid DNA was prepared from the 55 mutants and used to transform fresh *E. coli* HS4006 cells. Five of the mutants transferred the red colony phenotype to transformants, from which plasmid DNA was prepared for nucleotide sequence determination. Mutants that were selected for sequencing displayed altered fermentation of raffinose compared to cells containing wild-type RafB permease (Table 1).

Sequences of Maltose-Positive Mutants

Nucleotide sequencing of five stable maltose fermenting mutants revealed three types of site mutations in the *rafB* gene on plasmids (Fig. 1): Val-35 changed to Ala (MT-1), Ile-391 changed to Ser (MT-2), and Ser-138 changed to Asp, Ser-139 changed to Leu and Gly-389 changed to Ala (MT-3).

Maltose Transport by Mutants

Analysis of [^{3}H]-maltose downhill transport, in which transport is measured in cells containing a cytoplasmic maltose hydrolytic enzyme (invertase) such that the entry of sugar into cells is down its concentration gradient, showed cells harboring V-35→A (MT-1), and I-391→S mutations (MT-2) had significantly increased ($P \leq 0.05$) transport of maltose compared to cells harboring wild-type RafB (*see* Table 2). The MT-3 mutant that had a triple-mutation in RafB (S-138→D, S-139→L and G-389→A) also had significantly greater maltose uptake ($P \leq 0.05$) than cells harboring wild-type RafB permease.

Kinetic analysis of maltose downhill transport in the mutants showed apparent K_m values ranging 0.05–1.2 mM and apparent V_{max} values of 47–75 nmoles maltose/mg protein/min (*see* Table 3). Transport data for cells

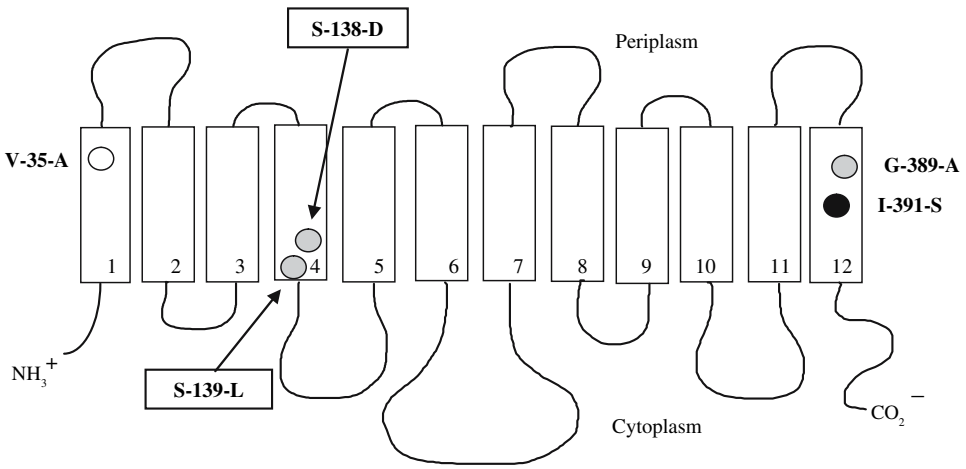

Fig. 1 Predicted two-dimensional structure of the RafB permease and mutations. Three classes of sugar selection mutations were found in RafB in this study. All three classes of mutations conferred changes in sugar selection and transport in RafB. MT-1 (*white circle*) showed Val-35 changed to Ala. MT-2 (*black circle*) showed Ile-391 changed to Ser. MT-3 (*gray circles*) showed Ser-138 changed to Asp, Ser-139 changed to Leu and Gly-389 changed to Ala. The locations of the mutations in RafB were determined according to the sequence data of RafB (Aslanidis et al., 1989) and, by extension, the crystal structure data of LacY from *E. coli* (Kaback, 2005; Guan & Kaback, 2006)

Table 2 Raffinose and maltose transport by mutants

Cell	Raffinose downhill transport[a] (% cells with RafB$^+$)	Maltose downhill transport[b] (% cells with RafB$^+$)
MT-1	26 ± 9.4%	297 ± 10.1%
MT-2	29 ± 2.8%	181 ± 8.5%
MT-3	17 ± 4.2%	170 ± 2.7%

[a] Bacterial transport assays of [^{14}C]-raffinose (0.4 mM final concentration) were conducted as described in "Methods." Cells harboring wild-type plasmid pRU600 had 4.25 ± 0.36 nmoles raffinose/mg protein. Student's *t*-test was performed, and all mutants tested had a significant decrease ($P \leq 0.05$) in the transport of raffinose compared to cells harboring wild-type RafB permease

[b] HS4006 containing wild-type pRU600 plasmid or plasmid derivatives were used to measure downhill entry of [^{3}H]-maltose (0.4 mM final concentration) by wild-type and mutated RafB, respectively, according to the procedures described in "Methods." Cells harboring wild-type pRU600 plasmid had 0.2 ± 0.015 nmoles maltose/mg protein. Student's *t*-test was performed, and all mutants differed significantly ($P \leq 0.05$) from cells containing wild-type RafB permease

harboring wild-type RafB permease, however, were at background radiation levels and too low to obtain reliable kinetic data for a kinetic measurement of maltose entry. The apparent K_i for maltose during raffinose transport by wild-type RafB permease was 0.30 ± 1.4 mM, which is significantly lower ($P \leq 0.005$) than the apparent K_m value for raffinose (Table 3). For raffinose transport (e.g., 0.1 mM) the apparent V_o (without maltose) was 24.2 ± 2.4 nmol/mg protein/min and the apparent V_i (with maltose) was 17.7 ± 19.3 nmol/mg protein/min, both of which were not significantly different ($P \geq 0.05$, Student's *t*-test) regardless of any of the four fixed maltose concentrations used.

The uphill transport (i.e., accumulation) of maltose was measured in transformant *E. coli* HS2053 cells harboring

Table 3 Kinetic analysis of maltose and raffinose entry by cells harboring wild-type or mutated RafB permeases

Cell	Raffinose		Maltose	
	Apparent V_{max} (nmol/mg protein/min)	Apparent K_m (mM)	Apparent V_{max} (nmol/mg protein/min)	Apparent K_m (mM)
RafB$^+$	219 ± 11	1.2 ± 0.35	—	—
MT-1	0.35 ± 0.03	0.53 ± 0.02	47 ± 6.2	0.2 ± 0.04
MT-2	1.2 ± 0.08	0.24 ± 0.22	55 ± 8	0.05 ± 0.04
MT-3	0.24 ± 0.61	0.52 ± 0.04	75 ± 6	1.2 ± 0.27

See "Methods" for details. Student's *t*-test was performed, and all mutants differed significantly in the transport of raffinose ($P \leq 0.05$) compared to cells containing wild-type RafB permease. Transport data of cells harboring wild-type RafB for [^{3}H]-maltose were too low to acquire reliable (repeatable) kinetic data

Table 4 Maltose uphill transport (accumulation) by cells with wild-type or mutated RafB permeases

Cell	Concentration ratio of maltose sugar in/out
RafB$^+$	0.05 ± 0.03
MT-1	2.6 ± 0.23
MT-2	3.3 ± 0.42
MT-3	4.7 ± 0.40

Mutant plasmids were transformed into *E. coli* HS2053 cells (Shinnick et al., 2003). Transport assays were initiated by addition of [^{3}H]-maltose to a final concentration of 0.4 mM for a 10-min incubation. Student's *t*-test was performed, and all mutants tested had a significant decrease ($P \leq 0.05$) in the cellular accumulation of maltose compared to cells harboring wild-type RafB permease

mutated RafB permeases MT1, MT2 and MT3 (*see* Table 4). In *E. coli* HS2053 cells, the *malB* locus is deleted and the *malPQ* operon is inactivated by a transposon (Tn5) insertion (Shinnick et al., 2003). Thus, the plasmid-free HS2053 cells are not capable of transport and utilization of maltose. Such cells are suitable hosts for the measurement of maltose accumulation upon cellular entry of the sugar. We observed significantly enhanced accumulation of maltose in HS2053 cells harboring mutated RafB permeases compared with HS2053 cells harboring wild-type RafB permease, which showed no maltose accumulation activity (Table 4).

Raffinose Transport by Mutants

The downhill transport of [^{14}C]-raffinose across the membrane was measured in cells harboring the mutated or wild-type RafB permeases (*see* Table 2). All three classes of mutations in RafB had significantly reduced entry of raffinose ($P \leq 0.05$) in cells harboring the mutations compared to cells harboring wild-type RafB permease. Analysis of the kinetics for raffinose cellular entry showed apparent K_m values of 1.2 mM for cells harboring wild-type RafB and K_m values of 0.24–0.53 for cells harboring mutated RafB (Table 3).

Discussion

Within the RafB permease of *E. coli*, we implicate amino acid residues that, when altered, confer reduced transport of a "native" substrate (i.e., raffinose) and enhanced transport of a novel substrate (i.e., maltose). We found three types of mutations that conferred enhanced transport of maltose by the RafB permease: Val-35→Ala (MT-1); Ile-391→Ser (MT-2); and Ser-138→Asp, Ser-139→Leu and Gly-389→Ala (MT-3).

Kinetic studies of downhill raffinose transport showed that the apparent K_m and V_{max} values were reduced in the mutants compared to the cells with wild-type RafB, suggesting tighter binding and reduced transport. Downhill maltose transport studies of the mutants showed that the apparent K_m and V_{max} properties are enhanced, indicating an improvement of binding and transport in cells harboring mutated RafB permease. Thus, taken together, during the transport cycle, the binding and release properties of raffinose could reduce RafB turnover, whereas those of maltose may enhance the turnover. It is not surprising that the structural differences between raffinose (a trisaccharide) and maltose (a disaccharide) would have distinct binding and transport properties. The apparent K_i value of maltose is lower than the apparent K_m value of raffinose for RafB$^+$. This indicates a competitive relationship between maltose and raffinose within the RafB permease. Therefore, maltose inhibits the transport of raffinose in wild-type RafB.

Our analysis of maltose entry in HS2053 cells harboring mutated RafB permeases indicates that the affected sites within RafB play a functional role in mediating energy-dependent maltose accumulation (uphill transport). This observation is striking in light of the case with wild-type RafB, for which no detectable maltose transport of any type can be measured. The role of protons in symport, however, remains poorly understood, particularly when one considers residues previously established to play a critical role in proton translocation, such as Glu-325 in LacY of *E. coli* (Franco & Brooker, 1994; Guan & Kaback, 2006) and, by extension, Glu-328 in the RafB permease, a residue for which we found no mutations conferring changes in sugar recognition in this study.

Mutations at Val-35, Ser-138, Ser-139, Gly-389 and Ile-391 in RafB were found to result in significantly reduced transport of raffinose, suggesting that these residues confer structures within RafB that play a functional role in the recognition and transport of this sugar. The implication of a Val residue in transmembrane 1 suggests that hydrophobic interactions are involved in sugar selection and transport. By analogy with the known structure of LacY (Abramson et al., 2003), we predict that the interaction between Val-35 of RafB and raffinose is a direct one. Likewise, because the crystal structure of LacY indicates that residues of helix 4 play a role in sugar binding and transport (Johnson & Brooker, 2003; Abramson et al., 2004b) and because we observed reduced raffinose transport and increased maltose transport when altered by mutation, we suggest that Ser-138 and Ser-139 in the RafB permease are involved in the binding and transport of raffinose and that, when altered by mutation, these positions allow binding and transport of maltose. Regarding a possible role for residues in transmembrane domain 12 of the RafB permease, a previous study using deletion mutational analysis of helix 12 of LacY suggested that this transmembrane helix functions in the stabilization of newly inserted and properly folded permeases within the membrane (McKenna et al., 1992). Since our mutational data show enhanced maltose transport, it is suggested that residues in transmembrane domain 12 of RafB permease, when altered, allow changes in substrate recognition to occur.

A sequence comparison of RafB with the other members of the OHS family (*see* Fig. 2) indicates that Val-35 in RafB is conserved only in the lactose permease (LacY) from *K. pneumoniae* (McMorrow et al., 1988) while the remaining three members (CscB, MelY and LacY from *C. freundii*) have an Ile residue in this position (Bockmann et al., 1992; Lee et al., 1994; Okazaki et al., 1997a, 1997b, 1997c). These predict that conversion of Val-37 to an Ala in LacY from *K. pneumoniae* would enhance transport of maltose. It is noted that substitution of residues in this particular region of helix 1 in LacY and MelY confers changes in sugar recognition and transport (Varela et al., 1997; Shinnick & Varela, 2002).

According to the multiple amino acid sequence alignment of transmembrane domain 4 in Figure 2b, Ser-138 from RafB is not conserved in the members of the OHS family. The LacY permeases from *C. freundii* and *E. coli* possess an Arg (Buchel et al., 1980; Lee et al., 1994), while LacY from *K. pneumoniae* (McMorrow et al., 1988) has an Ala and CscB from *E. coli* (Bockmann et al., 1992) has an Asn residue in the corresponding location. The Ser-139 residue from RafB, however, is conserved only in the LacY permeases from *C. freundii* and *E. coli*, whereas the LacY permease from *K. pneumoniae* has an Asn and CscB from *E. coli* has a Phe residue. The Gly-389 residue in RafB is not completely conserved in all members of the OHS family; e.g., CscB harbors a Ser in this location instead of Gly (Fig. 2c). Likewise, Ile-391 of RafB is not completely conserved in all OHS family members; e.g., LacY from *E. coli* and MelY from *E. cloacae* have a Val and a Phe in their corresponding positions, respectively. The lack of conservation of residues at positions 138, 389 and 391 suggests that evolution has preferred these sites for dictating altered substrate selection.

We observe that alteration of several amino acid residues within RafB resulted in enhanced transport of the sugar maltose in our transport studies. One may consider these residues within the context of three-dimensional structure; i.e., the residues may all be part of a single structure within the RafB permease, as explained in the carrier mechanism (Guan & Kaback, 2006). Alternatively, one may invoke the recent Brownian ratchet model in that the substituted residues enhance transport of maltose in RafB by dictating secondary structures (Naftalin et al., 2007). According to the predicted topology of LacY, and

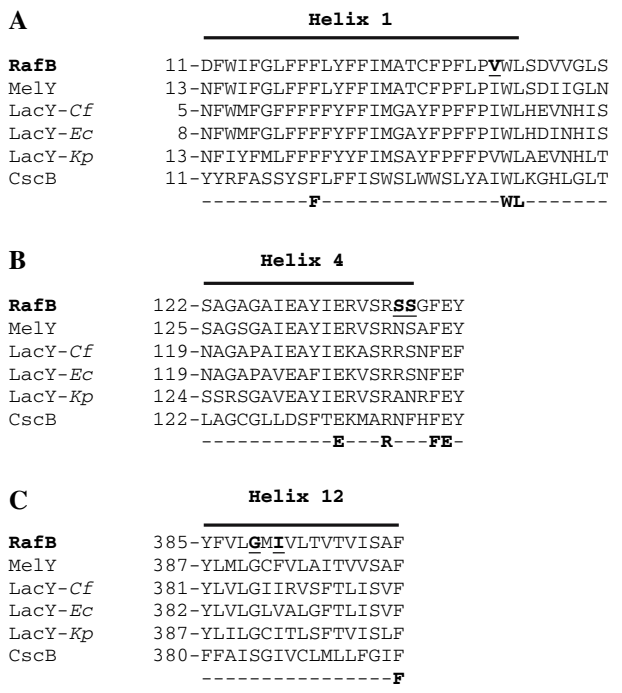

Fig. 2 Multiple amino acid sequence alignments of members of OHS family 5. The multiple sequence alignments of amino acids for helix 1 (transmembrane domain, **a**), helix 4 (**b**) and helix 12 (**c**) of the lactose permease (LacY-*Ec*) of *E. coli* are indicated by the *bold horizontal bars* and were determined by crystal structure and homology threading data (Kaback, 2005; Kasho et al., 2006) and extension to its homologous bacterial sugar transporters, the raffinose permease (RafB) of *E. coli* (Aslanidis et al., 1989), the melibiose permease (MelY) from *E. cloacae* (Okazaki et al., 1997b), the lactose permease (LacY-*Cf*) from *C. freundii* (Lee et al., 1994), the lactose permease (LacY-*Kp*) from *K. pneumoniae* (McMorrow et al., 1988) and the sucrose permease (CscB) from *E. coli* (Bockmann et al., 1992). The alignments were adapted from previous work (Varela & Wilson, 1996; Pao et al., 1998; Saier et al., 1999) as modified by the inclusion of the deduced amino acid sequence of MelY (Okazaki et al., 1997b). The residues that were found in this study to confer changes in sugar selection and transport are indicated within the alignments by *underlining*. *Bold residues* underneath each alignment indicate conserved amino acid residues

by extension RafB (Fig. 1), it is possible that the residues near the periplasmic half (Val-35, Gly-389) of the permease have distinct roles during the transport cycle from the residues near the cytoplasmic half (Ser-138, Ser-139). In any case, because position 35 of RafB was changed from a Val (bulky) to an Ala (less bulky) residue, we predict that steric hindrance plays a role in mediating substrate selection and transport in RafB. Conversion of Ser to Asp (position 138) and Leu (139) suggests that the mutated RafB structures interact with maltose by charged and hydrophobic interactions, respectively. Alteration of Gly-389 to Ala in transmembrane 12 of RafB suggests an indirect involvement in substrate selection via steric hindrance. Likewise, the conversion of Ile to Ser at position 391 in transmembrane 12 of RafB suggests an indirect involvement for this residue (Ser) in substrate selection and that this role is polar in nature or involves H-bonding properties.

Because three residues in RafB were simultaneously found to alter sugar selection and transport properties in MT-3, one could conceivably argue that not all three residues are necessary for the enhanced maltose transport that we observed here. The relative functional roles for these three residues remain unclear. Future work involving a careful dissection of MT-3 and transport studies is necessary in order to resolve whether this alternative conclusion has validity. Such studies are currently in progress using single and double mutations in RafB.

We note that Cys-scanning mutagenesis of corresponding residues in LacY (Arg-135 and Ser-136) results in the loss of transport by roughly 20% and 60%, respectively (Frillingos et al., 1997). We predict that Ser-139 in RafB (equivalent to Ser-136 in LacY) plays a functional role in sugar transport. The replacement of Gly-386 with Cys in LacY (corresponds to Gly-389 in RafB) results in severe loss of initial lactose transport and loss of expression in the membrane (Jung et al., 1995), suggesting an indirect role for residues in helix 12 of LacY. Although it is presently impossible to determine protein expression levels without anti-RafB antibodies, the observation that the mutants transport maltose implies sufficient RafB expression in this particular case. Because we observed changes in sugar selection, we surmise that Gly-389 of the RafB permease has an indirect role in substrate selection.

In summary, we provide the first mutational and physiological evidence for changes in substrate selection and transport in the RafB permease of *E. coli*. Future studies will be aimed at testing mutational and functional predictions that were made by this study in RafB within other homologues of the OHS family of bacterial sugar transporters.

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