Published in final edited form as:

J Membr Biol. 2007 December ; 220(1-3): 87–95.

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

Bonnie M. Van Camp, Robert R. Crow, Yang Peng, and Manuel F. Varela *

Eastern New Mexico University, Portales, NM 88130

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 red appeared. 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 [3H]-maltose showed 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 Acids; Bacteria; Maltose; Mutation; Permease; Raffinose; Secondary Active Transport; Sugar; Symport

Introduction

Secondary active transport of the sugar raffinose across the cytoplasmic membrane of *Escherichia coli* is mediated by the raffinose permease (RafB) (Schmid and Schmitt 1976; Aslanidis, Schmid et al. 1989; Titgemeyer, Mason et al. 1994), which is a member of the oligosaccharide: H^+ symporter (OHS) family 5 of the major facilitator superfamily (MFS) of transporters (Saier, Beatty et al. 1999). Other homologous members of the OHS family include the sucrose permease (CscB) of *E. coli* (Bockmann, Heuel et al. 1992; Sahin-Toth, Frillingos

*Correspondence to: M.F. Varela; Manuel.Varela@enmu.edu. Eastern New Mexico University, Department of Biology, Station 33, Portales, NM 88130 USA.

et al. 1995), the melibiose permease (MelY) of *Enterobacter cloacae* (Okazaki, Jue et al. 1997) and the lactose permeases (LacY) of *Citrobacter freundii*, *Klebsiella pneumoniae* and *E. coli* (Buchel, Gronenborn et al. 1980; McMorro, Chin et al. 1988; Lee, Okazaki et al. 1994; Varela and Wilson 1996; Abramson, Iwata et al. 2004). Because of shared similarities in their amino acid sequences, predicted 2-dimensional structures and phylogenetic relationships, the 3-dimensional structures of the transporters within OHS family are believed to be similar (Griffith, Baker 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, Bradley et al. 1984; Griffith, Baker et al. 1992; Hirai, Heymann 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, Smirnova et al. 2003). Studies of mutants have been useful for the study of sugar selection profiles in MelY (Shinnick, Perez et al. 2003) and in LacY (Shuman and Beckwith 1979; Brooker, Fiebig et al. 1985; Brooker and Wilson 1985; Brooker and Wilson 1985; King and Wilson 1990; Brooker 1991; Olsen, Greene et al. 1993; Varela, Brooker et al. 1997; Shinnick and Varela 2002). Primarily, transmembrane-associated amino acid residues have been implicated to play a role 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 and Beckwith 1979; Brooker, Fiebig et al. 1985; Markgraf, Bocklage et al. 1985) and Leu-88, Leu-91 and Ala-182 in MelY confer enhanced transport of maltose. Additional mutations in LacY that result in the enhancement of transport for other distinct sugars have been found and are well-documented (Brooker, Fiebig et al. 1985; Brooker and Wilson 1985; Brooker and Wilson 1985; Collins, Permeth et al. 1989; Franco, Eelkema et al. 1989; Olsen and Brooker 1989; Brooker 1990; King and Wilson 1990; Brooker 1991; Eelkema, O'Donnell et al. 1991; Gram and Brooker 1992; Goswitz and Brooker 1993; Olsen, Greene et al. 1993; Varela and Wilson 1996; Varela, Brooker et al. 1997; Varela, Wilson et al. 2000; Shinnick and 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 and Lee 2005; Kasho, Smirnova et al. 2006; Vadyvaloo, Smirnova et al. 2006; Yin, Jensen et al. 2006; Jensen, Yin et al. 2007; Klauda and Brooks 2007). In addition, studies of maltoporin (LamB) have been tremendously helpful in the elucidation the molecular basis of maltose transport across the membrane via outer membrane porin channels (Boos and Shuman 1998). Little or no such substrate selection or physiological data exists, however, for the transport of maltose in any 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, Iwata et al. 2004; Abramson, Kaback et al. 2004; Kasho, Smirnova et al. 2006; Mirza, Guan et al. 2006; Yin, Jensen et al. 2006; Jensen, Yin 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 ($\Delta malB101 \Delta melAB \Delta lac$), a gift from Dr. Howard A. Shuman (Columbia University, USA), was used as a background strain as it is deleted for its maltose transport system but can inductively express the maltose invertase enzyme (Shuman and Beckwith 1979; Brooker, Fiebig et al. 1985). Plasmid pRU600, containing an intact Raf operon (*rafR*, *rafA*, *RafB*, *rafD*) cloned into the plasmid vector pACYC184 (Chang and Cohen 1978), was a

gift from Dr. Rudiger Schmidt (Regensburg University, Republic of Germany) (Schmid and Schmitt 1976). The HS4006 bacterial strain was transformed with pRU600 plasmid using the CaCl₂ method as described previously (Shinnick, Perez 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 the 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 were incubated at 37 °C, 30 °C or 25 °C for approximately one week. Initial colonies (approximately 24–48 hr incubation) had white colony phenotypes on the indicator plates. However, after one to two 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, re-streaked 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 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 selections 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, Inc., using an ABI automated sequencer and primers complementary to F1 (5'-GGTGGTTGTTTAGAGATGCAGA-3') and F2 (5'-CGTGAACCTCCACGTTGAGATGTCA-3') promoter elements flanking the *rafB* insert (Aslanidis, Schmid et al. 1989).

SUGAR TRANSPORT ASSAYS

“Downhill” sugar transport assays of [³H]-maltose and [¹⁴C]-raffinose were performed as previously described (Varela, Brooker et al. 1997; Varela, Wilson et al. 2000; Shinnick and Varela 2002; Shinnick, Perez et al. 2003). Briefly, *E. coli* cells of the HS4006 strain containing wild-type or the 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 30mM raffinose (inducer of *rafB* expression), was seeded with approximately 0.5ml of the overnight cultures. The cells were then grown to mid-log phase of growth (OD₆₀₀ approximately 0.4). The cells were washed twice with 100 mM MOPS (pH 7.0) by centrifugation at 5000 rpm in a Beckman JA-120 rotor for 5 min., and then re-suspended such that 0.45 mg of protein were present in each sample. The transport reactions were initiated by the addition of [³H]-maltose or [¹⁴C]-raffinose to a final sugar concentration of 0.4mM. Samples (0.2 ml) were removed after 10 min., filtered and immediately washed with 100 mM MOPS (pH 7.0) containing 0.5 mM HgCl₂, which prevents sugar efflux and traps transported sugar within the cell (King and Wilson 1990). The filters were dissolved in LiquiScintTM (National Diagnostics) 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, Brooker et al. 1997). Initial transport rates for [³H]-maltose or [¹⁴C]-raffinose were determined with various sugar concentrations (0.1, 0.2, 0.4, and 2.0 mM) after cells were

incubated 15 s and 45 s in the presence of raffinose. Samples of cell suspension (0.2 ml) were removed for filtering through 0.65 μ m 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 μ g/ml overnight at 37 °C. These cells (0.5ml) were then used to seed a sub-culture at the mid-log phase of growth (OD_{600} approximately 0.4–0.6) in LB broth containing 30 μ g/ml chloramphenicol and 30mM raffinose as inducer. The cells were washed and resuspended twice using 100mM MOPS (pH 7.0) containing 0.5mM $MgSO_4$ by centrifugation at 5000 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 of protein/ml. The inhibition reactions were initiated by the 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 0.1mM, 0.2mM, 0.4mM or 2.0mM. After 15 and 45 s incubation in the presence of unlabeled maltose, 250 μ l samples were removed, filtered through a 0.65 μ m pore size filter, and washed with 3 ml 100mM MOPS (pH 7.0) buffer containing 0.5mM $HgCl_2$. The filters were dissolved in 4 ml LiquiScintTM (National Diagnostics) 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, Perez 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). *E. coli* HS2053 cells harboring the mutated pRU600 plasmids were grown overnight to saturation in 50 ml of LB broth containing 30 μ g/ml chloramphenicol with shaking at 37°C. Overnight grown cells were used to inoculate fresh LB broth containing 30 μ g/ml chloramphenicol and raffinose as an inducer of the RafB transporter, and were grown at 37°C with shaking to mid-log phase ($\Delta OD = 0.3$ –0.6). The cells were then harvested by centrifugation at 5000 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 of protein/ml and placed on ice. After the cells and radioactive sugar had equilibrated to room temperature (25 °C) for 20 min., the transport assays were initiated by the addition of 50 μ l of radioactive [3 H]-maltose to the HS2053 cells. After 10 min. incubation in the presence of [3 H]-maltose, 50 μ l samples were removed, filtered through a 0.65 μ m 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 LiquiScintTM (National Diagnostics) 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 and Schmitt 1976; Shuman and Beckwith 1979). After 1 to 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 re-streaking 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 when compared to cells containing wild-type RafB permease (Table 1).

SEQUENCES OF MALTOSE POSITIVE MUTANTS

Nucleotide sequencing of 5 stable maltose-fermenting mutants revealed three types of site mutations in the *rafB* gene on plasmids (Figure 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 [³H]-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 M-390→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-398→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 between 0.05 and 1.2 mM and apparent V_{max} values between 47 and 75 nmoles maltose/mg protein/min (see Table 3). Transport data for cells 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 the 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 values 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 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; see ref. (Shinnick, Perez 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 the wild-type RafB permease, which showed no maltose accumulation activity (Table 4).

RAFFINOSE TRANSPORT BY MUTANTS

The downhill transport of [¹⁴C]-raffinose across the membrane was measured in cells harboring the mutated or wild-type RafB permeases (see Table 3). 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 between 0.24 and 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 the 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 so when one considers residues previously established to play a critical role in proton translocation, such as Glu-325 in LacY of *E. coli* (Franco and Brooker 1994; Guan and 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.

Since 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, it suggests 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, Smirnova 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 and Brooker 2003; Abramson, Kaback et al. 2004), 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 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 in which deletion mutational analysis of helix 12 of LacY suggested that this transmembrane helix functions in stabilization of newly inserted and properly folded permeases within the membrane (McKenna, Hardy 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 Figure 2) indicates that Val-35 in RafB is conserved only in the lactose permease (LacY) from *K. pneumoniae* (McMorrow, Chin et al. 1988) while the remaining 3 members (CscB, MelY and LacY from *C. freundii*) have an Ile residue in this position (Bockmann, Heuel et al. 1992; Lee, Okazaki et al. 1994; Okazaki, Jue et al. 1997; Okazaki, Kuroda et al. 1997) (Figure 2A). 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 confer changes in sugar recognition and transport (Varela, Brooker et al. 1997; Shinnick and 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, Gronenborn et al. 1980; Lee, Okazaki et al. 1994); while LacY from *K. pneumoniae* (McMorrow, Chin et al. 1988) has an Ala, and CscB from *E. coli* (Bockmann, Heuel 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, for example, CscB harbors a Ser in this location instead of Gly (Figure 2C). Likewise, Ile-391 of RafB is not completely conserved in all OHS family members; for example, 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 result in enhanced transport of the sugar maltose in our transport studies. One may consider these residues within the context of 3-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 and 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, Green et al. 2007). According to the predicted topology of LacY, and by extension RafB (Figure 1), it is possible that the residues residing near the periplasmic half (Val-35, Gly-389) of the permease have distinct roles during transport cycle from the residues that reside 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 are interacting 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 because 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,

Gonzalez 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 the loss of expression in the membrane (Jung, 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.

Acknowledgements

We are indebted to Dr. Rudiger Schmidt of Regensburg University, Republic of Germany for plasmid pRU600, and to Dr. Howard A. Shuman of Columbia University, for *E. coli* strains HS4006 and HS2053. We thank Dr. Jeffrey Griffith of the University of New Mexico for critical evaluation of the unpublished manuscript. We thank Johnny Sena for technical assistance. This publication was made possible by NIH Grants 1 R15 GM070562-01 and P20 RR016480, the latter of which is from the NM-INBRE program of the National Center for Research Resources, as well as an Internal Research Grant awarded by Eastern New Mexico University.

References

- Abramson J, Iwata S, et al. Lactose permease as a paradigm for membrane transport proteins (Review). *Mol Membr Biol* 2004;21(4):227–36. [PubMed: 15371012]
- Abramson J, Kaback HR, et al. Structural comparison of lactose permease and the glycerol-3-phosphate antiporter: members of the major facilitator superfamily. *Curr Opin Struct Biol* 2004;14(4):413–9. [PubMed: 15313234]
- Abramson J, Smirnova I, et al. Structure and mechanism of the lactose permease of *Escherichia coli*. *Science* 2003;301(5633):610–5. [PubMed: 12893935]
- Aslanidis C, Schmid K, et al. Nucleotide sequences and operon structure of plasmid-borne genes mediating uptake and utilization of raffinose in *Escherichia coli*. *J Bacteriol* 1989;171(12):6753–63. [PubMed: 2556373]
- Bockmann J, Heuel H, et al. Characterization of a chromosomally encoded, non-PTS metabolic pathway for sucrose utilization in *Escherichia coli* EC3132. *Mol Gen Genet* 1992;235(1):22–32. [PubMed: 1435727]
- Boos W, Shuman H. Maltose/maltodextrin system of *Escherichia coli*: transport, metabolism, and regulation. *Microbiol Mol Biol Rev* 1998;62(1):204–29. [PubMed: 9529892]
- Brooker RJ. Characterization of the double mutant, Val-177/Asn-322, of the lactose permease. *J Biol Chem* 1990;265(7):4155–60. [PubMed: 2406272]
- Brooker RJ. An analysis of lactose permease “sugar specificity” mutations which also affect the coupling between proton and lactose transport. I. Val177 and Val177/Asn319 permeases facilitate proton uniport and sugar uniport. *J Biol Chem* 1991;266(7):4131–8. [PubMed: 1999407]
- Brooker RJ, Fiebig K, et al. Characterization of lactose carrier mutants which transport maltose. *J Biol Chem* 1985;260(30):16181–6. [PubMed: 3905809]
- Brooker RJ, Wilson TH. Isolation and nucleotide sequencing of lactose carrier mutants that transport maltose. *Proc Natl Acad Sci U S A* 1985;82(12):3959–63. [PubMed: 3889919]
- Brooker RJ, Wilson TH. Isolation, characterization, and nucleotide sequences of lactose permease mutants that have acquired the ability to transport maltose. *Ann N Y Acad Sci* 1985;456:350. [PubMed: 3911842]
- Buchel DE, Gronenborn B, et al. Sequence of the lactose permease gene. *Nature* 1980;283(5747):541–5. [PubMed: 6444453]

- Chang AC, Cohen SN. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J Bacteriol* 1978;134(3):1141–56. [PubMed: 149110]
- Collins JC, Permeth SF, et al. Isolation and characterization of lactose permease mutants with an enhanced recognition of maltose and diminished recognition of cellobiose. *J Biol Chem* 1989;264(25):14698–703. [PubMed: 2670925]
- Eelkema JA, O'Donnell MA, et al. An analysis of lactose permease “sugar specificity” mutations which also affect the coupling between proton and lactose transport. II. Second site revertants of the thiodigalactoside-dependent proton leak by the Val177/Asn319 permease. *J Biol Chem* 1991;266(7):4139–44. [PubMed: 1999408]
- Franco PJ, Brooker RJ. Functional roles of Glu-269 and Glu-325 within the lactose permease of *Escherichia coli*. *J Biol Chem* 1994;269(10):7379–86. [PubMed: 7907327]
- Franco PJ, Eelkema JA, et al. Isolation and characterization of thiodigalactoside-resistant mutants of the lactose permease which possess an enhanced recognition for maltose. *J Biol Chem* 1989;264(27):15988–92. [PubMed: 2674122]
- Frillingos S, Gonzalez A, et al. Cysteine-scanning mutagenesis of helix IV and the adjoining loops in the lactose permease of *Escherichia coli*: Glu126 and Arg144 are essential. *off. Biochemistry* 1997;36(47):14284–90. [PubMed: 9400367]
- Goswitz VC, Brooker RJ. Isolation of lactose permease mutants which recognize arabinose. *Membr Biochem* 1993;10(1):61–70. [PubMed: 8510563]
- Gram CD, Brooker RJ. An analysis of the side chain requirement at position 177 within the lactose permease which confers the ability to recognize maltose. *J Biol Chem* 1992;267(6):3841–6. [PubMed: 1740432]
- Griffith JK, Baker ME, et al. Membrane transport proteins: implications of sequence comparisons. *Curr Opin Cell Biol* 1992;4(4):684–95. [PubMed: 1419050]
- Guan L, Kaback HR. Lessons from lactose permease. *Annu Rev Biophys Biomol Struct* 2006;35:67–91. [PubMed: 16689628]
- Henderson PJ, Bradley S, et al. Sugar-proton transport systems of *Escherichia coli*. *Biochem Soc Trans* 1984;12(2):146–8. [PubMed: 6327421]
- Hirai T, Heymann JA, et al. Structural model for 12-helix transporters belonging to the major facilitator superfamily. *J Bacteriol* 2003;185(5):1712–8. [PubMed: 12591890]
- Jensen MO, Yin Y, et al. Sugar transport across lactose permease probed by steered molecular dynamics. *Biophys J* 2007;93(1):92–102. [PubMed: 17434947]
- Johnson JL, Brooker RJ. Role of glutamate-126 and arginine-144 in the lactose permease of *Escherichia coli*. *Biochemistry* 2003;42(4):1095–100. [PubMed: 12549931]
- Jung K, Jung H, et al. Role of glycine residues in the structure and function of lactose permease, an *Escherichia coli* membrane transport protein. *Biochemistry* 1995;34(3):1030–9. [PubMed: 7827019]
- Kaback HR. Structure and mechanism of the lactose permease. *C R Biol* 2005;328(6):557–67. [PubMed: 15950162]
- Kasho VN I, Smirnova N, et al. Sequence alignment and homology threading reveals prokaryotic and eukaryotic proteins similar to lactose permease. *J Mol Biol* 2006;358(4):1060–70. [PubMed: 16574153]
- King SC, Wilson TH. Characterization of *Escherichia coli* lactose carrier mutants that transport protons without a cosubstrate. Probes for the energy barrier to uncoupled transport. *J Biol Chem* 1990;265(17):9645–51. [PubMed: 2161839]
- King SC, Wilson TH. Identification of valine 177 as a mutation altering specificity for transport of sugars by the *Escherichia coli* lactose carrier. Enhanced specificity for sucrose and maltose. *J Biol Chem* 1990;265(17):9638–44. [PubMed: 2190983]
- Klauda JB, Brooks BR. Sugar binding in lactose permease: anomeric state of a disaccharide influences binding structure. *J Mol Biol* 2007;367(5):1523–34. [PubMed: 17320103]
- Lee JJ, Okazaki N, et al. Cloning and sequencing of the gene for the lactose carrier of *Citrobacter freundii*. *Biochem Biophys Res Commun* 1994;203(3):1882–8. [PubMed: 7945341]
- Maloney PC. Bacterial transporters. *Curr Opin Cell Biol* 1994;6(4):571–82. [PubMed: 7986535]

- Markgraf M, Bocklage H, et al. A change of threonine 266 to isoleucine in the lac permease of *Escherichia coli* diminishes the transport of lactose and increases the transport of maltose. *Mol Gen Genet* 1985;198(3):473–5. [PubMed: 3892229]
- McKenna E, Hardy D, et al. Evidence that the final turn of the last transmembrane helix in the lactose permease is required for folding. *J Biol Chem* 1992;267(10):6471–4. [PubMed: 1551862]
- McMorrow I, Chin DT, et al. The lactose carrier of *Klebsiella pneumoniae* M5a1; the physiology of transport and the nucleotide sequence of the lacY gene. *Biochim Biophys Acta* 1988;945(2):315–23. [PubMed: 3056524]
- Mirza O, Guan L, et al. Structural evidence for induced fit and a mechanism for sugar/H⁺ symport in LacY. *Embo J* 2006;25(6):1177–83. [PubMed: 16525509]
- Naftalin RJ, Green N, et al. Lactose permease H⁺-lactose symporter: mechanical switch or Brownian ratchet? *Biophys J* 2007;92(10):3474–91. [PubMed: 17325012]
- Okazaki N, Jue XX, et al. A melibiose transporter and an operon containing its gene in *Enterobacter cloacae*. *J Bacteriol* 1997;179(13):4443–5. [PubMed: 9209070]
- Okazaki N, Jue XX, et al. Sequence of a melibiose transporter gene of *Enterobacter cloacae*. *Biochim Biophys Acta* 1997;1354(1):7–12. [PubMed: 9375783]
- Okazaki N, Kuroda M, et al. Characteristics of the melibiose transporter and its primary structure in *Enterobacter aerogenes*. *Biochim Biophys Acta* 1997;1326(1):83–91. [PubMed: 9188803]
- Olsen SG, Brooker RJ. Analysis of the structural specificity of the lactose permease toward sugars. *J Biol Chem* 1989;264(27):15982–7. [PubMed: 2674121]
- Olsen SG, Greene KM, et al. Lactose permease mutants which transport (malto)-oligosaccharides. *J Bacteriol* 1993;175(19):6269–75. [PubMed: 8407799]
- Pao SS I, Paulsen T, et al. Major facilitator superfamily. *Microbiol Mol Biol Rev* 1998;62(1):1–34. [PubMed: 9529885]
- Park H, Lee S. Prediction of the mutation-induced change in thermodynamic stabilities of membrane proteins from free energy simulations. *Biophys Chem* 2005;114(2–3):191–7. [PubMed: 15829352]
- Sahin-Toth M, Frillingos S, et al. Active transport by the CscB permease in *Escherichia coli* K-12. *Biochem Biophys Res Commun* 1995;208(3):1116–23. [PubMed: 7535526]
- Saier MH Jr, Beatty JT, et al. The major facilitator superfamily. *J Mol Microbiol Biotechnol* 1999;1(2):257–79. [PubMed: 10943556]
- Schmid K, Schmitt R. Raffinose metabolism in *Escherichia coli* K12. Purification and properties of a new alpha-galactosidase specified by a transmissible plasmid. *Eur J Biochem* 1976;67(1):95–104. [PubMed: 786627]
- Shinnick SG, Perez SA, et al. 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. *J Bacteriol* 2003;185(12):3672–7. [PubMed: 12775706]
- Shinnick SG, Varela MF. Altered sugar selection and transport conferred by spontaneous point and deletion mutations in the lactose carrier of *Escherichia coli*. *J Membr Biol* 2002;189(3):191–9. [PubMed: 12395284]
- Shuman HA, Beckwith J. *Escherichia coli* K-12 mutants that allow transport of maltose via the beta-galactoside transport system. *J Bacteriol* 1979;137(1):365–73. [PubMed: 368019]
- Titgemeyer F, Mason RE, et al. Regulation of the raffinose permease of *Escherichia coli* by the glucose-specific enzyme IIA of the phosphoenolpyruvate:sugar phosphotransferase system. *J Bacteriol* 1994;176(2):543–6. [PubMed: 8288553]
- Vadyvaloo V I, Smirnova N, et al. Conservation of residues involved in sugar/H⁺ symport by the sucrose permease of *Escherichia coli* relative to lactose permease. *J Mol Biol* 2006;358(4):1051–9. [PubMed: 16574149]
- Varela MF, Brooker RJ, et al. Lactose carrier mutants of *Escherichia coli* with changes in sugar recognition (lactose versus melibiose). *J Bacteriol* 1997;179(17):5570–3. [PubMed: 9287014]
- Varela MF, Wilson TH. Molecular biology of the lactose carrier of *Escherichia coli*. *Biochim Biophys Acta* 1996;1276(1):21–34. [PubMed: 8764889]

- Varela MF, Wilson TH, et al. Mutants of the lactose carrier of *Escherichia coli* which show altered sugar recognition plus a severe defect in sugar accumulation. *J Membr Biol* 2000;174(3):199–205. [PubMed: 10758173]
- Yin Y, Jensen MO, et al. Sugar binding and protein conformational changes in lactose permease. *Biophys J* 2006;91(11):3972–85. [PubMed: 16963502]

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 (open 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-289 changed to Ala. The locations of the mutations in RafB were determined according to the sequence data of RafB (Aslanidis, Schmid et al. 1989) and, by extension, the crystal structure data of LacY from *E. coli* (Kaback 2005; Guan and Kaback 2006).

A**Helix 1**

RafB	11-DFWIFGLFFFLYFFIMATCFPFLP V WLSDVVGLS
MelY	13-NFWIFGLFFFLYFFIMATCFPFLPIWLSDIIGLN
LacY- <i>Cf</i>	5-NFWMFGFFFFFYFFIMGAYFPFFPIWLHEVNHIS
LacY- <i>Ec</i>	5-NFWMFGLFFFFFYFFIMGAYFPFFPIWLHDINHIS
LacY- <i>Kp</i>	13-NFIYFMLFFFFFYFIMSAYFPFFPVWLAENVHILT
CscB	11-YYRFASSYSFLFFISWSLWWSLYAIWLKGHLGLT
	----- F ----- WL -----

B**Helix 4**

RafB	122-SAGAGAIEAYIERVSR SS GFEY
MelY	125-SAGSGAIEAYIERVSRNSAFEY
LacY- <i>Cf</i>	119-NAGAPAIEAYIEKASRRSNFEF
LacY- <i>Ec</i>	119-NAGAPAVEAFIEKVSRRSNFEF
LacY- <i>Kp</i>	124-SSRSGAVEAYIERVSRANRFEY
CscB	122-LAGCGLLDSFTEKMARNHFHEY
	----- E --- R --- FE ---

C**Helix 12**

RafB	385-YFVL GM I I VLTVTVISAF
MelY	387-YLMLGCFVLAITVVSFAF
LacY- <i>Cf</i>	381-YLVLGII R VSFTLISVF
LacY- <i>Ec</i>	382-YLVGLVALGFTLISVF
LacY- <i>Kp</i>	387-YLILGCITLSFTVISLF
CscB	380-FFAISGIVCLMLLFGIF
	----- F

Fig. 2.

Multiple amino acid sequence alignments of members of the 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 determined by crystal structure and homology threading data (Kaback 2005; Kasho, Smirnova et al. 2006) and extended to its homologous bacterial sugar transporters, the raffinose permease (RafB) of *E. coli* (Aslanidis, Schmid et al. 1989), the melibiose permease (MelY) from *E. cloacae* (Okazaki, Jue et al. 1997), the lactose permease (LacY-*Cf*) from *C. freundii* (Lee, Okazaki et al. 1994), the lactose permease (LacY-*Kp*) from *K. pneumoniae* (McMorrow, Chin et al. 1988), and the sucrose permease (CscB) from *E. coli* (Bockmann, Heuel et al.

1992). The alignments were adapted from previous work (Varela and Wilson 1996; Pao, Paulsen et al. 1998; Saier, Beatty et al. 1999) as modified by the inclusion of the deduced amino acid sequence of MelY (Okazaki, Jue et al. 1997; Okazaki, Jue et al. 1997). The residues that were found in this study to confer changes in sugar selection and transport are indicated within the alignments with underlines. The bold residues underneath each alignment indicate conserved amino acid residues.

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 MT1 and MT3 mutants originally arose on the indicator plates after incubation at 37 °C, whereas the MT2 mutants arose after incubation at 30 °C.

^aIndicates colony phenotype before mutant isolation.

^bIndicates colony phenotype subsequent to isolation and archival of mutants.

Table 2**Raffinose and Maltose Transport by Mutants**

Cell	Raffinose “Downhill” Transport ^a (Percent of Cells with RafB ⁺)	Maltose “Downhill” Transport ^b (Percent of Cells with RafB ⁺)
MT1	26 ± 9.4 %	297 ± 10.1 %
MT2	29 ± 2.8 %	181 ± 8.5 %
MT3	17 ± 4.2 %	170 ± 2.7 %

^aBacterial transport assays of [¹⁴C]-raffinose (0.4 mM final concentration) were conducted as described in the Methods. Cells harboring wild-type plasmid pRU600 had 4.25 ± 0.36 nmoles raffinose/mg protein. The 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 the wild-type RafB permease.

^bHS4006 containing wild-type pRU600 plasmid or plasmid derivatives were used to measure “downhill” entry of [³H]-maltose (0.4 mM final concentration) by wild-type and mutated RafB, respectively, according to the procedures described in the Methods section. 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.

Table 3

Kinetic Analysis of Maltose and Raffinose Entry by Cells Harboring Wild-Type or Mutated RafB Permeases

Cell	Raffinose		Maltose	
	Apparent Vmax (nmol/mg protein/ min)	Apparent Km (mM)	Apparent Vmax (nmol/mg protein/ min)	Apparent Km (mM)
RafB+	219 ± 11	1.2 ± 0.35	-	-
MT1	0.35 ± 0.03	0.53 ± 0.02	47 ± 6.2	0.2 ± 0.04
MT2	1.2 ± 0.08	0.24 ± 0.22	55 ± 8	0.05 ± 0.04
MT3	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 [^3H]-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
MT1	2.6 ± 0.23
MT2	3.3 ± 0.42
MT3	4.7 ± 0.40

Mutant plasmids were transformed into *E. coli* HS2053 cells; see ref. (Shinnick, Perez et al. 2003). The transport assays were initiated by addition of [3H]-maltose to a final concentration of 0.4 mM for a 10 min. incubation. The 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 the wild-type RafB permease.