JOURNAL OF BACTERIOLOGY, Sept. 1997, p. 5570–5573 Vol. 179, No. 17
0021-9193/97/$04.00+0

Lactose Carrier Mutants of *Escherichia coli* with Changes in Sugar Recognition (Lactose versus Melibiose)

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Received 7 April 1997/Accepted 11 June 1997

The purpose of this research was to identify amino acid residues that mediate substrate recognition in the lactose carrier of *Escherichia coli*. The lactose carrier transports the α-galactoside sugar melibiose as well as the β-galactoside sugar lactose. Mutants from cells containing the *lac* genes on an F factor were selected by the ability to grow on succinate in the presence of the toxic galactoside β-thio-*o*-nitrophenylgalactoside. Mutants that grew on melibiose minimal plates but failed to grow on lactose minimal plates were picked. In sugar transport assays, mutant cells showed the striking result of having low levels of lactose downhill transport but high levels of melibiose downhill transport. Accumulation (uphill) of melibiose was completely defective in all of the mutants. Kinetic analysis of melibiose transport in the mutants showed either no change or a greater than normal apparent affinity for melibiose. PCR was used to amplify the *lacY* DNA of each mutant, which was then sequenced by the Sanger method. The following six mutations were found in the *lacY* structural genes of individual mutants: Tyr-26→Asp, Phe-27→Tyr, Phe-29→Leu, Asp-240→Val, Leu-321→Gln, and His-322→Tyr. We conclude from these experiments that Tyr-26, Phe-27, Phe-29 (helix 1), Asp-240 (helix 7), Leu-321, and His-322 (helix 10) either directly or indirectly mediate sugar recognition in the lactose carrier of *E. coli*.

Sugar-cation cotransport (symport) in bacteria is achieved by specialized integral membrane proteins called secondary active transporters or carriers (18, 22, 28). The lactose carrier of *Escherichia coli* catalyzes the accumulation of various sugars in the cell, using the energy of the proton motive force (24). The lactose carrier has been cloned (30), sequenced (2), purified (26), extensively mutagenized (12, 13, 14, 32), and subjected to structural analyses using a variety of genetic (3, 31) and biophysical (8, 12, 13, 14) approaches. Therefore, the lactose carrier is one of the best characterized of the sugar transporters and thus a good model for the study of solute transport across the membrane.

Evidence from the hydropathy profile (6) and from several biochemical studies (3, 6, 33) suggests that the lactose carrier of *E. coli* consists of 12 transmembrane α-helices with the N and C termini residing on the cytoplasmic side of the inner membrane. Although the three-dimensional structure of the lactose carrier has not been solved, the discovery of interhelical salt bridges (17) has lent insight into the three-dimensional arrangement of several of the α-helices. For example, Asp-240→Ala revertants showed a second-site revertant at Lys-319 (to Gln) (20), whereas Lys-319→Asn revertants revealed second-site mutations at Asp-240 (to Val) (19). In each case, both the positive and negative charges were neutralized in the revertants. This finding suggested that Asp-240 and Lys-319 form a salt bridge. Thus, helix 7 is near helix 10 of the carrier. In addition to several interhelical salt bridges (14, 32), intrahelical salt bridges have been found (11, 21). For example, Glu-325→Ser revertants revealed changes three residues upstream at His-322 (changed to a neutral residue), indicating that Glu-325 and His-322 interact to form a salt bridge (21). Biophysical studies have been useful in further defining the structure of the lactose carrier protein (14).

The lactose carrier transports a variety of structurally distinct sugars, such as α-galactosides (e.g., melibiose) or β-galactosides (e.g., lactose, *o*-nitrophenylgalactoside [ONPG], thiodigalactoside, and thiomethylgalactoside [TMG]) and the monosaccharide galactose. Shuman and Beckwith (29) first isolated a lactose carrier mutant with the ability to transport the sugar maltose. Alterations in sugar recognition have since been found in a variety of lactose carrier mutants (1, 4, 5, 7, 9, 16, 23, 27). From such studies, several amino acid residues in the carrier have been implicated directly or indirectly in sugar binding. However, the molecular basis for distinguishing between lactose and melibiose recognition and transport is unknown.

Here we present data on mutants that show more severe defects in lactose transport than in melibiose transport. Mutants chosen for study were those which grew on melibiose minimal plates but failed to grow on lactose minimal plates. In sugar transport assays, mutant cells showed the striking result of having low levels of lactose downhill transport but high levels of melibiose downhill transport. DNA sequencing of these melibiose-positive, lactose-negative mutants revealed the following point mutations in the *lacY* structural genes of individual mutants: Tyr-26→Asp, Phe-27→Tyr, Phe-29→Leu, Asp-240→Val, Leu-321→Gln, and His-322→Tyr (Fig. 1).

MATERIALS AND METHODS

Reagents. Bacteriological media were from Difco. The unlabeled sugars lactose, melibiose, TMG, α-methylgalactoside (α-MG), isopropylthiogalactopyranoside (IPTG), thio-*o*-nitrophenylgalactoside (TONPG), and ONPG were obtained from Sigma. Reagents for DNA cycle sequencing and PCR were purchased from Perkin-Elmer. Reagents for DNA manipulation were obtained from Qiagen and Pharmacia Biotech. Radiolabeled [glucose-1-^{14}C]lactose was from Amersham. [*methyl*-^{14}C]TMG was from Du Pont-New England Nuclear. [^{3}H]melibiose was the generous gift of G. Leblanc. Radiolabeled sugars were purified by descending paper chromatography (Whatman no. 3MM paper), using 3:1 propanol:water. Morpholinepropanesulfonic acid (MOPS) buffer was purchased from Sigma.

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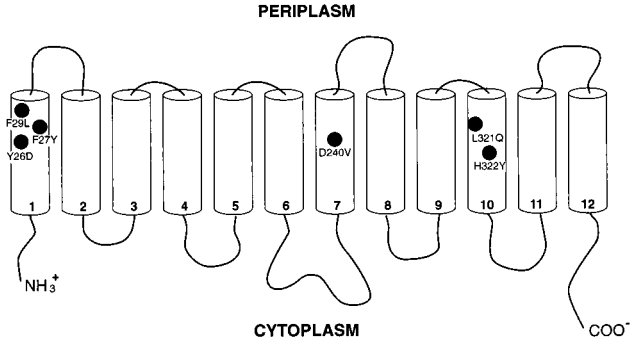

FIG. 1. Two-dimensional model showing locations of point mutations in the lactose carrier of *E. coli.*

Bacterial strains. All strains are *E. coli* K-12. The relevant genotypes are given in Table 1. Growth of *E. coli* cells on LB liquid or solid medium was always at 37°C in the presence of nalidixic acid (20 μg/ml).

Isolation of mutants. Mutants were isolated on minimal succinate plates containing 3 mM TONPG plus 0.5 mM IPTG and incubated at 37°C (25). Mutants which grew on these plates were streaked on melibiose MacConkey plates (plus IPTG). Colonies that were red were picked. These were streaked on lactose MacConkey and lactose minimal plates. Cells that were lactose negative and melibiose positive were chosen for further study. Two of the mutants (AJ36 and MUB7) were from the laboratory of B. Müller-Hill (10, 23). These also showed the lactose-negative, melibiose-positive phenotype.

To determine whether the phenotype was due to a mutation on the F′ factor, we transferred the F′ factor to *E. coli* DW2 (Δ*lac*) by mating and examined the resulting phenotype of the resulting strain, DW2/F′-*lac*$^+$. In every case, the original lactose-negative melibiose-positive phenotype was observed.

Sequencing of mutants. The *E. coli* cells selected as described above, which contain the *lacY* gene on an F′ factor, were grown to saturation in liquid LB medium and used for genomic DNA preparation (35). The DNA encoding the *lacY* gene on the F′ factor was amplified by PCR using *Taq* DNA polymerase, 100 to 200 ng of the genomic DNA preparation, and 1 μM each of two DNA primers which flank either end of the *lacY* gene. The primers were made by the Harvard Medical School Oligonucleotide Synthesis Facility. The DNA sequence of the primer flanking the transcription start site of the *lacY* gene is 5′-AAGTC ATCTGAATTCCATTACCAGTTGGTCTGGTGTC-3′ and contains an *Eco*RI restriction endonuclease cleavage site. The sequence of the primer flanking the termination end of the *lacY* gene is 5′-AAGTCATCTAAGCTTAAGCGACTT CATTCACCTGAC-3′ and contains a *Hin*dIII restriction site. The amplified *lacY* DNA was purified by using a QIAquick PCR purification kit (Qiagen) and was completely sequenced by cycle sequencing (Perkin-Elmer).

Sugar transport assays. The *E. coli* DP90 cells used in each transport study were grown at 37°C to inactivate the normal melibiose carrier, which is temperature sensitive. As a control, the uptake by DP90-ΔY was subtracted from each experimental value. Cells from a culture grown overnight at 37°C in LB plus nalidixic acid were diluted 100-fold into the same medium containing the inducers indicated for the relevant figures and tables. Cells were grown to mid-log phase at 37°C and harvested by centrifugation for 5 min at 5,000 rpm in a Sorvall SS-34 rotor at 4°C. Cells were washed twice in an equal volume of 100 mM MOPS buffer (pH 7.0) containing 0.5 mM $MgSO_4$ and 1 mM dithiothreitol. The cells were then resuspended in the same buffer to a concentration of approximately 0.45 mg of protein/ml. After equilibration for 20 min at 25°C, cells were incubated in the presence of radioactive sugar. Samples (0.2 ml) were periodically removed and vacuum filtered through 0.65-μm-pore-size filters (Sartorius). After a wash with 4 to 5 ml of 100 mM MOPS buffer (pH 7.0) containing 0.5 mM $HgCl_2$, the filters were dissolved in 4 ml of Liquiscint (National Diagnostics) plus 10% water. Radioactivity was counted in a scintillation counter.

Kinetics of transport. Melibiose transport was measured in normal or mutant cells of DP90/F′ *lacI*^{Q}*Z*$^+$*Y* with various concentrations (0.2, 0.33, 0.5, 1, 2, and 5 mM) of the sugar. Since the cells were grown in the presence of α-MG, melibiose is metabolized by α-galactosidase, and transport occurs down the sugar concentration gradient. To measure initial transport rates, radioactive sugar was mixed with the cell suspension, and samples (0.2 ml) were removed at 15 and 30 s. K_m and V_{max} were determined by the Lineweaver-Burk double-reciprocal plot.

TABLE 1. Bacterial strains and genotypes used

Strain	Genotype (chromosome/F′ factor/plasmid)	Reference or source
DW2	*lacI*$^+$ Δ(*lacZY*)*melA*$^+$ Δ*B rspL*/−/−	34
DP90-Y$^+$	Δ*lac-pro* Nal^r/F′ *lacI*^{Q}*Z*$^+$*Y*$^+$ *proAB*/−	10
DP90-ΔY	Δ*lac-pro* Nal^r/F′ *lacI*^{Q}*Z*$^+$Δ*Y proAB*/−	This work
DP90-Y26D[a]	Δ*lac-pro* Nal^r/F′ *lacI*^{Q}*Z*$^+$*Y* (Tyr-26→Asp) *proAB*/−	10, 23
DP90-F27Y	Δ*lac-pro* Nal^r/F′ *lacI*^{Q}*Z*$^+$*Y* (Phe-27→Tyr) *proAB*/−	This work
DP90-F29L	Δ*lac-pro* Nal^r/F′ *lacI*^{Q}*Z*$^+$*Y* (Phe-29→Leu) *proAB*/−	This work
DP90-D240V	Δ*lac-pro* Nal^r/F′ *lacI*^{Q}*Z*$^+$*Y* (Asp-240→Val) *proAB*/−	This work
DP90-L321Q	Δ*lac-pro* Nal^r/F′ *lacI*^{Q}*Z*$^+$*Y* (Leu-321→Gln) *proAB*/−	This work
DP90-H322Y[b]	Δ*lac-pro* Nal^r/F′ *lacI*^{Q}*Z*$^+$*Y* (His-322→Tyr) *proAB*/−	10

[a] Müller-Hill strain MUB7.
[b] Müller-Hill strain AJ36.

TABLE 2. Colony phenotypes of lactose carrier mutants[a]

Strain	Colony phenotype			
	Lactose minimal	Melibiose minimal	Lactose MacConkey	Melibiose MacConkey
Y$^+$	+	+	Red	Red
ΔY	−	−	White	White
Y26D	−	+	Red center	Red center
F27Y	−	+	White	Red
F29L	−	+	White	Red
D240V	−	+	White	Red center
L321Q	−	+	White	Red
H322Y	−	+	White	Red

[a] Growth of DW2 derivatives on minimal plates was measured in the presence of either 5 mM lactose or 5 mM melibiose plus IPTG for 3 days at 37°C. + indicates growth, and − indicates no growth. Fermentation studies were carried out on MacConkey agar plates containing either 30 mM lactose or 30 mM melibiose plus 1 mM IPTG in *E. coli* DP90/F′ *lacI*^{Q}*Z*$^+$*Y*$^{\text{normal or mutant}}$.

RESULTS

Isolation and sequencing of mutants. Mutants of *E. coli* DP90 containing the *lac* genes on an F factor were selected by the ability to grow on succinate in the presence of the toxic galactoside TONPG. The normal lactose carrier accumulates the sugar TONPG by cotransport with a proton into the cell (25). Because of the hydrophobicity of the sugar, it diffuses back out of the cell. This futile cycle is quite rapid and reduces the proton motive force, causing ATP levels to fall, and the cell fails to grow. Mutants that grow on TONPG plates either are transport negative or fail to couple proton entry to sugar accumulation. Of 35 mutants found by this method, 4 grew on melibiose minimal plates but failed to grow on lactose minimal plates (Table 2). Two more mutants (MUB7 and AJ36) were from the laboratory of B. Müller-Hill (10, 23). The six mutants fermented melibiose (red on melibiose MacConkey plates) (Table 2). The fermentation of lactose was negative (white colonies on lactose MacConkey plates) or defective (white with red center). The DNA encoding the mutant *lacY* gene present on the F′ factor of each mutant was isolated, amplified by PCR, and sequenced (see Materials and Methods).

Sequence analysis revealed that each mutant had a single-point mutation in the *lacY* structural gene: Tyr-26→Asp, Phe-27→Tyr, Phe-29→Leu, Asp-240→Val, Leu-321→Gln, or His-322→Tyr (Fig. 1). Three of the amino acids residues found to be mutated are located in helix 1 of the lactose carrier (Tyr-26, Phe-27, and Phe-29). One of the altered amino acids lies in helix 7 (Asp-240). Lastly, two affected residues lie in helix 10 (Leu-321 and His-322).

Transport of melibiose and of lactose. Melibiose entry was measured in cells grown in the presence of α-MG, an inducer

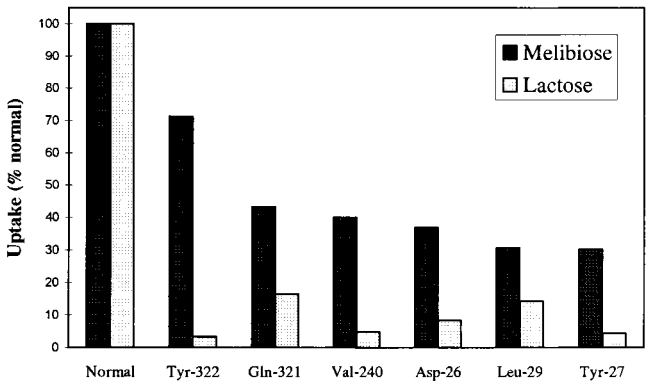

FIG. 2. Comparison of melibiose and lactose transport for mutants of the lactose carrier of *E. coli*. DP90/F′ *lacI*^{O}*Z*$^{+}$*Y*$^{\text{normal or mutant}}$ cells were grown at 37°C (this inactivates the normal melibiose carrier) in the presence of α-MG and IPTG. Under these conditions, both α-galactosidase and β-galactosidase are induced; thus, melibiose or lactose entering the cell is rapidly metabolized. Hence, the internal concentration of sugar is kept very low and sugar entry is thermodynamically downhill. Washed cells were exposed to 0.4 mM [^{3}H]melibiose or 0.4 mM [^{14}C]lactose and filtered for either 30 s or 1 min at 25°C. The normal lactose carrier showed the transport of 8.8 ± 2.5 nmol of lactose/mg of protein and 11.4 ± 3.3 nmol of melibiose/mg of protein during the downhill transport experiment after 1 min. Data represent the averages of three or more independent experiments.

TABLE 3. Transport of sugars by mutants of the lactose carrier

Strain	% of normal		
	ONPG entry[a]	Melibiose accumulation[b]	TMG accumulation[c]
Y^{+}	100	100	100
Y26D	35	5	8
F27Y	21	2	3
F29L	35	3	17
D240V	25	5	5
L321Q	35	9	50
H322Y	16	3	2

[a] Cells of *E. coli* DP90/F′ *lacI*^{O}*Z*$^{+}$*Y*$^{\text{normal or mutant}}$ were grown in the presence of 1 mM IPTG to induce the *lac* operon. ONPG entry was measured in the presence of 2 mM ONPG at 25°C for 1 h. Data represent the averages of three independent experiments.

[b] Melibiose accumulation was measured in the presence of 0.4 mM [^{3}H] melibiose at 25°C after 6 min. *E. coli* DP90/F′ *lacI*^{O}*Z*$^{+}$*Y*$^{\text{normal or mutant}}$ cells were grown with 1 mM TMG, which induces the *lac* system without inducing the *mel* system (36). Normal cells accumulated melibiose 11-fold. Data represent the averages of three independent experiments. Less than 10% of normal indicates no accumulation.

[c] Cells of *E. coli* DP90/F′ *lacI*^{O}*Z*$^{+}$*Y*$^{\text{normal or mutant}}$ were grown in 1 mM IPTG. Transport of TMG was measured by using *E. coli* DP90/F′ *lacI*^{O}*Z*$^{+}$*Y*$^{\text{normal or mutant}}$ in the presence of 0.1 mM [*methyl*-^{14}C]TMG after 10 min at 25°C. Normal cells accumulated TMG 30-fold. Data represent the averages of three independent experiments.

of the enzyme α-galactosidase (Fig. 2). In these cells, melibiose is metabolized as it enters the cells so that the internal concentration of the sugar remains low. Therefore, the entry of the sugar is thermodynamically downhill and the accumulation of melibiose does not occur. In this transport experiment, the external melibiose concentration was 0.4 mM. All mutants showed significant downhill entry of melibiose. For example, the lowest melibiose transport activity observed was 30% of the normal level (for the mutant Phe-27→Tyr), whereas the highest activity of melibiose transport for any of the mutants was 71% of the normal level (His-322→Tyr).

The uptake of lactose was studied in cells grown in the presence of IPTG where the enzyme β-galactosidase was induced (Fig. 2). Lactose enters the cell on the carrier and is metabolized. The external lactose concentration was 0.4 mM. In the mutants, the transport of lactose was greatly reduced and ranged between 3% (for His-322→Tyr) and 16% (for Leu-321→Gln) of the normal lactose carrier level. Thus, every lactose carrier mutant in this study had the striking result of showing a significantly higher rate of melibiose downhill entry (30 to 71%) than lactose downhill entry (3 to 16%) compared to the parent.

Other transport properties of mutants. Entry of the lactose analog ONPG was measured in each of the mutants and ranged between 16 and 35% of the normal lactose carrier level (Table 3). Accumulation of melibiose against a concentration gradient was absent for all mutants (Table 3). Accumulation of the lactose analog TMG was completely defective for the mutants Tyr-26→Asp, Phe-27→Tyr, Asp-240→Val, and His-322→Tyr. On the other hand, TMG accumulation was 17 and 50% of the normal level for the mutants Phe-29→Leu and Leu-321→Gln, respectively.

Kinetics of melibiose transport. A kinetic analysis of melibiose transport was conducted for all mutants in the study (Table 4). It is interesting that two mutants, Phe-29→Leu and Leu-321→Gln, showed an enhanced apparent affinity for melibiose. In contrast, the apparent affinity for melibiose was approximately the same as that of normal cells for the mutants Tyr-26→Asp, Phe-27→Tyr, Asp-240→Val, and His-322→Tyr. In all cases, the apparent V_{max} for melibiose ranged between 17 and 38% of the normal level. The rate of transport of lactose by the mutants was too low for reliable kinetic data to be obtained.

DISCUSSION

The results of this study indicate that Tyr-26, Phe-27, Phe-29, Asp-240, Leu-321, and His-322 are important for mediating substrate recognition in the lactose carrier protein of *E. coli* (Fig. 1). The normal carrier transports lactose and melibiose at about the same rate (Fig. 2). On the other hand, all of the mutants show a defect in lactose transport which is much more severe than that for melibiose (Fig. 2). This finding suggests that these six residues of the carrier are directly or indirectly involved in the structure of the lactose binding site. In addition, the apparent affinity for melibiose is enhanced (Table 4) in two of the mutants (Phe-29→Leu and Leu-321→Gln), which suggests that residues in helices 1 and 10 play a role in forming the structure that binds melibiose.

Previous studies have found mutants with altered sugar recognition. Mutants selected for growth on maltose (1) showed two sites of mutation: Ala-177 (changed to Val or Thr) and Tyr-236 (changed to Phe, Ser, or His). When cells were selected for growth on sucrose, a cell was isolated with the Ala-177→Val mutation (16). A mutant studied by Markgraf et al. (23) with Thr-266→Ile showed reduced lactose transport

TABLE 4. Kinetics of downhill transport of melibiose[a]

Strain	K_m (mM)	V_{max} (nmol/mg of protein/min)
Y^{+}	0.83 ± 0.24	53 ± 2.5
Y26D	0.58 ± 0.41	14 ± 1.0
F27Y	0.72 ± 0.42	13 ± 5.9
F29L	0.32 ± 0.03	15 ± 0.5
D240V	0.77 ± 0.31	9 ± 3.5
L321Q	0.19 ± 0.06	13 ± 2.7
H322Y	0.92 ± 0.15	20 ± 4.6

[a] See Materials and Methods for details.

and enhanced maltose transport. When the maltose-positive Ala-177→Val cells were incubated in maltose plus the inhibitor cellobiose, second-site revertants could be isolated (4). One of these revertants showed the original mutation plus His-322→Tyr as the second mutation. Thus, mutations which cause changes in sugar recognition were found in helices 6, 7, and 10.

It is interesting that one of the mutants in the present study (His-322→Tyr) is identical to the second-site mutation in the maltose-cellobiose revertant Ala-177→Val/His-322→Tyr mentioned above. In addition, the data in this study for the His-322→Tyr expressed on an F factor confirm the observation of high melibiose transport found with a high-copy-number plasmid containing this mutation (15). Three of the mutants found in this study were located in either helix 7 (Asp-240→Val) or helix 10 (His-322→Tyr and Leu-321→Gln), both regions previously identified as important for sugar recognition (4, 15, 23) and in forming salt bridges (19, 21). The three mutations in helix 1 are unique, as this helix had not previously been implicated in sugar recognition. This finding suggests that these residues in helix 1 are involved directly or indirectly in sugar binding. Furthermore, it is striking that the Tyr-26 residue is completely conserved in the lactose carriers in three different microorganisms but is different in the raffinose and sucrose carriers (32).

Despite the fact that the mutants show moderately good melibiose entry in the downhill assay, no uphill accumulation against a concentration gradient of melibiose was observed in any mutant. This indicates that in addition to having alterations in sugar binding, these mutants are defective in the energy coupling to melibiose accumulation. Two of these mutants (Leu-29 and Gln-321) which failed to accumulate melibiose showed significant accumulation of TMG. Thus, the mechanism of energy coupling to different sugars must be regulated somewhat differently.

In summary, the data presented here identify amino acid residues in the lactose transport protein that play a role in distinguishing between the sugar substrates lactose and melibiose. These residues are found in transmembrane helices 1, 7, and 10 of the lactose carrier. This finding confirms and extends previous studies implicating several amino acids of the carrier protein in sugar recognition and suggests that the sugar binding site contains charged amino acids (Asp-240 and His-322) that are known to form salt bridges.

ACKNOWLEDGMENTS

The research in this laboratory was supported by NIH grant DK05736.

We thank Mikkaa Vikkula for helpful discussions.

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