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Lactose Transport System of Streptococcus thermophilus

THE ROLE OF HISTIDINE RESIDUES*

Bert Poolman‡, Roel Modderman‡, and Jonathan Reizer†

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The lactose transport protein (LacS) of Streptococcus thermophilus is a chimeric protein consisting of an amino-terminal carrier domain and a carboxyl-terminal phosphoenolpyruvate:sugar phosphotransferase system (PTS) IIA protein domain. The histidine residues of LacS were changed individually into glutamine or arginine residues. Of the 11 histidine residues present in LacS, only the His-376 substitution in the carrier domain significantly affected sugar transport. The region around His-376 was found to exhibit sequence similarity to the region around His-322 of the lactose transport protein (LacY) of Escherichia coli, which has been implicated in sugar binding and in coupling of sugar and H+ transport. The H376Q mutation resulted in a reduced rate of uptake and altered affinity for lactose (King and Wilson, 1989a, 1989b, 1990). Moreover, the H322N mutation in the IIA protein domain of LacS corresponds to His-91 of the IIA protein of E. coli, which has been shown to be phosphorylated by HPr-P (Dorschug et al., 1984). The lactose transport protein (LacS) is a secondary active transport system that catalyzes the uptake of various α- and β-galactosides in symport with a proton as well as exchange of galactosides. The heterologous exchange of lactose for galactose most likely occurs during metabolism of lactose by S. thermophilus and Lactobacillus bulgaricus (Poolman, 1990).

Although the overall similarity between the carrier domain of LacS and the lactose carrier (LacY) of E. coli is not significant (Poolman et al., 1989), a region (H376/E379) in LacS can be identified that is similar to the H322/E325 region in LacY (Fig. 1). Evidence has been presented indicating that His-322, Glu-325 (putative helix X), and Arg-302 (putative helix IX) of LacY are in close contact with each other and that these residues could form a "charge relay" system that participates in the coupled transmembrane movement of protons and galactosides (Püttnner et al., 1986, 1989; Carrasco et al., 1986, 1989; Menick et al., 1987; Lee et al., 1988). Recently, the requirement of an ionizable histidine residue at position 322 in LacY for galactoside/proton symport has been questioned (King and Wilson, 1989a, 1989b; Franco and Brooker, 1991). It has been shown that some His-322 mutants of LacY, which do not build up a galactoside concentration gradient, catalyze galactoside-dependent proton transport (King and Wilson, 1989a, 1989b, 1990). Moreover, the H322N mutant of LacY still accumulates lactose against a concentration gradient, although the levels of accumulation are low compared to those of the wild-type strain (Franco and Brooker, 1991).

In this study, we assess the role of the histidine residues in the carrier and IIA protein domains of the lactose transport protein of S. thermophilus. The results indicate that His-552 is phosphorylated by HPr(His-P) from Bacillus subtilis, but that the conserved histidine residues in the IIA protein domain of LacS are not essential for transport. His-376 in the carrier domain of LacS could serve a role similar to that of His-322 in LacY. The similarities and differences between the proposed active-site residues of LacS and LacY are discussed.

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† Supported by a fellowship from the Royal Netherlands Academy of Arts and Sciences. To whom correspondence should be addressed. Tel.: 31-50-632170; Fax: 31-50-630298.

‡ The abbreviations used are: PTS, phosphoenolpyruvate:sugar phosphotransferase system; TMG, methyl-β-D-thio-galactopyranoside.

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EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids

Bacterial strains are described in Table I. The cells were grown aerobically in Luria broth at 37 °C (Sambrook et al., 1989) unless indicated otherwise. Plasmid pEK88 containing a 4.1-kilobase EcoRI chromosomal DNA fragment from S. thermophilus, encoding the lactose transport protein (LacS), has been described (Poolman et al., 1989, 1990). For the construction of pSKE8, the 4.1-kilobase EcoRI fragment of pEK88 was ligated in the EcoRI site of pBluescript IISK+ (Stratagene). Plasmid pAVL1 is pACYC177 that carries a 9.5-kilobase HindIII chromosomal DNA fragment of Klebsiella pneumoniae KAY2026, carrying the nag operon, including nagE, which codes for IICA-N-acetylglucosamine (Vogler et al., 1988).

Site-directed Mutagenesis

The mutagenic primers are listed in Table II. Site-directed mutagenesis was carried out by the method of Kunkel et al. (1987).

Mutations in pSKE8—Single-stranded uracil-containing DNA of pSKE8 was isolated from E. coli CJ236 (dur-, ung-)/pSKE8 after infection with helper phage M13KQ7 (Sambrook et al., 1989). Closed-circular heteroduplex DNA with the desired mutations was synthesized in vitro as described (Kunkel et al., 1987) and transformed into E. coli JM101 (ung-). Plasmid DNA was isolated from a number of transformants, and plasmids bearing the desired mutation(s) were identified by digestion with restriction enzymes for which a site was lost or created by the mutagenesis (see Table II). Subsequently, mutations were verified by nucleotide sequencing of double-stranded DNA using the dideoxy chain termination method (Sanger et al., 1977) and a set of primers complementary to a region of lacS located 50–100 bases downstream or upstream of the mutation site. Each mutant was isolated independently at least twice.

Mutations in pEK88—The 648-base pair KpnI-EcoRI fragment of pEK88, comprising the carboxyl-terminal 323-base pair region of lacS, was ligated in the replicative form of M13mp18 or M13mp19. Recombinant phage DNA was used to transfect E. coli CJ236, and single-stranded uracil containing M13mp18 or M13mp19 DNA, including the KpnI-EcoRI fragment, was isolated. Synthesis of the complementary strand and screening of mutants were carried out as described above. The entire region of lacS was sequenced. Subsequently, the KpnI-EcoRI fragment of pEK88 was exchanged for the corresponding fragment containing the desired mutation that was isolated from the replicative form of the M13 DNA. Preparation of Cell Suspensions

Overnight cultures or exponentially grown cells (A600 = 0.4–0.8) were harvested by centrifugation, washed twice, and resuspended to a final protein concentration of 20–80 mg/ml in 50 mM potassium phosphate (pH 6.5), 5 mM magnesium sulfate (KPM buffer). Measurements of the membrane potential and analysis of the effects of ionophores (valinomycin, nigericin) on transport were performed with E. coli cells that were treated with EDTA essentially as described previously (Sarkar et al., 1988). Concentrated cell suspensions were stored on ice until use.

Transport Assays

Transport experiments were performed at 20, 30, or 37 °C as specified in the figure legends. Active Transport—Cells were diluted to a final protein concentration of 0.6–1.2 mg/ml in KPM buffer containing 10 mM L-lactate as the electron donor. After 2 min of pre-energization in the presence of oxygen, radiolabeled substrate was added; and at appropriate time intervals, the uptake reaction was stopped by addition of 2 ml of ice-cold 100 mM LiCl. Cells were collected by filtration on a 0.45-μm cellulose nitrate filter (Millipore Corp.) and washed with 2 ml of ice-cold LiCl.

Efflux and Exchange—Preloading of cells with sugars was achieved by incubation (overnight at 4 °C) of the concentrated washed cell suspensions with the appropriate concentration of radiolabeled galactoside in the presence of deoxyribonuclease I (20 μg/ml). The next day, potassium azide and carbonyl cyanide m-chlorophenylhydrazone were added to final concentrations of 30 mM and 50 μM, respectively; and the cells were incubated for another 2 h at room temperature essentially as described (King and Wilson, 1990). For efflux and exchange, 1-μl aliquots of concentrated cell suspension (50–80 mg/ml) were diluted into 500 μl of KPM buffer containing no substrates and unlabeled galactosides, respectively. The transport reaction was stopped by rapid filtration as described above.

Isolation of Membranes

For the isolation of inside-out membrane vesicles of E. coli T184, T184/pEKSS, T184/pEKSS(H552Q), T184/pEKSS(H537A), and T184/pEKSS(is48E/O555D), cells were lysed by a 2-fold passage through a French pressure cell (20,000 p.s.i.), and membranes were collected as described (Poolman et al., 1983). The membranes were washed and resuspended to 20–30 mg of protein/ml in 10 mM Tris-HCl (pH 7.5) containing 0.5 mM phenylmethylsulfonyl fluoride.

Protein Purification

Enzyme I and HPr of B. subtilis and E. coli were purified as described previously (Reizer et al., 1988, 1992).

Phosphorylation Assay

Inside-out membrane vesicles (5 mg/ml) were incubated in 50 mM Tris-HCl (pH 7.5) containing 5 mM MgCl2, 0.1 mM dithiothreitol, 0.5 mM NaN3, EDTA, purified HPr (0.5 mg/ml) and Enzyme I (0.35 mg/ml), and [32P]P-enolpyruvate (Reizer et al., 1984). The phosphorylation reaction was carried out at 37 °C for 15 min in a total volume of 15 μl. The reaction was stopped by addition of an equal volume of Laemmli sample buffer (Laemmli, 1970), and proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5% polyacrylamide). TABLE I

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DW1</td>
<td>lac*, Δlac(ZY), Δmel(AB), strA</td>
<td>Wilson and Wilson (1987)</td>
</tr>
<tr>
<td>DW2</td>
<td>lac+Δlac(ZY), melAΔ, ΔmelB, strA</td>
<td>Wilson and Wilson (1987)</td>
</tr>
<tr>
<td>PPA209</td>
<td>DW2, pts1001 rfa: Tn10</td>
<td>Postmaa</td>
</tr>
<tr>
<td>T184</td>
<td>lacO ΔZY(A), rpsL, met, thr, recA, hsdR, hsdM(F', lacO ΔZY(11)K′(Y+A+))</td>
<td>Teather et al. (1980)</td>
</tr>
<tr>
<td>LR2-162</td>
<td>thi-1, hisG1, argG6, melB1, galT8, xyl-7, supE44, rpsL104,ΔphoA5, tonA2, ptsM, manI, man-162, amyB: Tn5 F'</td>
<td>Vogler et al. (1988)</td>
</tr>
<tr>
<td>LR2-167</td>
<td>LR2-162, nagE</td>
<td>Vogler et al. (1988)</td>
</tr>
<tr>
<td>LM1</td>
<td>LR2-162, nagE, crp-1</td>
<td>Vogler et al. (1988)</td>
</tr>
<tr>
<td>JM101</td>
<td>supE, thi, Δlac-pro(A), F', trd36, proAB, lacI73ΔM15</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>CJ236</td>
<td>dut-1, ung-1, thi-1, relA1/pCJ105 (CmR)</td>
<td>Kunke et al. (1987)</td>
</tr>
<tr>
<td>HB101</td>
<td>hsdS20(rm ma), recA13, ara14, proA2, lacY1, galk2, rps (smR), xyl-5, mitl-1, supE44, λ, F'</td>
<td>Boyer and Roulland-Dussoix (1969)</td>
</tr>
</tbody>
</table>

a P. W. Postma, unpublished data.
that were obtained from the main chain torsion angle distributions.

Phenotype of each of the mutants appeared to be indistinguishable from that of the wild-type. Similar results were obtained when transport studies were carried out with the levels of lactose, melibiose, and TMG were determined over a wide range of concentrations (Table II). The data show that the H376Q mutant had a lower K_T and V_max for lactose, whereas the kinetic properties of the other mutants were not significantly affected.

Since LacS transports lactose as well as melibiose (α-galactoside), TMG, galactose, and other galactosides, the effects of the H376Q mutation on the uptake of these carbohydrates were compared (Fig. 3). To avoid metabolism of lactose and melibiose, transport studies were carried out with E. coli DW1 bearing the wild-type or H376Q mutant proteins. The kinetic parameters for lactose uptake by E. coli DW1 bearing the wild-type or H376Q mutant proteins were similar to those obtained with E. coli DW2 bearing the corresponding transport protein (Table II). The data presented in Fig. 3 show that uptake of melibiose and TMG was markedly more affected by the H376Q mutation than uptake of lactose. In fact, the uptake rates were too low to determine accurately the kinetic parameters of the H376Q mutant (Fig. 3, B and C, insets). The K_T values for uptake of lactose, melibiose, and TMG by LacS (wild type) were 0.7, 0.53, and 0.27 mM, respectively. The V_max values for uptake of lactose, melibiose, and TMG were 270, 140, and 60 nmol/min/mg of protein, respectively.

In contrast to transport of lactose (Fig. 3A), TMG was not accumulated against a concentration gradient by the H376Q mutant (Fig. 3C). To establish whether this difference indeed reflected differences in the ability to accumulate various sugars or whether the apparent failure to accumulate TMG was due to higher efflux by passive and/or facilitated diffusion, a number of experiments were carried out. First, efflux of TMG was analyzed by diluting [14C]TMG-loaded cells into buffer without TMG. No significant differences were observed between the wild-type and H376Q mutants (Fig. 4). Second, the level of accumulation of TMG by the wild-type and H376Q proteins was determined over a wide range of concentrations (5–165 μM) and at different pH values (5.5–8.0). If passive efflux of TMG, in combination with a reduced rate of uptake, affects the accumulation level, one might observe a significant

**Materials**

[14C]Benzoic acid (50 mCi/mmol), methyl-α-2,3,4-[14C]glucopyranoside (50 mCi/mmol), and D-glucose-1-[14C]lactose (57 mCi/mmol) were obtained from the Radiochemical Centre (Amersham, United Kingdom). [14C]Methyl-β-D-thiogalactopyranoside (50 mCi/mmol) was obtained from Du Pont-New England Nuclear. [14C]Melibiose (3.2 Ci/mmol) was a generous gift of Dr. G. LeBlanc (Bassilana et al., 1987).

**RESULTS**

**Histidine Mutagenesis**—The lactose transport protein (LacS) of *S. thermophilus* contains 11 histidine residues (circled in Fig. 1A), of which 5 are present in the carrier domain and 6 in the IIA protein domain. Each of the histidine residues was replaced by site-directed mutagenesis with glutamine or arginine (Table II). Glutamine and arginine were chosen as replacements on the basis of amino acid similarity coefficients that were obtained from the main chain torsion angle distributions of highly resolved protein structures (Niefeld and Schomburg, 1991). Plasmids with the histidine mutations were used to transform *E. coli* HB101, and transformants were streaked on lactose MacConkey agar plates. The lac phenotype of each of the mutants appeared to be indistinguishable from that of the wild-type strain, i.e. HB101/pSKE8 or HB101/pEKS8.

**Lactose Transport by Histidine Mutants**—Each of the plasmids bearing a mutation resulting in the replacement of a single histidine residue was used to transform *E. coli* DW2 (ΔlacZY). Lactose transport by DW2/pSKE8 (wild-type and histidine mutants) is shown in Fig. 2. With the exception of the H376Q (and to some extent, H155Q) mutant protein(s), the initial rates of uptake and the steady-state levels of lactose accumulation of the histidine mutants were similar to those of the wild-type. Similar results were obtained when transport of TMG by the mutant proteins was assayed (data not shown). To ensure that secondary mutations did not contribute to the observed phenotype, the transport activity of each mutant was determined with two independent isolates. The data from a second set of mutants did not differ significantly from those presented in Fig. 2. To analyze the transport properties of each of the mutants in more detail, the affinity constant (K_T) and maximal rate of uptake (V_max) for lactose were determined (Table III). The data show that the H376Q mutant had a lower K_T and V_max for lactose, whereas the kinetic properties of the other mutants were not significantly affected.

Since LacS transports lactose as well as melibiose (α-galactoside), TMG, galactose, and other galactosides,2 the effects of the H376Q mutation on the uptake of these carbohydrates were compared (Fig. 3). To avoid metabolism of lactose and melibiose, transport studies were carried out with *E. coli* DW1 bearing the wild-type or H376Q mutant proteins. The kinetic parameters for lactose uptake by *E. coli* DW1 bearing the wild-type or H376Q mutant proteins were similar to those obtained with *E. coli* DW2 bearing the corresponding transport protein (Table II). The data presented in Fig. 3 show that uptake of melibiose and TMG was markedly more affected by the H376Q mutation than uptake of lactose. In fact, the uptake rates were too low to determine accurately the kinetic parameters of the H376Q mutant (Fig. 3, B and C, insets). The K_T values for uptake of lactose, melibiose, and TMG by LacS (wild type) were 0.7, 0.53, and 0.27 mM, respectively. The V_max values for uptake of lactose, melibiose, and TMG were 270, 140, and 60 nmol/min/mg of protein, respectively.

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2 B. Poolman, unpublished data.
Fig. 1. A, secondary structure model of lactose transport protein of *S. thermophilus* based on hydrophathy plot of deduced amino acid sequence (Poolman et al., 1989). Histidine residues are circled, and His-376 is also indicated by number. B, conserved residues in carrier domain of LacS and lactose, melibiose, and raffinose transport proteins of *E. coli*. The region corresponds to the loop that connects helixes X and XI of LacS. Sources of sequences are as follows: *E. coli* (ec) RafB, Aslanidis et al. (1989); *K. pneumoniae* (kp), LacY, Memorrow et al. (1988); *E. coli* LacY, Büchel et al. (1988); *S. thermophilus*, (st) LacS, Poolman et al. (1989); *B. Vogesiacus* (bs) LacS, Poolman et al. and *E. coli* MelB, Yazyu et al. (1984). C, Conserved residues in IIA protein domain of LacS. The region shown corresponds to the proposed phosphorylation site of LacS. Sources of sequences are as follows (see also B): Crt, Nelson et al. (1983); NagE, Rogers et al. (1988); HgS (also indicated as BglF), Schneitz et al. (1997); Streptococcus mutans (sm) SerA, Sato et al. (1989); and *B. subtilis* (bs) PtsG, Gonzy-Tréboul et al. (1989) and Sutrina et al. (1990). Residues corresponding to His-537 and His-552 in LacS are indicated by arrows 1 and 2, respectively. Shaded arrows indicate residues that are conserved in the PTS proteins, but not in LacS.

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The data presented above describe the effect of H376Q substitution on uphill (Ap-driven) transport by the lactose carrier protein. Under these conditions, a complete translocation cycle includes binding and release of H⁺ and galactoside as well as reorientation of the loaded and unloaded binding sites. Since LacS also catalyzes an exchange reaction involving only binding and release of galactoside and reorientation of loaded binding sites, nonequilibrium exchange of TMG and lactose by the wild-type and H376Q transport proteins were compared (Figs. 4 and 5). For the exchange reaction, cells were preloaded with [14C]lactose (47 µM final concentration), whereas maltose (α-glucoside) and sucrose (fructofuranoside) did not. The pattern of inhibition of lactose uptake by the unlabeled sugars does not indicate major differences in sugar recognition of the wild-type and H376Q transport proteins, although raffinose (trisaccharide/galactoside) was a more effective inhibitor of LacS(H376Q) compared with LacS(wild type).

* B. Poolman, S. Yoast, and B. F. Schmidt, unpublished data.

**Concentration gradient** ([TMG]ex > [TMG]out) at low concentrations only. Some accumulation (2-3-fold) of TMG was observed at concentrations <50 µM and at pH 5.5–6.0 (data not shown); the level of TMG accumulation by the wild-type strain also decreased with increasing TMG concentration. In conclusion, the data suggest that the H376Q mutation affects sugar recognition by LacS as well as the coupling between galactoside and proton transport.

To assess the sugar recognition properties of the wild-type and H376Q proteins in more detail, the effect of an 85-fold excess of unlabeled sugar on uptake of lactose was determined. The data presented in Table IV show that the galactosides lactose, melibiose, TMG, β-D-galactopyranosyl-1-thio-β-D-galactopyranoside, isopropyl-α-thio-β-D-galactopyranoside, and galactose inhibited uptake of [14C]lactose (47 µM final concentration), whereas maltose (α-glucoside) and sucrose (fructofuranoside) did not. The pattern of inhibition of lactose uptake by the unlabeled sugars does not indicate major differences in sugar recognition of the wild-type and H376Q transport proteins, although raffinose (trisaccharide/galactoside) was a more effective inhibitor of LacS(H376Q) compared with LacS(wild type).

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Lactose Transport in S. thermophilus

Fig. 2. Lactose uptake by E. coli DW2/pSKE8 wild-type and histidine LacS mutants. Cells were suspended in KPM buffer (pH 6.5) supplemented with 10 mM D-lithium lactate to a final protein concentration of 0.8 mg/ml. After 2 min of pre-energization, [14C] lactose was added to a final concentration of 10 μM, and uptake was assayed for different time intervals. Cells harvested in the stationary phase of growth were used; the assay temperature was 30 °C. HxQ, histidine mutants except H155Q and H376Q. ○, wild-type (WT) LacS; □, H155Q mutant; ◇, H376Q mutants. The shaded area denotes the results obtained with all other histidine mutants of LacS.

Table III

Kinetic parameters of lactose transport by wild-type and histidine LacS mutants

Experimental conditions were similar to those described in the legend to Fig. 3, except that lactose concentrations ranged from 10 μM to 1.6 mM.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>K_T (mM)</th>
<th>V_max (nmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0.8</td>
<td>210</td>
</tr>
<tr>
<td>H38Q</td>
<td>1.0</td>
<td>163</td>
</tr>
<tr>
<td>H51Q</td>
<td>1.2</td>
<td>180</td>
</tr>
<tr>
<td>H155Q</td>
<td>1.1</td>
<td>215</td>
</tr>
<tr>
<td>H376Q</td>
<td>0.27</td>
<td>40</td>
</tr>
<tr>
<td>H423Q</td>
<td>0.1</td>
<td>214</td>
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<td>H577Q</td>
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<td>235</td>
</tr>
<tr>
<td>H466Q</td>
<td>0.6</td>
<td>150</td>
</tr>
<tr>
<td>H502Q</td>
<td>0.8</td>
<td>160</td>
</tr>
<tr>
<td>H523Q</td>
<td>0.9</td>
<td>170</td>
</tr>
<tr>
<td>H537R</td>
<td>1.0</td>
<td>174</td>
</tr>
<tr>
<td>H612Q</td>
<td>0.9</td>
<td>180</td>
</tr>
</tbody>
</table>

the observed isotope exit reaction reflects the real exchange of galactoside. Fig. 4 shows that initially the release of [14C] TMG from the cell was monoexponential with a slope that depends upon the external TMG concentration. The exchange reaction catalyzed by the H376Q mutant was somewhat slower than that of the wild-type strain (the K_T values for external TMG were very similar, i.e. between 0.6 and 1.0 mM). This can be seen more readily from the kinetic analysis of lactose in/ lactose out exchange (Fig. 5). At an internal lactose concentration of 4.5 mM (see legend to Fig. 5), the exchange kinetics yielded K_T values for external lactose of ~10 mM, which is about an order of magnitude higher than that of Δp-driven lactose uptake (Fig. 3 and Table III).

Role of II A Protein Domain—The homology between the II A protein domain of LacS and the II A protein (domain) of the glucose, N-acetylgalactosamine, β-glucoside, and sucrose PTSs (Fig. 1C) suggests that Enzyme I and HPr can catalyze P-enolpyruvate-dependent phosphorylation of His-552 of the lactose transport protein. The data presented in Fig. 2 and

Fig. 3. Uptake of lactose, melibiose, and TMG by E. coli DW1/pSKE8 LacS(wild type) and LacS(H376Q). Experimental conditions were the same as described in the legend to Fig. 2, except that for the time course of galactoside uptake, cells harvested in the exponential phase of growth were used; [14C]lactose, [3H]melibiose,
varying from 10 pM to 1 mM. The assay temperature was 37 °C; the pEKS8 (ptsI-) were compared. The results obtained with the from uptake after wild type.

ary phase of growth were used. Initial rates of uptake were estimated 

ation of the protein is not essential for transport activity. To 

phosphorylation of His-552 does not occur or that phosphoryl-

ation does not affect transport activity. This suggests that 

Table III, on the other hand, indicate that the H552Q mutation does not affect transport activity. This suggests that phosphorylation of His-552 does not occur or that phosphorylation of the protein is not essential for transport activity. To assess the functional activities of the IIA protein domain of LacS, several approaches were undertaken. First, Δp-driven uptake and exchange by DW2/pEKS8 and PFA209/ pEKS8 (pSp1-) were compared. The results obtained with the and [3H]TMG were added to final concentrations of 45, 44, and 43 μM, respectively, and the assay temperature was 37 °C. For the kinetics of galactoside uptake (insets), cells harvested in the stationary phase of growth were used. Initial rates of uptake were estimated from uptake after 10 s (in triplicate) at galactoside concentrations varying from 10 μM to 1 mM. The assay temperature was 37 °C; the final protein concentrations were between 0.8 and 1.2 mg/ml. WT, wild type.

pT1 mutant were similar to those of the wild-type mutant (data not shown). Second, manipulation of the level of PTS phosphoprotein intermediates by addition of nonmetabolizable sugar analog methyl-α-D-glucopyranoside and 2-deoxyglucose (Nelson et al., 1986) did not affect the lactose transport activities. Third, pEKS8 (lacS+) was unable to complement E. coli LM1 (crr-, nagE) with respect to transport of α-methylglucoside. In accordance with previous observations (Vogler et al., 1988), pAVL1 containing nagE was able to restore the uptake of α-methylglucoside in E. coli LM1 to ~20% of the wild-type activity (strain LR2-167). Fourth, by examining the sequences of the IIA protein(s) (domains) of the lactose transport proteins of S. thermophilus and L. bulgaricus and the PTS proteins indicated in Fig. 1C, two positions were identified at which the lactose transport proteins differed significantly from the PTS proteins, i.e. Ile-548 and Gly-556. Three residues are close to proposed phosphorylation site residue (His-552); and the crystal structure of the IIAGc protein domain of the glucose PTS of B. subtilis indicates that the equivalent residues, i.e. Glu-79 and Asp-87, can assume a role in recognition of the interactive PTS proteins (the IIB protein domain or HPr) and facilitate the phosphotransfer reaction (Liau et al., 1991). The double mutant LacS(1548E/556D) was constructed, but the 1548E/556D mutations did not affect lactose transport activity (data not shown).

Phosphorylation of Lactose Transport Protein—To establish whether LacS could be phosphorylated by HPr(His-P), inside-out membrane vesicles of E. coli T184/pKK223-3 (lacS, vector control) and T184/pEKS8 (lacS+) and various mutants...
to form a \( H^+ \) relay system that could function as the chemical pathway for \( H^+ \) movement through the lactose carrier (Carrasco et al., 1986, 1989; Puttnet al., 1986, 1989; Menick et al., 1987; Lee et al., 1989). Although evidence against this proposal has been presented (King and Wilson, 1989a, 1989b; Franco and Brooker, 1991), it is evident that this region is crucial for energy transduction by the carrier protein. Mutations at position 322 in LacY also affect the sugar recognition properties of LacY (Franco et al., 1989; Collins et al., 1989). Indeed, some properties of the LacY(H322) mutants resemble those of LacS(H376Q). For instance, active accumulation of lactose, melibiose, and TMG is highly compromised in cells bearing the H376Q protein. Furthermore, the affinity for galactosides (lactose) is altered by the H376Q substitution. The histidine residue corresponding to His-376 in LacS and His-322 in LacY is not conserved in MelB. Recent experiments in which the histidine residues of MelB were replaced with arginine residues indicated that only His-94 is important for transport activity (Pourcher et al., 1990a). The H94R mutation caused complete loss of sugar binding and transport, whereas melB was expressed normally.

Interestingly, the residue corresponding to Glu-325 of LacY (Fig. 1B, arrow 4) is conserved in the lactose transport proteins of \( E. \ coli \), \( K. \ pneumoniae \), \( S. \ thermophilus \), and \( L. \ bulgaricus \); the raffinose transport protein of \( E. \ coli \); and the melibiose carrier of \( E. \ coli \). Substitution of Glu-325 in LacY leads to a transport protein that does not catalyze lactose/\( H^+ \) symport or lactose efflux, but catalyzes exchange and counterflow at normal rates (Carrasco et al., 1986). Similarly, substitution of the corresponding residue in MelB (Glu-361) for glycine, aspartate, or alanine affects sugar and cation translocation, but not recognition of the substrates (Pourcher et al., 1990b).

Mutations of the conserved lysine (Fig. 1B, arrow 2) have been isolated for LacY after selection for enhanced recognition of maltose (\( \alpha \)-glucoside) and resistance for TDG (Collins et al., 1989). The LacY(K319N) mutant protein exhibits diminished recognition of \( \beta \)-galactosides and \( \beta \)-glucosides. The conserved acidic residue corresponding to arrow 1 (Fig. 1B) has not been investigated in any of the transport proteins. Overall, comparison of the sequences presented in Fig. 1B and the data obtained from mutagenesis experiments suggests that the region connecting helices X and XI of LacS is important for substrate recognition.

A difference worth noting between the LacS and LacY regions is the higher polarity of the residues around His-376 (LacS) in comparison to the region around His-322 (LacY). As a consequence, hydropathy analysis predicts the His-322 region of LacY to be in the membrane, whereas the His-376 region of LacS is predicted to be in a cytoplasmic loop. Nevertheless, the local environment of the important residues in both protein segments might be similar if the regions are located near the head groups of the lipid bilayer.

The effect of the H376Q substitution on galactoside transport by LacS has been analyzed by measuring \( \Delta p \)-driven uptake and exchange under nonequilibrium conditions. Assuming that the \( V_{\text{max}} \) of the exchange reaction reflects the level of expression of the lactose transport protein, the effect of the H376Q mutation on the \( k_{\text{cat}} \) of \( \Delta p \)-driven uptake is \( \sim 2 \)-fold smaller than indicated by the \( V_{\text{max}} \) values for uptake of lactose, melibiose, and TMG (Fig. 3). The observation that \( \Delta p \)-driven transport of galactosides is more severely affected by the His-376 mutation than the exchange reaction suggests that the histidine at position 376 is important for energy transduction, i.e. coupled movement of galactosides and protons. The results also indicate that His-376 is not obligatorily conserved in the Lacs protein of \( S. \ bulgaricus \), the Lacy protein of \( S. \ thermophilus \), and the lacS protein of \( S. \ thermophilus \).

DISCUSSION

On the basis of hydropathy analysis and a comparison of the primary structures of LacS and MelB, a secondary structure model of the lactose transport protein is proposed (Fig. 1A). The polypeptide is organized into 12 hydrophobic transmembrane \( \alpha \)-helical domains that are connected alternately by periplasmic (OUT) and cytoplasmic (IN) hydrophilic charged segments. The topology of the periplasmic loops that connect helixes I and II, III and IV, and V and VI as well as the cytoplasmic loop that connects helixes VI-VII has been confirmed by analyzing lacS-alkaline phosphatase gene (phoA) fusions (Manoil and Beckwith, 1986): The IIIA protein was not detectable with Enzyme I and HPr isolated from \( E. \ coli \) (data not shown). Altogether, these results indicate that PTS-catalyzed phosphorylation of His-552 of Lacs can occur, but that it does not serve an essential functional role in the transport of lactose under the conditions employed in this study.

\( ^{3} \) V. Swarte and B. Poolman, unpublished data.
required for the active accumulation of galactosides.

The in vitro phosphorylation assays indicated that LacS can be phosphorylated by HPr(His-P) of *B. subtilis* and that His-562 in the IIA protein domain is most likely the phosphorylation site. Nevertheless, the in vivo transport experiments that were performed in *E. coli* bearing LacS did not provide an indication regarding the functional and/or regulatory role(s) of the phosphorylation reaction. Although *B. subtilis* IIA *<sup>Δ</sup>* can readily replace *E. coli* IIA *<sup>Δ</sup>* with various strains and for helpful discussions and Wil N. Konings for critical reading of the manuscript.

Acknowledgments—We thank Pieter W. Postma for providing us with various *E. coli* strains and for helpful discussions and Wil N. Konings for critical reading of the manuscript.

REFERENCES


