JOURNAL OF BACTERIOLOGY, Jan. 1989, p. 244–253
0021-9193/89/010244-10$02.00/0

Vol. 171, No. 1

Lactose Transport System of *Streptococcus thermophilus*: a Hybrid Protein with Homology to the Melibiose Carrier and Enzyme III of Phosphoenolpyruvate-Dependent Phosphotransferase Systems

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Received 12 August 1988/Accepted 3 October 1988

The gene responsible for the transport of lactose into *Streptococcus thermophilus* (*lacS*) was cloned in *Escherichia coli* as a 4.2-kilobase fragment from an *Eco*RI library of chromosomal DNA by using the vector pKK223-3. From deletion analysis, the gene for lactose transport mapped to two *Hin*dIII fragments with a total size of 2.8 kilobases. The gene was transcribed in *E. coli* from its own promoter. Functional expression of lactose transport activity was shown by assaying for the uptake and exchange of lactose both in intact cells and in membrane vesicles. The nucleotide sequence of *lacS* and 200 to 300 bases of 3′ and 5′ flanking regions were determined. The gene was 1,902 base pairs long, encoding a 69,454-dalton protein with an NH_2-terminal hydrophobic region and a COOH-terminal hydrophilic region. The NH_2-terminal end was homologous with the melibiose carrier of *E. coli* (23% similarity overall; >50% similarity for regions with at least 16 amino acids), whereas the COOH-terminal end showed 34 to 41% similarity with the enzyme III (domain) of three different phosphoenolpyruvate-dependent phosphotransferase systems. Among the conserved amino acids were two histidyl residues, of which one has been postulated to be phosphorylated by HPr. Since sugars are not phosphorylated during translocation by the lactose transport system, it is suggested that the enzyme III-like region serves a regulatory function in this protein. The *lacS* gene also appears similar to the partially sequenced lactose transport gene of *Lactobacillus bulgaricus* (*lacL*; >60% similarity). Furthermore, the 3′ flanking sequence of the *S. thermophilus* lactose transport gene showed approximately 50% similarity with the N-terminal portion of the β-galactosidase gene of *L. bulgaricus*. In both organisms, the lactose transport gene and the β-galactosidase appear to be separated by a 3-base-pair intercistronic region.

Lactose transport in bacteria generally proceeds via either the phosphoenolpyruvate-dependent sugar transferase system (PEP-PTS) or a proton motive force (pmf)-linked transport system (W. N. Konings, B. Poolman, and A. J. M. Driessen, Crit. Rev. Microbiol., in press). In the former system, lactose enters the cell as lactose 6-phosphate, which is subsequently cleaved into glucose and galactose 6-phosphate by a β-D-phosphogalactoside galactohydrolase. Lactose accumulated by the pmf-driven transport system enters the cell as free sugar and is hydrolyzed into galactose and glucose by a β-D-galactoside galactohydrolase (β-galactosidase).

Dairy lactic acid bacteria, including species of both the lactic streptococci and lactobacilli, utilize lactose as their primary energy source. In a number of species which transport lactose by a PEP-PTS, the *lac* genes involved, their organization on the chromosome or plasmid, and their regulation are well documented (1, 4, 33). In contrast, very little is known about the *lac* genes in lactic acid bacteria that encode a pmf-linked lactose transport system in conjunction with a β-galactosidase. Representatives of this group of organisms are *Streptococcus thermophilus*, the homofermentative lactobacilli like *Lactobacillus bulgaricus* and *L. helveticus*, and the heterofermentative lactobacilli like *L. brevis* and *L. buchneri* (14, 15, 37). In this paper, we report the cloning of the pmf-linked lactose transport system of *S. thermophilus* and provide information on the arrangement of the *lac* genes in this organism as well as in *L. bulgaricus*. We also show that the carboxy-terminal end of the lactose transport protein in *S. thermophilus* and *L. bulgaricus* is homologous to the enzymes III (domains) of various PEP-PTSs. We speculate that the degree of phosphorylation of the conserved active-site histidine in this region might regulate lactose transport in *S. thermophilus* (and *L. bulgaricus*) in a manner similar to that in which the lactose carrier of *Escherichia coli* is regulated by enzyme III^{Glc} (28, 29). In this respect, it is important to note that *S. thermophilus* and *L. bulgaricus* do possess functional (glucose) PTSs (14) which allow phosphorylation of enzymes III in principle.

MATERIALS AND METHODS

Bacterial strains and cultivation of organisms. *E. coli* HB101 [*hsdS20* (r^-_B m^-_B) *recA13 ara-14 proA2 lacY1 galK2 rps* (Sm^r) *xyl-5 mtl-1 supE44* λ^- F^-], MC1061 [Δ*lac* (IPOZYA) *araD139* Δ(*ara-leu*)*7697 galU galK rpsL* r_K m_k], JM101 (45), T206 (42), and AZ-1 {*recA thi* Δ(*lac-proAB*) [F′ lac^+ Δ(*lacY-lacA*) pro^+]} (J. Lee and H. R. Kaback, unpublished results) were grown in LB or minimal medium (23) supplemented with essential nutrients as indicated by the auxotrophic markers. *E. coli* P678-54 [*thr-1 leuB6 azi-8 tonA2 lacY1 minA1 supE44*? *gal-6* λ^- *minB2 rfbD1 mgl-50 galP63 rpsL135 malA1* (λ^r) *xyl-7 mtl-2 thi-1*] was grown in M9 medium containing 2 g of Casamino Acids per liter (23). When appropriate, the medium was supplemented with carbenicillin (50 μg/ml), tetracycline (15 μg/ml), and/or streptomycin (25 μg/ml). All *E. coli* strains were grown with vigorous aeration at 37°C. *S. thermophilus* A147 and *L. bulgaricus* B131 (Centre International de Recherche Daniel Carasso, BSN group) were grown in MRS broth (Difco

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Laboratories, Detroit, Mich.) supplemented with 1% (wt/vol) lactose or glucose at 42°C.

Cloning of the transport system. Chromosomal DNA isolated (B. F. Schmidt, R. M. Adams, C. Requadt, S. Power, and S. E. Mainzer, J. Bacteriol., in press) from *S. thermophilus* A147 was partially digested with restriction enzyme *Eco*RI, *Hin*dIII, or *Pst*I. The cleaved DNA was fractionated by polyacrylamide gel (5% wt/vol) electrophoresis, after which fragments 1 to 15 kilobases (kb) in length were electroeluted from the gel. The chromosomal DNA fragments were mixed with vector pKK223-3 (Pharmacia Inc., Piscataway, N.J.) that had been linearized with the corresponding restriction enzymes and dephosphorylated. After ligation, the resulting chimeric plasmids were used to transform *E. coli* cells which were made competent by the $CaCl_2$-RbCl method (23). The transformed organisms were spread on MacConkey agar (Difco) plates containing 1% (wt/vol) lactose and 50 μg of carbenicillin per ml (plus 25 μg of streptomycin per ml, in the case of strain HB101).

Transport assays. Cells grown to an A_{600} of 0.5 to 1.0 were harvested by centrifugation, washed two times, and suspended in buffer at 10 to 60 mg of total cellular protein per ml. Transport of [D-glucose-1-^{14}C]lactose (57 mCi/mmol; Radiochemical Centre, Amersham, England) and [^{14}C] methyl-β-D-thiogalactopyranoside (TMG) (50 mCi/mmol; New England Nuclear Research Products, Boston, Mass.) was assayed at 30°C in 200-μl incubation mixtures as described previously (32). Details of these measurements are specified in the legend to Fig. 2. For transport studies in cytoplasmic membranes, right-side-out membrane vesicles were isolated from *E. coli* AZ-1 (pEKS8) and *E. coli* AZ-1, as described elsewhere (17). Transport experiments with membrane vesicles were carried out in 50 mM potassium-phosphate (pH 6.6)–5 mM $MgSO_4$ buffer in the presence and absence of the electron donor D-lactate (Na-salt; 10 mM final concentration). Further manipulations were the same as those described for transport measurements in intact cells.

DNA-DNA hybridization. Chromosomal DNA was digested with the appropriate restriction enzymes and fractionated by agarose gel electrophoresis. DNA transfer to Nytran (Schleicher & Schuell, Inc., Keene, N.H.) filters and nick translation of plasmids or plasmid fragments were carried out as described previously (23).

DNA sequencing. DNA fragments were cloned into M13tg130, M13tg131 (Amersham Corp., Arlington Heights, Ill.), M13mp18, and M13mp19 (New England BioLabs, Inc., Beverly, Mass.) by using *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I, and *Hin*cII restriction sites. The nucleotide sequences of the fragments were determined by using the dideoxy-chain termination method (39). When necessary, synthetic oligonucleotide primers (17-mers) were synthesized (Genentech Inc.) for sequencing of selected regions of the gene fragments. Most of the DNA sequencing was done with guanosine and inosine (25) in the sequencing reactions.

Minicells. Transformation of the minicell-producing *E. coli* P678-54 was performed as described above. Minicells were purified by three subsequent sucrose gradient centrifugations (24), and the in vivo-labeled ([^{35}S]methionine, 1,100 Ci/mmol; Amersham) proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The ^{35}S-labeled proteins were identified by autoradiography.

Miscellaneous. Plasmid DNA was isolated by the alkaline lysis method of Birnboim and Doly (3). Protein was measured by the method of Lowry et al. (21) with bovine serum albumin as the standard.

RESULTS

Cloning of the lactose transport gene. For shotgun cloning, fractionated *S. thermophilus* chromosomal DNA was ligated in the expression vector pKK223-3. pKK223-3 was used as the cloning vector since DNA fragments could be inserted downstream of the *tac* promoter (7) in case the cloned lactose transport gene did not possess a promoter or in case the *S. thermophilus* promoter was not functional in *E. coli*. The ligation mixtures were used to transform *E. coli* HB101, after which the cells were spread on MacConkey-lactose plates containing carbenicillin and streptomycin. Colonies growing on these plates are red when the *lacY1* mutation of *E. coli* HB101 is functionally complemented by the lactose transport gene of *S. thermophilus* and are white without the gene or when its complementation is ineffective. From one shotgun cloning experiment with *Eco*RI-digested *S. thermophilus* DNA, one colony out of approximately 2,000 transformants was found to be red, even after repeated transfer from liquid medium to MacConkey plates. The latter procedure was carried out to discriminate true *lac*$^+$ clones from false positives (pink-red colonies) which appeared among the transformants at low frequencies. Analysis of plasmid DNA from the red colony showed that the pKK223-3 vector contained a 4.2-kb *Eco*RI insert. This chimeric plasmid was designated pEKS8.

Since the *E. coli* HB101 *lacY* gene has only a point mutation rendering it inactive, pEKS8 was used to transform *E. coli* AZ-1 in which the *lac* operon was deleted from the chromosome but which contained the β-galactosidase gene (*lacZ*) on an F factor. Although the transformation efficiency of *E. coli* AZ-1 was low compared with that of *E. coli* HB101, pEKS8 was able to complement the *lacY* deletion of *E. coli* AZ-1 (growth on lactose minimal medium; red colony phenotype on MacConkey plates), confirming that the lactose transport system was indeed located on pEKS8.

Construction of deletion derivatives and subcloning of fragments. A restriction enzyme map of the *S. thermophilus* 4.2-kb *Eco*RI insert was constructed by double and triple digestions. The map is shown together with flanking regions of the pKK223-3 vector in Fig. 1A. No restriction sites were detected within the insert for the enzymes *Asu*II, *Bam*HI, *Bgl*II, *Hpa*I, *Mlu*I, *Nsi*I, *Pvu*I, *Sac*I, *Sac*II, *Sal*I, *Sma*I, *Stu*I, *Xba*I, and *Xho*I.

The restriction map was used to locate the lactose transport gene on pEKS8 by subcloning fragments. Subclones of pEKS8 were generated by restriction and random ligation (pEKS8-11, pEKS8-17, pEKS8-4, pEKS8-7, and pEKS8-24) or religation of one or two isolated fragments in the appropriate vectors (pEKS8-100, pEKS8-115, and pEBS8). Maps of the plasmid derivatives and the phenotypes of *E. coli* HB101 transformed with these plasmids on MacConkey plates are shown in Fig. 1B. Where necessary, i.e., when isolated fragments were religated (pEKS8-7, pEKS8-24, pEKS8-100, and pEKS8-115), the orientations of the fragments with respect to the *tac* promoter or with respect to each other were determined in separate experiments. The data indicate that the gene for the lactose transport system can be located more precisely to two *Hin*dIII fragments with a combined length of 2.8 kb (pEKS8-100). Furthermore, insertion of the 4.2-kb *Eco*RI fragment in pBR322 at the *Eco*RI site containing no promoter yields a Lac$^+$ phenotype, indicating that the gene for the lactose transport system uses its own promoter.

DNA-DNA hybridization. To confirm that the gene for lactose transport originated from the *S. thermophilus* chro-

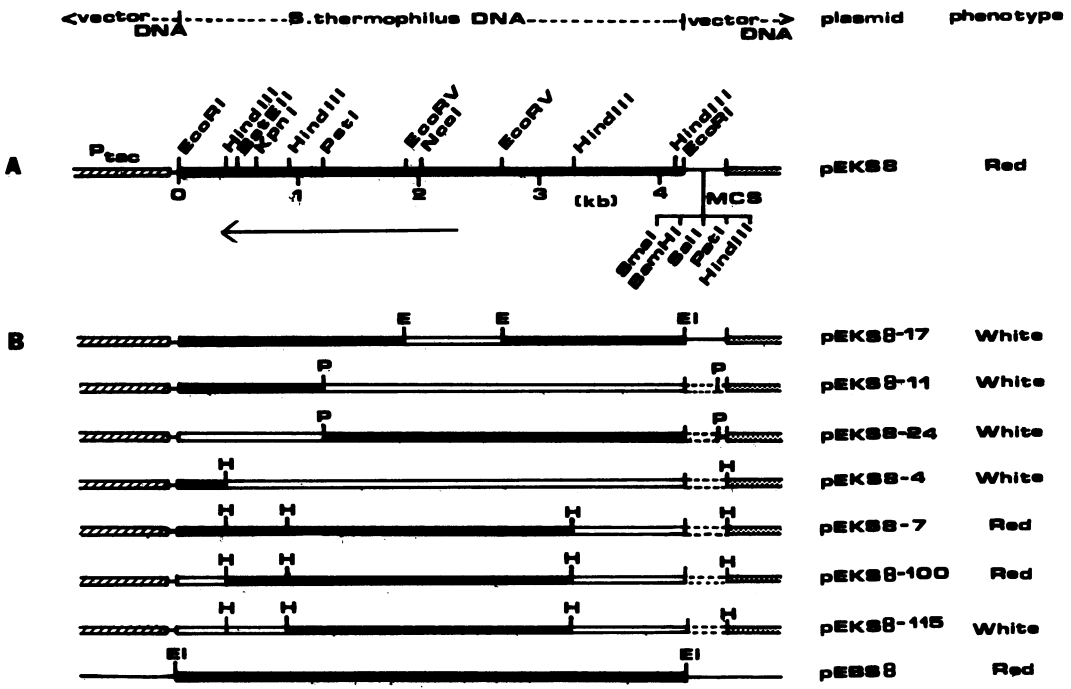

FIG. 1. Restriction endonuclease map (A) and characterization of deletion derivatives (B) of pEKS8. The construction of the plasmids is described in the text. Phenotypes of *E. coli* HB101 transformed with these plasmids were determined by growing the organisms on MacConkey plates and screening for red and white colony types. Vector DNA indicates plasmid pKK223-3 for all derivatives except pEBS8, for which pBR322 was used as cloning vehicle. P_{tac} and MCS refer to the *tac* promoter and the multiple cloning site of pKK223-3, respectively. Symbols: ▭, deletions in the lactose transport gene; ▨ and ▩, P_{tac} and vector DNA, respectively. The position and the direction of transcription of the lactose transport gene are indicated by the arrow. EI, *Eco*RI; E, *Eco*RV; H, *Hin*dIII; P, *Pst*I.

mosome, chromosomal DNA of *S. thermophilus* was digested with *Eco*RI or *Hin*dIII and fractionated by agarose gel electrophoresis. A nick-translated pEKS8 fragment hybridized with a 4.2-kb *Eco*RI fragment and with 2.2-, 0.85-, and 0.55-kb *Hin*dIII fragments (data not shown) in accordance with the restriction map (Fig. 1A). The same probe did not hybridize to *E. coli* chromosomal DNA fragments corresponding to *lacY* (6, 42). These experiments suggest that the *S. thermophilus* and *E. coli* lactose transport genes are not very similar, although in *E. coli* functional complementation occurred.

Expression of the lactose transport gene. To demonstrate that the lactose transport gene of *S. thermophilus* is functionally expressed in *E. coli*, lactose transport activity was assayed in *E. coli* MC1061 *lacS*$^+$ (pEKS8), which lacks the *E. coli lac* operon but contains the lactose transport gene of *S. thermophilus*. Lactose uptake in this strain proceeded with an initial rate of about 3 nmol/min × (milligrams of protein) in the presence of glucose (Fig. 2). The maximum accumulation level of lactose ($[lactose]_{in}/[lactose]_{out}$) in the steady state was about 40. Without glucose as a source of metabolic energy in the medium, lactose uptake was significantly reduced. Importantly, uptake of lactose was not observed in *E. coli* MC1061 *lacS*, i.e., the parent strain without plasmid pEKS8 (Fig. 2).

Accumulated lactose could be chased from the cell upon addition of excess unlabeled lactose. This exchange reaction indicates that lactose is present intracellularly as free sugar, supporting the contention that net uptake proceeds via a pmf-linked mechanism. Further evidence for a role of the pmf as the driving force for lactose transport comes from studies with membrane vesicles prepared from *E. coli* AZ-1 *lacS*$^+$ (pEKS8). In the presence of the electron donor D-lactate, the membrane vesicles accumulated lactose, whereas no significant amount of lactose was taken up in the absence of D-lactate (data not shown). At a lactose concentration of 50 μM and in the presence of D-lactate, the initial rate of uptake was 1.4 nmol/min × (milligrams of protein). Lactose transport in membrane vesicles was inhibited by the ionophores valinomycin plus nigericin, which dissipate the pmf.

Results similar to those observed for *E. coli* MC1061 *lacS*$^+$ (pEKS8) were obtained with *E. coli* AZ-1 *lacS*$^+$ (pEKS8) cells by monitoring the uptake of the nonmetabolizable lactose analog TMG (data not shown). On the basis of the extent of (competitive) inhibition of TMG uptake, these experiments indicated that the affinities of the transport system for various β-galactosides were, in descending order, for galactose, lactose, and TMG.

Nucleotide sequence of the lactose transport gene and its flanking regions. The nucleotide sequence of the lactose transport gene (*lacS*) and about 500 base pairs (bp) of flanking sequences are shown in Fig. 3. *lacS* consists of 1,902 bp which correspond to a protein monomer of 634 amino acids with a molecular mass of 69,454 daltons. The GC content of *lacS* is 38.1%. The translation initiation site at position 194 is proposed (Fig. 3) on the basis of the start of the open reading frame (at bp 182), the position of the putative ribosome-binding site (RBS) (AGGA at bp 180), and the amino acid sequence homology between the lactose and melibiose transport systems (Fig. 4A). Another potential start site (at bp 215) seems less likely since the spacing between the RBS (AGG at bp 208) and the ATG codon is somewhat short, only 4 bases (12, 41).

Since the lactose transport gene in *E. coli* is transcribed from its own promoter, nucleotide sequences upstream of

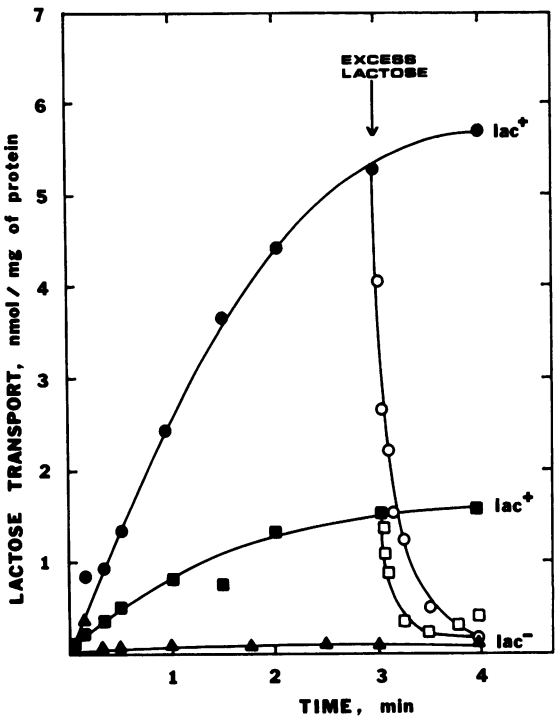

FIG. 2. Transport of lactose in *E. coli* MC1061 with (lac$^+$) and without (lac$^-$) pEKS8 which contains the *S. thermophilus* lactose transport gene. Concentrated cell suspensions were diluted to a final protein concentration of about 0.5 mg/ml into 60 mM K-PIPES [piperazine-*N*,*N*'-bis(2-ethanesulfonic acid); pH 7.2]–3 mM $MgSO_4$ buffer in the absence (■ and □) or presence (●, ○, and ▲) of 10 mM glucose. After 1 min of incubation, [D-glucose-1-^{14}C]lactose was added to a final concentration of 50 μM. The transport reaction was stopped at different time intervals as described in Materials and Methods. Open symbols indicate results of an excess of unlabeled lactose (2 mM, final concentration) added to the cells.

lacS were searched for −10 and −35 promoter regions by using consensus sequences reported for gram-positive and gram-negative bacteria (13). Strong consensus promoter regions, supported by additional conserved A clusters at −42 to −40 (nucleotides 126 to 128) and −5 to −3 (nucleotides 162 to 164) that are characteristic for gram-positive bacteria (13), were found (Fig. 3). Clearly, these promoter elements are in accordance with those recognized by RNA polymerases from both gram-negative and gram-positive bacteria. The possibility that different promoters are recognized in *S. thermophilus* and *E. coli* cannot, however, be ruled out.

The stop codon of the lactose transport gene is found 32 bp downstream of a *Hin*dIII site (bp 2058 to 2063) (Fig. 3). The nucleotide region downstream of this *Hin*dIII site appeared not to be essential for functional lactose metabolism in *E. coli* HB101 since it was absent in pEKS8-100 (Fig. 1), indicating that the last 11 amino acids of the lactose transport protein could be removed without inactivating the system. In the construction of pEKS8-100, a stop codon is found 38 bp downstream of the *Hin*dIII site, i.e., in the *tac* promoter region of pKK223-3.

By aligning the amino acid sequence derived from the 3' flanking nucleotide sequence of *lacS* with that of β-galactosidase of *L. bulgaricus* (Schmidt et al., in press) (Fig. 4B), evidence was obtained that the lactose transport gene and the β-galactosidase of *S. thermophilus* are present in a single operon (see also below). The intercistronic region between the stop codon of the lactose transport gene and the translation initiation codon of the β-galactosidase consists of three nucleotides. Furthermore, 8 nucleotides upstream of the β-galactosidase start codon an RBS (AGGAG at bp 2148 to 2152) is found which is indicative of a translational reinitiation mechanism (12).

Amino acid sequence homology. The amino acid sequence of the lactose transport system of *S. thermophilus* has been compared with that of various transport proteins, including cation-substrate symporters and enzymes II of PEP-PTSs. Homology was found only between the NH_2-terminal end of the *lacS* protein and the melibiose carrier (*melB* protein) of *E. coli* (46) (Fig. 4A). The overall similarity between these two transport systems is 23%. Extensive similarity, i.e., >50%, was found in the regions of residues 8 to 24, 86 to 107, and 136 to 159 as well as in a number of smaller segments. The significance of the sequence homology was demonstrated by a high score in the Lipman and Pearson algorithm (20). For this test, the sequence homology score was compared with that for a large number of random amino acid sequence alignments of the proteins. No significant sequence similarity was found either between the *lacS* protein of *S. thermophilus* and the transport proteins for lactose (*lacY*) (6), arabinose (*araE*) (22), and proline (*putP*) (26) of *E. coli* or between the *lacS* protein and the PEP-PTS enzymes II or the NH_2-terminal ends of the enzymes II-III pairs for lactose of

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                10        20        30        40        50        60
                .         .         .         .         .         .
1   TAATCACAAAGGTTTTGGAGATATTATCTTAGATAAAGGTCATAGTGTAAACTATGAAAT
2   TGGCTTCCAATACTTTAATTCATCAAGATAACCATGTATTAGTAAAATTTTAGTAAAAAA

                -35               -10
3   CACTGAAATTATTGACTGCATAAACCAATTTTCATATAATGTAAACGTATTCAAATAATA

    RBS         start lactose carrier
4   GGATGTTTCCGAAATGGAAAAATCTAAAGGTCAGATGAAGTCTCGTTTATCCTACGCAGC

5   TGGTGCTTTTGGTAACGACGTCTTCTATGCAACCTTGTCAACATACTTTATCATGTTTGT
6   GACAACTCACTTGTTTAACACAGGTGATCCAAAGCAAAATAGTCACTACGTACTATTAAT
7   CACTAACATTATCTCTATTTTGCGTATCTTGGAAGTATTTATCGATCCATTGATCGGTAA
8   TATGATTGATAACACTAATACTAAGTATGGTAAATTCAAACCATGGGTAGTTGGTGGTGG
9   TATCATCAGTTCTATCACCTTGTTGCTTCTCTTCACCGATTTAGGTGGTTTGAATAAAAC
10  AAATCCTTTCTTGTACCTTGTACTTTTTGGAATTATCTACCTTGTAATGGATGTCTTCTA
11  CTCGATTAAAGATATCGGTTTCTGGTCAATGATTCCTGCCTTGTCTCTTGATAGTCACGA
12  ACGTGAAAAAATGGCAACTTTTGCCCGTATTGGTTCTACGATTGGTGCCAATATTGTAGG
13  TGTTGCCATCATGCCAATCGTTTTGTTCTTCTCTATGACGAACAATAGTGGCTCTGGAGA
14  TAAATCTGGATGGTTCTGGTTTGCATTTATCGTTGCTCTCATTGGTGTAATTACATCAAT
15  TGCTGTTGGTATTGGTACACGTGAAGTTGAGTCAAAAATTCGTGATAATAACGAAAAAAC
16  TAGCCTTAAACAAGTCTTTAAGGTTCTTGGTCAAAACGACCAATTGATGTGGTTATCTCT
17  TGGATATTGGTTCTATGGTCTTGGTATTAATACACTTAATGCTCTTCAACTTTATTATTT
18  CACATTTATCCTTGGTGATTCAGGTAAATACTCAATTCTTTACGGATTGAATACAGTTGT
19  TGGTTTGGTTTCAGTTTCACTCTTCCCTACCCTAGCTGATAAATTCAACCGTAAACGTTT
20  GTTCTACGGATGTATTGCAGTAATGCTCGGGGGTATCGGAATATTTAGTATTGCAGGTAC
21  ATCACTTCCAATAATCTTGACTGCAGCTGAACTCTTCTTCATTCCACAACCTCTTGTGTT
22  CCTTGTTGTCTTTATGATTATCTCTGACTCAGTAGAATATGGTCAATGGAAAACGGGACA
23  CCGTGATGAATCACTTACTTTGTCAGTTCGTCCACTTATTGATAAACTTGGTGGTGCGAT
24  GTCAAACTGGCTTGTTTCTACATTTGCCGTAGCTGCCGGTATGACAACAGGTGCCTCAGC
25  ATCAACAATTACAACACATCAACAGTTTATCTTTAAGCTTGGCATGTTTGCTTTCCCAGC
26  AGCAACAATGCTTATCGGTGCCTTCATTGTTGCTCGTAAAATCACTTTGACTGAAGCACG
27  TCACGCTAAAATTGTTGAAGAATTGGAACATCGCTTTAGCGTAGCAACTTCTGAAAATGA
28  AGTTAAAGCTAACGTCGTATCTCTTGTAACCCCTACAACTGGTTATTTGGTTGATCTCTC
29  AAGTGTTAATGATGAACACTTTGCTTCAGGTAGCATGGGTAAAGGTTTCGCCATTAAACC
30  TACTGATGGAGCTGTCTTTGCACCAATTAGTGGTACCATTCGTCAAATTCTTCCTACTCG
31  CCATGCAGTTGGTATTGAAAGTGAAGATGGTGTCATTGTTCTTATCCACGTTGGCATCGG
32  AACAGTTAAACTTAATGGTGAAGGATTCATTAGTTACGTAGAACAAGGTGATCGTGTTGA
33  AGTTGGACAAAAACTTCTTGAGTTCTGGTCACCAATTATTGAGAAAAATGGTCTTGATGA
34  CACAGTACTTGTCACTGTAACTAATTCAGAAAAATTCAGTGCTTTCCATCTTGAACAAAA

                                                    RBS  stop
35  AGTTGGAGAAAAGGTAGAAGCTTTGTCTGAAGTTATTACCTTCAAAAAAGGAGAATAATC

    start ß-galactosidase
36  TATGAACATGACTGAAAAAATTCAAACTTATTTAAACGATCCAAAGATTGTTAGCGTTAA

37  TACTGTTGATGCTCACTCAGATCATAAGTATTTTGAATCTCTTGAAGAATTTTCTGAAGG
38  GGAGATGAAGTTAAGACAATCTCTTAATGGAAAATGGAAAATTCACTATGCTCAGAATAC
39  AAATCAGGTTTTAAAAGACTTTTATAAAACAGAATTTGATGAAACTGATTTGAATTTCAT
40  CAATGTACCAGGTCATTTAGAGCTTCAAGGTTTTGGTTCTCCACAATATGTGAATACCCA
41  ATATCCTTGGGATGGTAAAGAATTC
                .         .         .         .         .         .
                10        20        30        40        50        60
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FIG. 3. The nucleotide sequence of the *S. thermophilus* lactose transport gene and flanking regions. The start and stop codons as well as the putative promoter elements and the possible RBSs are underlined.

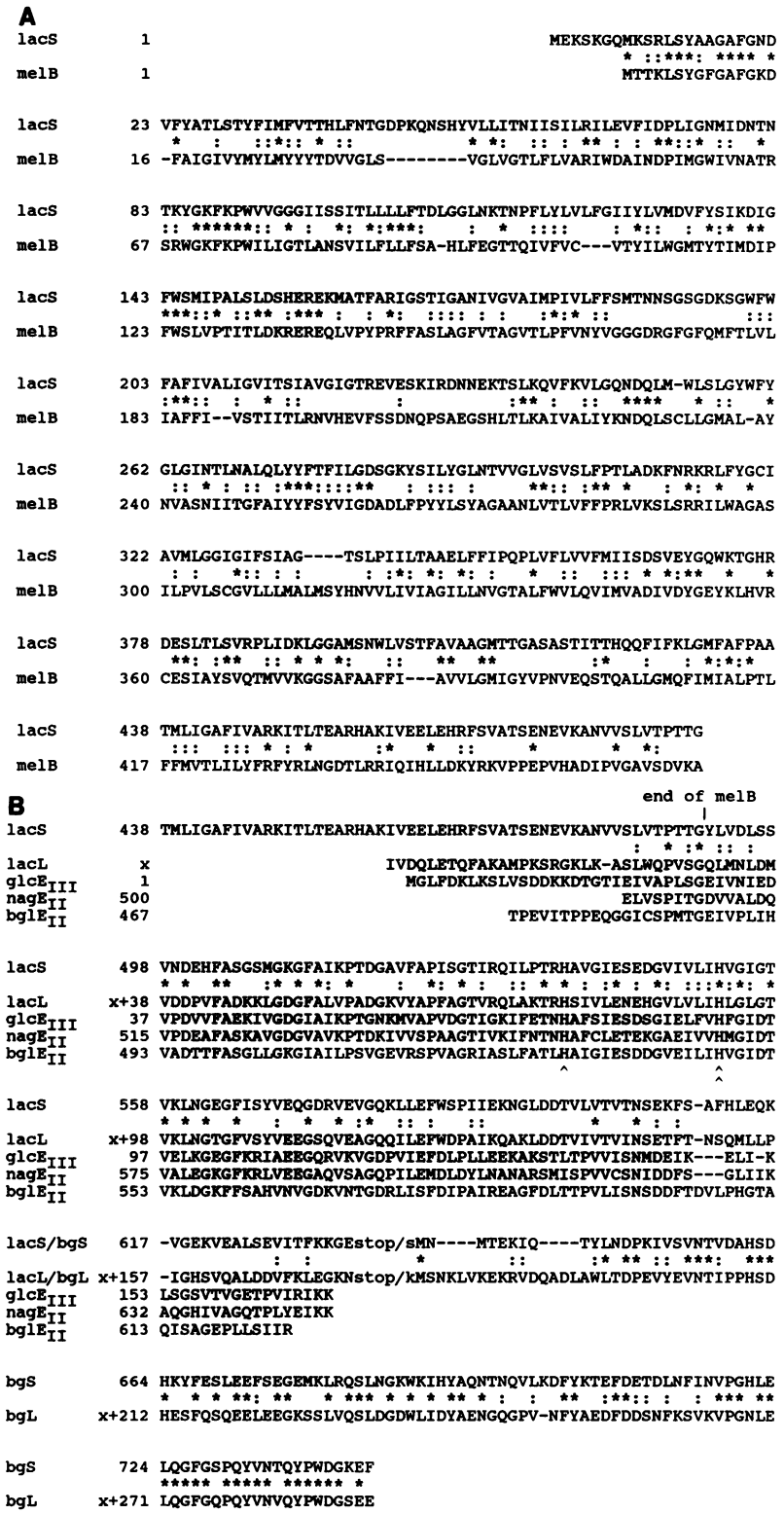

FIG. 4. Computer alignment of the amino acid sequences of the NH_2-terminal (A) and the COOH-terminal (B) ends of the *S. thermophilus* lactose transport system with various proteins. (A) Alignment of the sequences of the NH_2-terminal 490 amino acids of the *S. thermophilus* lactose transport system protein (*lacS*) and the *E. coli* melibiose carrier (*melB*). (B) Alignment of the amino acid sequences of the COOH-terminal ends of the lactose transport systems of *S. thermophilus* and *L. bulgaricus* (*lacL*) and the enzymes III (domains) of the PEP-PTSs for glucose ($glcE_{III}$) from *S. typhimurium* and for *N*-acetylglucosamine ($nagE_{II}$) and β-glucoside ($bglE_{II}$) from *E. coli*, and of the NH_2-terminal regions of the β-galactosidases from *S. thermophilus* (*bgS*) and *L. bulgaricus* (*bgL*). Identical (*) or conserved (:) amino acids in the sequences are indicated. Since the *lacL* protein has not yet been fully sequenced, the starting number of the amino acid sequence is designated x. Conserved His residues are indicated by arrows. The double arrow indicates the corresponding His that has been shown to be phosphorylated in $glcE_{III}$. The *lacS* protein sequence from residues 438 to 497 is shown in both panels A and B; the region where the alignment with the *melB* protein ends is indicated in panel B.

TABLE 1. Amino acid identity between pairs of proteins[a]

Protein	% Amino acid identity with:				
	lacS-c	*lacL*-c	$glcE_{III}$	$nagE_{II}$-c	$bglE_{II}$-c
lacS-c					
lacL-c	48.3				
$glcE_{III}$	34.3	30.4			
$nagE_{II}$-c	35.9	32.9	45.0		
$bglE_{II}$-c	41.1	34.0	35.9	35.9	

[a] *lacS*, Lactose transport system of *S. thermophilus*; *lacL*, lactose transport system of *L. bulgaricus*; $glcE_{III}$, enzyme III of the *Salmonella typhimurium* PTS (27); $nagE_{II}$, enzymes II-III pair of the *E. coli* *N*-acetylglucosamine PTS (36); $bglE_{II}$, enzymes II-III pair of the *E. coli* β-glucoside PTS (40); c, the COOH-terminal region of the corresponding protein. The sequence comparisons of the enzymes III (domains) have been published before (38).

Staphylococcus aureus (5) and for glucose (10), mannose (11), glucitol (44), β-glucoside (40), *N*-acetylglucosamine (36), and mannitol (19) of *E. coli*.

The COOH-terminal end of the lactose transport protein showed strong similarity to enzyme III of the *Salmonella typhimurium* glucose PEP-PTS and to the enzymes III domains of the enzymes II-III pairs of the *E. coli* *N*-acetylglucosamine and β-glucoside PEP-PTS (Fig. 4B and Table 1). The percentage of amino acid identity between the *lacS* protein and the individual enzymes III (domains) was between 34 and 41%, which is similar to the homology among the different enzymes III (domains) (Table 1). Preliminary work on the sequencing of the lactose transport system of *L. bulgaricus* (*lacL*) (Fig. 4B; B. F. Schmidt et al., unpublished results) revealed that this protein also contained a hydrophilic domain at the COOH-terminal end with homology to enzymes III of PEP-PTSs. The percent similarity between the COOH-terminal ends of the *lacS* and *lacL* transport proteins was 48.3 (Table 1), whereas the NH_2-terminal ends appeared to be more than 60% similar (B. F. Schmidt et al., unpublished results). Interestingly, two histidine residues (His-537 and His-552 in *lacS*) were conserved in all five proteins (Fig. 4B). The histidine corresponding to His-552 in *lacS* has been shown to be phosphorylated in vitro in enzyme III^{Glc} (8). With the exception of the amino acids at positions 548 and 556, the region around His-552 was well conserved in the enzymes III (domains) and the lactose transport proteins.

No significant similarity was found between *lacS* and the enzymes III (domains) of the PEP-PTSs for lactose of *S. aureus* (5), for lactose of *L. casei* (1), and for mannose of *E. coli* (11). Finally, the amino acid sequence deduced from the nucleotide sequence of the open reading frame 3′ of *lacS* showed approximately 50% similarity with the β-galactosidase of *L. bulgaricus* (Fig. 4B) (Schmidt et al., in press).

Identification of the lactose transport protein in minicells. Minicell-producing *E. coli* P678-54 was transformed with pKK223-3 (cloning vector), pEKS8-7, and pEKS8 (Fig. 1). Compared with extracts of control cells carrying pKK223-3, extracts prepared from cells containing pEKS8 showed additional protein bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis of apparent molecular masses of 55 and 38 kilodaltons (data not shown). Since the 38-kilodalton band was absent in extracts prepared from cells containing pEKS8-7, the 55-kilodalton protein most likely corresponds to the lactose transport system. The discrepancy between the calculated (deduced from the nucleotide sequence) and the apparent molecular masses is probably due to the abnormally high binding of sodium dodecyl sulfate to the lactose transport system, similar to that observed for other hydrophobic proteins (6, 11, 19, 26, 46).

TABLE 2. Amino acid composition of the lactose transport system of *S. thermophilus* and the melibiose transport system of *E. coli*

Amino acid residue	No. (%) of amino acids in transport system		
	Lactose	Lactose (NH_2 end)	Melibiose[a]
Polar			
Asp	23 (3.6)	16 (3.3)	17 (3.6)
Glu	30 (4.7)	16 (3.3)	9 (1.9)
Arg	16 (2.5)	13 (2.7)	17 (3.6)
Lys	34 (5.4)	24 (4.9)	13 (2.8)
His	11 (1.7)	7 (1.4)	7 (1.5)
Ser	49 (7.7)	38 (7.8)	26 (5.5)
Thr	46 (7.3)	38 (7.8)	27 (5.8)
Asn	26 (4.1)	22 (4.5)	14 (3.0)
Gln	14 (2.2)	10 (2.0)	11 (2.4)
Nonpolar			
Ala	40 (6.3)	33 (6.7)	38 (8.1)
Gly	60 (9.5)	44 (9.0)	36 (7.7)
Cys	1 (0.2)	1 (0.2)	4 (0.9)
Pro	17 (2.7)	13 (2.7)	17 (3.6)
Met	17 (2.7)	16 (3.3)	16 (3.4)
Leu	65 (10.3)	54 (11.0)	58 (12.4)
Ile	59 (9.3)	47 (9.6)	40 (8.5)
Val	54 (8.5)	37 (7.6)	51 (10.9)
Phe	45 (7.1)	37 (7.6)	34 (7.3)
Tyr	18 (2.9)	16 (3.3)	26 (5.5)
Trp	9 (1.4)	8 (1.6)	8 (1.7)

[a] Data taken from reference 46. The percentages of polar amino acids are 39.3, 37.5, and 30.1 for the lactose, lactose (NH_2 end), and melibiose transport systems, respectively.

Amino acid composition and hydropathy of the lactose transport system. The amino acid composition of the NH_2-terminal end as well as of the entire lactose transport system is shown in Table 2. The polarity of the *lacS* protein is significantly higher than that of the melibiose carrier, even when only the NH_2-terminal end is considered (Table 2). The most abundant amino acids of the lactose transport system are leucine, isoleucine, valine, and glycine, similar to those of the melibiose transport system. Of the 490 amino acids in the NH_2-terminal end of the *lacS* protein, 32 are acidic and 37 are basic (when ignoring the His residues), giving an excess of five positive charges at neutral pH. The net charge of the melibiose transport system is +4.

The higher polarity of the lactose transport system relative to that of the melibiose transport system is also reflected in the overall hydropathy (hydrophilicity and hydrophobicity) of the proteins (Fig. 5). In contrast to those of the melibiose transport system, most of the hydrophobic stretches of the lactose transport system are connected by relatively long highly hydrophilic regions. Interestingly, the two hydrophilic domains of the melibiose transport system, i.e., around Gly-70 and Leu-132, are the regions most conserved in comparison with the lactose transport system. The lactose transport system contains eight segments of 19 residues (or longer), with an average hydropathy exceeding 1.6. Most likely these hydrophobic segments span the cytoplasmic membrane. For five stretches with an average hydropathy of 1.0 to 1.6, it is difficult to predict whether they span the membrane or whether they merely pass through the inside of the protein itself (18). One 19-residue hydrophobic segment with an average hydropathy of 1.28 is present in the COOH-terminal end of the lactose transport system. This segment is

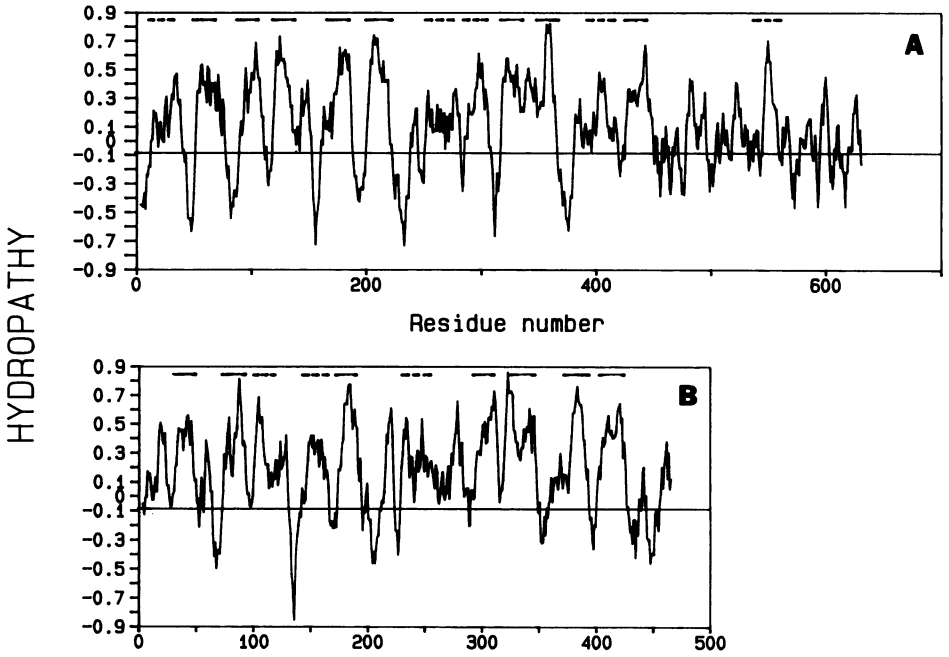

FIG. 5. Hydropathy patterns of the *S. thermophilus* lactose transport system (A) and the *E. coli* melibiose transport system (B). Hydropathy was calculated by the method of Kyte and Doolittle (18) with a span setting of 7 amino acid residues. Hydropathy values given at the *y* axis are normalized from −1 (hydrophilic) to +1 (hydrophobic). The midpoint line indicates the average hydropathy of a large number of sequenced soluble proteins. Possible membrane-spanning 19-residue segments with an average hydropathy exceeding 1.6 are indicated by bars at the top of each panel. Dashed lines indicate hydrophobic regions with an average hydropathy between 1.0 and 1.6.

unlikely to be membrane spanning, on the basis of the homology between this region and enzyme III and the fact that enzyme III is a cytoplasmic protein. Therefore, the number of membrane-spanning segments is most likely between 8 and 12.

Codon usage in the lactose transport gene. The codon usage for the lactose transport system is shown in Table 3. Analysis of the codon usage for the lactose transport system indicates a strong preference for A or U in the third position. Six sense codons are not used at all, of which four code for arginine residues. CUG, which is the predominant codon in *E. coli* (43), is also not used in *lacS*. In general, the codon usage of *S. thermophilus* resembles that of *E. coli*, with the codons for Leu and Pro being the major exceptions.

DISCUSSION

In this paper, we have described the cloning, functional expression, and nucleotide sequence of the lactose transport system of *S. thermophilus*. Some of the properties of this transport system, the possible implications of the homology with the melibiose carrier of *E. coli* and the enzymes III (domains) of PEP-PTSs, and the arrangement of the *lac* operon in *S. thermophilus* (and in *L. bulgaricus*) will be discussed.

Experiments measuring the transport of lactose in whole cells and in membrane vesicles have indicated that the mechanism of energy coupling for the *lacS* protein is similar to that of the *E. coli* lactose transport system (encoded by

TABLE 3. Codon usage in the lactose transport gene of *S. thermophilus*

No. of times used	Codon	Amino acid	No. of times used	Codon	Amino acid	No. of times used	Codon	Amino acid or mutation	No. of times used	Codon	Amino acid or mutation
20	UUU	Phe	15	UCU	Ser	8	UAU	Tyr	1	UGU	Cys
25	UUC	Phe	1	UCC	Ser	10	UAC	Tyr	0	UGC	Cys
4	UUA	Leu	19	UCA	Ser	1	UAA	Ochre	0	UGA	OP
21	UUG	Leu	1	UCG	Ser	0	UAG	Amber	9	UGG	Trp
32	CUU	Leu	7	CCU	Pro	4	CAU	His	14	CGU	Arg
6	CUC	Leu	0	CCC	Pro	7	CAC	His	2	CGC	Arg
2	CUA	Leu	10	CCA	Pro	12	CAA	Gln	0	CGA	Arg
0	CUG	Leu	0	CCG	Pro	2	CAG	Gln	0	CGG	Arg
34	AUU	Ile	16	ACU	Thr	16	AAU	Asn	10	AGU	Ser
23	AUC	Ile	7	ACC	Thr	10	AAC	Asn	3	AGC	Ser
2	AUA	Ile	20	ACA	Thr	28	AAA	Lys	0	AGA	Arg
17	AUG	Met	3	ACG	Thr	6	AAG	Lys	0	AGG	Arg
27	GUU	Val	16	GCU	Ala	19	GAU	Asp	42	GGU	Gly
8	GUC	Val	9	GCC	Ala	4	GAC	Asp	3	GGC	Gly
17	GUA	Val	14	GCA	Ala	27	GAA	Glu	14	GGA	Gly
2	GUG	Val	1	GCG	Ala	3	GAG	Glu	1	GGG	Gly

lacY). Most likely, lactose is taken up in symport with a proton (or cation) and enters the cell as free sugar. This latter point discriminates the sugar-cation symporters from PEP-PTSs which phosphorylate the sugar concomitantly with the translocation step. As a consequence, sugars accumulated by PEP-PTSs are not directly exchangeable with sugars in the medium. Exchange of sugars via the lactose transport system of *S. thermophilus* has been shown upon the addition of excess unlabeled lactose to cells which had accumulated [^{14}C]lactose (Fig. 2).

Amino acid sequence comparisons have revealed a significant degree of similarity between the NH_2-terminal region of the lactose transport system of *S. thermophilus* and the melibiose transport system of *E. coli*. Both transport systems facilitate the translocation of TMG and perhaps other common substrates which have also been shown to be transported by the lactose carrier of *E. coli* (2). In this respect, it is worthwhile to note that the melibiose carrier is capable of utilizing H^+, Na^+, and Li^+ as coupling cations for cotransport, depending on the sugar transported (2, 46). Future studies are required to clarify whether a similar situation holds for the lactose transport system of *S. thermophilus*. Since *S. thermophilus* (and *L. bulgaricus*) metabolize only the glucose moiety of lactose and excrete galactose stoichiometrically into the medium during growth on lactose (14, 16), the physiological transport reaction may not involve a coupling cation but may simply be driven by the exchange of lactose for galactose (9, 31). In fact, preliminary experiments have already indicated that galactose efflux from *L. bulgaricus* is stimulated almost 100-fold by the presence of lactose in the external medium, suggesting that heterologous exchange is a favorable transport reaction (B. Poolman et al., unpublished results).

Charged amino acid residues that are found in highly hydrophobic regions are often considered to be catalytically important. To accommodate a charged (hydrophilic) amino acid residue in a hydrophobic segment of a protein, one faces a large loss in free energy of the hydrophobic force that, among other parameters, determines the native structure of the protein (18). As a result, the number of charged residues in membrane-spanning segments of a protein is usually low. In the case of the lactose transport system of *S. thermophilus*, three charged amino acids, i.e., Arg-64, Glu-67, and Asp-133 (Fig. 4A), are present in hydrophobic domains, i.e., in the 19-residue segments of the hydropathy analysis (Fig. 5). Of these charged amino acids, Arg-64 is conserved whereas Glu-67 is replaced by an Asp in the corresponding region of the melibiose transport system of *E. coli*. Although His residues have been shown to be important in the function of the lactose transport system of *E. coli* as well as in other proteins (34), none of the His residues of the *S. thermophilus* lactose transport system are conserved in the melibiose transport system. The only Cys residue (Cys-320) in the lactose transport system of *S. thermophilus* also appears not to be conserved.

A surprising finding in the analysis of the amino acid sequence of the lactose transport system is the homology between the COOH-terminal ends and the enzymes III (domains) of three different PEP-PTSs. Since the proposed active-site histidine residue is conserved in the lactose transport proteins of both *S. thermophilus* and *L. bulgaricus* (Fig. 4B), the question of what effect possible phosphorylation of this residue will have on transport arises. Lactose is transported as free sugar by the *S. thermophilus* transport system, in contrast to PEP-PTSs in which the phosphoryl group is transferred from the enzyme III (domain) to the sugar most likely via a cysteine residue in enzyme II (30). Phosphorylation of the conserved histidine (His-552 in *lacS*), if it occurs, may have a regulatory function. Site-directed mutagenesis of this residue may aid in understanding the function of the enzyme III domain of the lactose transport system. The role of the hydrophilic region, i.e., the part with homology to enzyme III, of the *S. thermophilus* and *L. bulgaricus* lactose transport systems is reminiscent of that of enzyme III^{Glc} in regulating lactose transport via the *lacY* protein in *E. coli* (28). It has been shown that enzyme III^{Glc} can interact with the lactose carrier of *E. coli*, resulting in the inhibition of galactoside translocation (28, 29). Since the phosphorylated form of enzyme III^{Glc} does not bind to the lactose carrier, the degree of phosphorylation of enzyme III^{Glc} can determine the level of lactose transport activity. This type of regulation of β-galactoside transport has been proposed as the basis for the phenomenon of inducer exclusion (28, 29; Konings et al., in press). The in vivo consequence of this mechanism is that the lactose carrier is active only when enzyme III^{Glc} is in the phosphorylated form, i.e., when glucose is absent from the medium.

For the heterofermentative lactobacilli *L. brevis* and *L. buchneri*, it has been proposed that β-galactoside transport is regulated by HPr(Ser-P), the only PTS protein found in these organisms (35, 37). In the presence of glucose, HPr(Ser) is phosphorylated by an ATP-dependent protein kinase (35). Whether HPr(Ser-P) is involved in the regulation of β-galactoside (lactose) transport in *S. thermophilus* and *L. bulgaricus* is unknown. Since HPr(Ser-P) is not present in gram-negative bacteria, the presumptive HPr(Ser-P)-mediated glucose effect on β-galactoside transport will not be observed in *E. coli*. In fact, contrary to the inhibition of TMG uptake by glucose in *L. brevis*, glucose stimulates TMG and lactose uptake via the *S. thermophilus* lactose transport system in *E. coli* (Fig. 2). This stimulation may be due either to an increase of the pmf or to a regulatory effect, e.g., dephosphorylation of His-552, on the transport system.

Recently, the genes encoding the β-galactosidase and flanking regions of *L. bulgaricus* have been sequenced (Schmidt et al., in press). From these sequences and the nucleotide sequence of the lactose transport gene and flanking regions of *S. thermophilus* presented in this paper, it becomes evident that the *lac* genes in both organisms are organized in operons. The order of the lactose transport and the β-galactosidase gene is opposite to that of the *lac* operon in *E. coli*. The intercistronic region between the genes is 3 nucleotides both in *S. thermophilus* and in *L. bulgaricus*, and in both cases the ATG of the β-galactosidase is preceded by an RBS at the proper distance. This suggests that the translation reinitiation frequency for the β-galactosidase could be close to 1, as proposed for other cases in which the termination codon lies between the ATG and the RBS (12).

Codon usage can be considered as a means of regulating protein expression at the level of translation (43). Since lactose is the preferred carbohydrate for growth of *S. thermophilus* and carbohydrates are the only source of metabolic energy for this organism, it can be expected that the *lac* proteins are well expressed and that the major codons in *S. thermophilus* are used. At the moment, the codon usage presented in Table 3 cannot be compared with that of other *S. thermophilus* genes. Comparison of the codon usage in the *S. thermophilus* lactose transport protein and in the *L. bulgaricus* β-galactosidase (Schmidt et al., in press) yields remarkable differences for most of the amino acids.

ACKNOWLEDGMENTS

We thank Fernando Valle and Bruce Chassy for helpful discussions throughout the preparation of the manuscript and Scott Power for the preparation of the hydropathy plots. We thank H. R. Kaback and J. Lolkema from the Roche Institute of Molecular Biology for providing us with *E. coli* AZ-1 and T206.

This work was supported in part by the Centre International de Recherche Daniel Carasso, BSN group.

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