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The Lactose Transporter in *Leuconostoc lactis* Is a New Member of the LacS Subfamily of Galactoside-Pentose-Hexuronide Translocators

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The gene encoding the lactose transport protein (*lacS*) of *Leuconostoc lactis* NZ6009 has been cloned from its native lactose plasmid, pNZ63, by functional complementation of lactose permease-deficient *Escherichia coli* mutants. Nucleotide sequence analysis revealed an open reading frame with the capacity to encode a protein of 639 amino acids which had limited but significant identity to the lactose transport carriers (LacS) of *Streptococcus thermophilus* (34.5%) and *Lactobacillus bulgaricus* (35.6%). This similarity was present both in the amino-terminal hydrophobic carrier domain, which is homologous to the *E. coli* melibiose transporter, and in the carboxy-terminal enzyme IIA-like regulatory domain. The flanking regions of DNA surrounding *lacS* were also sequenced. Preceding the *lacS* gene was a small open reading frame in the same orientation encoding a deduced 95-amino-acid protein with a sequence similar to the amino-terminal portion of β-galactosidase I from *Bacillus stearothermophilus*. The *lacS* gene was separated from the downstream β-galactosidase genes (*lacLM*) by 2 kb of DNA containing an IS*3*-like insertion sequence, which is a novel arrangement for *lac* genes in comparison with that in other lactic acid bacteria. The *lacS* gene was cloned in an *E. coli-Streptococcus* shuttle vector and was expressed both in a *lacS* deletion derivative of *S. thermophilus* and in a pNZ63-cured strain, *L. lactis* NZ6091. The role of the LacS protein was confirmed by uptake assays in which substantial uptake of radiolabeled lactose or galactose was observed with *L. lactis* or *S. thermophilus* plasmids harboring an intact *lacS* gene. Furthermore, galactose uptake was observed in NZ6091, suggesting the presence of at least one more transport system for galactose in *L. lactis*.

Leuconostocs contribute significantly to the dairy industry, in which they are used together with acid-producing lactococci for the production of cultured buttermilk, cottage cheese, sour cream, and certain cheese types (50). The main functions of the leuconostocs in these fermentations are to cause the development of flavor by metabolic conversion of the citrate in milk to diacetyl and other volatile compounds and to produce CO_2, which is responsible for hole formation in certain cheese types (21). During bacterial growth in milk, lactose is the primary energy source. Two systems for lactose transport and metabolism have been established for dairy lactic acid bacteria: (i) a phosphoenolpyruvate-lactose phosphotransferase system (PEP-PTS) with a phospho-β-galactosidase enzyme, found in the lactococci and *Lactobacillus casei*, and (ii) a lactose permease system with a β-galactosidase, found in the thermophilic *Lactobacillus bulgaricus*, *Lactobacillus helveticus*, and *Streptococcus thermophilus* and the mesophilic *Leuconostoc lactis* (9, 44).

At the genetic level, genes involved in lactose transport and hydrolysis from lactic acid bacteria have been cloned and in many cases are linked to the genes necessary for galactose metabolism (12). The *lac* operon of *Lactococcus lactis* may be plasmid encoded or reside on the chromosome and includes genes encoding the PEP-PTS^{lac}, phospho-β-galactosidase, and enzymes in the tagatose-6-phosphate pathway (10, 46). In both *S. thermophilus* and *Lactobacillus bulgaricus*, the chromosomal lactose transport genes (*lacS*) are similarly organized in an operon with the downstream β-galactosidase (*lacZ*) genes, and their respective proteins are highly homologous (23, 34). The LacS proteins are classified as a subgroup of members of the galactoside-pentose-hexuronide family based on the primary sequence identity of their amino-terminal hydrophobic carrier domains (31, 32, 38). Other members of this family include the MelB and GusB subfamilies, represented by the melibiose and glucuronide transporters, respectively, of *Escherichia coli*. The LacS proteins are chimeric, as they also possess a carboxy-terminal hydrophilic region that is homologous to IIA domains of various PTS enzymes. Phosphorylation of the IIA domain in the presence of PEP, enzyme I, and heat-stable HPr results in an inhibition of transport of lactose (30). The energetics and kinetic mechanism of transport by the *S. thermophilus* LacS (LacSSt) have been studied in detail and indicate that the LacS carrier can mediate both galactoside/H^+ symport and galactoside exchange, in particular, lactose-galactose exchange (16). It has been proposed that the observed excretion of galactose by most strains of *S. thermophilus* when grown in excess lactose is due to the latter reaction of LacSSt, which is favored under physiological conditions (28). By extrapolation, a similar system may operate in *Lactobacillus bulgaricus*, which also has a galactose-negative phenotype. Upstream of *lacS* in *S. thermophilus* lie genes encoding enzymes for the Leloir pathway of galactose metabolism (3, 35).

L. lactis NZ6009 contains a β-galactosidase that is encoded by two partially overlapping genes, *lacL* and *lacM*, which were cloned from the native lactose plasmid, pNZ63 (7). Similar split β-galactosidase genes were also later discovered in various lactobacilli, including *Lactobacillus casei*, *Lactobacillus helveticus*, and *Lactobacillus plantarum* (7, 12). However, the ge-

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TABLE 1. Bacterial strains, plasmids, and phages used in this study

Strain, plasmid, or phage[a]	Relevant feature	Source or reference
Escherichia coli		
BY1	*lacY* derivative of JC5412 obtained by NTG mutagenesis[b]	University of Groningen
C600	F^- *rec*$^+$ *thi thr leuB lacY tonA supE*	40
HB101	*recA13 proA leuB hsdM hsdR rpsL supE thi lacY galK*	40
TG1	(*lac-pro*) *supE thi hsdR5* F′ [*traD36 proA*$^+$*B*$^+$*lacI*^{q} *lac*M15]	40
Leuconostoc lactis		
NZ6009	Lac$^+$, harbors lactose plasmid pNZ63	6
NZ6091	Lac$^-$, cured of pNZ63	6
Streptococcus thermophilus		
ST11	Lac$^+$	Nestle Research Centre
ST11 (Δ*lacS*)	Lac$^-$	30
pACYC184	4.2-kb *E. coli* vector, Cm^r	4
pHSG575	3.6-kb *E. coli* vector, Cm^r	43
pNZ601	16-kb *Bam*HI fragment of pNZ63 cloned in pACYC184	7
pGKV210	4.4-kb *E. coli*, *Lactococcus*, and *Streptococcus* vector, Em^r	45
pNZ2102	3.3-kb, Cm^r, source of *lac* promoter	27
M13mp18/19	Phage-cloning vector	51

[a] All plasmids constructed during this study are described in the text.
[b] NTG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine.

netic determinants for transport of unmodified lactose have not yet been characterized in these lactic acid bacteria. In contrast to *S. thermophilus* and *Lactobacillus bulgaricus*, *L. lactis* and the lactobacilli utilize both free galactose and the moiety that is hydrolyzed from lactose. To gain some insight into this important difference, we have cloned and characterized the lactose transport protein of *L. lactis* that showed remarkable similarity to the chimeric $LacS^{St}$ and $LacS^{Lb}$ proteins.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and culture conditions. The strains and plasmids used in this study are listed in Table 1. *L. lactis* strains were routinely propagated in MRS (Difco Laboratories, Detroit, Mich.) at 30°C, and *S. thermophilus* strains were propagated in M17 broth (Difco, Surrey, United Kingdom) or Elliker broth (14) containing 0.5% beef extract (Belliker medium) at 42°C. *E. coli* was grown in TY broth (40) with aeration at 37°C. Agar media were prepared by adding 1.5% agar to broth. *E. coli* TG1 was used for the propagation of M13 chimeras, and *E. coli* C600 was used for the preparation and complementation of pGKV210-derived plasmids. MacConkey lactose agar (Difco Laboratories) was used to detect lactose-positive *E. coli* strains. The API 50CH system was used to determine carbohydrate fermentation patterns (API System SA, Montalieu Vercieu, France). Brain heart infusion agar was supplemented with 150 μg of erythromycin per ml for the selection of Em^r *E. coli*. The other antibiotics used for selection in media were chloramphenicol (15 μg/ml) and ampicillin (40 μg/ml) for *E. coli* and erythromycin (10 μg/ml for *L. lactis* and 2.5 μg/ml for *S. thermophilus*).

DNA manipulations and transformation. Plasmid and M13 DNA was isolated from *E. coli* essentially by using established protocols (40). The Anderson and McKay (1) lysis procedure was used to detect plasmid DNA in *L. lactis* and *S. thermophilus*. Chromosomal DNA from *L. lactis* was extracted by the procedure of Hayes et al. (18). DNA was digested with restriction enzymes (Gibco BRL Life Technologies, Gaithersburg, Md., and New England Biolabs Inc., Beverly, Mass.) as recommended by the manufacturer. Calf intestinal alkaline phosphatase and the Klenow fragment of *E. coli* DNA polymerase I were obtained from Boehringer GmBH, Mannheim, Germany. DNA fragments were recovered from agarose gels with the GlassMatrix DNA Isolation System (Gibco BRL).

For the expression of *L. lactis lacS* in a gram-positive background, the 2.4-kb *Nru*I-*Cla*I fragment of pNZV5 containing the *lacS* gene was isolated, the 3′-recessed terminus of the *Cla*I site was filled, and the fragment was subsequently ligated into the calf intestinal alkaline phosphatase-treated *Sca*I site in the shuttle vector pGKV201 (45). Analysis of the resulting plasmid constructions indicated that the *lacS* gene was cloned in both orientations, and the plasmids were named pNZV55-1 and pNZV55-2 (Fig. 1). In the event that the insert did not include the *lacS* promoter, plasmid pNZ2102 was used as a source for the isolation of an 0.5-kb *Eco*RI fragment containing the *lacR* promoter of the lactose operon of *Lactococcus lactis* (46), and this region was cloned in front of the *lacS* gene in pNZV55-1. The resulting construct was designated pNZV641 (Fig. 1).

E. coli was electrotransformed and transfected with plasmid and phage DNA by using a Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, Calif.) as specified by the manufacturer. *L. lactis* was electrotransformed as previously described (5). Electroporation of *S. thermophilus* was performed by the procedure of Mollet et al. (24), but the harvested cells were washed and resuspended in 0.5 M sucrose supplemented with 0.5 mM $MgCl_2$.

Nucleotide sequence analysis. Nucleotide sequences were determined by the dideoxy-chain termination method (41) adapted for Sequenase version 2.0 (U.S. Biochemical Corp., Cleveland, Ohio) with single-stranded or alkali-denatured double-stranded M13 templates or plasmids. The M13 universal primer and specifically synthesized primers (Eurogentech, Gent, Belgium) were used to determine the sequences of both strands. The sequence data were assembled with PC/GENE program version 6.6 (Genofit, Geneva, Switzerland). The amino acid comparisons were performed with the Swiss-Prot (release 28.0) and NBRF/PIR (release 40.0) databases, through the facilities of the CAOS/CAMM Center, Nijmegen, The Netherlands.

Carbohydrate transport assays. *L. lactis* cells were grown at 30°C in MRS broth, made from first principles (8) and supplemented with 0.5% lactose, glucose, or galactose, until the sugar was depleted. Following harvesting by centrifugation, the cells were washed twice, resuspended to a final protein concentration of 50 to 75 mg/ml in 100 mM potassium phosphate buffer (pH 6.5), and stored on ice. In preparation for the transport assay, the cells were diluted to a protein concentration of 7 to 13 mg/ml in 1.0 ml of the same buffer at room temperature and preenergized with 0.5% sugar for 4 min; the radiolabeled substrate was added, and at appropriate time intervals, a 100-μl aliquot was diluted into 2 ml of ice-cold LiCl to stop the uptake reaction. Cells were collected by filtration on a 0.45-μm-pore-size cellulose filter (Millipore Corp.) and washed with 2 ml of ice-cold LiCl. The final concentrations of [^{14}C]lactose and [^{14}C]galactose in the assays were 46 and 41 μM, respectively. D-[Glucose-1-^{14}C]lactose (54 mCi/mmol) and D-[1-^{14}C]galactose (61 mCi/mmol) were obtained from The Radiochemical Centre, Amersham, United Kingdom. Bradford's reagent (Bio-Rad Laboratories) was used to estimate protein concentrations (2).

Nucleotide sequence accession number. The GenBank accession number of the sequences in Fig. 2 and 3 is U47655.

RESULTS

Cloning and functional expression of the lactose transport gene. Plasmid pNZ601 contains a 16-kb *Bam*HI fragment of the *L. lactis* lactose plasmid pNZ63, including the β-galactosi-

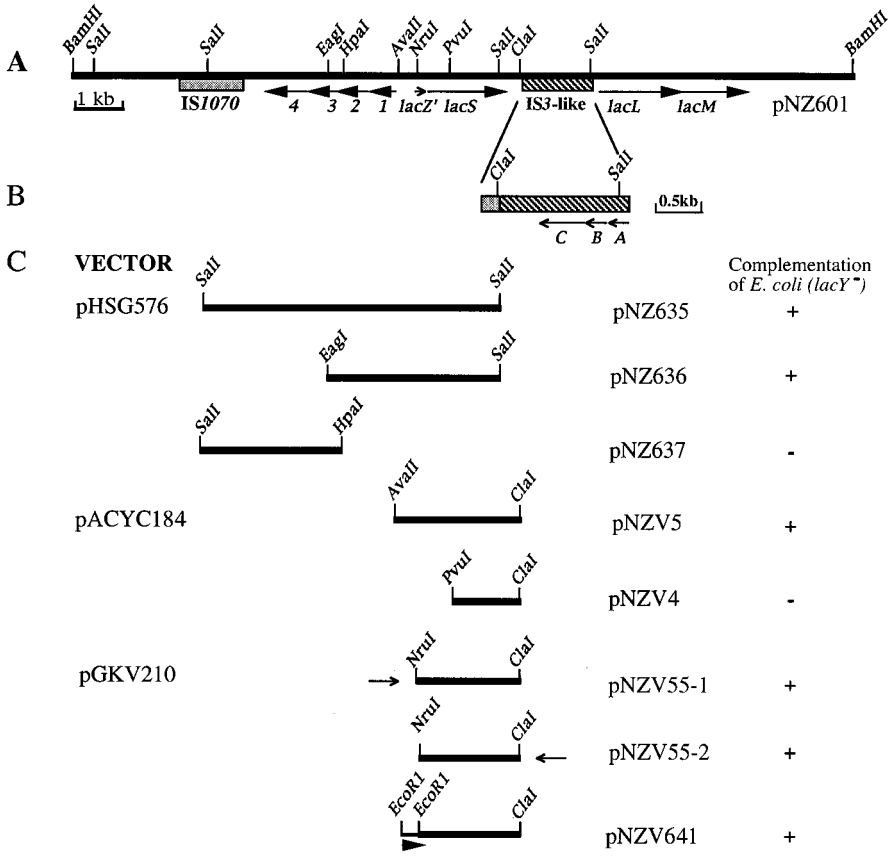

FIG. 1. (A) Physical map of the 16-kb *Bam*HI fragment in pNZ601, showing the position of ORFs, IS-like sequences, and relevant restriction sites. The directions of transcription of the *lacS*, *lacZ'*, and *lacLM* genes and ORFs 1, 2, 3, and 4 (47) are indicated by arrows. The locations of IS*1070* and IS*3*-like insertion sequences are indicated by dotted and hatched boxes, respectively. (B) Coding capacity of the IS*3*-like sequence; the directions of orientation of ORFA, ORFB, and ORFC are indicated by arrows. The 201-bp segment homologous to IS*1070* DNA that is located upstream of the IS*3*-like sequence is depicted like IS*1070*. (C) Schematic representation of the constructed plasmids and their ability to complement *lacY*-deficient *E. coli* mutants (right-hand columns). The vectors used are indicated on the left. The arrows indicate the direction of transcription of the *lacS* gene in plasmids pNZV55-1 and pNZV55-2. The *lacR* promoter preceding *lacS* in pNZV641 is indicated by the arrowhead.

dase genes (*lacLM*), cloned in pACYC184 (Fig. 1) (7). Transformation of pNZ601 into lactose permease-deficient *E. coli* HB101 or BY1 resulted in Cm^r red colonies on MacConkey lactose agar, indicating complementation of their *lacY* mutations.

To further locate the lactose permease determinant, subclones of the 16-kb *Bam*HI fragment were generated and examined for their ability to confer a Lac^+ phenotype on *E. coli* BY1 (Fig. 1). Complementation was observed with pNZ635, which was constructed by inserting a 6.5-kb *Sal*I fragment in the *Sal*I site of the low-copy-number pHSG576 vector. Deletion of the left-hand side of pNZ635 to the *Eag*I site resulted in pNZ636, which maintained the ability to complement the *lacY* mutation, while in contrast, the deletion at the right-hand side of pNZ635 to the *Hpa*I site (pNZ637) failed to complement. These results suggested that the coding region for the lactose carrier was located on the 3.3-kb *Eag*I-*Sal*I fragment. Nucleotide sequence analysis was initiated at the *Sal*I site of pNZ636 and revealed part of an open reading frame (ORF) with a predicted amino acid sequence similar to that of the lactose transport proteins (LacS) of *S. thermophilus* and *Lactobacillus bulgaricus* (see below). The sequencing also indicated that there was no termination codon in the ORF present in the insert of pNZ636. Thus, the complete ORF was cloned from pNZ601 by inserting a 2.8-kb *Ava*I-*Cla*I fragment into pACYC184 (Fig. 1). The resulting plasmid, pNZV5, conferred a Lac^+ phenotype on *E. coli* BY1, while a 1-kb deletion to the *Pvu*I site of this insert (as in pNZV4) gave a Lac^- phenotype, confirming the requirement for this region.

Nucleotide sequence of the *lacS* gene and flanking sequences that include an IS*3*-like sequence. The lactose transport determinant of *L. lactis* has been designated *lacS* on the basis of its deduced amino acid sequence homology to LacS of *S. thermophilus* and *Lactobacillus bulgaricus*. The complete nucleotide sequences of *lacS* and its flanking regions were determined and are presented in Fig. 2 and 3. The *lacS* gene has a size of 1,917 bp and contains a translational initiation site at position 631 which corresponds to a predicted protein of 639 amino acids with a calculated molecular weight of 70,153. A putative ribosome-binding site (3′ AGGAG 5′) complementary to the 3′ end of 16S rRNA of lactic acid bacteria (11) is positioned 7 bp upstream from the start codon. Consensus −35 and −10 promoter regions, separated by 16 bp, were present upstream of the ribosome-binding site at positions 528 (−35) and 550 (−10). A small ORF, which could encode a protein of 95 amino acids initiating at position 314 and terminating at position 598, was detected upstream of *lacS*. The potential expression product of the ORF showed 65 and 59% similarity (46 and 38% identity) to the 90-amino-acid N-terminal portions of the β galactosidase I of *Bacillus stearothermophilus* (19) and the

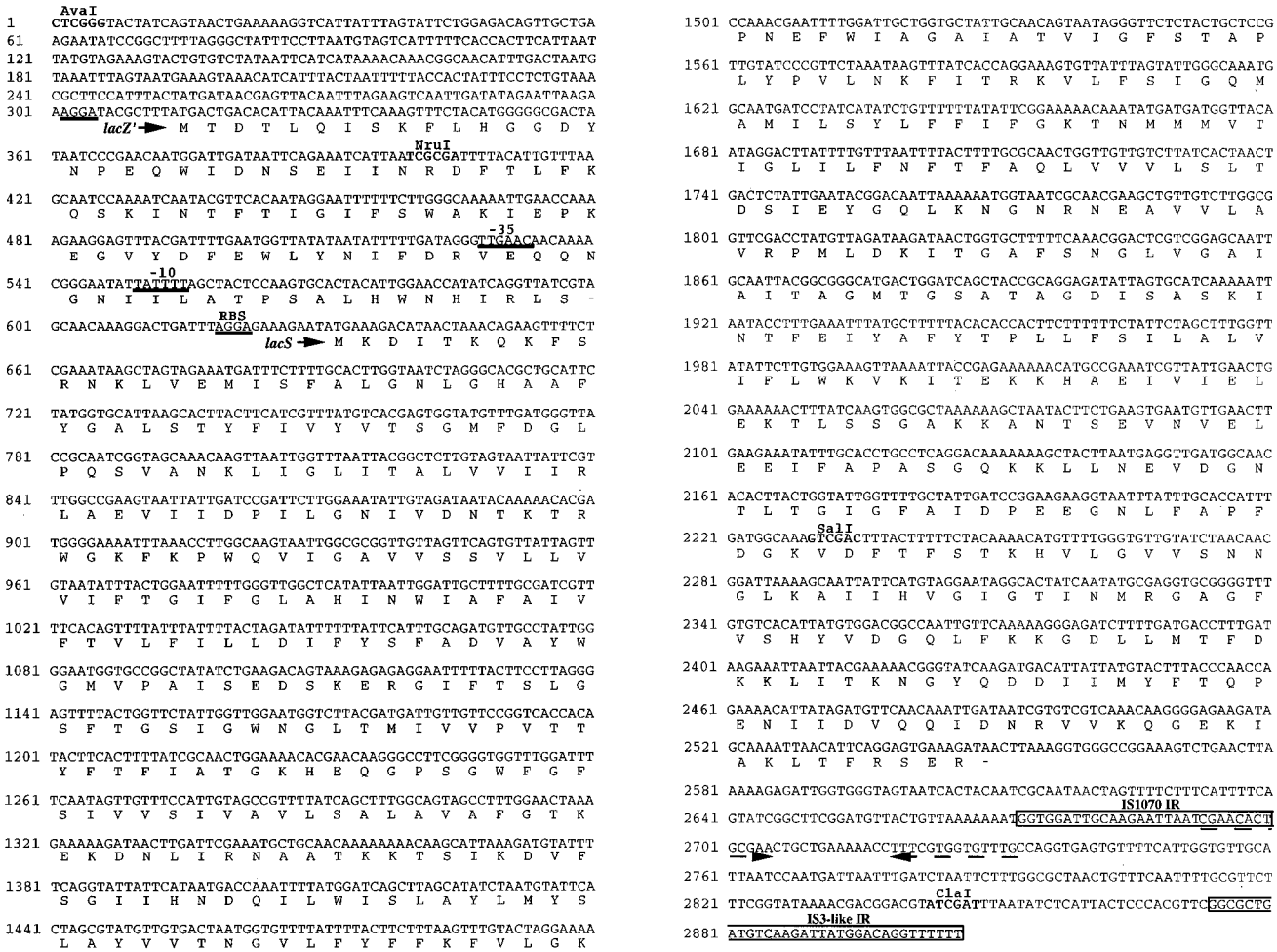

FIG. 2. Nucleotide sequence of the *L. lactis lacS* gene and flanking sequences. The translational start sites of *lacS* and *lacZ′* are indicated by arrows. Putative promoter regions and ribosome-binding sites are underlined. Relevant restriction sites are in boldface type. The inverted repeat (IR) of the IS*1070*-like segment of DNA is boxed. Dashed lines indicate the stem structure of a potential rho-independent terminator which lies within the IS*1070*-like DNA. The inverted repeat which starts the IS*3*-like element at the 3′ end of the sequence is also boxed.

putative β-D-galactosidase of *Bacillus circulans* (L03424), respectively. A putative ribosome-binding site was found 8 bp upstream of the start codon of this ORF, designated *lacZ′* on the basis of homology, but regions that are similar to consensus transcription initiation sequences were not detected over 300 bp upstream of *lacZ′*.

Downstream (120 bp) of *lacS* and including the *Cla*I site, a 201-bp segment of DNA with 94% identity to the 3′ end of IS*1070* was present, beginning with the 28-bp inverted repeat and including only the C-terminal portion of the putative transposase (49). An inverted repeat which could function as a rho-independent terminator of transcription for *lacS* was found in this sequence (142 bp 3′ to the stop codon at position 2693) (26).

Immediately beyond the IS*1070* segment of homologous DNA, a series of three ORFs (ORFA, ORFB, and ORFC) were found on the complementary strand (Fig. 3A); their predicted amino acid sequences demonstrated homology to potential proteins that could be encoded by the IS*3* family of IS elements, in particular with ORF1 of IS*1076* (20). The largest ORF (ORFC), which could encode a protein of 181 amino acids, shared 56% similarity with the C-terminal portion of ORF1 of IS*1076* consisting of residues 184 to 371. The two smaller ORFs present upstream of ORFC demonstrated homology to the N-terminal portion of the IS*1076* ORF1. The deduced 86-amino-acid residue peptide of ORFA was 52% similar to the first 93 amino acids of ORF1, and the deduced 43-amino-acid peptide of ORFB had 59% homology with residues 110 to 150. The ORFs were flanked by weakly matching 33-bp inverted repeats (12 mismatches), giving the 1,417-bp fragment an IS-like structure. Both inverted repeat sequences fitted poorly with the consensus sequence derived for the IS*3* group of insertion sequences, and only the left-hand inverted repeat contained the strongly conserved terminal dinucleotides, 5′ TG....CA 3′ (Fig. 3B) (15, 36). The translational start of the *lacL* gene lies 101 bp on the complementary strand preceding the 5′ inverted repeat of the IS*3*-like sequence (7).

Amino acid sequence homology. The deduced amino acid sequence of LacS from *L. lactis* demonstrated strong similarity throughout the whole sequence to the LacS proteins of *S. thermophilus* (64.1%) and *Lactobacillus bulgaricus* (62.7%) and the putative raffinose permease (RafP) of *Pediococcus pentosaceus* (63.2%; Z32771). However, the LacS of *L. lactis* shares 34 to 36% identity with the other LacS proteins and RafP, which is considerably lower than the values shared by pairs of the latter proteins (54 to 63%). An alignment is presented in Fig.

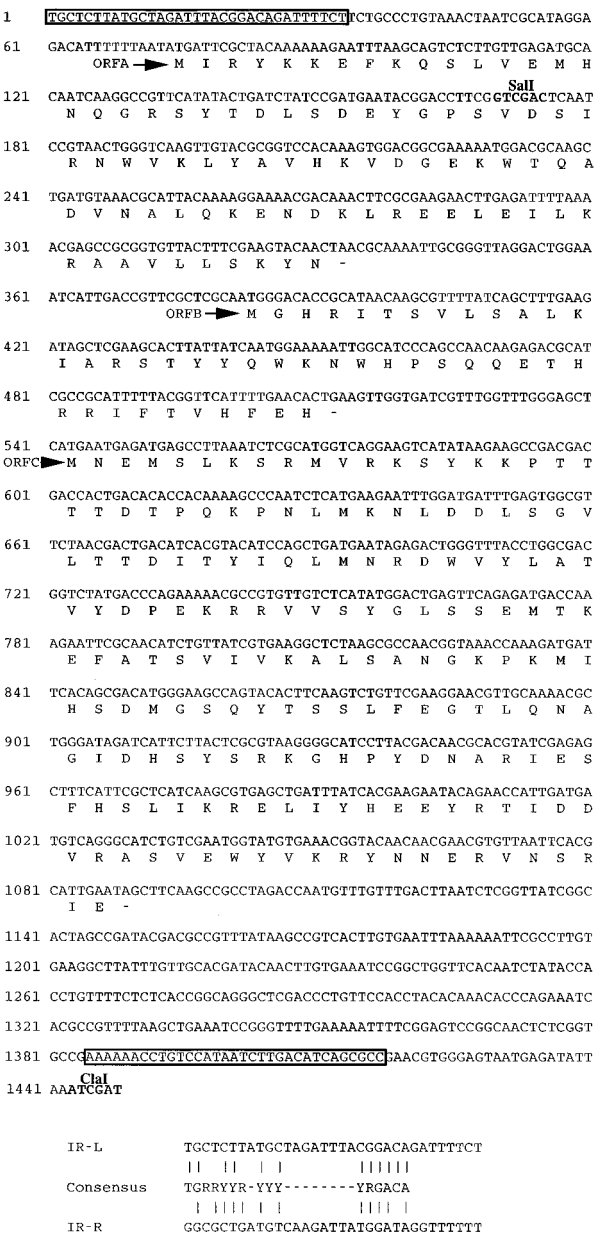

FIG. 3. (A) Nucleotide sequence and deduced amino acid sequence of the IS*3*-like insertion sequence which separates the *lacS* and *lacLM* genes. The three ORFs are indicated by arrows. The inverted repeats flanking the sequence are boxed. Relevant restriction sites are in boldface type. The inverted repeat at the 3′ end corresponds to the inverted repeat at the 3′ end of the nucleotide sequence presented in Fig. 2. (B) Alignment of the right- and left-hand inverted repeat sequences with the consensus inverted repeat sequence derived for the IS*3* group of inverted repeat elements.

4. Like $LacS^{St}$ and $LacS^{Lb}$, the amino-terminal 500 residues of $LacS^{Lc}$ are homologous to the melibiose carrier proteins of several members of the family *Enterobacteriacae* such as MelB of *E. coli* (52). While the overall identity between these transport systems is low, i.e., 26% between $LacS^{Lc}$ and MelB of *E. coli*, they share greater than 50% similarity. Alignment of the lactose permease (LacY) amino acid sequence of *E. coli* along its length does not show significant homology with $LacS^{Lc}$ or with the other LacS proteins. However, the proposed interhelix loop between hydrophobic segments X and XI of the LacS proteins and the corresponding segment in RafP share some conserved residues with a segment of amino acids in helix X of LacY, i.e., Lys-319, His-322, and Glu-325 (Fig. 4), which are believed to be significant for the function of LacY (22, 33). This conserved motif (Lys-X-X-His-X-X-Glu) is a feature of members of the galactoside-pentose-hexuronide family (31). Glu-379 in $LacS^{St}$ (Glu-325 in LacY), which is required for the coupled uptake of lactose and protons (29), is conserved in $LacS^{Lc}$. The conserved His-376 of $LacS^{St}$ (His-322 in LacY) was found to be important but not essential for the cotransport of protons and galactosides (33), although it is not present in $LacS^{Lc}$, where it is replaced by Asn. Transporters of the galactoside-pentose-hexuronide family also possess conserved acidic residues in different amino-terminal helices, particularly in helix II. Glu-67 and Asp-71 of $LacS^{St}$ are important for galactoside/H^+ symport activity in $LacS^{St}$ (31), and both these residues are also present in $LacS^{Lc}$ (Fig. 4).

The 160 amino acids at the carboxy-terminal end of $LacS^{Lc}$ were similar to several enzyme IIA domains of PEP-PTS systems, the most similar being the β-glucosidase-specific PTS enzyme II of *E. coli* (25), the glucose-specific PTS enzyme II of *Bacillus subtilis* (17), and the sucrose-specific PTS enzyme II of *Streptococcus mutans* (42). The amino acid identity ranged from 28 to 35%, which is about 10% lower than the homology among the different enzyme II domains of PEP-PTS systems (39) and with $LacS^{St}$ and $LacS^{Lb}$. Significantly, His-557 in $LacS^{Lc}$, which corresponds to His-552 in $LacS^{St}$, is conserved (Fig. 4). There are strong indications that phosphorylation of His-552 in the enzyme IIA domain of $LacS^{St}$ by PEP-dependent enzyme I and HPr has an inhibitory effect on the activity of the lactose carrier (30). Notably, pNZ635, which lacks most of the carboxy-terminal enzyme IIA domain of the $LacS^{Lc}$, including His-557, maintains the ability to functionally complement *E. coli lacY* mutants. By analogy, carboxy-terminal deletion mutants of $LacS^{St}$ which lack the entire enzyme IIA domain retain the basic properties of the transport reaction, as regards lactose/H^+ symport and galactoside equilibrium exchange (30).

Uptake assays and specificity of *L. lactis* LacS. The role of LacS in *L. lactis* was deduced from uptake studies with radiolabeled lactose (Fig. 5). Initial experiments indicated that lactose and galactose were not efficiently accumulated in *L. lactis* NZ6009 when glucose was used as the energizing source (data not shown). Fructose-energized cells of galactose-grown NZ6009 accumulated significant amounts of lactose and galactose (Fig. 5), and therefore fructose was used to energize the *L. lactis* cells used in the uptake studies. In contrast to the parental strain, galactose-grown cells of the pNZ63-cured derivative NZ6091 could not transport lactose efficiently (Fig. 5A). However, *L. lactis* NZ6091 transformed with plasmid pNZV641 carrying the *lacS* gene showed rapid accumulation of the lactose to at least a 10-fold higher level than the parental strain did (Fig. 5B). Similar results were obtained with NZ6091 harboring pNZV55-1 or pNZV55-2 (data not shown). Since there is no known promoter preceding the *Sca*I cloning site in pGKV210, it is likely that the *lacS* gene is transcribed from its own promoter on the *Nru*I-*Cla*I insert.

The lactose plasmid, pNZ63, of *L. lactis* 6009 harbors the β-galactosidase genes in addition to the lactose carrier gene. Consequently, it was not possible to check for *lacS* expression by restoration of the Lac^+ phenotype to the cured NZ6091 Lac^- strain. Therefore, pNZV641 was transformed into *S. thermophilus* ST11 (Δ*lacS*) and examined for complementation of the *lacS* chromosomal deletion. The *lacS* gene was expressed in this host, as demonstrated by growth of the Em^r transformants on M17 with lactose as the only carbon source.

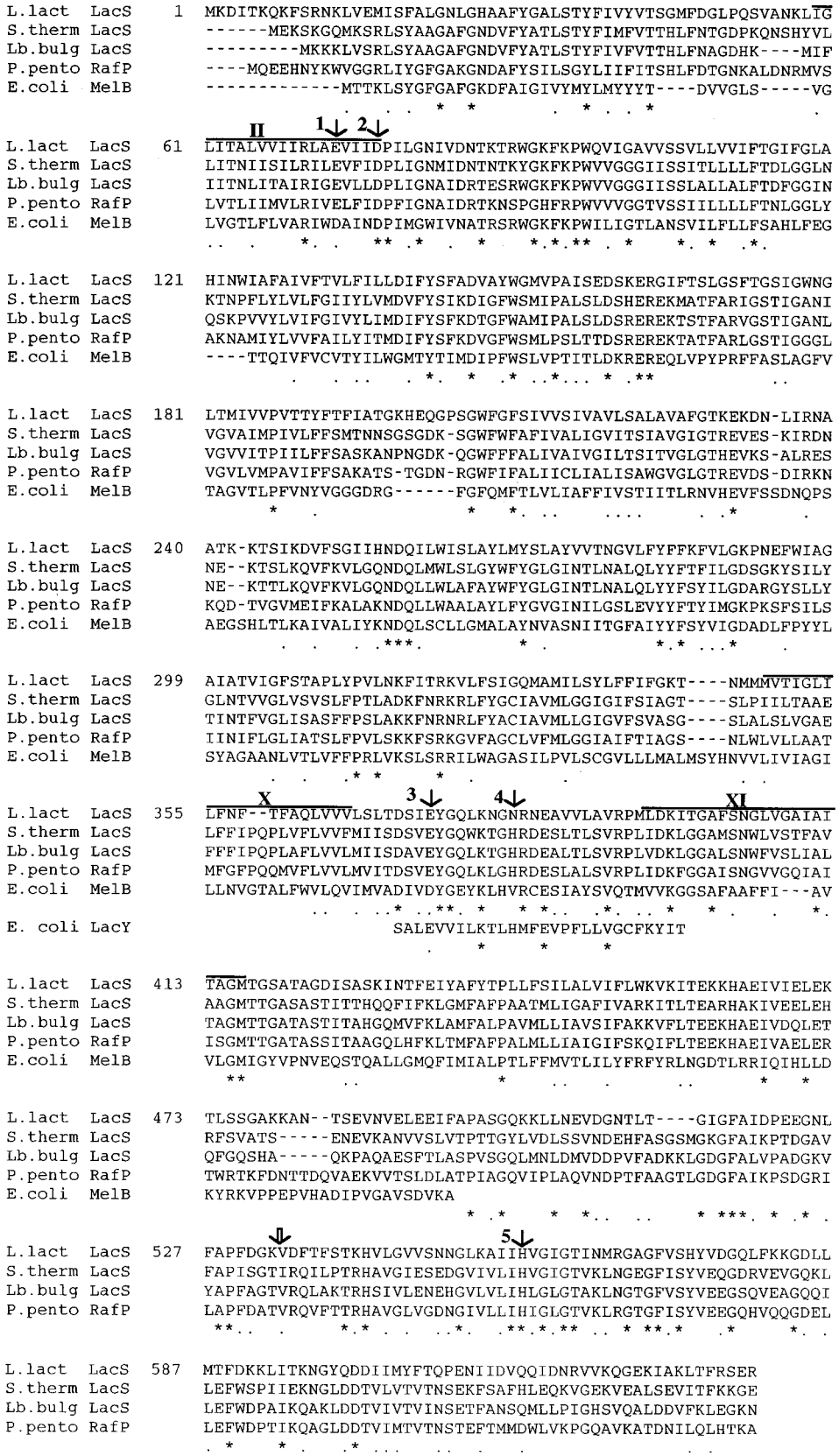

FIG. 4. Homology between the deduced amino acid sequences of *L. lactis*, *S. thermophilus*, and *Lactobacillus bulgaricus* LacS and *P. pentosaceus* RafP proteins from lactic acid bacteria and MelB of *E. coli*. Identical (.) and functionally related (*) amino acids are indicated. Gaps were introduced to maximize identity. The numbers on the left correspond to the amino acid positions of the *L. lactis* LacS. The conserved Glu and Asp in helix II (shown as 1 and 2, respectively), the conserved Glu and the Asn residue diverged from His-376 of $LacS^{St}$ between helices X and XI in the carrier domain (shown as 3 and 4, respectively), and the conserved His residue in the EIIA domain (shown as 5) are indicated. An alignment of the conserved region of helix X of *E. coli lacY* is included. The position at the *Sal*I site in the DNA from which the carboxy-terminal amino acids are deleted in pNZ635 is indicated by an open arrow.

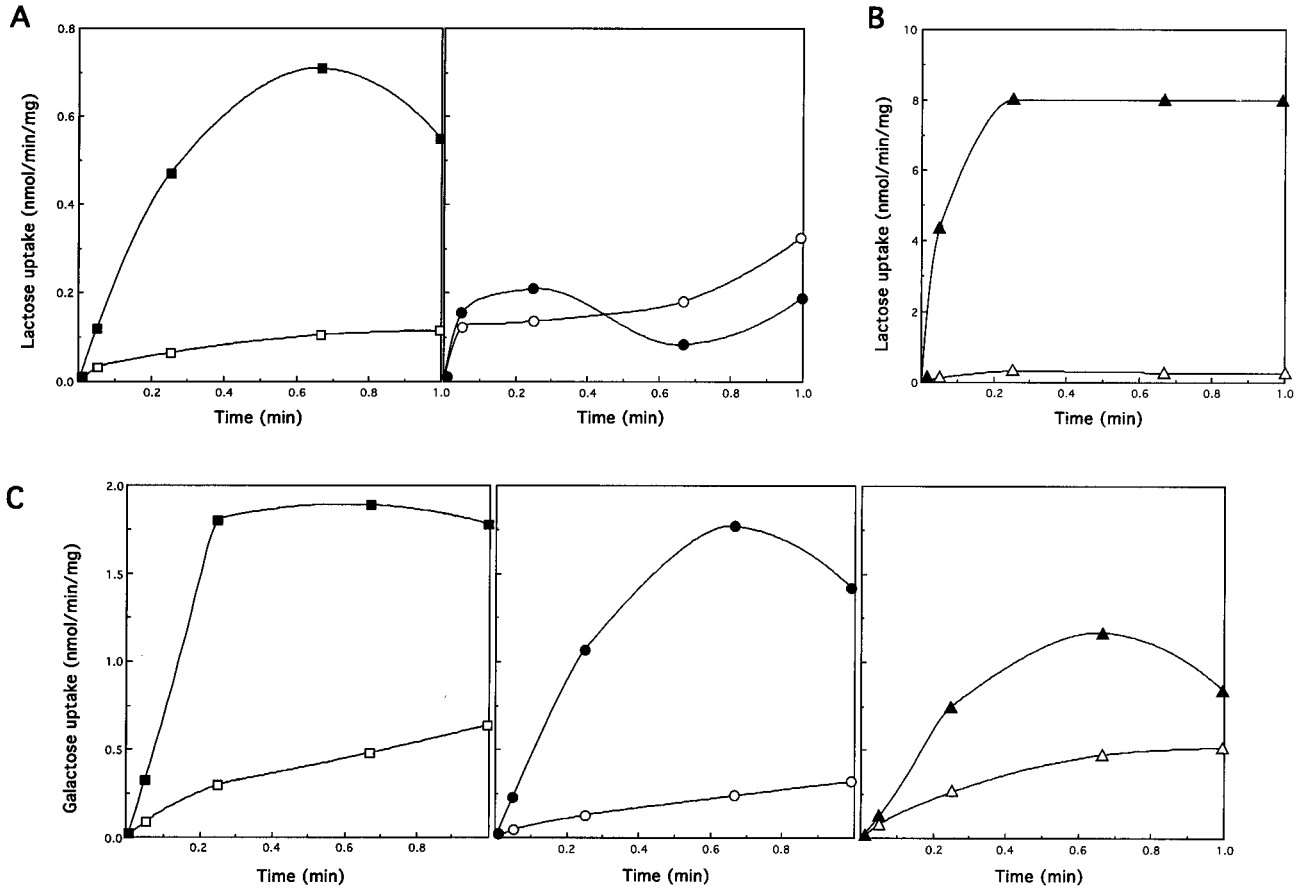

FIG. 5. (A and B) Uptake of [^{14}C]lactose by galactose-grown cells of *L. lactis* NZ6009 (□, ■) and NZ6091 (○, ●) (A) and NZ6091/pNZV641 (△, ▲) (B). (C) Uptake of [^{14}C]galactose by galactose-grown cells of *L. lactis* NZ6009 (□, ■), NZ6091 (○, ●), and NZ6091/pNZV641 (△, ▲). The solid symbols indicate energization of the cells with fructose.

The LacS protein of *S. thermophilus* is the only galactoside-transporting activity in this organism and is specific not only for lactose but also for galactose, melibiose, and, to a lesser extent, raffinose (30, 33). The Lac$^-$ *L. lactis* NZ6091 still has the ability to grow with galactose, melibiose, or raffinose as the sole carbon source, indicating the presence of a further transport system(s) for these carbohydrates. Galactose-grown cells of NZ6009 and NZ6091 efficiently transported [^{14}C]galactose as expected, although NZ6091 accumulated the label more slowly than the parent strain did (Fig. 5C). However, NZ6091 harboring pNZV641 transported galactose to a lower internal concentration than the other two strains did (Fig. 5C). Moreover, this reduction in galactose uptake contrasted with its ability for lactose uptake (Fig. 5B). To establish the role of LacS^{Lc} in galactose uptake, *S. thermophilus* ST11(Δ*lacS*)/pNZV641 was used, since the deletion in the chromosomal *lacS* gene renders the cells unable to transport galactose. When ST11(Δ*lacS*) expressed the *L. lactis lacS* gene on pNZV641, substantial galactose accumulation was observed upon energization of the cells, whereas no galactose uptake was detected in the absence of *lacS* (Fig. 6). These results provide strong evidence that LacS of *L. lactis* also has an affinity for galactose.

DISCUSSION

In this study, the cloning, nucleotide sequence, and characterization of the *L. lactis lacS* gene and its surrounding DNA regions have been described. Remarkably, lactose transport in the galactose-fermenting *L. lactis* was shown to be mediated by a lactose transport protein, LacS, that has significant similarity to other LacS proteins from lactic acid bacteria that do not utilize the galactose moiety of lactose. The *L. lactis lacS* gene was separated from the downstream *lacLM* encoding a β-galactosidase by 2 kb of DNA, which is a novel arrangement for lactose genes in lactic acid bacteria. The intergenic region consisted of an insertion sequence-like element related to the IS*3* family and a segment of another insertion sequence almost identical to IS*1070* (15, 49). In comparison, in both *S. thermophilus* and *Lactobacillus bulgaricus*, which possess LacS proteins similar to that of *L. lactis*, the *lac* genes are organized in an operon-like structure on the chromosome (23, 34). In these lactic acid bacteria, the *lacS* gene is located directly upstream of *lacZ* with just a 3-bp intergenic region and they are cotranscribed from a promoter preceding *lacS*. Upstream of the *lacS* gene in *S. thermophilus* are located genes involved in galactose metabolism (3, 35). In contrast, the galactose metabolic genes of *L. lactis* are not associated with the *lac* genes and are not encoded by the lactose plasmid pNZ63, as was demonstrated by curing the plasmid (data not shown).

On the basis of homology between ORFs and inverted repeats, the IS*3* family includes a variety of insertion sequences that are widely spread in both gram-negative and -positive genera. ORFC of the *L. lactis* IS*3*-like sequence shared 25 to 34% identity with the carboxy-terminal portions of most puta-

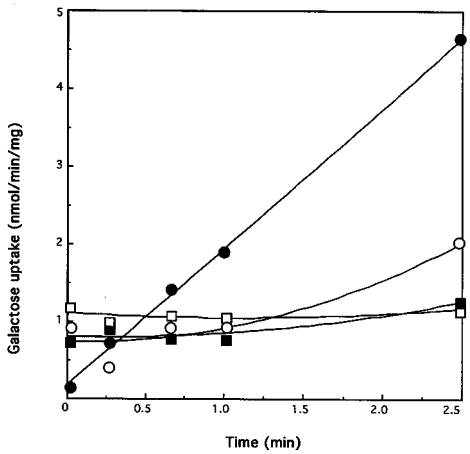

FIG. 6. Uptake of [^{14}C]galactose by *S. thermophilus* ST11(Δ*lacS*) (□, ■) and ST11(Δ*lacS*)/pNZV641 (○, ●). *S. thermophilus* cells were grown in Belliker broth supplemented with 0.5% sucrose and 0.2% galactose. The washed cells were diluted to a final protein concentration of 2 to 2.5 mg/ml, and 47 μM [^{14}C]galactose was added to initiate uptake. The solid symbols indicate energization of the cells with sucrose.

tive transposases from this family, such as IS*3* of *E. coli*, IS*600* of *Shigella sonnei*, and IS*6110* of *Streptococcus agalactiae*, but the preceding ORFA and ORFB shared homology only with the amino-terminal portion of the putative transposase of IS*1076* from *Lactococcus lactis*. It is interesting that three closely related members of the IS*3* family have been identified in the lactic acid bacterium *Lactococcus lactis*, namely, IS*1068* and IS*904*, which are associated with the nisin-sucrose conjugative transposon, and IS*1076*, which was found on a lactose plasmid (13, 20, 36, 37). The inverted repeats of the IS*3*-like element in *L. lactis* demonstrated poor conservation with the consensus derived for the IS*3* family; furthermore, there were no direct repeats bordering the structure. From these data, it is possible that this structure is the remnant of a former IS and is probably no longer an active element. The presence of this IS-like structure and the partial segment of IS*1070* between such metabolically linked genes is intriguing, and one can speculate whether evolution is in the process of joining or separating the genes. It is noteworthy that a complete copy of IS*1070* lies approximately 6 kb upstream of the partial copy encompassing the *lacS* gene and four other genes (Fig. 1). This conformation resembles that of a transposable element, since IS*1070* and its partial copy flank the intervening sequence, resulting in an inverted repeat arrangement.

The *L. lactis lacS* gene was expressed both in the original host background and in *S. thermophilus*. The rapid accumulation to a high level of the [^{14}C]lactose by *L. lactis* NZ6091 containing the cloned *lacS* gene in comparison with the parent strain is likely to be due to a combination of the increased copy number of the gene on this plasmid and/or the increased expression under the *lacR* promoter. The low uptake of labeled sugars by the deenergized cells may be due to energization by the added labeled sugar or to further transport systems for lactose and galactose. Interestingly, lactose- or galactose-grown cells of *L. lactis* NZ6009 simultaneously expressed transport systems for lactose and galactose uptake (data not shown). Uptake of lactose was also observed when cells were cultured on glucose- or fructose-containing medium, indicating constitutive expression for lactose transport. Expression of the β-galactosidase gene(s) is also constitutive in *L. lactis* NZ6009 (48).

The G+C content of the *lacS* gene is 35.4%, which is lower than the genomic G+C content of its host (43 to 44%) and also substantially lower than the value for the *lacLM* genes (47%). These variable values suggest that the *lac* genes may be recent immigrants in *L. lactis*, which could be tied in with the intervening IS-like element, and the sources of the *lac* genes might be quite different.

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