

Characterization, Expression, and Mutation of the *Lactococcus lactis* *galPMKTE* Genes, Involved in Galactose Utilization via the Leloir Pathway

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A cluster containing five similarly oriented genes involved in the metabolism of galactose via the Leloir pathway in *Lactococcus lactis* subsp. *cremoris* MG1363 was cloned and characterized. The order of the genes is *galPMKTE*, and these genes encode a galactose permease (GalP), an aldose 1-epimerase (GalM), a galactokinase (GalK), a hexose-1-phosphate uridylyltransferase (GalT), and a UDP-glucose 4-epimerase (GalE), respectively. This genetic organization reflects the order of the metabolic conversions during galactose utilization via the Leloir pathway. The functionality of the *galP*, *galK*, *galT*, and *galE* genes was shown by complementation studies performed with both *Escherichia coli* and *L. lactis* mutants. The GalP permease is a new member of the galactoside-pentose-hexuronide family of transporters. The capacity of GalP to transport galactose was demonstrated by using *galP* disruption mutant strains of *L. lactis* MG1363. A *galK* deletion was constructed by replacement recombination, and the mutant strain was not able to ferment galactose. Disruption of the *galE* gene resulted in a deficiency in cell separation along with the appearance of a long-chain phenotype when cells were grown on glucose as the sole carbon source. Recovery of the wild-type phenotype for the *galE* mutant was obtained either by genetic complementation or by addition of galactose to the growth medium.

Understanding the mechanisms involved in carbohydrate metabolism and its regulation in lactic acid bacteria (LAB) is a prerequisite for improving the industrial properties of these organisms by metabolic engineering (16, 29). The primary function of LAB in industrial dairy fermentations is conversion of lactose to lactic acid. Utilization of this disaccharide can occur in LAB via several mechanisms (18, 47, 48). The glucose moiety resulting from lactose hydrolysis is metabolized via glycolysis, whereas the galactose moiety follows different pathways depending on the LAB. While some thermophilic strains of LAB are known to release the galactose moiety of lactose into the medium (16, 30, 37), other LAB metabolize this disaccharide via two possible pathways depending on the mode of transport of the sugar (18, 48). When lactose or galactose is transported via a phosphoenolpyruvate-dependent phosphotransferase system (PTS), the resulting galactose 6-phosphate is metabolized by the enzymes of the tagatose 6-phosphate pathway (3, 51). In the case of galactose or lactose entry via a permease (18, 48), the sugar is not phosphorylated, and the galactose is degraded via the Leloir pathway (24).

The Leloir pathway is among the first metabolic pathways that were discovered and includes three enzymes, galactokinase (GalK; EC 2.7.1.6), hexose-1-phosphate uridylyltransferase (GalT; EC 2.7.7.12), and UDP-glucose 4-epimerase (GalE; EC 5.1.3.2), which are involved in the conversion of

galactose to glucose 1-phosphate. Aldose 1-epimerase or mutarotase (GalM; EC 5.1.3.3) is an additional enzyme required for rapid lactose metabolism and was characterized more recently (7, 35, 40). GalM catalyzes the interconversion of the α - and β -anomers of galactose, and the presence of this enzyme was found to be essential for efficient lactose utilization in *Escherichia coli*, as cleavage of this β -galactoside by β -galactosidase yields glucose and β -D-galactose, while α -D-galactose is the sole substrate for GalK (7). Genes encoding Leloir pathway enzymes have been characterized in different LAB, and their molecular organization has been discussed (27).

Previous reports on the related strains *Lactococcus lactis* NCDO 712 and ML₃ focused on characterization of their efficient lactose metabolic pathway (18, 47, 48). It was shown that lactose is transported in these strains via a phosphoenolpyruvate-dependent PTS and that enzymes of the tagatose 6-phosphate pathway, which are encoded by the *lac* operon that resides on a lactose plasmid, are involved in degradation of the galactose 6-phosphate (13, 34, 51). In addition, it was found that galactose was catabolized via two distinct routes, the tagatose 6-phosphate and the Leloir pathways (47, 48). Although it was shown that the lactose PTS was able to transport galactose with low affinity, an additional galactose PTS activity was detected in a derivative of strain ML₃ lacking a lactose plasmid (18, 36). However, the well-studied plasmid-free NCDO 712 derivative *Lactococcus lactis* MG1363 (25) was found to utilize galactose via the Leloir pathway, which requires a galactose permease (18). A chromosomally encoded galactose permease system was partially characterized and was found to be highly specific for galactose and to have a low

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TABLE 1. Bacterial strains used in this study

Strain	Relevant features ^a	Source or reference(s)
<i>Escherichia coli</i> strains		
MC1061		12
HB101	GalK-deficient strain (carrying <i>galK2</i> mutation)	8
DW2	MelB-deficient strain ($\Delta melB$)	58
LE392	GalK- and GalT-deficient strain (carrying <i>galK2</i> and <i>galT22</i> mutations)	44
CGSC4467	GalT-deficient strain (carrying <i>galT22</i> mutation)	CGSC ^b
CGSC4498	GalE-deficient strain (carrying <i>galE28</i> mutation)	CGSC ^c
<i>Lactococcus lactis</i> strains		
MG1363	Lac ⁻ , Gal ⁺ , plasmid-free and prophage-cured derivative of NCDO712	25
MG1363 $\Delta galK$	Lac ⁻ , Gal ⁻ , MG1363 derivative with 380-bp deletion in the coding sequence of <i>galK</i> , obtained after a replacement-recombination event using pNZ8402	This study ^d
MG1363:: <i>galP1</i>	Lac ⁻ , Gal ⁻ , Em ^r , MG1363 derivative obtained by insertion mutagenesis, <i>galP</i> gene disrupted by integration of pNZ8451 in the coding sequence	This study ^d
MG1363:: <i>galP2</i>	Lac ⁻ , Gal ⁻ Em ^r , MG1363 derivative obtained by insertion mutagenesis, <i>galP</i> gene disrupted by integration of pNZ8452 in the coding sequence	This study ^d
MG1363:: <i>galE</i>	Lac ⁻ , Gal ⁻ Em ^r , MG1363 derivative obtained by insertion mutagenesis, <i>galE</i> gene disrupted by integration of pNZ8460 in the coding sequence	This study ^d

^a Lac⁻, non-galactose-fermenting phenotype; Gal⁺, galactose-fermenting phenotype; Gal⁻, non-galactose-fermenting phenotype; Em^r, resistance to erythromycin.

^b CGSC, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.

^c See reference 11.

^d See Materials and Methods, Table 2, and Fig. 1.

affinity for lactose, suggesting that the Leloir pathway is the major route for galactose utilization (48).

Here we describe molecular and functional characterization of the *gal* gene cluster of *Lactococcus lactis* MG1363, whose gene organization and CcpA-dependent expression were reported previously (27, 33). The *gal* genes identified encode galactose transport via a galactose permease (GalP; previously called GalA) (27, 33) that belongs to the galactoside-pentose-hexuronide (GPH) family of transporters, a mutarotase (GalM), and the Leloir pathway enzymes (GalK, GalT, and GalE). Gene inactivation experiments showed that the *galP* and *galK* genes are essential for growth on galactose, while *galE* gene disruption not only eliminated growth on galactose but also resulted in the appearance of a long-chain phenotype when the organism was grown on glucose.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The strains and plasmids used in this study are shown in Tables 1 and 2, respectively. *E. coli* MC1061 (12) was used as a host strain for cloning experiments and was grown in Luria broth-based medium with aeration at 37°C (44). MacConkey agar (Difco Laboratories, Detroit, Mich.) was used to detect sugar fermentation by *E. coli* strains. *Lactococcus lactis* strains were grown without aeration at 30°C in M17 broth (Merck, Darmstadt, Germany) supplemented with 1% sugar. For screening of the galactose-fermenting phenotype, *Lactococcus lactis* was plated on galactose indicator agar (GIA) plates containing Elliker medium (21) supplemented with 1% galactose and 0.004% bromocresol purple (Merck). Ampicillin (50 µg/ml), erythromycin (2.5 µg/ml), and chloramphenicol (10 µg/ml) were used when appropriate.

DNA isolation, library construction, and other manipulations. Isolation of *E. coli* plasmid DNA and standard recombinant DNA techniques were performed by using established protocols (44). Total DNA was isolated from protoplasts of *Lactococcus lactis* as described previously (55). Large-scale isolation of *E. coli* plasmid DNA for nucleotide sequence analysis was carried out by performing

TABLE 2. Plasmids used in this study

Plasmid	Relevant features ^a	Source or reference(s)
pBR322	Ap ^r Tc ^r ; cloning vector	5
pUC19	Ap ^r ; cloning vector	59
pUC18Ery	Ap ^r Em ^r ; derivative of pUC18 carrying an <i>ery</i> gene	50
pUC19Ery	Ap ^r Em ^r ; derivative of pUC19 carrying an <i>ery</i> gene	32
pNZ8020	Cm ^r ; inducible expression vector carrying the <i>nisA</i> promoter	14
pNZ8400	Ap ^r ; 2.4-kbp <i>SstI</i> fragment of <i>Lactococcus lactis</i> cloned in pUC19	This study ^b
pNZ8401	Ap ^r ; pNZ8400 derivative with a <i>Scal/StuI</i> 380-bp deletion in the <i>galK</i> reading frame	This study ^b
pNZ8402	Ap ^r Em ^r ; <i>XbaI/PstI</i> fragment of pUC19Ery containing the <i>ery</i> gene and cloned in pNZ8401	This study ^b
pNZ8410	Ap ^r ; 7.3-kbp <i>SalI/EcoRI</i> fragment of <i>Lactococcus lactis</i> cloned in pBR322	This study ^b
pNZ8421	Ap ^r ; 1.8-kbp <i>EcoRI</i> fragment of pNZ8410 containing <i>galE</i> cloned in pBR322, orientation 1	This study ^b
pNZ8422	Ap ^r ; same as pNZ8421, orientation 2	This study ^b
pNZ8442	Ap ^r ; 2.5-kbp <i>SalI/NheI</i> fragment of pNZ8410 containing <i>galP</i> cloned in pUC18	This study ^b
pNZ8451	Ap ^r Em ^r ; 1.1-kbp internal <i>HpaII</i> fragment of <i>galP</i> cloned in pUC18Ery, orientation 1	This study ^b
pNZ8452	Ap ^r Em ^r ; same as pNZ8451, orientation 2	This study ^b
pNZ8460	Ap ^r Em ^r ; internal 0.8-kbp fragment of <i>galE</i> cloned in pUC18Ery	This study ^b
pNZ8560	Cm ^r ; 1.8-kbp <i>EcoRI</i> fragment of pNZ8410 containing <i>galE</i> cloned in pNZ8020	This study ^b

^a Em^r, Ap^r, Cm^r, and Tc^r, resistance to erythromycin, ampicillin, chloramphenicol, and tetracycline, respectively.

^b See Materials and Methods and Fig. 1.

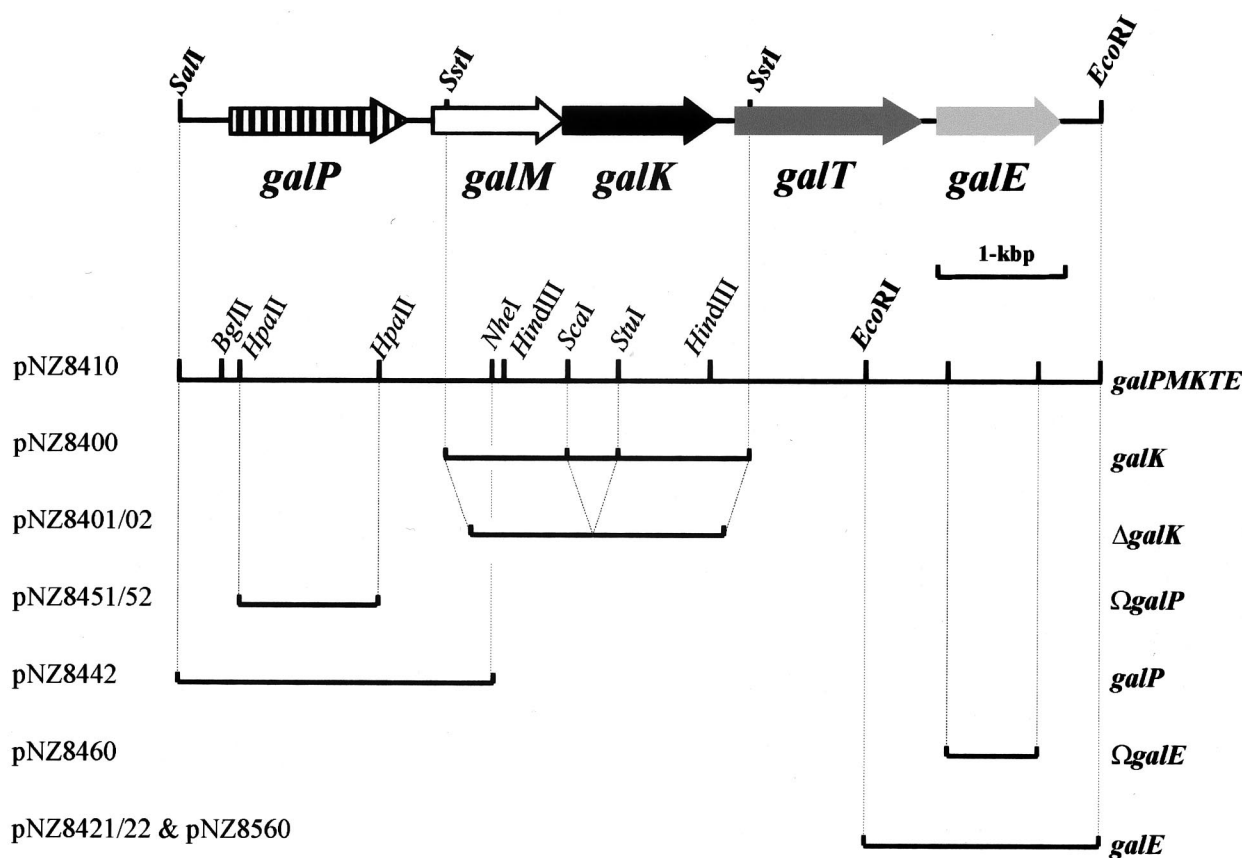


FIG. 1. Arrangement of the *gal* gene cluster of *Lactococcus lactis* MG1363 (accession no. AJ011653) and schematic diagram of the inserts of relevant plasmids.

isopycnic centrifugation in CsCl-ethidium bromide gradients. Restriction endonucleases and other enzymes used for DNA manipulations were used according to the instructions of the suppliers (GIBCO-BRL, Gaithersburg, Md.; New England Biolabs Inc., Beverly, Mass.; and Boehringer GmbH, Mannheim, Germany). DNA fragments were recovered from agarose gels by using the Glass-MAX system (GIBCO-BRL). Both *E. coli* and *Lactococcus lactis* were transformed by electroporation by using a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.) as specified by the manufacturer in the case of *E. coli* and as previously described in the case of *Lactococcus lactis* (28, 57). A library of *Lactococcus lactis* MG1363 DNA was constructed in *E. coli* HB101 by inserting *Sst*I fragments that were approximately 2 to 3 kb long into *Sst*I-linearized pUC19, since it had been observed that a *Lactococcus lactis galK*-like gene was located on a 2.4-kb *Sst*I fragment (R. D. Pridmore, unpublished data).

Nucleotide sequence analysis. Nucleotide sequence analysis of double-stranded plasmid DNA was performed by the dideoxy chain termination method (45) by using an ALF automatic sequencer in combination with T7 DNA polymerase autoread kits (Pharmacia Biotech). Sequencing reactions were initiated by using fluorescein-labeled universal and reverse primers (in the case of pUC-based constructs) and were continued with synthetic primers in combination with fluorescein-dATP as recommended by the manufacturer (Pharmacia Biotech). The synthetic primers used for sequencing from the *Eco*RI and *Sal*I sites of plasmid pBR322 were pBREco (5'-ATAAAGAAGACAGTCATA-3') and pBRSal (5'-ATCATGACATTAACCTATA-3'), respectively. Sequence data were assembled and analyzed by using the PC/GENE program (version 6.70; IntelliGenetics) and the Clone Manager program (version 4.0; Scientific and Educational Software).

Construction of strains and plasmids. The relevant properties of the constructed strains and plasmids are shown in Tables 1 and 2. Plasmids pNZ8400 and pNZ8410 carrying part of the *gal* gene cluster and the complete *gal* gene cluster, respectively, were used for several subcloning experiments and for construction of the integration plasmids in *E. coli* (Fig. 1). Plasmid pNZ8401 is a derivative of pNZ8400 having a 380-bp *Sca*I/*Stu*I deletion in the *galK* reading

frame. Integration plasmid pNZ8402 was obtained after cloning of a pUC19Ery *Xba*I/*Pst*I fragment containing the erythromycin resistance gene (*ery*) in the corresponding restriction sites of pNZ8401. In order to do this, plasmid pNZ8402 containing a deleted *galK* gene and the *ery* gene was introduced into *Lactococcus lactis* MG1363. Following transformation, erythromycin-resistant (Em^r) colonies which contained a copy of the plasmid integrated in the chromosome were obtained, as confirmed by Southern hybridization (data not shown). These integrants were grown for at least 200 generations in the absence of erythromycin. Colonies sensitive to erythromycin and having a galactose-minus phenotype on GIA plates were analyzed by PCR, and Southern analysis confirmed that the intact *galK* gene was replaced by the deleted *galK* gene copy (Δ *galK*) (Fig. 1). Integration plasmids pNZ8451 and pNZ8452, used for disruption of the *galP* gene, contained an internal *Hpa*II fragment of *galP* cloned in the *Acc*I site of plasmid pUC18Ery, in the same orientation as the orientation of the *ery* gene for pNZ8451 and in the opposite orientation for pNZ8452. For disruption of the *galE* gene, an integration vector designated pNZ8460 was constructed, and it contained an internal 0.8-kbp *galE* gene fragment that was PCR amplified, digested with restriction enzymes *Eco*RI and *Bam*HI, and cloned in plasmid pUC18Ery (50) previously linearized with the same enzymes. The PCR primers used were primers DE1 (5'-GCACGAATTCAGTAACAGGTCATCGCGAG G-3') and DE2 (5'-GCACGGATCCGCTACAAGTTCATCTGGATC-3') carrying *Eco*RI and *Bam*HI restriction sites (underlined). Plasmids pNZ8421 and pNZ8422 were pBR322 derivatives carrying a 1.8-kbp *Eco*RI fragment containing the complete *galE* gene and its upstream region cloned in the two possible orientations. The same fragment was used for construction of the lactococcal complementation plasmid pNZ8560 after cloning in the expression vector pNZ8020 (15). Plasmid pNZ8442 is a pUC18 derivative carrying the *Sal*I/*Nhe*I fragment containing the complete *galP* gene and cloned between the pUC18 *Sal*I and *Xba*I restriction sites.

Hybridization analysis. DNA was transferred from a 0.8% agarose gel to GeneScreen Plus membranes (Du Pont, NEN Research Products, Boston, Mass.) as described previously (44, 46). Radioactively labeled oligonucleotide

primers or DNA fragments were used as probes; these probes were obtained either by end labeling with [γ -³²P]ATP or by nick translation with [α -³²P]ATP (44).

Galactokinase and galactose uptake assays. Galactokinase activity was measured by using a procedure adapted from the procedure described by Ajdić et al. (1). Cells of *Lactococcus lactis* were grown in M17 medium supplemented with the appropriate sugar (at a concentration of 1%) to an optical density at 600 nm of 1, washed twice with ice-cold 50 mM Tris-HCl (pH 7.3) buffer, and finally resuspended at a concentration of 30% in the same buffer. Cell lysates were prepared by disrupting the cells by using a Bead Beater (Biospec Products, Bartlesville, Okla.) and an equal volume of zirconia-silica beads (diameter, 0.1 mm). The protein concentration in the cell extract was determined by using Bradford's reagent (Bio-Rad Laboratories) (9). For galactokinase activity measurement, 20 μ l of appropriately diluted cell extract was analyzed in a 100- μ l reaction mixture containing 100 mM Tris-HCl (pH 7.3), 4 mM MgCl₂, 3.2 μ M NaF, 1.6 mM ATP, and 1 mM [¹⁴C]galactose (0.5 μ Ci ml⁻¹; Amersham). The assay was started by adding the radiolabeled galactose, and galactose phosphorylation was determined at periodic intervals during 15 min of incubation at 30°C. Aliquots were transferred onto Whatman DE81 filter discs and air dried for 5 min. The filters, which bound sugar phosphates but not free galactose, were washed twice with water and oven dried. The radioactivity bound to the discs was determined by using a liquid scintillation counter (LS7500; Beckman Instruments Inc., Palo Alto, Calif.). Galactose uptake was measured essentially as described by Russel et al. (42).

SEM sample preparation. Cells grown overnight were fixed in 0.2 M phosphate-buffered saline (pH 7.4) containing 2% glutaraldehyde for 30 min. Samples of cells were dehydrated by using a graded series of ethanol concentrations and were subsequently washed twice with absolute ethanol. The samples were critical point dried and coated with gold-palladium prior to observation with a Zeiss DSM950 scanning electron microscope (SEM).

Nucleotide sequence accession number. The nucleotide sequence of the 7,309-bp *SalI/EcoRI* fragment containing the galactose gene cluster of *Lactococcus lactis* MG1363 has been deposited in the EMBL, GenBank, and DDBJ databases under accession number AJ011653.

RESULTS

Cloning, nucleotide sequence, and organization of the *gal* gene cluster of *Lactococcus lactis*. A library of *Lactococcus lactis* subsp. *cremoris* MG1363 chromosomal DNA in pUC19 was used to complement the *galK*-deficient *E. coli* strain HB101. Transformants able to ferment galactose were selected on MacConkey agar plates containing galactose. A red colony, indicating galactose utilization, was isolated, and it contained a pUC19 derivative (pNZ8400) carrying a 2.4-kb *SstI* fragment derived from the *Lactococcus lactis* MG1363 chromosomal DNA, as shown by hybridization. Sequence analysis of the fragment revealed the presence of one complete gene, designated *galK*, preceded by an incomplete gene, designated *galM*, oriented in the same direction (Fig. 1). Comparison of the deduced amino acid sequences with the sequences available in the databases showed that there was significant homology between the complete *GalK* sequence and several galactokinase sequences, while the incomplete *GalM* sequence displayed homology with the sequences of mutarotases. Since the cloned fragment appeared to contain part of a *gal* gene cluster with a new gene order (*galM-galK*), we used a second step to clone a larger fragment using the same complementation strategy, based on preliminary restriction enzyme mapping of the chromosomal region (data not shown). An approximately 7.5-kbp *SalI-EcoRI* fragment was cloned and stably maintained in the medium-copy-number vector pBR322, resulting in pNZ8410. Sequencing of the insert in pNZ8410 revealed the presence of three additional genes, one located upstream from *galM* and designated *galP* and two situated downstream from *galK* and designated *galT* and *galE*, all oriented in the same direction

(Fig. 1). *GalT* and *GalE* exhibited high sequence homologies with previously described *GalT* and *GalE* sequences, respectively, and the sequence of *GalP* showed homology with sequences of membrane proteins belonging to the GPH transporter family (38). This subfamily of transporters is called TC 2.A.2 in the recent transporter classification (TC) system (43). Thus, the *galPMKTE* gene cluster of *Lactococcus lactis* MG1363 (Fig. 1) encodes all the proteins required for transport and utilization of galactose via the Leloir pathway.

Amino acid homologies and identity of *GalP* with transport proteins. Comparison of the predicted amino acid sequences of the *gal* gene products with previously described equivalent protein sequences showed a high degree of identity. The enzymes of the Leloir pathway showed the greatest predicted similarities to the enzymes identified in related gram-positive bacteria; *GalK* exhibited 59% identity to *Streptococcus thermophilus GalK* (54), *GalT* exhibited 53% identity to *Lactobacillus casei GalT* (2), and *GalE* exhibited 61% identity to *S. thermophilus GalE* (40). *Lactococcus lactis* subsp. *cremoris* MG1363 *GalM* exhibited a high level of identity (86%) only with the predicted enzyme from the related strain *Lactococcus lactis* subsp. *lactis* IL-1403 (6). Lower levels of identity were observed when *GalM* was compared to *E. coli GalM* (31%) (7) and also to any other *GalM* from a gram-positive bacterium; *S. thermophilus GalM* (40) exhibited 26% identity. Low levels of conservation similar to those of *Lactococcus lactis GalM* were not observed among bacteria when the other Leloir enzymes (*GalK*, *GalT*, and *GalE*) were considered.

Analysis of the deduced sequence of *Lactococcus lactis GalP* revealed a level of identity of 51% with the galactose permease (*GalP*) recently characterized by Djordjevic and coworkers for the heterofermentative LAB *Lactobacillus brevis* (19). The galactose permease-encoding gene of *Lactococcus lactis* was therefore designated *galP* instead of *galA*, which was the designation used previously (26, 27, 33). Another putative lactose permease, which is designated LacS and exhibits 96% identity with *GalP*, is present in *Lactococcus lactis* strain IL-1403 (6). *GalP* is also 45 and 44% identical to the lactose permeases (LacS) of *Leuconostoc lactis* (52) and *S. thermophilus* (39), respectively. Both *Leuconostoc lactis* LacS (639 amino acids) and *S. thermophilus* LacS (634 amino acids) are chimeric, as they possess a carrier domain homologous to the melibiose carrier (MelB) of *E. coli* (60) and an extra enzyme IIA-like domain attached to the C-terminal end of the carrier domain (39, 52). In contrast, *Lactococcus lactis* MG1363 *GalP* (462 amino acids), *Lactococcus lactis* IL-1403 LacS (462 amino acids), and *Lactobacillus brevis GalP* (474 amino acids) lack this C-terminal enzyme IIA-like domain and are composed of a carrier domain that is approximately the same size as *E. coli* MelB (462 residues for *GalP*, compared with 469 residues for MelB). The level of identity between the *GalP* and MelB proteins was 26%. Prediction of membrane-spanning helices revealed the presence of 12 putative helices in the *GalP* protein, supporting the hypothesis that its putative function is as a transmembrane protein.

Complementation of mutations in *E. coli* by *Lactococcus lactis gal* genes. In order to show the functionality of the *gal* genes, we complemented several *E. coli gal*-deficient strains using pNZ8400 or pNZ8410 or some of their derivatives (Fig. 1). The complementation test was performed on MacConkey

TABLE 3. Complementation of lesions in *E. coli* genes with *Lactococcus lactis* genes

<i>E. coli</i> strain	Sugar ^a	Deficient gene(s)	Complementation plasmid ^b	Intact <i>gal</i> gene(s) on plasmid
HB101	Gal	<i>galK</i>	pNZ8400 pNZ8410	<i>galK</i> <i>galPMKTE</i>
LE392	Gal	<i>galK</i> , <i>galT</i>	pNZ8410	<i>galPMKTE</i>
CGSC4467	Gal	<i>galT</i>	pNZ8410	<i>galPMKTE</i>
CGSC4498	Gal	<i>galE</i>	pNZ8421 pNZ8422	<i>galE</i> <i>galE</i>
DW2	Mel	<i>melB</i>	pNZ8442	<i>galP</i>

^a Gal, galactose; Mel, melibiose.

^b For construction and descriptions of the plasmids used see Table 2 and Fig. 1.

agar plates supplemented with 1% galactose or melibiose. Plasmid integrity in the complementing colonies was confirmed by restriction enzyme analysis of the plasmid contents (data not shown). All *Lactococcus lactis gal* genes tested (*galK*, *galT*, and *galE*) could complement the galactose utilization defect in the corresponding *E. coli gal* mutant (Table 3). GalE complementation was observed with both pNZ8421 and pNZ8422, two plasmids that carry the *galE* gene in opposite orientations, suggesting that a functional *E. coli* promoter was present in front of the *galE* gene. As expected, pNZ8401, which had a deletion in the *galK* gene, in contrast to pNZ8400 (Fig. 1), did not show complementation of galactose utilization in *E. coli* HB101, which also confirmed the functionality of *galK*. Because we did not have *E. coli* mutants that were deficient in galactose transport, we were not able to investigate the ability of GalP to transport galactose in *E. coli*. However, we demonstrated that GalP was able to complement the chromosomal mutation in the MelB melibiose transport system of *E. coli* DW2 (Table 3). MelB also is a member of the GPH (TC 2.A.2) transporter family. Thus, the *Lactococcus lactis galP* gene was able to complement the *E. coli melB* gene, indicating that the encoded transmembrane protein GalP is also involved in transport of the α -galactoside melibiose.

Functional analysis of the *galP* and *galK* genes in *Lactococcus lactis*. We investigated the ability of lactococcal cells to accumulate galactose and to exhibit galactokinase activity when they were grown on glucose, galactose, maltose, or a glucose-galactose mixture. In the wild-type strain *Lactococcus*

lactis MG1363, three different levels of activity were detected, and they were subject to strong regulation (Table 4). Whereas the levels of galactose uptake and the galactokinase activities were very low on glucose, large increases were observed on galactose. A third, intermediate level of expression was detected in cells grown on maltose or on a galactose-glucose mixture (Table 4). These results suggest that expression of both *galK* and *galP* is subject to glucose repression (catabolite repression) or galactose activation or both.

The chromosomal copy of the *galP* gene was inactivated by single-crossover integration of the nonreplicating plasmid pNZ8451 or pNZ8452 (Fig. 1). These two plasmids carry an internal fragment of the *galP* gene cloned in two orientations, which allows integration of the erythromycin resistance-encoding gene (*ery*) of pUC18Ery in both directions. As the *ery* gene is not followed by a terminator and has its own promoter, readthrough transcription allows expression of the downstream genes provided that the *ery* gene is cloned in the same orientation (50). Therefore, pNZ8451 integration in *galP* allows expression of the downstream genes (*galMKTE*), while pNZ8452 integration probably leads to a loss of expression of these downstream genes. Strains were selected in which pNZ8451 (*Lactococcus lactis* MG1363::*galP1*) or pNZ8452 (*Lactococcus lactis* MG1363::*galP2*) was integrated, and these strains were analyzed by PCR and by Southern hybridization (data not shown). This analysis confirmed that the plasmids were integrated in the *galP* gene, which resulted in disruption of the gene into two truncated parts. The phenotypes of the different integrants were analyzed on GIA plates and in M17 liquid medium containing galactose. Both *Lactococcus lactis* MG1363::*galP1* and MG1363::*galP2* appeared to be unable to use galactose as a sole carbon source. These results show that the *galP* gene is necessary for galactose metabolism in *Lactococcus lactis* MG1363. Enzymatic assays in which *Lactococcus lactis* MG1363::*galP2* was used showed that there was a strong decrease in the ability to import galactose concomitant with a complete loss of detectable galactokinase activity (Table 4). In contrast, *Lactococcus lactis* MG1363::*galP1* showed significant galactokinase activity when it was grown on glucose (data not shown), confirming the expected readthrough from the *ery* gene, as reported previously for *Lactococcus lactis* (50). The absence of galactokinase activity in strain *Lactococcus lactis* MG1363::*galP2* suggests that a functional promoter down-

TABLE 4. Galactose uptake and galactokinase activities in wild-type and mutant strains grown on different sugars

Sugar(s) ^a	MG1363		MG1363:: <i>galP2</i>		MG1363 Δ <i>galK</i> galactokinase activity ^f
	Gal uptake ^b	Galactokinase activity ^c	Gal uptake ^b	Galactokinase activity ^c	
Glu	0.56 \pm 0.05	0.44	0.29 \pm 0.07	<0.1	<0.1
Gal	102.7 \pm 12.2	281	NG ^d	NG	NG
Glu + Gal	2.12 \pm 0.06	74	0.30 \pm 0.03	<0.1	ND ^e
Mal	2.52 \pm 0.18	55	0.63 \pm 0.05	<0.1	<0.1

^a Glu, glucose; Gal, galactose; Mal, maltose.

^b Expressed as nanomoles of labeled galactose accumulated per minute per milligram of protein. The values are the means \pm standard deviations for at least triplicate determinations.

^c Specific activity in cell extracts, expressed as nanomoles of phosphorylated galactose per minute per milligram of protein. The values are means for duplicate determinations.

^d NG, no growth.

^e ND, not determined.

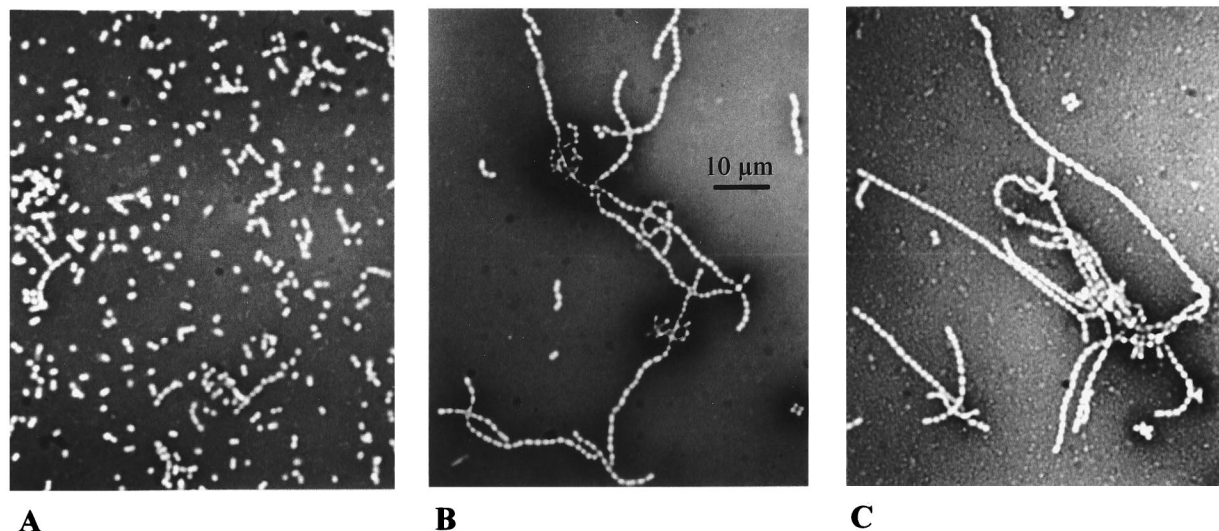


FIG. 2. Light micrographs of the wild-type strain *Lactococcus lactis* MG1363 (A) and the *galE* mutant strain *Lactococcus lactis* MG1363:*galE* (B and C). Both strains were grown overnight in M17 medium supplemented with glucose. Bar = 10 μ m.

stream of the *galP* gene is not present and therefore that an operon-like organization of the *gal* genes is likely.

A 380-bp deletion was introduced into the *galK* gene by a standard two-step homologous recombination procedure (Fig. 1). The resulting strain, *Lactococcus lactis* MG1363 Δ *galK*, was not able to use galactose as a carbon source, as determined both on GIA plates and in M17 liquid medium. Furthermore, no galactokinase activity could be detected in this strain after growth on maltose (Table 4).

Inactivation of chromosomal *galE* by gene replacement and effect on the phenotype of the recombinants. An internal fragment of the *galE* gene was PCR amplified and cloned in plasmid pUC18Ery. The resulting nonreplicating plasmid, pNZ8460 (Fig. 1), was introduced into *Lactococcus lactis* MG1363 by electroporation, and recombinant strains were selected after plating on M17 agar plates supplemented with glucose and containing erythromycin. Integration of pNZ8460 into the *galE* gene following a single recombination event was confirmed by Southern hybridization (data not shown), and a clone showing the expected genotype was designated *Lactococcus lactis* MG1363:*galE* and was analyzed further. As expected, *Lactococcus lactis* MG1363:*galE* was not able to use galactose as a sole carbon source. Moreover, the *galE* mutant strain showed a strongly reduced growth rate when it was grown in a liquid medium containing glucose or maltose as the carbon source (data not shown). This growth defect was characterized by cell flocculation, followed by sedimentation of the cells in the bottoms of the culture tubes. The mutant cells were examined by light microscopy, and the results suggested that the growth defect was linked to formation of long chains of cells containing up to 50 cocci (Fig. 2). Analysis of the cells by SEM revealed a normal shape but the presence of a possible peptidoglycan bridge between unseparated cells (Fig. 3). This could reflect an unfinished separation mechanism, while the elongation and septation steps were not affected, as observed by transmission electron microscopy (data not shown). In order to fully associate the phenotype of the *galE* mutant with the *galE* disruption and not with a possible polar effect of insertion

of the knockout plasmid pNZ8460, genetic complementation of the *galE* gene was performed. We constructed a plasmid that contained the intact *galE* gene and was able to replicate in lactococcal cells. Introduction of this plasmid, pNZ8560, into the *galE* mutant strain resulted in restoration of the ability of the cells to grow on galactose and in the disappearance of the long-chain phenotype when cells were grown on glucose (data not shown). No nisin induction was required for such genetic complementation when the pNZ8020 expression vector was used, suggesting that a functional promoter was present upstream of the *galE* gene. Remarkably, disappearance of the long-chain phenotype was also observed when *galE* mutant cells were grown on a mixture of glucose and galactose. The galactose was probably used by the cells to restore a sufficient level of UDP-galactose even when the *gal* genes were expressed at a low level because of the presence of glucose (catabolite repression of the *gal* genes). Similarly, when cells of the *galE* mutant were grown on maltose, they showed the long-chain phenotype, which was not observed during growth on a mixture of maltose and galactose. A minor amount of galactose appeared to be necessary for such substrate complementation, as 0.005% galactose was enough to prevent the long-chain phenotype in the presence of 1% glucose. This finding is consistent with the previous observation that the *gal* genes were not fully repressed in the presence of glucose and with the observation that galactose transport and galactokinase activities were detectable in a culture grown on a mixture of glucose and galactose (Table 4).

DISCUSSION

To investigate the genetics of galactose utilization via the Leloir pathway in *Lactococcus lactis*, we cloned and characterized the chromosomal *gal* gene cluster, which has the order *galP-galM-galK-galT-galE*. This cluster organization is novel compared to the organization of previously described *gal* gene clusters and features the presence of a galactose permease gene (*galP*) located upstream of the genes for the Leloir path-

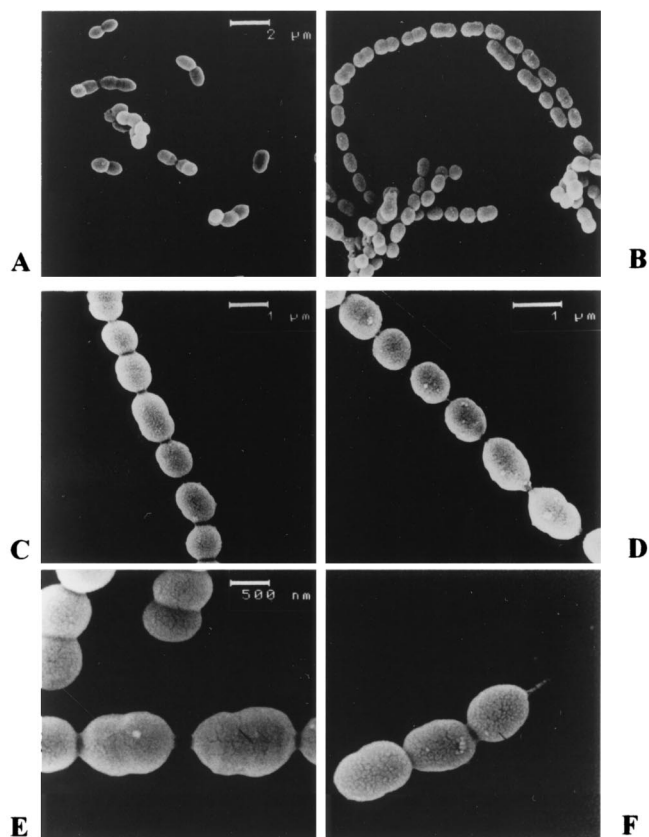


FIG. 3. SEM micrographs of the wild-type strain *Lactococcus lactis* MG1363 (A) and the *galE* mutant strain *Lactococcus lactis* MG1363:*galE* (B to F). Both strains were grown overnight in M17 medium supplemented with glucose.

way, reflecting the order of galactose utilization. The functionality of the *gal* gene products was established based on complementation studies, transport experiments, and analysis of strains having mutations in the *gal* gene cluster.

A unique feature of this *gal* gene cluster is the presence of the *galP* gene, encoding a galactose permease. The mutant strains *Lactococcus lactis* MG1363:*galP1* and MG1363:*galP2* were constructed and used to show the relationship between galactose accumulation and the presence of a functional *galP* gene. Galactose transport was strongly reduced in the mutant strains but was not completely eliminated. The observed residual uptake could be related to nonspecific transporters. The functionality of GalP was also confirmed with the complementation in *E. coli* DW2 of a transporter belonging to the same family, the melibiose transport protein MelB. These results corroborate previous observations which suggested the presence in *Lactococcus lactis* ATCC 7962 of a galactose permease with higher affinity for galactose than for lactose (31). The low capacity of *Lactococcus lactis* MG1363 to transport lactose has also been reported previously (17). Moreover, a DNA fragment containing a putative lactose permease-encoding gene was described, but it was characterized only by restriction enzyme digestion, which showed that it is clearly different from the *galP* gene described here (41). Hence, it seems likely that *Lactococcus lactis* MG1363 contains a lactose transport system

with low efficiency that differs from the highly efficient galactose permease that is encoded by the *galP* gene. Based on sequence comparisons and functionality data, the GalP galactose permease of *Lactococcus lactis* MG1363 was classified as a new member of the GPH family of transporters (38), which is also called TC 2.A.2 (43), and clustered with the recently characterized GalP protein of *Lactobacillus brevis* (19). While sequence identity comparisons predict that the GalP proteins belong to the LacS subfamily of the GPH family (38), these proteins do not contain the C-terminal extra enzyme IIA-like domain which is a characteristic and functionally important feature of the LacS subfamily (38). Thus, *Lactococcus lactis* GalP and *Lactobacillus brevis* GalP are the only members of a new subfamily in the GPH family of transporters and probably have the same properties.

Comparison of the organization of the *gal* genes with the organization of previously described clusters revealed some similarities but also important differences (27). Among other gram-positive bacteria, high degrees of identity were found when the Leloir enzymes, GalK, GalT, and GalE, were considered (more than 50% identity). The gene order *galK-galT-galE* has been observed in five different LAB or closely related bacteria (*S. thermophilus*, *Streptococcus mutans*, *Streptococcus salivarius*, *Streptococcus equi*, and *Lactococcus lactis* MG1363) (27, 49). The complementation studies performed with specific *E. coli* mutants demonstrated the functionality of the Leloir enzyme genes, *galK*, *galT*, and *galE*. We show here that a *galM* gene is present in the *gal* gene cluster of *Lactococcus lactis* MG1363. So far, this gene has also been identified in other LAB (1, 2, 40). However, its function has been studied only in *E. coli*, where it is related to efficient lactose utilization (7). The presence of *galM* in a *gal* cluster like that of *Lactococcus lactis* MG1363 may reflect possible involvement of GalM in efficient galactose metabolism. Construction of *Lactococcus lactis* mutant strains deficient in GalP, GalK, or GalE showed the functionality of the corresponding genes and also confirmed that the Leloir pathway is the only functional pathway for galactose utilization in *Lactococcus lactis* MG1363.

A remarkable feature of the *gal* gene cluster of *Lactococcus lactis* subsp. *cremoris* MG1363 is that it does not consist of a combination of *gal* and *lac* genes. Genes related to lactose metabolism are often found together with *gal* genes in LAB (18, 27). This is the case for *Lactobacillus helveticus*, in which the *galE* gene is separated from the *galKTM* genes and is associated with the *lacL* and *lacM* genes encoding the subunits of the β -galactosidase, and for *S. thermophilus*, in which there is an association among the *gal* operon, the lactose permease gene (*lacS*), and the β -galactosidase gene (*lacZ*) (18). Remarkably, another lactococcal strain, *Lactococcus lactis* ATCC 7962, was found to have a different organization, in which *galT* and *galE* are separated by two inserted *lac* genes, *lacA* encoding a putative thiogalactosidase and *lacZ* encoding the β -galactosidase (53). This organization is also found in *Lactococcus lactis* subsp. *lactis* IL-1403, in which the predicted gene order is *lacS-galM-galK-galT-lacA-lacZ-galE* (6). Thus, the *gal* gene cluster of *Lactococcus lactis* MG1363 appears to be highly specific for galactose, containing all the structural genes needed for galactose utilization via the Leloir pathway in an uninterrupted form at the same locus. This is not the case in other dairy LAB, in which the *gal* gene clusters are parts of

lactose utilization systems or do not contain galactose-specific transporters (27). The *gal* operon of *Lactobacillus casei* also contains only *gal* genes (*galKETRM*), but it lacks a galactose permease (2).

The data provided here confirm that galactose metabolism in *Lactococcus lactis* MG1363 is strongly regulated and subject to catabolite repression. The involvement of *Lactococcus lactis* catabolite control protein CcpA in control of *gal* gene expression has been shown recently (33). This study also showed that the *galP* promoter contains a consensus *cre* site. Galactokinase and galactose uptake assays performed under different growth conditions demonstrated that the two activities are similarly regulated. This observation is consistent with the transcriptional organization of the *gal* genes that showed the presence of a *galPMKTE* polycistronic transcript which was present only in galactose-grown cells (26; Grossiord, unpublished data). The lack of GalK activity in the *Lactococcus lactis* MG1363::*galP2* mutant also confirms the possible operon organization of the *Lactococcus lactis* MG1363 *gal* gene cluster. However, notably, the complementation studies performed with *Lactococcus lactis* suggested that an additional functional promoter is present in the *gal* gene cluster and is located in front of the *galE* gene; this suggestion is supported by the role of *galE* in glucose-grown cells and is analogous with previous observations that indicated the role of *galE* in more general metabolic properties (1, 4, 40). A promoter was mapped upstream from the *galE* gene in *Lactococcus lactis* NCDO2054 (53) and was also characterized in *Lactococcus lactis* MG1363 (26). In *Lactococcus lactis* MG1363, the role of the *galE* gene product in general metabolism was observed when the *galE* mutant was grown on glucose, which resulted in the long-chain phenotype. The inability of the *galE* mutant cells to separate after division is reminiscent of the phenotype of *Lactococcus lactis* deficient in *Acma*, the major autolysin (10). A similar observation was described by Duwat et al. (20) for a strain deficient in *dltD*, a gene involved in D-alanylation of the lipoteichoic acids (LTAs). Evaluation of the biological functions and the fate of UDP-galactose in the cells (14) showed that it is used in *Lactococcus lactis* for substitution of LTAs, which are molecules of polyglycerol-phosphate that are linked to the membrane and extend through the cell wall (22). LTAs have been shown to play a key role in the regulation of autolysis and are often branched with D-alanine or, in some cases, with monosaccharides, such as glucose or galactose (23). The composition of the LTAs has been studied previously, and these molecules have been shown to contain galactose in *Lactococcus lactis* NCDO712 (23), the parental strain of *Lactococcus lactis* MG1363 (25). The level of alanine substitution in the LTAs of *Bacillus subtilis* was found to be important for the function of these molecules in the regulation of autolysis (56). Similarly, galactose substitution in the LTAs in *Lactococcus lactis* could link this process, which requires UDP-galactose, with the regulation of autolysis.

In conclusion, we present evidence that the *galPMKTE* gene cluster is functional and allows efficient galactose utilization via the Leloir pathway in *Lactococcus lactis* MG1363. The organization of this cluster is unique among the known *gal-lac* gene clusters, and there is a *galP* gene encoding a galactose permease which is a new member of the GPH family of transporters. We also demonstrate the essential role of the *galE* gene

product for normal growth, which is related to the function of UDP-galactose in cell wall biosynthesis.

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