APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 2003, p. 4049–4056 Vol. 69, No. 7
0099-2240/03/$08.00+0 DOI: 10.1128/AEM.69.7.4049–4056.2003

Characterization of Genes Involved in the Metabolism of α-Galactosides by *Lactococcus raffinolactis*

Isabelle Boucher, Christian Vadeboncoeur, and Sylvain Moineau*

Département de Biochimie et de Microbiologie, Faculté des Sciences et de Génie, Groupe de Recherche en Écologie Buccale (GREB), Faculté de Médecine Dentaire, Université Laval, Québec, Canada G1K 7P4

Received 9 December 2002/Accepted 31 March 2003

***Lactococcus raffinolactis*, unlike most lactococci, is able to ferment α-galactosides, such as melibiose and raffinose. More than 12 kb of chromosomal DNA from *L. raffinolactis* ATCC 43920 was sequenced, including the α-galactosidase gene and genes involved in the Leloir pathway of galactose metabolism. These genes are organized into an operon containing *aga* (α-galactosidase), *galK* (galactokinase), and *galT* (galactose 1-phosphate uridylyltransferase). Northern blotting experiments revealed that this operon was induced by galactosides, such as lactose, melibiose, raffinose, and, to a lesser extent, galactose. Similarly, α-galactosidase activity was higher in lactose-, melibiose-, and raffinose-grown cells than in galactose-grown cells. No α-galactosidase activity was detected in glucose-grown cells. The expression of the *aga-galKT* operon was modulated by a regulator encoded by the upstream gene *galR*. The product of *galR* belongs to the LacI/GalR family of transcriptional regulators. In *L. lactis*, *L. raffinolactis* GalR acted as a repressor of *aga* and lowered the enzyme activity by more than 20-fold. We suggest that the expression of the *aga* operon in lactococci is negatively controlled by GalR and induced by a metabolite derived from the metabolism of galactosides.**

The gram-positive lactic acid bacteria (LAB) comprise 11 bacterial genera, including *Lactococcus* (28). Several strains of *Lactococcus lactis* are commonly used in the production of fermented dairy products to convert the milk sugar lactose into lactic acid (17). *Lactococcus raffinolactis,* formerly known as *Streptococcus raffinolactis,* is a LAB naturally found in raw milk, but this lactococcal species is not currently used by the dairy industry, mainly because of its lack of caseinolytic activity (15, 17, 26). *L. raffinolactis* cells are ovoid, and they are found in pairs or short chains (17). This species does not grow at 40°C, at pH 9.2, or in the presence of 4% NaCl and cannot hydrolyze arginine. To our knowledge, no genetic studies are available on this LAB.

L. raffinolactis has also the peculiar property of being able to ferment α-galactosides, such as melibiose and raffinose. This characteristic is attributed mainly to the activity of an α-galactosidase (Aga). Following the hydrolysis of α-galactosides by Aga, the α-galactose subunit is released and can be degraded through two distinct metabolic pathways: the tagatose 6-phosphate pathway and the Leloir pathway. The substrate for the tagatose 6-phosphate pathway is the galactose 6-phosphate, resulting from the transport and phosphorylation of melibiose by the sugar phosphotransferase transport system (PTS). Galactose 6-phosphate is metabolized to triose-phosphates by three enzymes: galactose 6-phosphate isomerase (LacA and LacB), tagatose 6-phosphate kinase (LacC), and tagatose 1,6-bisphosphate aldolase (LacD). On the other hand, intracellular nonphosphorylated galactose molecules are degraded by the Leloir pathway. α-Galactose is first transformed into galactose 1-phosphate by a galactokinase (GalK). Then, two additional enzymes, namely, galactose 1-phosphate uridylyltransferase (GalT) and UDP-galactose 4-epimerase (GalE), are responsible for converting the galactose 1-phosphate to glucose 1-phosphate, a glycolysis precursor. Both pathways are found in *L. lactis* (2, 29, 30), and their genetic determinants usually form an operon (9, 13, 14, 32).

A few α-galactosidase genes have also been cloned and sequenced in LAB. In *Streptococcus mutans*, the α-galactosidase gene is associated with the multiple-sugar metabolism operon (24). This Aga is essential for growth in the presence of melibiose and raffinose. The expression of *S. mutans* α-galactosidase is activated by a transcriptional regulator of the AraC/XylS family. In *Streptococcus pneumoniae*, Aga is essential for raffinose utilization and its activity is stimulated by raffinose and catabolite repressed by sucrose but not glucose (23). The expression of Aga is also stimulated by two gene products, one of which belongs to the AraC/XylS family. In *Carnobacterium piscicola*, the α-galactosidase determinant is grouped with two β-galactosidase genes and both enzymatic activities are repressed in the presence of glucose or lactose during growth (6). Similarly, the α-galactosidase gene from *Lactobacillus plantarum* (*melA*) is clustered with *lacA* and *lacM*, encoding a heterodimeric β-galactosidase (21, 27). Transcription of *melA* is independent of surrounding genes and is induced by melibiose and partially repressed by glucose (27).

In this work, we present the characterization and transcriptional analysis of a 12-kb chromosomal segment of *L. raffinolactis* ATCC 43920 (also known as NCDO617) containing the α-galactosidase gene and its transcriptional regulator as well as genes involved in the Leloir pathway of galactose degradation. This study provides the first genetic analysis of *L. raffinolactis* through its α-galactosides metabolism.

* Corresponding author. Mailing address: Groupe de Recherche en Écologie Buccale (GREB), Faculté de Médecine Dentaire, Université Laval, Québec, Canada G1K 7P4. Phone: (418) 656-3712. Fax: (418) 656-2861. E-mail: Sylvain.Moineau@bcm.ulaval.ca.

TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristic(s)[a]	Source[b] or reference(s)
Strains		
Escherichia coli		
DH5α	*supE44* Δ*lac* U169 (f80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Invitrogen Life Technologies
MC1061	F^-*araD139* Δ(*ara-leu*)*7696 galE15 galK16* Δ(*lac*)*X74 rpsL* (Str^r) *hsdR2* (r_K^- m_K^+) *mcrA mcrB1*	ATCC
Lactococcus lactis		
MG1363	Laboratory strain, plasmid free, Lac^-	12
SMQ-754	Industrial strain, Lac^+	This study
KF292	Isolated from soya sprouts, Raf^+	19, 20
Lactococcus raffinolactis		
ATCC 43920	Plasmid free	ATCC
Streptococcus thermophilus		
SMQ-301	Industrial strain, Lac^+	31, 32
Plasmids		
pBS	Cloning vector for DNA sequencing, Ap^r	Stratagene
pNZ123	Shuttle cloning vector, Cm^r	8
pRAF102	pBS plus 5-kb *Cla*I/*Spe*I fragment from *L. raffinolactis* ATCC 43920 encoding *galT,* Ap^r	This study
pRAF110	pBS plus 8-kb *Kpn*I/*Nhe*I fragment from *L. raffinolactis* ATCC 43920 encoding *aga,* Ap^r	This study
pRAF300	pNZ123 plus 4-kb *Eco*RI/*Hin*dIII fragment from *L. raffinolactis* ATCC 43920 encoding *aga,* Cm^r	5
pRAF301	pNZ123 plus 2.5-kb *aga* amplicon from *L. raffinolactis* ATCC 43920, Cm^r	5

[a] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Str^r, streptomycin resistance; Lac, lactose fermentation; Raf, raffinose fermentation.
[b] ATCC, American Type Culture Collection, Manassas, Va.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown at 37°C in Luria broth (LB), and lactococci were grown at 30°C in M17 medium (0.5% casein peptone, 0.5% soy peptone, 0.5% meat peptone, 0.25% yeast extract, 0.05% ascorbic acid, 0.025% magnesium sulfate, 1.9% sodium β-glycerophosphate) (Quélab) supplemented with the appropriate sugar. Carbohydrate fermentation was tested in bromocresol purple medium (2% tryptone, 0.5% yeast extract, 0.4% NaCl, 0.15% Na-acetate, 40 mg of purple bromocresol/liter). Sugars were filter sterilized and added to autoclaved media at a final concentration of 0.5%. When required, antibiotics (Sigma-Aldrich) were added as follows: for *E. coli*, 50 μg of ampicillin per ml, and for *L. lactis*, 5 μg of chloramphenicol per ml.

DNA techniques. Routine DNA manipulations were carried out according to standard procedures (25). Restriction enzymes, alkaline phosphatase, RNase-free DNase, RNase inhibitor (Roche Diagnostics), and T4 DNA ligase (Invitrogen Life Technologies) were used according to the suppliers' instructions. All primers used in this study were obtained from Invitrogen Life Technologies and are listed in Table 2. Transformations of *E. coli* (25) and *L. lactis* (16) were performed as described elsewhere. Plasmid DNA from *E. coli* and *L. lactis* was isolated as previously described (5, 11). Total lactococcal DNA was obtained as described by Boucher et al. (5). When needed, large amounts of *E. coli* plasmid DNA were isolated with a Qiagen Plasmid Maxi kit.

Sequencing of the *aga* locus from *L. raffinolactis* ATCC 43920. The 4-kb *Eco*RI/*Hin*dIII DNA fragment from *L. raffinolactis* containing the *aga* gene (5) was used as a probe in Southern hybridizations to target a larger chromosomal region. The fragment was labeled using a DIG High-Prime DNA labeling kit (Roche Diagnostics). Prehybridization, hybridization, and posthybridization washes and detection by chemiluminescence were performed as suggested by the manufacturer (Roche Diagnostics). An 8-kb *Kpn*I/*Nhe*I fragment was isolated as previously described (5) and cloned into pBS to construct pRAF110, and the DNA sequences on both strands were determined by primer walking. Primer raf34, complementary to the *galT* gene, was then used to target additional chromosomal restriction fragments covering the area downstream of this gene on Southern hybridizations. A 5-kb *Cla*I/*Spe*I fragment was cloned into pBS to construct pRAF102, and the cloned DNA was sequenced on both strands by primer walking. Finally, a 3-kb *Kpn*I fragment was used as the substrate for inverse PCR (4), and the resulting amplicon was also sequenced. DNA sequencing was carried out by the DNA sequencing service at the Université Laval by means of an ABI Prism 3100 apparatus. Sequence analyses were performed using the Wisconsin Package software (version 10.3) of the Genetics Computer Group (7).

Transcriptional analysis of the *aga* locus. Total RNA was isolated from *L. raffinolactis* by means of the same procedure used with *L. lactis* (5). Approximately 5 μg of material was separated on agarose-formaldehyde electrophoresis gel as described by Sambrook and Russell (25). Nucleic acids were transferred to a positively charged nylon membrane (Roche Diagnostics) and fixed by UV exposure. PCR amplicons were labeled with ^{32}P by using High Prime (Roche Diagnostics) and used as probes. Prehybridization (3 h) and hybridization (overnight) were performed in DIG Easy Hyb (Roche Diagnostics) at 50°C, and two posthybridization washes were made at the same temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate)–0.1% sodium dodecyl sulfate prior to film exposure (Kodak X-Omat AR). Stripping of the membrane was

TABLE 2. Primers used in this study

Primer	Sequence (5′–3′)
CB3	GAATTCGAATTCCCCCCCCCCCCCCCCCC
raf5	AGTGCAGCGCACCAAGGTGA
raf9	CATTGAAGGCACGATTCCCA
raf11	TTTAGCCACAGCACAAAGGA
raf12bam	GGATCCGGATCCATGACACTAATCACATTTGA
raf13	CCATCACCGAAGAGGGCTGT
raf32	TGCTGTGATCAAGACGCATT
raf33	AACGCACGATAAATCCCGAT
raf34	CGTTGAGTTCAAATTGCCAG
raf48	CCCTTAGAAGAGTTGCTTGG
raf57	CCTCCGGTCTAGCGTGCGGT
raf67	CGGTATTGTCCACGCGCCAA
raf73	CGCCGTGACCATAACTTGGA

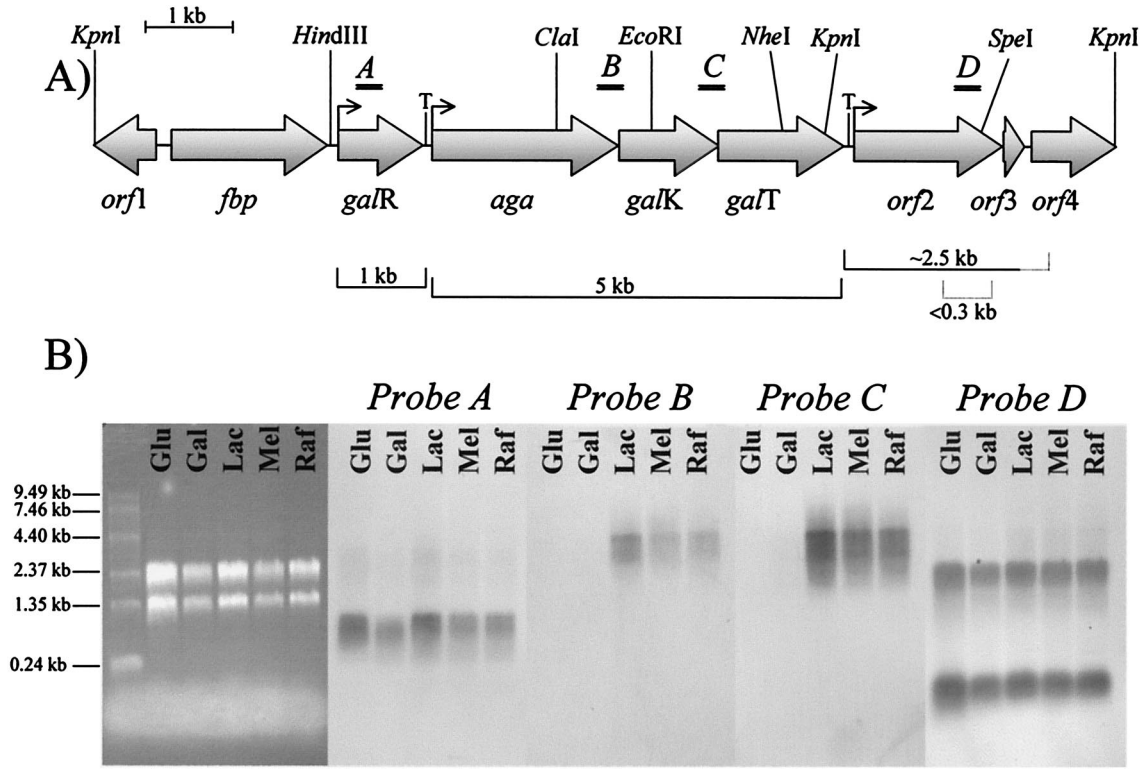

FIG. 1. Characterization of the *aga* locus of *L. raffinolactis* ATCC 43920. (A) Over 12 kb of genomic DNA of *L. raffinolactis* was analyzed. Nine ORFs were identified, and putative functions were assigned to corresponding proteins according to conserved motifs and similarity with proteins of known functions (Table 3). Brackets located under the figure identify transcripts, with dotted lines indicating the putative limits of transcription. Positions of restriction sites used for cloning or sequencing are indicated above the figure. Bent arrow, promoter; T, terminator. (B) Northern analysis of the *aga* locus. Total RNA was isolated from *L. raffinolactis* cultivated in the presence of glucose (Glu), galactose (Gal), lactose (Lac), melibiose (Mel), or raffinose (Raf). The agarose gel (left side) is presented to indicate the relative amount of RNA loaded per lane. mRNAs were detected using ^{32}P-labeled probes targeting *galR* (probe A), *aga-galK* (probe B), *galK-galT* (probe C), and *orf2* (probe D).

performed by immersion in boiling 0.1% sodium dodecyl sulfate (25). The membrane was successively hybridized with the different probes in the following order: B, D, A, and C (Fig. 1). Probes A, B, C, and D were obtained by PCR amplification of *L. raffinolactis* DNA with primers raf32 and raf33, primers raf5 and raf13, primers raf9 and raf11, and primers raf48 and raf57, respectively.

Localization of the transcriptional initiation site of *aga*. The transcriptional initiation site of *aga* was established through 5′ rapid amplification of cDNA ends (RACE) essentially as described by Sambrook and Russell (25). RNA was isolated from *L. raffinolactis* cells grown in raffinose. DNase treatments and cDNA synthesis were performed using 20 μg of total RNA and the *aga*-specific primer raf67 (5). Free nucleotides and primers were removed by precipitating the cDNA twice in 2.5 M ammonium acetate with 3 volumes of 95% ethyl alcohol. cDNA was then dissolved in double-distilled water to a final volume of 20 μl. cDNA (13 μl) was used for poly(dG) tailing with terminal deoxyribonucleotidyl transferase, as recommended by the manufacturer (Amersham Pharmacia Biotech). Tailed DNA was diluted to 1 ml with 10 mM Tris-HCl, pH 8.5, and 5 μl was used for PCR amplification with a poly(dC) primer (CB3) and the *aga*-specific primer raf73. The PCR product was purified with silica as previously described (10) and sequenced using primer raf73. The transcriptional initiation site of *aga* was also determined by primer extension analysis essentially as described by Sambrook and Russell (25).

α-Galactosidase assay. α-Galactosidase activity was evaluated as described by Boucher et al. (5). Briefly, 10-ml cell cultures (optical density at 600 nm, 0.5) were washed and lysed with glass beads. The cell lysate was cleared by centrifugation, and the protein content was determined with the Bio-Rad DC protein assay reagent. The α-galactosidase activity was assayed at 30°C and at pH 7.0 with *p*-nitrophenyl-α-D-galactopyranoside (Sigma) as the substrate (22).

β-Galactosidase and phospho-β-galactosidase assays. Both the β-galactosidase and phospho-β-galactosidase enzymatic activities were detected as described for the α-galactosidase assay but with cells grown in LM17 medium (optical density at 600 nm, 0.5). The enzyme assays were performed at 30°C and at pH 7.0 with *o*-nitrophenyl-β-D-galactopyranoside (Sigma-Aldrich) as the substrate for β-galactosidase and *o*-nitrophenyl-β-D-galactopyranoside 6-phosphate (Sigma-Aldrich) as the substrate for phospho-β-galactosidase. The presence of the enzyme activity was confirmed by the appearance of a yellow color after the cells were incubated overnight. The industrial strain *Streptococcus thermophilus* SMQ-301 (31) was used as a positive control for β-galactosidase, and *L. lactis* subsp. *cremoris* SMQ-754 was used as a positive control for phospho-β-galactosidase.

Nucleotide sequence accession number. The GenBank accession number assigned to the nucleotide sequence of the *aga* locus of *L. raffinolactis* ATCC 43920 is AY164273.

RESULTS AND DISCUSSION

Confirmation of *L. raffinolactis* ATCC 43920. The *L. raffinolactis* strain used in this study grew well in M17 medium and fermented the two α-galactosides raffinose and melibiose, as well as lactose (β-galactoside), galactose, and glucose. Sequencing of the 16S rRNA gene was performed on *L. raffinolactis* ATCC 43920, and the data matched the previously published 16S rRNA sequence for *L. raffinolactis* NCDO617 (GenBank accession no. X54261).

Sequence analysis of the *aga* locus from *L. raffinolactis* ATCC 43920. A 4-kb DNA region involved in α-galactoside fermentation was previously cloned and sequenced from *L. raffinolactis* ATCC 43920 and used as a dominant selectable marker in a food-grade cloning vector for the genetic modifi-

TABLE 3. Characterization of ORFs identified on the *L. raffinolactis* chromosome[a]

Product of ORF	Length (aa)	pI	Mol. mass (kDa)	Potential RBS/N terminus[b]	Proposed function	Characteristic(s)
Orf1	>241 (incomplete)			tattcgattatttGGAGGttt t**atg**/MTIGKDDFIR	Unknown	
Fbp	640	6.00	73.6	cccatcAGGAAAGaagacaag c**atg**/MQENKYLSLL	Fructose-1,6-bisphosphatase	Up to 51% aa identity over the entire protein sequence with orthologues found in various gram-positive organisms, including *L. lactis* subsp. *lactis* (GB no. AE006263)
GalR	345	5.86	38.4	gcaagtAAAGGAAttgtaag ct**atg**/MASIREIAKL	Transcriptional regulator	LacI/GalR family; up to 34% aa identity over the entire protein sequence with orthologues found in various gram-positive organisms, such as *S. thermophilus* (GB no. AAM28581.1) and *S. mutans* (GB no. JC5310)
Aga	735	5.02	83.3	aaatAAAGAAAGCGGG tcactc**atg**/MTLITFDENN	α-Galactosidase	Up to 54% aa identity over the entire protein sequence with orthologues found in various gram-positive organisms, such as *Geobacillus stearothermophilus*(GB no. AF130985, AY013287, AY013286)
GalK	392	4.98	42.7	aaatttACAGccctcttcggt g**atg**/MSTKEMKQEV	Galactokinase	Up to 66% aa identity over the entire protein sequence with orthologues found in various gram-positive organisms, such as *S. thermophilus* (GB no. AF152357) and *L. lactis* (GB no. AJ011653, AE006428)
GalT	492	5.24	55.0	ttgcAGATGGAGctagaaaa tt**atg**/MNISQAVIDF	Galactose 1-phosphate-uridylyltransferase	Up to 54% aa identity over the entire protein sequence with orthologues found in various gram-positive organisms, such as *S. pneumoniae* (GB no. AE007475) and *L. lactis* (GB no. AF082008, AE006428)
Orf2	608	5.33	70.0	aagtgtGAGGAGGAGAta aaat**atg**/MIRQAINLTE	Transcriptional regulator	Contains a PTS regulatory domain and a PTS EIIA domain from the fructose subfamily
Orf3	94	4.40	9.9	tcaagaAGGTGAGGaataaa at**atg**/MKLAAVCGSG	PTS EIIB component	Contains a putative phosphorylation site and a cysteine residue at position 7
Orf4	>297 (incomplete)			ctagattAGGAGAAtaatca ac**atg**/MKDVLDVLID	PTS EIIC component	Contains putative transmembrane helices

[a] aa, amino acids; RBS, ribosome binding site; GB no., GenBank accession number.
[b] Bold type indicates start codons; uppercase letters indicate A+G-rich stretches.

cation of *L. lactis* (5). In our study, this region was further characterized, and an additional 8 kb of DNA was sequenced (Fig. 1A) This DNA segment possessed a G+C content of 39.7%, which is in agreement with the previous estimated values of 40.3 to 41.5% (17). The analysis of the 12-kb DNA fragment revealed nine open reading frames (ORFs) (Fig. 1A and Table 3). Putative functions were attributed to products of eight ORFs based on motifs in the peptide chains, and/or extensive amino acid similarity with proteins of known function (see Table 3 for details).

Sequence analysis also revealed the presence of three putative promoters upstream from the genes *aga* (TTGACA-N_{17}-TATAAT) (5), *orf2* (TTGAAA-N_{17}-TAAAGT), and *galR* (TTGTTT-N_{20}-TAAAAT). Two regions containing inverted repeats, able to form a stem-loop structure and to act as intrinsic transcriptional terminators, were found downstream from *galR* ($\Delta G^{25°C} = -17.6$ kcal/mol) and *galT* ($\Delta G^{25°C} = -13.2$ kcal/mol). Two catabolite-responsive element sequences were also identified in the 12-kb fragment of *L. raffinolactis*. Catabolite-responsive element sequences were found in the −35 region of the promoters upstream from *aga* and *orf2*, indicating that these genes might be subjected to catabolite repression (18). Finally, a potential binding site for GalR was identified in the −10 box region of the *aga* promoter (Fig. 2). The N-terminal helix-turn-helix motif of GalR and its putative binding site are similar to cognate regions characterized for *S. mutans* (1) and *S. thermophilus* (34).

These results suggested that (i) *aga*, *galK*, and *galT* formed a

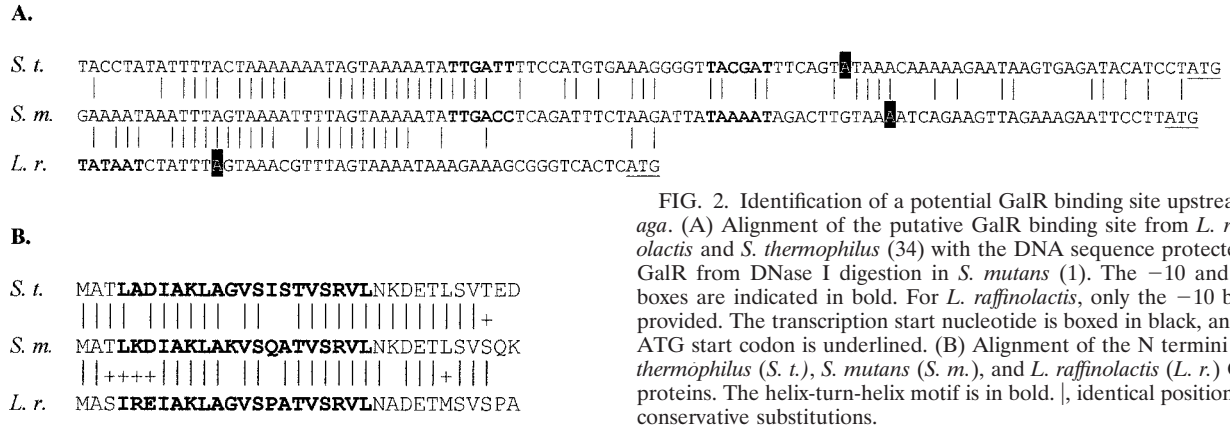

FIG. 2. Identification of a potential GalR binding site upstream of *aga*. (A) Alignment of the putative GalR binding site from *L. raffinolactis* and *S. thermophilus* (34) with the DNA sequence protected by GalR from DNase I digestion in *S. mutans* (1). The −10 and −35 boxes are indicated in bold. For *L. raffinolactis*, only the −10 box is provided. The transcription start nucleotide is boxed in black, and the ATG start codon is underlined. (B) Alignment of the N termini of *S. thermophilus* (*S. t.*), *S. mutans* (*S. m.*), and *L. raffinolactis* (*L. r.*) GalR proteins. The helix-turn-helix motif is in bold. |, identical positions; +, conservative substitutions.

transcriptional unit expressed from a promoter located upstream from *aga* and ending at the terminator identified downstream from *galT*; (ii) *galR* was transcribed independently from the *aga-galKT* operon and its transcription ended in the *galR-aga* intergenic region; and (iii) *orf2*, *orf3,* and *orf4* may also be expressed independently from at least one promoter located upstream from *orf2*.

mRNA analysis of the 12-kb DNA fragment from *L. raffinolactis* ATCC 43920. To determine the number and size of transcripts in this chromosomal region, Northern blot analyses were performed on *L. raffinolactis* cells grown on various sugars (Fig. 1B). The gene *galR* was expressed, under every condition tested, as a monocistronic mRNA 1 kb in size. The transcript size was in agreement with the gene size and suggested transcription from a promoter located upstream from *galR* and termination at the stem-loop structure identified in the *galR-aga* intergenic region.

The *aga-galKT* mRNA was 5 kb in size, which corresponded to a transcriptional unit that started at the proposed promoter upstream from *aga* and ended at the terminator downstream from *galT*. The transcription of this operon was stimulated by galactosides such as lactose, melibiose, and raffinose (Fig. 1B). Overexposure of the Northern membrane revealed a weak *aga-galKT* transcript of the same size in cells grown on galactose, while no signal was detected in glucose-grown cells (data not shown).

Finally, the three genes downstream from *galT* were expressed independently from the *aga* operon in cells grown on all the sugars tested (Fig. 1B).

Identification of the transcription start site of the *aga* operon. The RACE-PCR technique was used to identify the transcription initiation site of the *aga-galKT* operon (Fig. 3). The transcription was initiated at the A position located 7 bases downstream from the last T position of the −10 box identified on the DNA coding strand. An identical transcription initiation site was confirmed by primer extension analysis (data not shown).

α-Galactosidase activity in *L. raffinolactis* ATCC 43920. The α-galactosidase activity was measured in cell lysates of *L. raffinolactis* grown in the presence of various sugars (Table 4). Aga expression was strongly stimulated by the galactosides lactose, melibiose, and raffinose but poorly stimulated by galactose (Table 4), which induced fivefold less Aga activity than did melibiose. Virtually no Aga activity was detected in *L. raffinolactis* cells cultivated in the presence of glucose (Table 4). The finding that lactose induced *aga* in *L. raffinolactis* contrasted with the observation that lactose did not induce *aga* in the industrial lactose-positive strain *L. lactis* SMQ-741 (5). This difference most likely resulted from the fact that these two lactococcal species do not metabolize lactose the same way. *L. lactis* transports lactose by the PTS, and the galactose 6-phosphate produced following hydrolysis by phospho-β-galactosidase is metabolized via the tagatose 6-phosphate pathway (2, 29). Both β-galactosidase and phospho-β-galactosidase activities were detected in lactose-grown *L. raffinolactis* cells (data not shown), suggesting that the galactose moiety of lactose could be metabolized by the Leloir and the tagatose 6-phosphate pathways. Thus, the metabolism of lactose by *L. raffinolactis* generated intracellular galactose 6-phosphate as well as free galactose and galactose derivatives of the Leloir pathway. These results suggested that galactose or an intermediate of the Leloir pathway, but not galactose-6 phosphate, is involved in *aga* induction in lactococci. However, since extracellular galactose barely induced the *aga* operon in *L. raffinolactis* (Table 4), we suggest that *aga* induction in lactococci is optimally driven by a metabolite derived from galactoside metabolism.

α-Galactosidase activity in *L. lactis* MG1363 in the presence or absence of GalR. A potential GalR binding site was found in the promoter region of the *aga* operon. To determine whether GalR acted as a transcriptional regulator of the *aga* operon, gene inactivation and allelic replacement were attempted in *L. raffinolactis*. Despite several attempts using various strategies, we failed to obtain a *galR* mutant derivative in this species. Thus, the function of *L. raffinolactis* GalR was assessed in *L. lactis* subsp. *cremoris* MG1363, which does not display any α-galactosidase activity.

The *aga* gene was cloned into the high-copy-number plasmid vector pNZ123, alone (pRAF301) or in combination with *galR* (pRAF300). *aga* was cloned on a high-copy-number vector to minimize the potential effects of endogenous *L. lactis* regulators. The recombinant plasmids were separately introduced into *L. lactis* MG1363, and the α-galactosidase activity was evaluated after growth in the presence of glucose, galactose, and melibiose (Table 5). It is noteworthy that this *L. lactis* strain cannot utilize lactose. Interestingly, the α-galactosidase activity was at least 20-fold higher in cells containing pRAF301

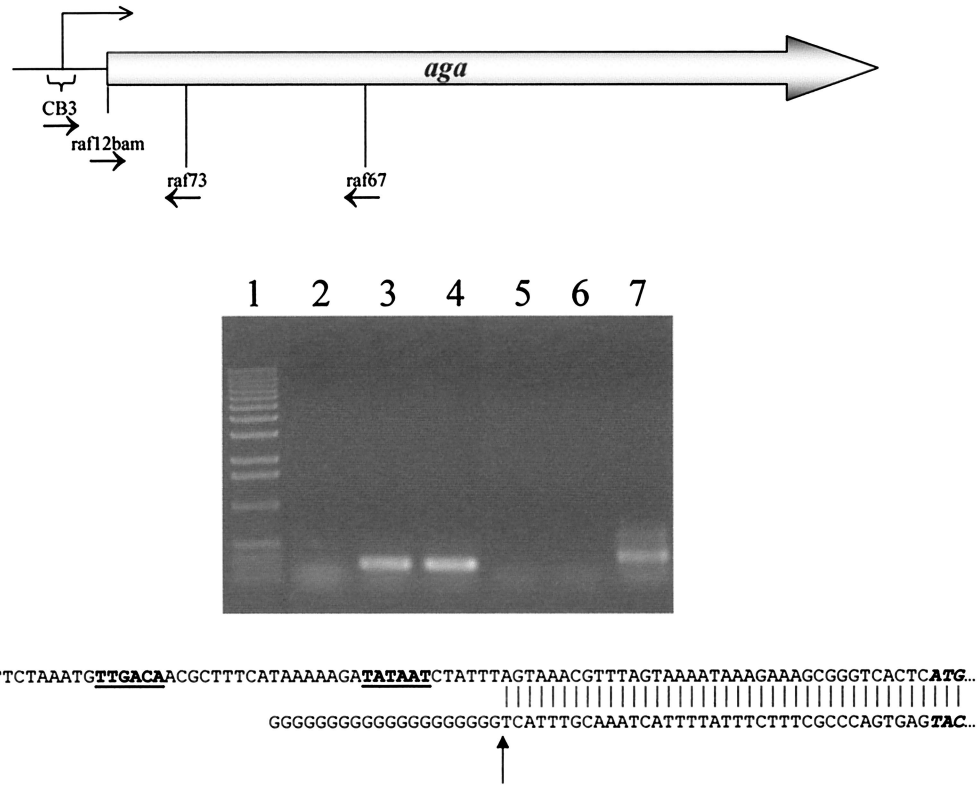

FIG. 3. Localization of the transcription initiation site of *aga* by RACE-PCR. Total RNA was isolated from *L. raffinolactis* grown in M17 medium plus 0.5% raffinose and used to synthesize cDNA with primer raf67. cDNA was tailed with dGTP using terminal transferase and PCR amplified with raf73 and a poly(C) primer (CB3) or primer raf12bam as a control. Lane 1, 1-kb DNA ladder (Invitrogen Life Technologies); lanes 2 to 4, controls with raf73 and raf12bam; lane 2, negative control (no DNA); lane 3, positive control (*L. raffinolactis* DNA); lane 4, positive control [poly(G)-cDNA]; lanes 5 to 7, amplification with raf73 and CB3; lane 5, negative control (no DNA); lane 6, CB3 specificity control (*L. raffinolactis* DNA); lane 7, poly(G)-cDNA. The amplicon obtained in lane 7 was sequenced with primer raf73, and its sequence was aligned with that of *L. raffinolactis* genomic DNA. The transcriptional initiation site is indicated by an arrow.

(*aga*) than in those with pRAF300 (*aga* and *galR*). Therefore, *aga* was expressed from its own promoter without the need for GalR-mediated activation, and GalR clearly repressed Aga activity in *L. lactis*. These results suggest that GalR acted as a repressor in *L. raffinolactis*.

Organization of the α-galactosidase genes in LAB. The genes coding for the Leloir pathway enzymes generally form operons (14, 32). In *L. lactis* subsp. *cremoris* MG1363, the order of the five genes required for galactose utilization is *galPMKTE*, where *galP* (formerly *galA*) encodes a galactose transporter (5, 13, 14). The *gal* genes of *L. lactis* subsp. *lactis* NCDO2054 and IL1403 are similarly organized but include the insertion of two genes (*lacA* and *lacZ*) between *galT* and *galE* (3, 33). In *L. raffinolactis* ATCC 43920, the *gal* genes are not grouped around *galM*, and *galE* genes could not be found on the 12-kb segment of the DNA sequenced.

Conversely, α-galactosidase determinants are heterogeneous in terms of their genetic positioning. In *L. raffinolactis*, the α-galactosidase gene is clustered with *galK* and *galT* to form an operon. The α-galactosidase gene organization is known for six other LAB genera (Fig. 4). In a general manner, α-galactosidase genes were found with genes coding for a di-

TABLE 4. Effect of the fermentation substrate on α-galactosidase activity in *L. raffinolactis* ATCC 43920

Sugar	Activity[a]
Glucose	16 ± 18
Galactose	191 ± 12
Lactose	643 ± 34
Melibiose	930 ± 93
Raffinose	513 ± 44

[a] Values are the means ± standard deviations of 12 measurements obtained with two cell extract quantities in two independent experiments. Activity is expressed in nanomoles of *p*-nitrophenol formed per milligram of protein per minute.

TABLE 5. Regulation of α-galactosidase activity by GalR in *L. lactis* subsp. *cremoris* MG1363

Recombinant plasmid	Sugar	Activity[a]
pRAF300 (*aga* plus *galR*)	Glucose	27 ± 27
	Galactose	372 ± 107
	Melibiose	314 ± 21
pRAF301 (*aga*)	Glucose	1807 ± 469
	Galactose	9998 ± 1713
	Melibiose	6719 ± 816

[a] Values are the means ± standard deviations of 12 measurements obtained with two cell extract quantities in two independent experiments. Activity is expressed in nanomoles of *p*-nitrophenol formed per milligram of protein per minute.

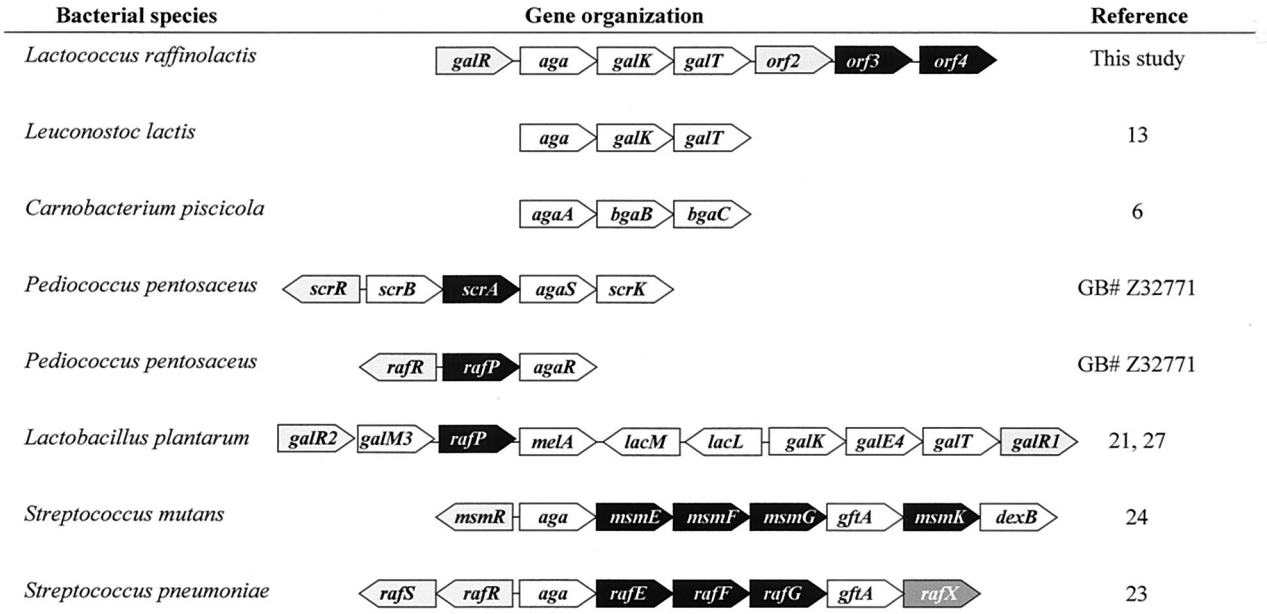

FIG. 4. Genetic organization of α-galactosidase genes in LAB. The genes are indicated by polygons of the same size regardless of their length. White, carbohydrate degradation enzymes; black, sugar transporters; gray, transcriptional regulators. The following genes code for the indicated proteins: *aga*, *melA* α-galactosidase; *galK*, galactokinase; *galT*, galactose 1-phosphate uridylyltransferase; *galM*, mutarotase; *bgaB*, *bgaC*, *lacL*, and *lacM* β-galactosidase; *scrB*, sucrose phosphorylase; *scrK*, fructokinase; *gftA*, sucrose 6-phosphate hydrolase; *dexB*, dextran glucosidase; *galR*, *scrR*, *rafR*, *rafS*, *msmR*, and *orf2* transcriptional regulators; *orf3* and *orf4*, putative PTS EIIB and EIIC domains; *scrA*, PTS EII^{Suc}; *rafP* (also named *lacS2* in *Lactobacillus plantarum* WCFS1), galactoside-pentose-hexuronide transporter; *msmEFGK* and *rafEFG*, ABC transporters; *rafX*, unknown. GB#, GenBank accession number.

versity of enzymes and transporters involved in the metabolism of various sugars. In *Lactobacillus plantarum*, the *melA* gene encoding α-galactosidase was also located near genes involved in the Leloir pathway of galactose metabolism, namely, *galM3*, *galK*, *galE4*, and *galT* (21, 27). Surprisingly, the organization found in *Leuconostoc lactis* appeared to be similar to that found in *L. raffinolactis* (13). Unfortunately, the DNA sequence required to support investigations concerning possible horizontal gene transfer between the two bacteria was not available.

α-Galactoside metabolism is also found in some *L. lactis* subsp. *lactis* strains isolated from fruits and vegetables where raffinose is readily available (19, 20). In the α-galactosides-fermenting strain KF292 of *L. lactis*, we have found a DNA region encompassing the *galR* and *aga* genes that is over 90% identical to a DNA segment of *L. raffinolactis* ATCC 43920 (data not shown). Most *L. lactis* strains used by the dairy industry are melibiose and raffinose negative, and they were isolated from raw milk or fermented milk products. On the other hand, *L. lactis* strains found on plant material are able to utilize these sugars. Therefore, the presence or absence of an α-galactosides metabolism in lactococcal strains is likely related to their distinct ecological adaptation.

Conclusion. Over the years, research on LAB genetics has focused mainly on the industrial applications of these organisms. Consequently, certain lactococcal species, such as *L. raffinolactis,* remain uncharacterized at the genetic level. This study provides the first genetic analysis of *L. raffinolactis.* In this species, α-galactoside metabolism was driven by an operon that contains the three genes *aga*, *galK*, and *galT*. The gene *aga* encoded an α-galactosidase, while *galK* and *galT* most likely encoded, respectively, a galactokinase and a galactose 1-phosphate-uridylyltransferase, two enzymes of the Leloir pathway. The expression of this operon is controlled by a gene located upstream, *galR*. The product of *galR* belonged to the LacI/GalR family of transcriptional regulators and acted as a repressor of the gene cluster.

ACKNOWLEDGMENTS

We thank the Natural Sciences and Engineering Research Council of Canada (Research Partnerships Program—Research Network on Lactic Acid Bacteria), Agriculture and Agri-Food Canada, Novalait, Inc., Dairy Farmers of Canada, and Institut Rosell-Lallemand, Inc., for their financial support. I.B. is a recipient of a Fonds FCAR graduate scholarship.

We are grateful to William Kelly for providing the α-galactoside-fermenting strain *L. lactis* KF292.

REFERENCES

1. **Ajdic, D., and J. J. Ferretti.** 1998. Transcriptional regulation of the *Streptococcus mutans gal* operon by the GalR repressor. J. Bacteriol. **180:**5727–5732.
2. **Bissette, D. L., and R. L. Anderson.** 1974. Lactose and D-galactose metabolism in group N streptococci: presence of enzymes for both the D-galactose 1-phosphate and D-tagatose 6-phosphate pathways. J. Bacteriol. **117:**318–320.
3. **Bolotin, A., P. Wincker, S. Mauger, O. Jaillon, K. Malarme, J. Weissenbach, S. D. Ehrlich, and A. Sorokin.** 2001. The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. lactis IL1403. Genome Res. **11:**731–753.
4. **Bouchard, J. D., and S. Moineau.** 2000. Homologous recombination between a lactococcal bacteriophage and the chromosome of its host strain. Virology **270:**65–75.
5. **Boucher, I., M. Parrot, H. Gaudreau, C. P. Champagne, C. Vadeboncoeur, and S. Moineau.** 2002. Novel food-grade plasmid vector based on melibiose

fermentation for the genetic engineering of *Lactococcus lactis*. Appl. Environ. Microbiol. **68:**6152–6161.
6. **Coombs, J., and J. E. Brenchley.** 2001. Characterization of two new glycosyl hydrolases from the lactic acid bacterium *Carnobacterium piscicola* strain BA. Appl. Environ. Microbiol. **67:**5094–5099.
7. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
8. **de Vos, W. M.** 1987. Gene cloning and expression in lactic streptococci. FEMS Microbiol. Rev. **46:**281–295.
9. **de Vos, W. M., and E. E. Vaughan.** 1994. Genetics of lactose utilization in lactic acid bacteria. FEMS Microbiol. Rev. **15:**217–237.
10. **Duplessis, M., and S. Moineau.** 2001. Identification of a genetic determinant responsible for host specificity in *Streptococcus thermophilus* bacteriophages. Mol. Microbiol. **41:**325–336.
11. **Émond, É., R. Lavallée, G. Drolet, S. Moineau, and G. LaPointe.** 2001. Molecular characterization of a theta replication plasmid and its use for development of a two-component food-grade cloning system for *Lactococcus lactis*. Appl. Environ. Microbiol. **67:**1700–1709.
12. **Gasson, M. J.** 1983. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. J. Bacteriol. **154:**1–9.
13. **Grossiord, B. P., E. J. Luesink, E. E. Vaughan, A. Arnaud, and W. M. de Vos.** 2003. Characterization, expression, and mutation of the *Lactococcus lactis galPMKTE* genes, involved in galactose utilization via the Leloir pathway. J. Bacteriol. **185:**870–878.
14. **Grossiord, B., E. E. Vaughan, E. Luesink, and W. M. de Vos.** 1998. Genetics of galactose utilization via the Leloir pathway in lactic acid bacteria. Lait **78:**77–84.
15. **Holler, B. J., and J. L. Steele.** 1995. Characterization of lactococci other than *Lactococcus lactis* for possible use as starter cultures. Int. Dairy J. **5:**275–289.
16. **Holo, H., and I. F. Nes.** 1989. High frequency transformation, by electroporation, of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. Appl. Environ. Microbiol. **55:**3119–3123.
17. **Holt, J. G., N. R. Krieg, P. H. A. Sneath, J. T. Staley, and S. T. Williams.** 1994. Bergey's manual of determinative bacteriology, 9th ed. The Williams & Wilkins Co., Baltimore, Md.
18. **Hueck, C. J., W. Hillen, and M. H. Saier, Jr.** 1994. Analysis of a *cis*-active sequence mediating catabolite repression in Gram-positive bacteria. Res. Microbiol. **145:**503–518.
19. **Kelly, W. J., G. P. Davey, and L. J. H. Ward.** 1998. Characterization of lactococci isolated from minimally processed fresh fruit and vegetables. Int. J. Food Microbiol. **45:**85–92.
20. **Kelly, W. J., G. P. Davey, and L. J. H. Ward.** 1998. Conjugative transfer of raffinose metabolism in *Lactococcus lactis*. FEMS Microbiol. Lett. **167:**145–149.
21. **Kleerebezem, M., J. Boekhorst, R. van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. Fiers, W. Stiekema, R. M. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. Groot, R. Kerkhoven, M. De Vries, B. Ursing, W. M. de Vos, and R. J. Siezen.** 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc. Natl. Acad. Sci. USA **100:**1990–1995.
22. **McCleary, B. V.** 1988. Alpha-galactosidase from luciferine and guar seed. Methods Enzymol. **160:**627–632.
23. **Rosenow, C., M. Maniar, and J. Trias.** 1999. Regulation of the α-galactosidase activity in *Streptococcus pneumoniae*: characterization of the raffinose utilization system. Genome Res. **9:**1189–1197.
24. **Russell, R. R. B., J. Aduse-Opoku, I. C. Sutcliffe, L. Tao, and J. J. Ferretti.** 1992. A binding protein-dependent transport system in *Streptococcus mutans* responsible for multiple sugar metabolism. J. Biol. Chem. **267:**4631–4637.
25. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
26. **Schleifer, K. H., J. Kraus, C. Dvorak, R. Kilpper-Balz, M. D. Collins, and W. Fischer.** 1985. Transfer of *Streptococcus lactis* and related streptococci into the genus *Lactococcus* gen. nov. Syst. Appl. Microbiol. **6:**183–195.
27. **Silvestroni, A., C. Connes, F. Sesma, G. Savoy de Giori, and J.-C. Piard.** 2002. Characterization of the *melA* locus for α-galactosidase in *Lactobacillus plantarum*. Appl. Environ. Microbiol. **68:**5464–5471.
28. **Stiles, M. E., and W. H. Holzapfel.** 1997. Lactic acid bacteria of foods and their current taxonomy. Int. J. Food Microbiol. **36:**1–29.
29. **Thompson, J.** 1979. Lactose metabolism in *Streptococcus lactis*: phosphorylation of galactose and glucose moieties in vivo. J. Bacteriol. **140:**774–785.
30. **Thompson, J.** 1980. Galactose transport systems in *Streptococcus lactis*. J. Bacteriol. **144:**683–691.
31. **Tremblay, D. M., and S. Moineau.** 1999. Complete genomic sequence of the lytic bacteriophage DT1 of *Streptococcus thermophilus*. Virology **255:**63–76.
32. **Vaillancourt, K., S. Moineau, M. Frenette, C. Lessard, and C. Vadeboncoeur.** 2002. Galactose and lactose genes from the galactose-positive bacterium *Streptococcus salivarius* and the phylogenetically related galactose-negative bacterium *Streptococcus thermophilus*: organization, sequence, transcription, and activity of the *gal* gene products. J. Bacteriol. **184:**785–793.
33. **Vaughan, E. E., R. D. Pridmore, and B. Mollet.** 1998. Transcriptional regulation and evolution of lactose genes in the galactose-lactose operon of *Lactococcus lactis* NCDO2054. J. Bacteriol. **180:**4893–4902.
34. **Vaughan, E. E., P. T. C. van den Bogaard, P. Catzeddu, O. P. Kuipers, and W. M. de Vos.** 2001. Activation of silent *gal* genes in the *lac-gal* regulon of *Streptococcus thermophilus*. J. Bacteriol. **183:**1184–1194.