Novel Food-Grade Plasmid Vector Based on Melibiose Fermentation for the Genetic Engineering of *Lactococcus lactis*

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The α -galactosidase gene (*aga*) and a gene coding for a putative transcriptional regulator from the LacI/GalR family (*galR*) of *Lactococcus raffinolactis* ATCC 43920 were cloned and sequenced. When transferred into *Lactococcus lactis* and *Pediococcus acidilactici* strains, *aga* modified the sugar fermentation profile of the strains from melibiose negative (Mel[−]) to melibiose positive (Mel⁺). Analysis of *galA* mutants of *L. lactis* subsp. *cremoris* MG1363 indicated that the putative galactose permease GalA is also needed to obtain the Mel⁺ phenotype. Consequently, GalA may also transport melibiose into this strain. We demonstrated that when *aga* was associated with the theta-type replicon of a natural *L. lactis* plasmid, it constituted the selectable marker of a cloning vector named pRAF800. Transcriptional analysis by reverse transcriptase PCR suggests that this vector is also suitable for gene expression. The α -galactosidase activity conferred by pRAF800 was monitored in an industrial strain grown in the presence of various carbon sources. The results indicated that the enzymatic activity was induced by galactose and melibiose, but not by glucose or lactose. The gene encoding the phage defense mechanism, *AbiQ*, was cloned into pRAF800, and the resulting clone (pRAF803) was transferred into an industrial *L. lactis* strain that became highly phage resistant. The measurements of various growth parameters indicated that cells were not affected by the presence of pRAF803. Moreover, the plasmid was highly stable in this strain even under starter production conditions. The *L. raffinolactis* *aga* gene represents the basis of a novel and convenient food-grade molecular tool for the genetic engineering of lactic acid bacteria.

Lactococcus lactis is a mesophilic lactic acid bacterium (LAB) used in starter cultures for the manufacture of several fermented dairy products, such as buttermilk, sour cream, and Cheddar cheese. Most *L. lactis* strains naturally contain numerous plasmids, some of them encoding phenotypes relevant to their industrial use, including lactose fermentation, proteolytic activities, and phage resistance (33). These intrinsic plasmids were used to develop an array of cloning devices adapted for the genetic engineering of lactococci. However, a good number of these devices are excluded from food applications because of the presence of antibiotic resistance genes and/or exogenous DNA.

Over the past several years, food-grade alternatives have been proposed for the genetic modification of *L. lactis* strains. These cloning options are based on selectable markers that can be catalogued as dominant or complementation markers (10, 16, 49). Dominant markers confer a novel phenotype that is exploited to differentiate transformed from nontransformed cells. Complementation markers require the prior development of a bacterial mutant with a specific deficiency that is later complemented by the marker to restore the original phenotype. While the limitation of dominant markers is linked to

the natural occurrence of the phenotype they confer, complementation markers are inconvenient to use. Recently, a third approach was proposed that capitalizes on the advantages of both systems and relies on two components (16). The first component is a cloning vector that carries a functional lactococcal replicon entirely made up of *L. lactis* DNA and that has no selection marker. The second element, the “companion,” is a replication-deficient plasmid that carries an erythromycin-resistance gene as a dominant selection marker. The companion construct can only replicate in *L. lactis* if complemented in *trans* by the cloning vector. Since only the cotransformants containing both the cloning vector and the companion can survive on plates containing erythromycin, the companion can be used transiently to select cells that have acquired the cloning vector. Given its defective replication, the companion can be readily cured from the cells grown on an antibiotic-free medium after the selection step (16).

For lactococcal food-grade cloning vectors, the ability to metabolize sugars such as sucrose and lactose was previously exploited so that the sugar fermentation phenotypes could serve as dominant or complementation markers, respectively (28, 42). However, both of these phenotypes are widespread in several *Lactococcus* strains, which limits their utilization. Still, LAB represent an exceptional reservoir of peculiar sugar fermentation phenotypes that could potentially be exploited as selectable markers on cloning vectors used for their genetic modification. Moreover, despite the diversity of organic compounds that can collectively be metabolized by LAB, most strains and species can only grow on a limited number of substrates (29, 50). Consequently, a dominant marker based on

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a rare sugar fermentation phenotype emerges as an attractive option for the construction of a novel food-grade vector. For instance, melibiose (6-*O*- α -D-galactopyranosyl-D-glucose) is an uncommon fermentation substrate for LAB. However, melibiose fermentation is a distinctive characteristic of *Lactococcus raffinolactis*, a nondairy organism.

Melibiose is analogous to lactose in terms of its galactose and glucose subunits. The difference resides in the chemical link between the two subunits. While lactose has a β -1-4 glycosidic bond that can be enzymatically hydrolyzed by a β -galactosidase, melibiose displays an α -1-6 glycosidic bond that can be broken down by an α -galactosidase. The monosaccharide subunits resulting from the intracellular hydrolysis of these sugars are subsequently degraded through metabolic pathways found in a wide variety of LAB. Consequently, α -galactosidase is a key enzyme in melibiose fermentation.

The exploitation of α -galactosidase activity has recently emerged in biotechnology for yeast research. A few strains of *Saccharomyces cerevisiae* possess a *mel* gene coding for an α -galactosidase and are capable of assimilating the melibiose released from the hydrolysis of raffinose (molasses sugar) by the enzyme invertase (38). Baker's yeast strains able to utilize melibiose by expressing the *mel1* gene have been constructed to improve the overall yield of either biomass or ethanol production from molasses, thereby reducing the biological oxygen demand of industrial waste (40, 45, 52). The yeast *mel1* gene, which encodes α -galactosidase, a secreted but largely cell-wall-associated enzyme (27), is also used as a reporter gene in the yeast two-hybrid system (1). In this particular system, the extracellular localization of the α -galactosidase is considered an advantageous characteristic for whole-cell enzymatic assays and sensitive screening of living cells on X- α -Gal (5-bromo-4-chloro-3-indolyl- α -D-galactopyranoside) indicator plates (1).

In this study, we describe the cloning and sequencing of the α -galactosidase gene from *L. raffinolactis* ATCC 43920, the isolation of a theta-replicon from an *L. lactis* plasmid, and their association to create a novel cloning vector for food-grade applications in lactococci and other LAB.

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MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. The bacterial strains, phages, and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown at 37°C in Luria broth (LB), *Lactococcus* was grown at 30°C, and *Streptococcus thermophilus* was grown at 42°C in M17 broth (Quélab, Montréal, Québec, Canada; and Difco, Detroit, Mich.) supplemented with the appropriate sugar. *Pediococcus acidilactici* was grown in MRS medium (Merck, Darmstadt, Germany) at 37°C. Carbohydrate fermentation was tested in BCP medium (2% tryptone, 0.5% yeast extract, 0.4% NaCl, 0.15% Na-acetate, 40 mg of purple bromocresol per liter). Enrichment of Mel⁺ transformants was usually performed in liquid EL1 medium (1% tryptone, 0.4% NaCl, 0.15% Na-acetate, 40 mg of purple bromocresol per liter). Sugars were filter sterilized and added to autoclaved medium at a final concentration of 0.5%. The BCP and EL1 formulations were based on the Elliker medium (13). When required, antibiotics were added at 50 μ g of ampicillin per ml for *E. coli* and 5 μ g of erythromycin or chloramphenicol per ml for *L. lactis*. All of the antibiotics were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Phages were amplified on their respective *L. lactis* hosts as described previously (15).

DNA techniques. Routine DNA manipulations were carried out according to standard procedures (46). Restriction enzymes, alkaline phosphatase, RNase-

free DNase, RNase inhibitor (Roche Diagnostics, Laval, Québec, Canada), and T4 DNA ligase (Invitrogen Life Technologies, Burlington, Ontario, Canada) were used according to the supplier's instructions. All primers were obtained from Invitrogen Life Technologies. Transformation of *E. coli* (46), *L. lactis* (24), *S. thermophilus* (24), and *P. acidilactici* (6) was performed as described elsewhere. Unless specified otherwise, plasmid DNA from *E. coli* and *L. lactis* was isolated as previously described (16). *L. raffinolactis* total DNA was isolated from a culture grown overnight at 30°C in 200 ml of GM17. Pelleted cells were resuspended in 10 ml of lysis solution (6.7% sucrose, 50 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0], 30 mg of lysozyme per ml) and incubated at 37°C for 20 min. Following the addition of 1.12 ml of 10% sodium dodecyl sulfate (SDS), the mixture was kept at 60°C for 10 min. After addition of 80 μ l of proteinase K (20 mg/ml; Roche Diagnostics), the lysate was incubated at 60°C for an additional 20 min. After three phenol-chloroform (1:1) extractions, the DNA was precipitated with a 1/10 volume of 3 M potassium acetate (pH 7.0) and 2 volumes of 95% ethanol. The DNA precipitate was washed with 70% ethanol, air dried, and dissolved in 1 ml of sterile double-distilled water containing RNase A (5 μ g/ml).

Cloning and sequencing of *aga* from *L. raffinolactis*. The oligonucleotide α -gal (Table 2) was used as a probe in Southern hybridizations to locate the α -galactosidase gene on specific restriction fragments of the *L. raffinolactis* chromosome. The probe was labeled with the digoxigenin 3'-end oligonucleotide labeling kit (Roche Diagnostics). Prehybridization, hybridization, posthybridization washes, and detection by chemiluminescence were performed as suggested by the manufacturer (Roche Diagnostics). Genomic DNA was double digested with *Hind*III and *Eco*RI, and restriction fragments of 3 to 5 kb were extracted from 0.8% agarose gel after electrophoresis. DNA was recovered from the gel as described by Duplessis and Moineau (11). Relevant DNA fragments were cloned into pBS (*Hind*III-*Eco*RI), and recombinants were selected by blue-white screening and restriction mapping as described elsewhere (46). The DNA sequences were determined on both strands with universal primers and the Tn1000 kit (Gold Biotechnology, St. Louis, Mo.). *E. coli* plasmid DNA was isolated with the Qiagen Plasmid Maxi kit (Chatsworth, Calif.). DNA sequencing was carried out by the DNA sequencing service at the Université Laval with an ABI Prism 3100 apparatus. Sequence analyses were performed by using the Wisconsin Package software (version 10.2) of the Genetics Computer Group (GCG) (8).

Construction of a *L. lactis* MG1363 *galA* mutant. Two primer sets (Table 2) carrying terminal restriction sites were used to amplify by PCR the DNA regions upstream (*galA5-galA6*) and downstream (*galA7-galA8*) from *galA* of *L. lactis* MG1363. Both amplicons were digested with *Eco*RI, ligated together with T4 DNA ligase, and reamplified by PCR with primers *galA5* and *galA8*. The resulting amplicon was digested with *Xba*I and *Xho*I and cloned into pGhost4 to generate pGalA2. Homologous integration of pGalA2 into the chromosome of *L. lactis* MG1363 was achieved at 37°C in the presence of erythromycin as described previously (31). A pGalA2 integrant was selected and grown at 30°C without selective pressure to favor excision and loss of the plasmid. Colonies were screened for erythromycin sensitivity, and the presence of the mutated allele was confirmed by PCR with primers *galA5* and *galA8*. For the complementation assay, the wild-type *galA* gene was amplified by PCR from MG1363 with primers *galA5* and *galA8*, and the PCR product was cloned into pTRKH2 to construct pGalA3. The sugar fermentation pattern was determined on API 50 CH strips with API 50 CHL medium (bioMérieux, Marcy l'Etoile, France).

Isolation of the minimal replicon of three *L. lactis* plasmids. *L. lactis* plasmid pSRQ800 (3) was subcloned into the vector pNC1, which can replicate in *E. coli*, but not in *L. lactis*. A double-stranded nested deletion kit (Amersham Pharmacia Biotech, Baie d'Urfé, Québec, Canada) was used to generate several deleted clones. These deletants were tested for their ability to replicate in *L. lactis*. The smallest replicative deletant was sequenced on both strands.

Construction of a food-grade vector. The minimal replicon of pSRQ800 was amplified by PCR with the primers IB800.21 and IB800.23 (Table 2) and with pSRQ800 as the template. The *aga* gene from *L. raffinolactis* was also amplified by PCR with the primers *raf12* and *raf13* and with *L. raffinolactis* total DNA as the template. The two PCR products were digested with *Xba*I and *Xho*I and joined together, and the ligation mixture was used directly to transform *L. lactis* MG1363 by electroporation. Cells were incubated for 2 h for recuperation in SM17MC medium (24) supplemented with 0.5% melibiose. After recuperation, electroporated cells were inoculated into 10 ml of Mel-EL1 medium and incubated at room temperature until acidification (which might take a few days), which was manifested by a color change (from purple to yellow) of the pH indicator purple bromocresol. Then, cells were diluted in sterile peptonized water, plated on Mel-BCP plates, and incubated for 24 h at 30°C. Plasmid DNA was recovered from the Mel⁺ (yellow) colonies and sequenced.

Expression profile of pRAF800. The transcription profiles of *aga* (α -galactosidase) and *repB* (plasmid replication initiator) encoded on pRAF800 were

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

Strain, phage, or plasmid	Relevant characteristics ^a	Source or reference ^b
Strains		
<i>E. coli</i> DH5 α	<i>supE44</i> Δ <i>lac</i> <i>U169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	Invitrogen Life Technologies
<i>L. lactis</i> subsp. <i>cremoris</i> MG1363	Laboratory strain, plasmid free, Mel ⁻	18
SMQ-741	Industrial strain, Mel ⁻	QI
<i>L. lactis</i> subsp. <i>lactis</i> IL1403	Laboratory strain, plasmid free, Mel ⁻	7
SMQ-561	Industrial strain, Mel ⁻	QI
<i>Lactococcus raffinolactis</i> ATCC 43920	Plasmid free, Mel ⁺	ATCC
<i>Streptococcus thermophilus</i> SMQ-301	Industrial strain, Mel ⁻	51
<i>Pediococcus acidilactici</i> SMQ-249	Industrial strain, Mel ⁻	QI
Phages		
c2	c2 species, infects <i>L. lactis</i> MG1363	23
p2	936 species, infects <i>L. lactis</i> MG1363	37
Q37	936 species, infects <i>L. lactis</i> SMQ-741	35
Plasmids		
pBS	Cloning vector for DNA sequencing, Ap ^r	Stratagene
pGhost4	Integration vector, Ts Em ^r	31
pNC1	Replicon-screening vector, Ap ^r Cm ^r	16
pNZ123	Shuttle cloning vector, Cm ^r	9
pTRKH2	Shuttle cloning vector, Em ^r	41
pGalA2	pGhost4 + <i>L. lactis</i> MG1363 truncated <i>galA</i> , Ts Em ^r	This study
pGalA3	pTRKH2 + <i>L. lactis</i> MG1363 <i>galA</i> , Em ^r	This study
pRAF100	pBS + 4-kb <i>EcoRI/HindIII</i> fragment of <i>L. raffinolactis</i> ATCC 43920 encoding <i>aga</i> , Ap ^r	This study
pRAF300	pNZ123 + 4-kb fragment of pRAF100	This study
pRAF301	pNZ123 + 2.5-kb <i>aga</i> amplicon from <i>L. raffinolactis</i> ATCC43920	This study
pRAF800	Food-grade cloning vector, Mel ⁺	This study
pRAF803	pRAF800 containing <i>abiQ</i> , AbiQ ⁺	This study
pSRQ800	Natural <i>L. lactis</i> plasmid, AbiK ⁺	3, 15
pSRQ835	pNC1 + replicon of pSRQ800	This study
pSRQ900	Natural <i>L. lactis</i> plasmid, AbiQ ⁺	3, 14

^a Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Em^r, erythromycin resistance; Mel, melibiose fermentation; Abi, phage abortive infection mechanism; Ts, thermosensitive replicon.

^b ATCC, American Type Culture Collection, Manassas, Va.; QI, Quest International, Rochester, Minn.

determined by reverse transcriptase PCR (RT-PCR). *L. lactis* was grown at 30°C in 10 ml of M17 broth supplemented with 0.5% melibiose to an optical density at 600 nm (OD₆₀₀) of 0.2. The culture was pelleted, and cell lysis was carried out in 100 μ l of Tris-EDTA (TE) containing 30 mg of lysozyme per ml (Elbex, Québec, Canada) at 37°C for 10 min. Total RNA was isolated by using the RNeasy kit (Qiagen) as described by the manufacturer. The DNA was eliminated from the isolated RNA by using RNase-free DNase in the presence of RNase inhibitor. Then, an additional RNeasy column was used for RNA cleanup. All of the reagents (RNA, RT buffer, dithiothreitol [DTT], deoxynucleoside triphosphates [dNTP], hexanucleotides, and RNase inhibitor), except RT for the cDNA synthesis, were mixed in microtubes. RNase-free DNase was added to the mixture for a second DNase treatment at 37°C for 30 min. The DNase was heat inactivated at 75°C (5 min), and tubes were cooled to 4°C. Expand RT (Roche Diagnostics) was added, and cDNA synthesis was performed essentially as recommended by the manufacturer. Two microliters of the cDNA was used for PCR amplification with various primer combinations. The PCR products were fractionated by electrophoresis on a 0.8% agarose gel, stained with ethidium bromide, and photographed under UV illumination with a gel documentation system (Bio-Rad, Mississauga, Ontario, Canada).

α -Galactosidase assay. Strains were grown in 10 ml of M17 broth supplemented with 0.5% of the appropriate sugar to an OD₆₀₀ of 0.5 to 0.6. Cell pellets

were washed twice in 50 mM sodium phosphate (pH 7.0) and resuspended in 500 μ l of the same buffer. Cells were disrupted at 4°C by shaking three times on a vortex (3 min each time followed by a 1-min rest on ice) in the presence of glass beads (106 μ m in diameter and finer [Sigma]). Cell debris was removed by centrifugation, and the supernatant (cell extract) was kept on ice until the enzymatic assay, which was completed within 2 h. The protein concentration was estimated by using 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 (PNPG) as a substrate (32). Essentially, 50 or 100 μ l of cell extract was added to the prewarmed reaction mixture containing 250 μ l of 3 mg of PNPG per ml (Sigma) and enough Na₂PO₄ (50 mM at pH 7.0) to complete the volume to 3 ml. Aliquots of 900 μ l were retrieved after 5, 10, and 15 min of incubation and added to 100 μ l of chilled 1 M Na₂CO₃. The OD₄₂₀ was determined with a Beckman DU530 spectrophotometer, and activity was calculated by using the extinction coefficient for PNPG at 420 nm of 18,300 M⁻¹ cm⁻¹.

Cloning of *abiQ* into pRAF800. The phage resistance gene *abiQ* (14) was amplified by PCR with the primers *abiQ1* and *abiQ2* and with pSRQ900 as the template. pRAF800 was digested with *Xba*I, dephosphorylated, and ligated to the *Xba*I-digested *abiQ* amplicon. The ligation mixture was used to transform *L. lactis* MG1363 by electroporation, and Mel⁺ transformants were obtained as

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5'→3') ^a
α-gal	TTTGTTYTWGATGATGGWTTGTTGGW
abiQ1	TCTAGATCTAGAACCCGTCCAAGGAATATACAA
abiQ2	TCTAGATCTAGATGTTTCTAATCTAAATGACTGGT
galA5	TCTAGATCTAGACAAGGTCGCTCTGATATTAG
galA6	GAATTCGAATTCGATCATGTCTAGTGCACCA
galA7	GAATTCGAATTCCTTTGTAGTCCCAGCGGTCT
galA8	CTCGAGCTCGAGCCAATCAACAATGCGAGCTC
IB800.6	ACATGACGATACCGCTACA
IB800.8	AATGCAAAAGACCGCTCTCA
IB800.21	TCTAGATCTAGAAGGGCTTGCCCTGACCGTCT
IB800.23	CTCGAGCTCGAGTTACACCTAACTCATCCGCA
raf12	TCTAGATCTAGAAGGTCGCTCTGACCGTCT
raf13	CTCGAGCTCGAGCCATCACCGAAGAGGGCTGT
raf39	ATGAGTACCTCTCGTGACCA
raf56	GCTGGGATTAATCCCTTTGG
raf63	GAATTCGAATTCGTCTGCTGGTCTTCAATATC
Forward	CGCTATTACGCCAGCTGGCGAAA
Reverse	TAGGCACCCAGGCTTTACACTT

^a Underlines indicate nucleotides participating in restriction sites.

indicated above. Resistance to phages c2 and p2 was assessed as described previously (36).

Evaluation of the effect of pRAF803 on the growth rate of an industrial *L. lactis* strain. Stock cultures of *L. lactis* strains SMQ-741 and SMQ-741 (pRAF803) were prepared by mixing 10 ml of a fresh culture with 25 ml of 20% skim milk and 25 ml of a 20% glycerol solution and then freezing the mixture at -70°C in 1-ml sterile cryovials (Nalgene, Rochester, N.Y.). Working cultures were prepared by inoculating 100 ml of LM17 broth with 1 ml of a thawed stock culture, and incubating this mixture at 30°C for 24 h. The growth of *L. lactis* strains in LM17 broth was evaluated by automated spectrophotometry with a Powerwave unit (Bio-Tek Instrument, Winooski, Vt.) as described previously (4). Media were prepared in test tubes, inoculated at 1% (vol/vol) from a fresh 100-ml LM17 working culture, and 200-μl samples were added aseptically to each well. Microplates were incubated at 30°C for 24 h. The OD₆₀₀ of each well was automatically recorded every 15 min. Prior to each reading, the plates were shaken at intensity setting 2 for 3 s.

Starter production in milk and in commercial medium. Starter production was achieved in either 6% (wt/wt) nonfat dry milk or in 6-Cess Plus medium (SKW Biosystems-Canada, Mississauga, Canada). Fermentations in milk were carried out without an external pH control, while a pH control was used in fermentations in 6-Cess Plus medium. All media were heat pasteurized at 90°C for 45 min. Two liters of media was inoculated at 1% (vol/vol) from a fresh LM17 culture. Production was carried out at 30°C in 2.5-liter New Brunswick Bioflo 3000 units (New Brunswick, Edison, N.J.). Agitation was kept at 60 rpm, except during addition of base (100 rpm). To reproduce industrial conditions, the pH-controlled fermentation was carried out by zone neutralization. In such a process, acidification is allowed to proceed until a pH of 5.8 is reached. At this pH set point, agitation is increased to 100 rpm, and base is added until a pH of 6.0 is attained. At this second pH set point, base addition is stopped and agitation is lowered to 60 rpm. If acidification continued, the cycle is reinitiated once the pH reaches 5.8. The neutralizing base was composed of a 5:1 mixture of 6 M KOH and 6 M NH₄OH. Fermentations performed with the pH control were stopped when the medium's sugar was depleted (e.g., when acidification stopped). In milk, fermentations were stopped when the pH of the medium reached 4.7. The time required to complete the various fermentations was registered and will be referred to hereafter as the "fermentation time."

Biomass production and acidification activity of the starters produced in milk or in commercial medium. Viable counts of the starters were obtained by plating appropriate dilutions (0.1% sterile peptone) on LM17 agar. The first dilution bottle contained approximately 2.5 g of glass beads in order to break cell chains. A modified Pearce test was used to determine the acidification activity of the starters (5). First, the milk powder was irradiated at 5 kG. Five hundred milliliters of 10% reconstituted nonfat dry milk was prepared. Milk solutions were kept for 16 h at 4°C prior to use. Tubes containing milk were inoculated at 1% (vol/vol) with samples taken at the end of starter production fermentations and incubated at 32°C for 5 h. A pH reading was made at the end of the incubation period. Three independent assays were carried out for each experiment.

Evaluation of the stability of pRAF803. The stability of pRAF803 was evaluated as the percentage of cells in the starter expressing the melibiose fermentation or phage resistance phenotypes conferred by the plasmid. Starter samples were streaked on LM17 plates and incubated at 30°C for 24 h. For each sample, melibiose fermentation was tested for 100 isolated colonies on Mel-BCP plates. Mel⁻ colonies were reinoculated on Mel-BCP plates and tested for phage resistance. Resistance to phage Q37 was also tested for another 100 isolated colonies as described elsewhere (36). Phage-sensitive colonies were tested again for phage resistance and for melibiose fermentation.

Accession numbers. The GenBank accession numbers assigned to the nucleotide sequences of *Lactococcus* plasmids pSRQ800 and pRAF800 are U35629 and AY091640, respectively.

RESULTS

Characterization of the α-galactosidase locus of *L. raffinolactis*. A stretch of conserved amino acids (FVLDDGWFG) was identified within bacterial α-galactosidases and used to design a degenerated oligonucleotide primer (α-gal, Table 2), based on lactococcal codon preference. Using this primer as a probe in Southern hybridization assays, the α-galactosidase gene was located on a 4-kb *EcoRI*-*HindIII* DNA fragment of *L. raffinolactis* ATCC 43920 (data not shown). This fragment was cloned into pBS (pRAF100), sequenced, and found to comprise two genes, *galR* and *aga* (Fig. 1). Based on amino acid sequence similarities and conserved motifs, these two genes likely encode an α-galactosidase (Aga, 735 amino acids) and a transcriptional regulator (GalR, 245 amino acids) from the LacI/GalR family. The product of *aga* displays up to 54% identity with bacterial α-galactosidases, particularly *Geobacillus stearothermophilus* AgaN (390 identical amino acids out of 722), AgaB (376 of 730 amino acids), and AgaA (372 of 730 amino acids) (GenBank accession no. AAD23585.1, AAG49421.1, and AAG49420.1, respectively). GalR is up to 34% identical to various transcriptional regulators, including those of the galactose operon from *Lactobacillus casei* (115 of 343 amino acids), *Streptococcus thermophilus* (112 of 340 amino acids), and *Streptococcus salivarius* (109 of 348 amino acids) (GenBank accession no. AAC19331.1, AAD00092.1, and AAL67295, respectively). An inverted repeat with the po-

FIG. 1. Genetic organization of the *aga* locus of *L. raffinolactis*. Genes are identified by shaded arrows oriented to indicate the direction of transcription. The gene *aga* codes for α -galactosidase, while the gene *galR* codes for a putative transcriptional regulator. The putative terminator is indicated by a stem-loop structure, and the putative promoter is indicated by an arrow upstream of *aga*. The sequence of the *galR-aga* intergenic region is presented in the box. The GalR stop codon and the Aga start codon are shaded in black. The -35 and -10 regions of the promoter are indicated in boldface. The CRE sequence is underlined. The consensus CRE sequence for gram-positive bacteria is presented in italic below the underline.

tential to form a stem-loop structure was found in the *galR-aga* intergenic region and could act as an intrinsic terminator. A canonical promoter sequence (TTGACA-N₁₇-TATAAT) was found upstream of *aga* and a putative catabolite-responsive element (CRE), involved in the regulation of sugar metabolism (25), overlaps the -35 region. Consequently, the expression of *aga* is likely regulated through catabolite repression in *L. raffinolactis*.

Cloning of *aga* in *L. lactis*. The 4-kb DNA fragment from *L. raffinolactis* was cloned into the lactococcal cloning vector pNZ123 (pRAF300) and transferred by electroporation into the laboratory strain *L. lactis* subsp. *cremoris* MG1363. The presence of pRAF300 gave *L. lactis* MG1363 the ability to ferment melibiose. This phenotype was easily observable on BCP plates containing melibiose, because the acidification resulting from melibiose fermentation led to the formation of yellow colonies surrounded by a yellow halo on the purple background. On this medium, melibiose-negative cells formed smaller purple colonies. A 2.5-kb fragment containing only the *aga* gene and its putative promoter was also amplified by PCR, cloned into pNZ123 (pRAF301), and transferred into the following five strains: *L. lactis* subsp. *cremoris* MG1363, *L. lactis* subsp. *lactis* IL1403 and SMQ-561, *Streptococcus thermophilus* SMQ-301, and *Pediococcus acidilactici* SMQ-249. The pres-

ence of pRAF301 was sufficient to give the melibiose fermentation phenotype to all strains except *S. thermophilus*.

Identification of the melibiose carrier in *L. lactis*. *L. lactis* subsp. *cremoris* MG1363 has a limited sugar fermentation pattern, including acid production from galactose. Because different galactosides can be imported by the same transporter (43), we hypothesized that the putative permease of galactose GalA (21) might also transport melibiose into *L. lactis* MG1363. Using the suicide vector pGalA2 (see Materials and Methods for details), a *galA*-deficient mutant (Δ *galA*) of *L. lactis* MG1363 was constructed by double crossover. After two homologous recombination events, 11 of the 24 Em^s clones tested contained a 600-bp truncated copy of *galA* instead of the 2-kb wild-type allele. Surprisingly, all of the Δ *galA* mutants conserved their ability to produce acid from galactose (Table 3). No difference between the sugar fermentation patterns of the wild-type and Δ *galA* *L. lactis* strains was observed on API 50 CH strips. One of the Δ *galA* mutants was selected and transformed with pRAF300. The 50 Cm^r transformants tested did not ferment melibiose (Table 3). The wild-type *galA* gene cloned into the cloning vector pTRKH2 (pGalA3) was then introduced into the *L. lactis* strain MG1363(Δ *galA*, pRAF300) to complement the mutation. All 24 Em^r Cm^r transformants tested were able to produce acid from melibiose, indicating

FIG. 2. Genetic organization of pRAF800. (A) Sequence of the replication origin of pRAF800. The replicon of the *L. lactis* plasmid pSRQ800 was used to construct pRAF800. The nucleotide boxed in black differs from pSRQ800 (T→G substitution). Direct repeats (DR) are underlined (continuous and discontinuous). Inverted repeats (IR) are indicated in boldface. The putative -35 and -10 boxes of the *repB* promoter are shaded. The *repB* start codon is italicized. (B) Map of pRAF800. Genes are identified by shaded arrows oriented to indicate the direction of transcription. *aga*, gene encoding α -galactosidase; *repB*, gene encoding the replication initiator protein. The positions of primers used for RT-PCR are indicated on the plasmid map. bp, base pairs. (C) Expression profile of pRAF800. RNA was isolated from SMQ-741 cells transformed with pRAF800 and grown in the presence of melibiose. RT-PCR was used to map the 3' ends of *aga* and *repB* transcripts. PCR products were obtained with an mRNA internal primer as well as one of four possible 3'-end primers. Amplicons were separated by electrophoresis on 0.8% agarose gel and stained with ethidium bromide. The targeted transcripts are identified by arrows A to H (indicating the direction of transcription), as seen on the gels. PCR products are aligned with their corresponding start and stop positions on the plasmid map. Bold line, transcript detected; normal line, transcript weakly detected; dotted line, transcript not detected. M, 1-kb DNA ladder (Invitrogen Life Technologies).

TABLE 3. Galactose and melibiose fermentation phenotypes of *L. lactis* subsp. *cremoris* MG1363 derivatives used in this study according to their genotype

Genotype ^a	Phenotype
<i>galA</i>	Gal ⁺ Mel ⁻
<i>galA aga</i>	Gal ⁺ Mel ⁺
$\Delta galA$	Gal ⁺ Mel ⁻
<i>aga</i> $\Delta galA$	Gal ⁺ Mel ⁻
<i>galA</i> [*] <i>aga</i> $\Delta galA$	Gal ⁺ Mel ⁺

^a *, supplemented in *trans*.

that *galA* is required to obtain the Mel⁺ phenotype provided by *aga* (Table 3). All of the transformants generated above, such as MG1363($\Delta galA$), MG1363($\Delta galA$, pRAF300), and MG1363($\Delta galA$, pRAF300, pGalA3), were confirmed by plasmid profile (data not shown), PCR amplification of *aga* and $\Delta galA$ (data not shown), and their sensitivity to phages c2 (c2 species) and p2 (936 species).

Isolation of lactococcal plasmid replicons. The minimal region essential for the maintenance of the natural lactococcal plasmid pSRQ800 was identified by carrying out successive deletions and sequence analysis. The minimal DNA segment consisted of a typical lactococcal theta replication module containing a replication origin (*repA*) and the gene encoding a replication initiator (*repB*). The replication origin includes the AT-rich stretch, iterons (22-bp direct repeats), and inverted repeats usually found in other lactococcal plasmids (Fig. 2A). The minimal region obtained by nested deletion that still allowed plasmid replication was 2,212 bp. This region encompassed positions 7196 through 1549 in the plasmid sequence (3). The replicon was further limited (1,767 bp) by PCR to positions -443 to +44 from the *repB* coding sequence to serve as the basis for a new plasmid vector.

Construction of a food-grade vector. The *aga* gene of *L. raffinolactis* with its putative promoter and the minimal replicon of *L. lactis* plasmid pSRQ800 were amplified by PCR and ligated together to form a functional vector with two cloning sites (*XhoI* and *XbaI*). The ligation mixture was transferred by electroporation into *L. lactis* MG1363, and after 4 days of enrichment by incubation at room temperature in liquid EL1 medium containing melibiose, Mel⁺ transformants were recovered on BCP plates. Plasmid DNA of a Mel⁺ transformant was isolated, digested, analyzed by agarose gel electrophoresis, and sequenced on both strands. The 4,245-bp constructed plasmid was named pRAF800 (Fig. 2B). This novel plasmid differed from the two parental DNA segments in four locations. The first difference is a nonconservative A/G substitution causing a T/A amino acid change at position 227 of the α -galactosidase enzyme. A second A/G substitution is found at position 2424, immediately downstream of the *aga* coding sequence. A single-nucleotide deletion was also found within the primer raf13 sequence, suggesting a likely error in the primer sequence itself. Finally, a T/G substitution is localized 358 nucleotides upstream of the *repB* start codon, in the replication origin region (Fig. 2A). These variations, which most likely resulted from PCR amplification, did not affect the functionality of the plasmid.

Expression profile of pRAF800 in the industrial strain

TABLE 4. α -Galactosidase activity in *L. lactis* SMQ-741 transformed with pRAF800 and grown in the presence of various sugars

Sugar	Activity (nmol of <i>p</i> -nitrophenol formed/mg of protein/min) ^a
Glucose	83.0 $\pm$ 18.0
Galactose	368.3 $\pm$ 30.3
Lactose	75.3 $\pm$ 17.2
Melibiose	441.1 $\pm$ 35.1

^a Values are the means $\pm$ standard deviations of 12 measurements performed with two cell extract quantities in two independent experiments. Note that no α -galactosidase activity was detected in the parental strain, SMQ-741.

SMQ-741. To determine the vector-driven transcriptional activity at the two proposed cloning sites (*XbaI* and *XhoI*) of pRAF800, the 3' ends of *aga* and *repB* transcripts were determined by RT-PCR with RNA isolated from *L. lactis* SMQ-741(pRAF800). Two overlapping transcripts were detected (Fig. 2C). The *repB* transcript overlaps most of the *aga* sequence and likely ends at the inverted repeat located immediately upstream of *aga* (Fig. 1). The *aga* transcript also extends beyond *repB* and is suspected to end at one of the multiple inverted repeats located within this gene, as shown by the weak signal obtained with primer IB800.8. These findings suggest that the *XhoI* site region is transcribed in both directions from the two promoters of *aga* and *repB*. Moreover, the *XbaI* site region does not appear to be transcribed from either of the two promoters located on pRAF800.

α -Galactosidase activity in *L. lactis*. The activity of α -galactosidase was measured in cell extracts from *L. lactis* SMQ-741(pRAF800) after growth at 30°C in the presence of various sugars (Table 4). Activity was measured under conditions in which the rate of reaction was constant with the time of incubation and proportional with the enzyme concentration. The results, summarized in Table 4, indicate that the α -galactosidase activity was four- to fivefold higher in galactose- and melibiose-grown cells than in glucose- or lactose-grown cells. Because no α -galactosidase activity could be detected with the parental strain SMQ-741, *aga* is clearly responsible for this activity in *L. lactis*.

Cloning of a phage resistance mechanism into pRAF800 and its transfer into an industrial *L. lactis* strain. To test the effectiveness of the novel cloning tool, the gene encoding the phage abortive infection mechanism, AbiQ (14), was cloned into pRAF800 at one of the two cloning sites available. The phage resistance determinant was obtained by a PCR amplification from pSRQ900 and inserted into the unique *XbaI* site of pRAF800 to generate pRAF803. The recombinant vector was first obtained in *L. lactis* MG1363. All 48 Mel⁺ colonies tested were resistant to phages c2 and p2. Plasmids pRAF800 and pRAF803 were then transferred by electroporation into the industrial *L. lactis* subsp. *cremoris* strain SMQ-741. Because this strain did not grow well in the EL1 medium, the posttransformation enrichment was performed in liquid BCP medium containing melibiose. All Mel⁺ colonies tested contained pRAF803 and were resistant to phage Q37 (936 species), while Mel⁻ colonies did not contain pRAF803 and were sensitive to phage Q37.

Effect of pRAF803 on various growth parameters of the industrial *L. lactis* strain SMQ-741 and evaluation of its sta-

TABLE 5. Effect of pRAF803 on various growth parameters of *L. lactis* strain SMQ-741 and its stability under starter production conditions

Strain	Growth rate (h ⁻¹) ^a	Fermentation time (h) ^b		Biomass production (10 ⁸ CFU/ml) ^b		Acidification activity (pH) ^b		Plasmid stability (%) ^b	
		Milk	Commercial medium	Milk	Commercial medium	Milk	Commercial medium	Milk	Commercial medium
SMQ-741	0.16 ± 0.06	8.7 ± 0.5	16.3 ± 0.5	1.1 ± 0.1	9.5 ± 0.7	6.2 ± 0.0	5.5 ± 0.2	NA ^c	NA
SMQ-741 (pRAF803)	0.20 ± 0.04	9.0 ± 0.0	17.3 ± 1.0	1.1 ± 0.3	7.7 ± 1.5	6.3 ± 0.1	5.8 ± 0.2	97.5 ± 1.8	97.9 ± 1.5

^a Values are the means of two independent trials ± standard deviations.

^b Values are the means of three independent trials ± standard deviations.

^c NA, not applicable.

bility. The growth of *L. lactis* strain SMQ-741 was compared to that of its derivative carrying pRAF803 (Table 5). First, the growth rates of both strains were evaluated in LM17 broth, and no significant difference was observed. Then, both strains were produced in fermentation units by using milk or a commercial medium. The presence of pRAF803 did not disturb the starter production process in terms of fermentation time and biomass production (Table 5). The acidification activities of these starters were also tested in milk, and they were found equivalent for the two strains (Table 5). Finally, the prevalence of pRAF803 was measured after starter production. Over 95% of the cells displayed both of the pRAF803-encoded phenotypes, indicating that pRAF803 was very stable (Table 5).

DISCUSSION

α -Galactoside metabolism was chosen as a potential selectable marker for the development of a novel food-grade cloning vector, because it is rarely found in lactococci and in many LAB. Given its relatedness to food-grade bacteria, its non-pathogenicity, and its α -galactosidase activity, *L. raffinolactis* was selected as the source of the *aga* gene. Typically, strains of *L. raffinolactis* can be isolated from raw milk, various animals, and ready-to-eat foods (2, 44, 47). The genetic determinant of the α -galactosidase was located on a 4-kb chromosome fragment of *L. raffinolactis*. On the basis of protein similarities and of conserved motifs, functions were attributed to the two open reading frames found on this sequenced DNA segment, *aga* and *galR*. When transferred into various lactococci and *Pediococcus* strains, the *aga* gene gave them the ability to produce acid from the disaccharide melibiose. This disaccharide is not prevalent in the environment and is not a common fermentation substrate for lactococci. Thus, the Mel⁺ phenotype was regarded as a prospective dominant selection marker for a plasmid vector.

To be able to metabolize melibiose, cells require, in addition to α -galactosidase, a transporter to allow the sugar to reach the bacterial cytoplasm. The proposed permease GalA from the galactose operon (21) was suspected to act as the nonspecific carrier of melibiose in *L. lactis* MG1363. Inactivation of *galA* resulted in a loss of the Mel⁺ phenotype. This altered phenotype could be complemented and restored by plasmid-encoded GalA. Accordingly, GalA can now be regarded as the melibiose transporter in *L. lactis* MG1363. Surprisingly, *galA* inactivation did not visibly affect the capacity of MG1363 cells to produce acid from galactose. Thus, the exact role of the en-

coded transporter in the galactose fermentation phenotype remains to be elucidated.

In addition to a selectable marker, a plasmid-cloning vector requires a replicon to support its autonomous replication as well as its maintenance in a bacterial host. Because lactococcal plasmids containing a theta-type replicon are well known for their structural and segregational stability and their narrow host range, a vector using this mode of replication should be carried in a stable manner in lactococci with limited risk of heterologous transfer (12, 16, 19, 26, 30, 48, 53). The lactococcal plasmid pSRQ800 was selected as the source of theta replicon, because it is found in some *L. lactis* strains currently utilized in large-scale industrial milk fermentation and has a history of safe use (34). The minimal theta replicon of pSRQ800, obtained through the functional analysis of successive deletions, included two genetic features: a noncoding region (*repA*) and a coding region (*repB*) containing the replication initiator gene. The replicon of pSRQ800 was used for the construction of pRAF800.

To test this novel food-grade vector, the phage resistance mechanism AbiQ was cloned into pRAF800, and the resulting construct was successfully transferred into a phage-sensitive industrial *L. lactis* strain that became phage resistant. The pRAF800 derivatives coexisted with the wild-type plasmids of *L. lactis* SMQ-741, a finding that is consistent with previous studies (16, 17, 20, 22, 48). However, replicon homology is also recognized as the major cause of plasmid incompatibility that excludes coexistence (20, 39). Although this phenomenon was not observed in this study, plasmid incompatibility is likely to occur with other *L. lactis* strains. The replacement of the pSRQ800 theta replicon with those of other lactococcal plasmids could be an alternative to circumvent incompatibility.

Transcription profiling of the 3' ends of *aga* and *repB* transcripts indicated that the genetic determinants of pRAF800 were expressed on two overlapping and divergently oriented transcripts. Thus, one of the proposed cloning sites (*XhoI*) offers the opportunity to express cloned genes from one of either plasmid-localized promoters, one of which is inducible by carbohydrates, such as galactose and melibiose. The other cloning site (*XbaI*) appears to be adapted for the expression of genes from their own promoters. If needed, future versions of pRAF800 could carry features such as multiple cloning sites and a transcriptional terminator at the *XhoI* site, to avoid the production of an antisense RNA that could interfere with gene expression. Moreover, the analysis of α -galactosidase activity provided by pRAF800 in SMQ-741 indicated that lactose and

glucose did not induce the enzyme activity. Since lactose is present in the industrial conditions of starter culture production and milk fermentation, the presence of pRAF800 in an industrial *L. lactis* strain should not cause a metabolic burden. In fact, growth of *L. lactis* SMQ-741 was clearly not affected by the presence of pRAF803, as evaluated with four independent parameters. Moreover, the plasmid was found to be stable under conditions similar to those in industrial starter productions. To our knowledge, this is the first demonstration of the applicability of a food-grade plasmid vector for industrial purposes.

In conclusion, a novel small plasmid vector was constructed based on an *L. lactis* theta replication module and an *L. raffinolactis* *aga* gene encoding α -galactosidase as a selection marker. Constituted exclusively of lactococcal DNA and exempt from antibiotic resistance genes, the proposed vector should therefore be appropriate for safe use in foods. The Mel⁺ phenotype produced by the *L. raffinolactis* *aga* gene emerged as a convenient dominant selection marker operating with a practical melibiose-containing medium. Lactococcal α -galactosidases represent new molecular tools for the genetic modification of lactococci and other LAB that could be exploited for research purposes and as food-related applications.

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