Characterization of the *melA* Locus for α-Galactosidase in *Lactobacillus plantarum*

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Alpha-galactosides are abundant sugars in legumes such as soy. Because of the lack of α-galactosidase (α-Gal) in the digestive tract, humans are unable to digest these sugars, which consequently induce flatulence. To develop the consumption of the otherwise highly nutritional soy products, the use of exogenous α-Gal is promising. In this framework, we characterized the *melA* gene for α-Gal in *Lactobacillus plantarum*. The *melA* gene encodes a cytoplasmic 84-kDa protein whose enzymatically active form occurs as oligomers. The *melA* gene was cloned and expressed in *Escherichia coli*, yielding an active α-Gal. We show that *melA* is transcribed from its own promoter, yielding a monocistronic mRNA, and that it is regulated at the transcriptional level, i.e., it is induced by melibiose but is not totally repressed by glucose. Posttranscriptional regulation by the carbon source could also occur. Upstream of *melA*, a putative galactoside transporter, designated RafP, was identified that shows high homology to LacS, the unique transporter for both α- and β-galactosides in *Streptococcus thermophilus*. *rafP* is also expressed as a monocistronic mRNA. Downstream of *melA*, the *lacL* and *lacM* genes were identified that encode a heterodimeric β-galactosidase. A putative *galM* gene identified in the same cluster suggests the presence of a galactose operon. These results indicate that the genes involved in galactoside catabolism are clustered in *L. plantarum* ATCC 8014. This first genetic characterization of *melA* and of its putative associated transporter, *rafP*, in a lactobacillus opens doors to various applications both in the manufacture of soy-derived products and in probiotic and nutraceutical issues.

Human consumption of soy-derived products has been hampered by the presence of α-galactosides, mostly raffinose and stachyose, in soybeans. Alpha-galactosides are carbohydrate reserves in many plant tissues and particularly in seeds. They include one or several galactose units, linked together or to the glucose moiety of sucrose through α-1,6 linkages. Since humans and monogastric animals are deficient in pancreatic α-galactosidase (α-Gal), α-galactosides are not digested in the duodenum. Therefore, they pass into the large intestine where they are degraded by gas-producing intestinal bacteria, such as *Clostridium* spp. and *Bacteroides* spp., yielding considerable amounts of CH_4, CO_2, and H_2. The abnormal accumulation of flatulent rectal gas thus provokes gastrointestinal distress, such as abdominal pain, nausea, diarrhea, and increased peristalsis (46).

To overcome these drawbacks of soy products and to boost the consumption of these otherwise highly nutritional food products, many attempts have been made to eliminate the α-galactosides from soybeans. They consist of tedious physical methods, including bean soaking, bean germination, water extraction, and ultrafiltration (28, 30, 32). Alternatively, the use of α-Gal as a biotechnological approach for removal of α-galactosides from leguminous seeds has been proposed (13, 18, 51). The enzyme α-Gal (α-D-galactoside-galactohydrolase [EC 3.2.1.22]) catalyzes the hydrolysis of α-1,6-galactoside links present in α-galactosides, such as raffinose. In this framework, the use of microbial α-Gal offers a promising solution for the degradation of α-galactosides, especially during soymilk fermentation by lactic acid bacteria (LAB).

Several microorganisms are known to produce α-Gal (3, 13). In LAB, *Lactobacillus plantarum, Lactobacillus fermentum, Lactobacillus brevis*, and *Lactobacillus buchneri* used in vegetable fermentations can hydrolyze α-galactosides to digestible carbohydrates. However, little is known about LAB α-Gals. Recently, the α-Gal-encoding genes from two lactic acid bacteria, *Carnobacterium piscicola* and *Lactococcus raffinolactis*, were cloned and characterized (10; I. Boucher, M. Parrot, C. Vadeboncoeur, and S. Moineau, Abstr. Int. Anim. Agric. Food Sci. Conf. Am. Dairy Sci. Assoc., Indianapolis, Ind., abstr. 24, p. 6, 2001). In lactobacilli, α-Gals have been characterized at the biochemical and physiological levels (1, 21, 22, 38, 40, 52, 57). No data are available regarding the molecular characterization of α-Gal in lactobacilli. In order to fill this gap, with the aim of developing probiotic microorganisms with high α-Gal activity, we undertook the genetic characterization of the α-Gal system in *Lactobacillus*. In this study we describe the cloning, sequencing, and characterization of an α-Gal gene (*melA)* from *L. plantarum* ATCC 8014 and its expression in *Escherichia coli*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Lactobacilli were grown in MRS broth (15) supplemented with glucose, galactose, melibiose, or raffinose at a 2% (wt/vol) final concentration at 30 or 37°C without shaking. The α-Gal⁻ strain *E. coli* RA11r (Δ*lacZY melA recA*) (24) is a K12 derivative and was used

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TABLE 1. Primers used in this study

Primer	Sequence (5′-3′)[a,b]
P2	TTTGGTTMGARCCAGAAAWKATTTCA
Pal7R	CTAGTTTGAACGGGAGTCGTG
PXRb	GCGGATCCGGCTGGCTNAAATCAAGC
P2Rb	GCGGATCCGTATTCGTTCCGCGATAA
Pal11R	AACGACCATAACGGTATCATCC
Pal12R	TTGTACGGTGGTTCCTGAATC
Pal17	TCATAGCAGCAGGTACACCG
Pal18	CGGTTTCCAAGTGAGTTGACC
Pal19	ACGACTAAACTTCTTTGCTAATGG
Pal20	TAACAACTGCTAAGAAGAACA
Pal21	AAATTGCAAAAATAATTAAATATAGAATGGGG
Pal22	CACCAATGTTAGAGCCAACCCGGGCG
Raf1	GAAGGCATCGTGCCCAAACGCCCC
Raf2	CCACTACTCTTATCGAACAAGTGGGAGG
Raf5	GGGATACTTGAATGATAAGCGCC
Pal25	GTGGGCCAACACGTTGCCTGTCTCC
Pu02	GGCTTCATCCTCATCTTCGAC
Pu04	GGATGTTTGTTAGTCCGGATAAG
Lac1B	CAGCGGCGGTATAGGGAAGGC
Lac5B	CCAGGCCTATTCAGCGACGCCGCGG
4A	CGGACTTTGGTTCGAGCCAG
5AR	CGACCGTCCTAACAATTCATCCGG
Pal11b	GCGGATCCGGATGATACCGTTATGGTCGTT
Pal14Re	GCGAATTCTTATCAATCTACGGTGCCG

[a] M, R, W, and K represent A/C, A/G, A/T, and G/T, respectively.
[b] Sites for restriction enzymes (*Bam*HI and *Eco*RI) are underlined.

in expression experiments. *E. coli* DH5α [*supE44Δ(lac)U169 (φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] was used for all other genetic manipulations. *E. coli* strains were routinely grown in Luria-Bertani medium (49) at 37°C, in M9 minimal medium (49) supplemented with 1% melibiose (M9mel), or in M9 minimal medium enriched with 0.2% Casamino Acids–10 mM glycerol (50)–1% melibiose (EM9mel) at 30°C under aerobic conditions. When appropriate, ampicillin was added at a concentration of 100 μg/ml.

DNA manipulations. Chromosomal DNA was extracted from lactobacilli as previously described (45). *E. coli* CaCl₂-competent cells were used, and heat shock transformation and recombinant DNA techniques were performed according to the method of Sambrook et al. (49). Restriction endonucleases and DNA polymerases were purchased from New England Biolabs, (New England Biolabs, Inc., Beverly, Mass.) or Promega (Promega Co., Madison, Wis.) and used as recommended by the suppliers. For routine PCR amplification, *Taq* polymerase (Qbiogene, Illkirch, France) was used. For cloning and sequencing purposes, the high-fidelity *Vent* polymerase (New England Biolabs) was used. Amplified fragments were either cloned in pBluescript II SK⁺ (pBS) (Stratagene, La Jolla, Calif.) with *E. coli* DH5α as a host or directly sequenced using an automated DNA Sequencer 373 (Applied Biosystems Inc., Foster City, Calif.).

Probing, cloning, and sequencing of the gene for α-Gal and its flanking regions. Based on the nucleotide sequence of the *AGA*-1 (α-Gal R) gene of *Pediococcus pentosaceus* (GenBank sequence accession no. Z32771) and an *L. plantarum* C3.8 partial sequence (courtesy of Evaristo Suarez, Oviedo, Spain), the degenerate primer P2 and nondegenerate Pal7R (Table 1) were used to amplify the genes for α-Gal in lactobacilli. Amplicons were cloned in pBS and sequenced. The upstream genomic sequence of the amplified fragment was obtained by Uneven PCR (8). For this purpose, nested primers were combined with a set of arbitrarily selected 10-mer primers from the B series of Bioprobe (Bioprobe Systems, Montreuil-sous-Bois, France). The resulting DNA fragments were sequenced, and new nested primers were designed to continue walking. The following oligonucleotides pairs were used in first and second PCR rounds, respectively: PXRb and P2Rb, Pal11R and Pal12R, Pal17 and Pal18, Pal19 and Pal20, Pal21 and Pal22, and Raf1 and Raf2 (Table 1). After assembly of the sequences obtained in six independent uneven PCRs, the entire region was amplified using Raf5 and Pal25 primers and the high-fidelity *Vent* DNA polymerase. The amplicons obtained were subsequently sequenced. The downstream sequence of the original P2-Pal7R amplicon was obtained by inverse PCR: chromosomal *L. plantarum* ATCC 8014 DNA was digested to completion with *Eco*RI and self-ligated to yield the template used with the divergent primers Pu02 and Pu04. Once the sequences were assembled, three complete open reading frames (ORFs) were detected. The primers Pal11b and Pal14Re with

sites for restriction enzymes (*Bam*HI and *Eco*RI, respectively) were then used to amplify a fragment spanning the entire ORF 2, which was then cloned in pBS to yield the plasmid p1α. In order to characterize the sequence lying downstream of the gene for α-Gal, the primers Lac1B and Lac5B were designed on the basis of the highly homologous *lacL* and *lacM* genes from *Leuconostoc lactis* (GenBank sequence accession no. M92281) (14), and the resulting amplicon was sequenced.

DNA and protein sequences analyses. Nucleotide and amino acid sequence homology searches were performed using the BLAST facility at the National Center of Biotechnology Information (2). Promoters were searched using the Neural Network Promoter Prediction algorithm (www.fruitfly.org/seq_tools /promoter.html) (48). DNA secondary structures were analyzed using the GeneBee server (http://www.genebee.msu.su/genebee.html) (7). Protein alignments were made using the BOXSHADE 3.21 software (http://www.ch.embnet.org/software/BOX_form.html), and predictions of protein signals or structures were obtained using the ExPASy Molecular Biology Server (http://expasy.ch).

Preparation of cell extracts and α-Gal activity measurement. For lactobacilli, 7-ml cultures at an optical density at 600 nm (OD₆₀₀) of 1.5 were harvested by centrifugation at $10,000 \times g$ for 3 min at 4°C, washed twice with Mc Ilvaine buffer(Na_2HPO_4-citric acid, pH 5.8 [37]) and resuspended in 0.5 ml of the same buffer (final OD₆₀₀, 20). Cells were disrupted with 500 mg of glass beads (0.10 to 0.11 mm; Sigma) upon shaking at maximum speed on a vortex mixer during four cycles of 2 min each with 5-min pauses on ice. Cellular debris was removed by centrifugation at $10,000 \times g$ for 10 min at 4°C, and α-Gal activity in supernatants was determined by the method of Church et al. (9). One unit of α-Gal is defined as the amount of enzyme that releases 1.0 μmol of pNP per min. The protein concentration was determined by the method of Bradford using bovine serum albumin as a standard (6). The α-Gal activity in *E. coli* EM9mel was determined using 10-ml samples harvested at regular time intervals, washed with Mc Ilvaine buffer, and further processed as described for lactobacilli.

Protein analyses. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (33) using a Mini Protean II system (Bio-Rad, Hercules, Calif.). Proteins were stained using Coomassie brilliant blue R-250. Bovine serum albumin (New England Biolabs) was used as an internal standard for α-Gal quantitation in gels. Ultrafiltration studies were performed on *L. plantarum* ATCC 8014 cell extracts using Ultrafree-MC filters units as recommended by the manufacturer (Millipore, Guyancourt, France). Both α-Gal activity and the amount of protein were determined in the resulting retentates and ultrafiltrates, as indicated above.

RNA isolation, Northern hybridization, and 5′ RACE analysis. Total RNA was extracted from *L. plantarum* as previously described for *Lactococcus lactis* (47). A 535-bp internal fragment of *melA* amplified by PCR with primers 4A and 5AR was used as a probe. For Northern blot analysis, 25-μg RNA samples were denatured, separated by electrophoresis along with an RNA ladder (Invitrogen, Cergy-Pontoise, France) in 1.25% agarose gels, and transferred to a Hybond-N membrane (Amersham Biosciences, Saclay, France). RNA was stained with methylene blue (56). DNA probe labeling and signal detection were carried out using the ECL kit as recommended by the manufacturer (Amersham Biosciences). To determine the transcription start region, rapid amplification of 5′-cDNA ends (5′ RACE) experiments were performed according to the instruction provided by the manufacturer (Gibco-BRL, Invitrogen). The 5′ RACE system is a procedure for amplification of nucleic acid sequences from an mRNA template between a defined internal site and unknown sequences at the 5′ end of the mRNA. This system includes synthesis of first-strand cDNA, homopolymeric tailing of the cDNA, and PCR amplification using a gene-specific primer and an adapter primer complementary to the homopolymeric tail. Briefly, total RNA was isolated, DNase treated, and reverse transcribed using a reverse primer downstream of the putative transcription initiation point (Pal18 and Pal7R primers for *rafP* and *melA*, respectively). After RNase treatment, a poly(G) tail was added to the 5′ end of cDNA by terminal deoxynucleotidyl transferase in the presence of dGTP, and PCR was performed using a specific nested reverse primer (Pal17 and 5AR for *rafP* and *melA*, respectively) combined with a poly(C) primer provided by the kit.

Nucleotide sequence accession numbers. The DNA sequences described in this study are available in the GenBank database under accession numbers AY048859 (partial *galM* and *rafP*), AF189765 (*melA*), and AY048860 (partial *lacL* and *lacM*).

RESULTS

Selection of α-Gal producer strains. Several *Lactobacillus* strains were screened for their capacity to use melibiose and raffinose by using the API 50CH test (bioMérieux, Marcy
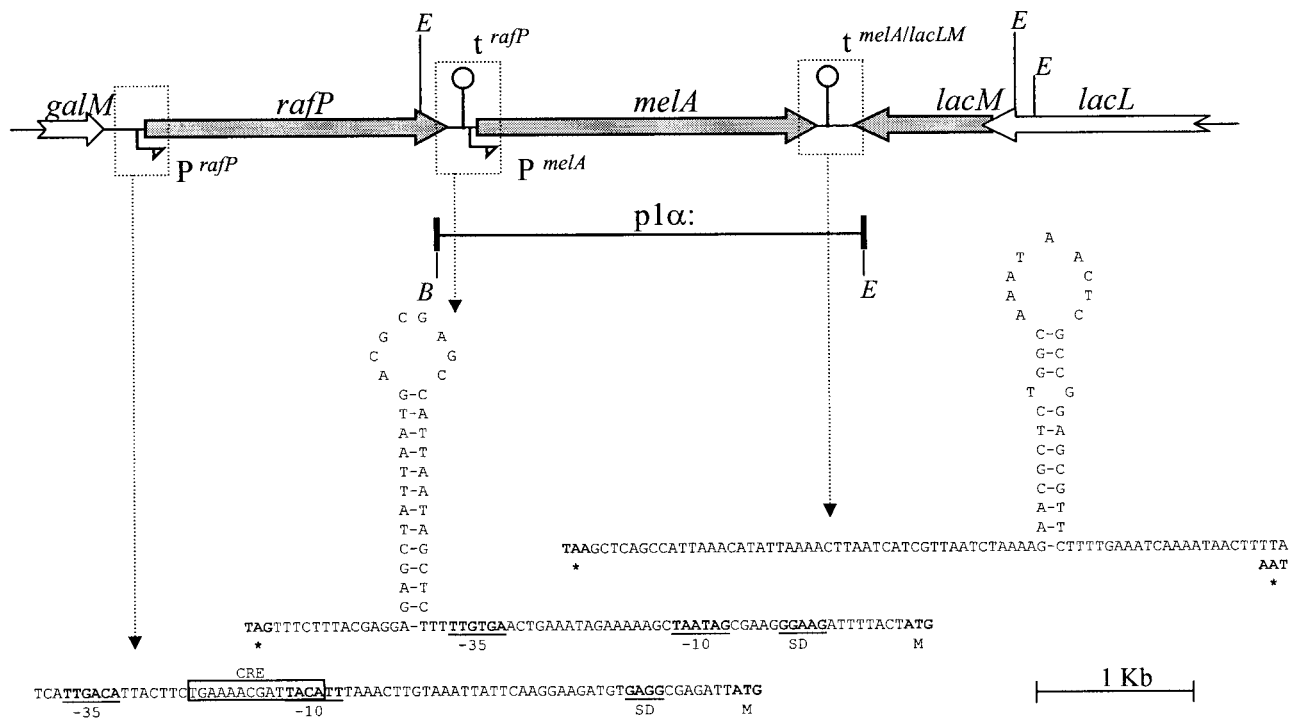
FIG. 1. Galactoside metabolism gene cluster of *L. plantarum* ATCC 8014. The gene order and nucleotide sequence of intercistronic regions are shown. White arrows correspond to incomplete ORFs. The region cloned in p1α is represented as a bold segment. Relevant restriction enzymes sites are indicated: B, *Bam*HI; E, *Eco*RI. Promoters (P) and terminators (t) are indicated. Secondary structures are given as corresponding DNA sequences. Stop (∗) and start codons (M) are in bold letters. Promoter sequences (−35, −10) and Shine Dalgarno sequence (SD) are underlined. The CRE site is boxed.

l'Etoile, France) (data not shown). A panel of positive strains including *L. plantarum*, *L. fermentum*, *L. brevis*, *L. buchneri*, and *Lactobacillus reuteri* was retained for assay of α-Gal activity. *L. fermentum* CRL251 and CRL722 showed the highest α-Gal activities. These two strains and the widely studied model strain *L. plantarum* ATCC 8014 were selected for further studies.

Organization and sequence analysis of the galactosidase gene cluster. The three α-Gal+ strains, *L. fermentum* CRL722 and CRL251 and *L. plantarum* ATCC 8014, were probed for the presence of the gene for α-Gal using PCR with degenerate oligonucleotides. In *L. plantarum* only, an amplicon of 800 bp was obtained using P2-Pal7R primers, and we decided to characterize the gene for α-Gal in this bacterium. Sequence analysis of the PCR product showed high homology with the genes for α-Gal from various microorganisms. Using uneven and inverse PCR techniques, we walked the genome of *L. plantarum* on both sides of this fragment. Analysis of the sequence revealed the gene organization illustrated in Fig. 1. The central gene encoding α-Gal was designated *melA*, according to the nomenclature in *E. coli* (*melA* stands for melibiase). Upstream of *melA*, a complete ORF (*rafP*) corresponding to a putative raffinose transporter and a partial ORF (*galM*) similar to the microbial galactose-1-epimerase (mutarotase) gene were identified. Inspection of the sequence downstream of *melA* revealed the presence of two putative genes on the opposite strand that encode the two subunits of the heterodimeric β-galactosidase (*lacL* and *lacM*).

(i) The *melA* structural gene for α-Gal. The 2,214-bp *melA* gene encodes a 738-amino-acid polypeptide with a deduced molecular weight of 84 kDa and an isoelectric point of 5.12. Based on its amino acid sequence, MelA belongs to family 36 of the glycosyl hydrolases according to the Henrissat classification (25, 26). Prediction of protein localization was performed using the Psort program from the ExPASy server. Neither a signal peptide nor a transmembrane segment was identified, suggesting that MelA is located in the cytoplasm. MelA and other α-Gals from family 36 of glycosyl hydrolases show strong conservation (>75%) in their central domain (Fig. 2).

Inspection of the sequence at the 5′ end of *melA* revealed a potential promoter sequence and a putative Shine-Dalgarno sequence (GGAAG) 8 nucleotides upstream of the ATG start codon of *melA*. Downstream of *melA*, inverted repeats were detected, indicative of a stem-loop structure with a free-energy change (ΔG_0) value of −17 Kcal/mol. This structure could act as a transcriptional terminator for both the *melA* and the *lacLM* genes since it is preceded by a stretch of A bases and followed by a stretch of T bases (see below).

(ii) The *rafP* gene for a raffinose transporter. The *rafP* gene specifies a 652-residue protein with a calculated molecular weight of 73 kDa. No putative signal peptide was detected in the RafP sequence, while 9 to 12 transmembrane segments (according to the prediction program used) are predicted, suggesting that it is an integral membrane protein. RafP exhibits a high level of homology with the raffinose transporter of *P. pentosaceus* (71% similarity) (GenBank sequence accession no

Gene Organism

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melA  L.plan     AAPLGIEMFVLDDGWFGIRNDDNSSLGDWIVN   394
aga1  P.pent     AKQLGIEMFVLDDGWFGIRIDDNSSLGDWQVI   388
agaN  B.stea     AAELGIELFVLDDGWFGIKRIDDHSSLGDWIVN   386
melA  T.etha     AKDLGIEIFVLDDGWFGIKRNSDNSSLGDWIVN   283
agaA  C.pisc     ISSALGIEIFVLDDGWFGIKRNSDKISLGDWEIN   386
rafA  E.coli     AAALGVIERFIIDDGWFIKGRNDDRAALGDWYTI   354
aga   B.adol     AVESGIVIERFVIDDGWFGARRDDTIGLGDWQIS   400
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FIG. 2. Multiple alignment analysis of putative active sites of α-Gal sequences from microorganisms producing family 36 glycosyl hydrolases. Sequences from *Lactobacillus plantarum* ATCC 8014 (*melA L.plan*), *Pediococcus pentosaceus* (*aga1 P.pent*), *Bacillus stearothermophilus* (*agaN B.stea*), *Thermoanaerobacter ethanolicus* (*melA T.etha*), *Carnobacterium piscicola* (*agaA C.pisc*), *Escherichia coli* (*rafA E.coli*), and *Bifidobacterium adolescentis* (*aga B.adol*) are shown. The gene denomination precedes the species acronym at the beginning of each line. The last amino acid number is at the right end of each sequence.

L32093/Z32771). Also, RafP shows high homology (>70% similarity) with and a structural organization similar to that of LacS, the bifunctional lactose and α-galactoside transporter from *Streptococcus thermophilus* (44). Similar proteins have been found for *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus salivarius*, and *Leuconostoc lactis* (34, 53, 54).

Inspection of the sequence upstream of the *rafP* gene revealed a putative promoter sequence and a consensus catabolite responsive element (CRE) located between the −10 and −35 boxes (Fig. 1). This organization suggests that this gene is subject to catabolic repression (27). A potential Shine-Dalgarno sequence (GAGG) is located 7 bp upstream of the ATG start codon. Downstream of *rafP* and just upstream of the −35 box of the potential *melA* promoter, the sequence shows a putative mRNA transcriptional terminator consisting of a stem-loop structure with a ΔG_0 of −12.6 Kcal/mol followed by a stretch of T.

(iii) The *lacL* and *lacM* genes for β-galactosidase. Downstream of *melA* and located on the opposite strand, the overlapping β-Gal genes *lacM* and *lacL* were identified. They showed 98% similarity with the LacL and LacM subunits of β-Gal from *Leuconostoc lactis* (14) (GenBank accession no. Q02603), 77% similarity with *lacLM* genes from *Lactobacillus acidophilus* (GenBank accession no. BAA20536/O07684), and 74% similarity with those from *Lactobacillus sakei* (41) (GenBank accession no 2208404A).

The *L. plantarum* ATCC 8014 *melA* gene can be highly expressed in *E. coli*. A 2,690-bp PCR fragment containing the *melA* gene from *L. plantarum* ATCC 8014 including the putative *rafP* terminator, *melA* promoter, and *melA* terminator was cloned in pBS in the same orientation as the *lacZ* promoter, yielding the plasmid p1α (Fig. 1). To determine whether the *melA* gene from *L. plantarum* ATCC 8014 was active in *E. coli*, p1α was introduced in the α-Gal⁻ *E. coli* RA11r strain and grown in M9mel. In contrast with the native strain, *E. coli* RA11r(p1α) showed normal growth in M9mel, suggesting that *melA* is functional in *E. coli* (data not shown). In a second step, cultures of *E. coli* RA11r(pBS) and *E. coli* RA11r(p1α) were grown in the enriched EM9mel medium, allowing the growth of both strains (Fig. 3). Monitored α-Gal activity showed no

α-Gal production in *E. coli* RA11r(pBS) and an α-Gal production kinetics in *E. coli* RA11r(p1α) that paralleled the growth curve (Fig. 3). Interestingly, α-Gal production reached amounts about 15 times higher than those found with the native producer *L. plantarum* ATCC 8014 (Fig. 3; compare with Fig. 6). This increase could be partly due to a gene dosage effect, since p1α that derives from pBS is a high-copy-number plasmid. The high level of expression strongly suggests that the putative promoter sequence identified upstream of *melA* is active in *E. coli*. The observed high expression levels of *melA* were obtained in cultures without the inducer isopropyl-β-D-thiogalactopyranoside. In addition, p1α contains the putative *rafP* terminator located upstream of the *melA* promoter sequence, and we presume that this terminator would arrest any transcript synthesized from the *lacZ* promoter. Altogether, these observations strongly suggest that the *L. plantarum* ATCC 8014 *melA* gene is functional and that it is transcribed from its own promoter.

Transcriptional analyses of the *melA* and *rafP* genes of *L. plantarum* ATCC 8014. mRNA from *L. plantarum* ATCC 8014 was analyzed by both 5′ RACE and Northern blotting. The 5′RACE experiment was performed using reverse primers
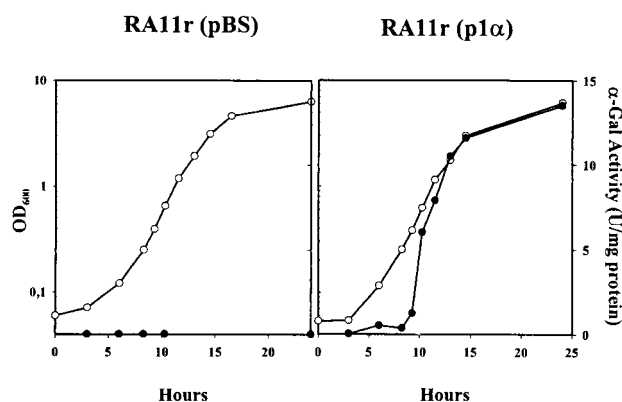

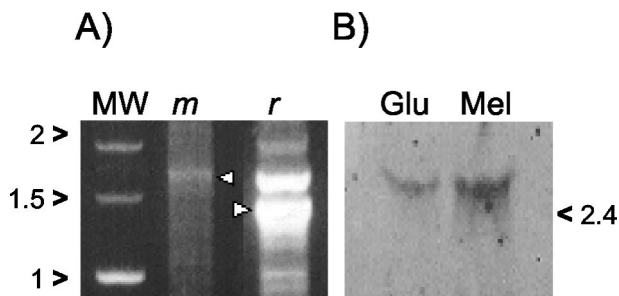FIG. 3. Growth (white) in EM9mel at 30°C and α-Gal activity (black) of α-Gal⁻ *E. coli* RA11r(pBS) and RA11r(p1α).

FIG. 4. (A) RNA analysis by 5′RACE experiment. PCR fragments were amplified from cDNA using internal primers for the *rafP* gene (r) or the *melA* gene (m). Arrowheads indicate specific products. MW, Marker Smart Ladder (Eurogentec). (B) Northern blot analysis of *L. plantarum* ATCC 8014 grown in glucose (Glu) or melibiose (Mel) using an internal *melA* fragment as a probe. Migration of molecular weight standards is indicated on the right edge of the figure.

Pal17 and 5AR, located 1.4 and 1.8 kb downstream of the putative promoter sequences of *rafP* and *melA*, respectively. Major amplicons of 1.4 kb (*rafP*) and 1.8 kb (*melA*), respectively, were obtained (Fig. 4A). These correspond in size to monocystronic mRNA. In the case of *rafP*, additional amplicons of greater and lesser size were detected. The larger amplicon could be due to unspecific annealing of the *rafP* primer, or it could reflect the presence of polycistronic mRNA. The smaller amplicons could be due to the presence of degradation products.

In order to assess whether the *melA* gene is regulated in *L. plantarum* ATCC 8014, mRNA was hybridized with a *melA* probe generated by labeling a 535-bp PCR fragment. An approximately unique 2.5-kb band corresponding to the *melA* transcript was detected irrespective of whether glucose or melibiose was used (Fig. 4B). This result confirms that *melA* is transcribed from its own promoter. Interestingly, *melA* transcription was induced at a level of approximately fourfold when *L. plantarum* ATCC 8014 was grown with melibiose compared to results with glucose.

Protein analyses. Comparison of the SDS-PAGE profiles from *E. coli* RA11r(p1α) with that of *E. coli* RA11r(pBS) (negative control) showed similar patterns except for one protein band migrating around 80 kDa that was absent in the negative control (Fig. 5). Most probably, this band represents α-Gal whose molecular weight deduced from the amino acid sequence is 84 kDa. Quantitation of α-Gal estimated by comparison with known amounts of bovine serum albumin indicates that the expression level is around 10 mg/liter of culture at an OD_{600} of 1 (data not shown).

The molecular weight of native α-Gal was assessed from cell extracts of *L. plantarum* ATCC 8014. Using ultrafiltration units with a 100-kDA cutoff, all α-Gal activity was recovered in the retentate, suggesting a molecular weight of more than 100 kDa for α-Gal (data not shown). Using units with a 300-kDa cutoff, only half the activity was retained by the membrane, suggesting that α-Gal occurs as oligomers. This hypothesis was supported by zymogram analysis that showed that MelA monomers are inactive (data not shown).

Galactosides induce α-Gal activity in *L. plantarum* ATCC 8014. The α-Gal activity was assessed for *L. plantarum* ATCC 8014 grown with various sugars at 30 and 37°C. With all sugars,
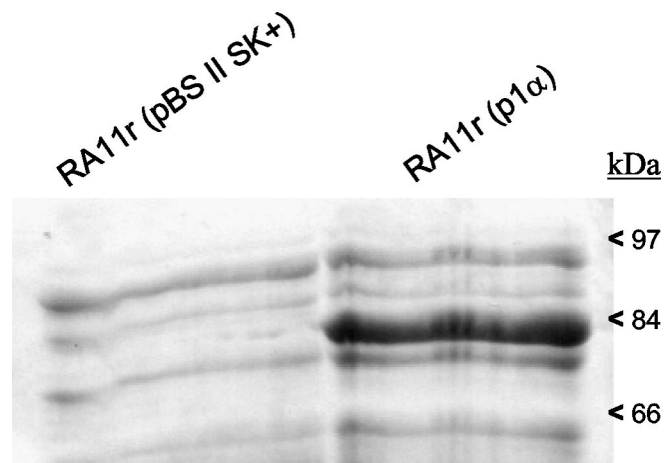

FIG. 5. SDS-PAGE analysis of total protein extracts from *E. coli* RA11r(p1α) expressing the *L. plantarum melA* gene. *E. coli* RA11r(pBS) was used as a negative control. The positions of molecular size standards are indicated on the right edge of the figure.

α-Gal activity in cultures of *L. plantarum* ATCC 8014 grown at 30°C was higher than in those at 37°C (Fig. 6). Apart from glucose, all other sugars used induced α-Gal activity. Raffinose exhibited the strongest induction, followed by galactose, melibiose, and lactose. The relative induction levels for the different galactosides were similar at 30 and 37°C.

DISCUSSION

LAB have long been used as starters in food fermentation, where they exert technologically important functions such as acidification, aroma formation, and modification of rheological properties of raw agricultural products. As such, LAB have
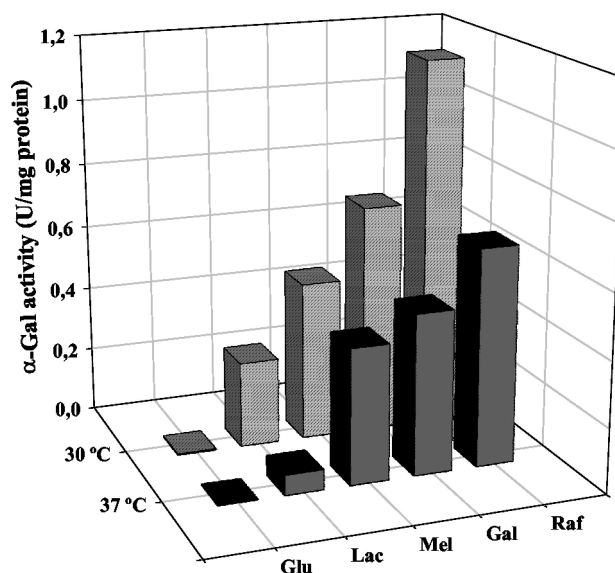

FIG. 6. α-Gal activity in cell extracts of *L. plantarum* ATCC 8014 grown at 30 or 37°C in MRS plus glucose (Glu), lactose (Lac), melibiose (Mel), galactose (Gal), and raffinose (Raf) until reaching an OD_{600} of 1.5.

been widely studied in the fields of physiology and genetics. The impressive amount of knowledge acquired on these bacteria now opens the way to new applications, one of which is the production by LAB of compounds beneficial to health. A priori, LAB are excellent candidates to deliver such compounds in the gastrointestinal tract of humans and animals, since these bacteria are commonly ingested along with food products, and some, including lactobacilli, can survive in the gut, where they could exert a given biological activity.

The production of α-Gal by LAB is a biological activity whose delivery in the gastrointestinal tract is of potential interest since neither humans nor monogastric animals are able to synthesize digestive α-Gal. Since lactobacilli had been reported to exhibit α-Gal activity and given that some lactobacilli are part of the native intestinal microflora, we focused this work on α-Gals of lactobacilli. Upon probing the α-Gal-encoding gene using degenerate primers, the α-Gal structural gene of *L. plantarum* ATCC 8014 could be amplified, and it was designated *melA*. The *melA* gene is present as one unique copy in *L. plantarum* ATCC 8014 as indicated by Southern blot analysis using an internal *melA* fragment as a probe (data not shown). However, we cannot rule out the existence of additional α-Gals with different sequences in *L. plantarum* ATCC 8014.

DNA sequence analysis of the *melA* locus in *L. plantarum* ATCC 8014 identified five putative genes, *galM-rafP-melA-lacM-lacL*. This result shows that the genes required for the utilization of both α- and β-galactosides are in close vicinity. *C. piscicola*, a LAB previously classified as *Lactobacillus carnosus*, is the only other LAB for which clustered α- and β-Gals have been characterized so far. In contrast with *L. plantarum* ATCC 8014, all genes are on the same strand (10, 11). In several thermophilic bacteria, such as *Thermus* strain T2 (31), *Bacillus stearothermophilus* KVE39 (19), *Thermoanaerobacter ethanolicus* (GenBank sequence accession no. Y08557; unpublished data), and *Thermotoga maritima* (35), clustered α- and β-Gals have also been identified.

The structural *melA* gene for α-Gal of *L. plantarum* ATCC 8014 encodes an 84-kDa protein belonging to family 36 glycosyl hydrolases. This family is one of the three glycosyl hydrolase families defined for α-Gals, and it gathers α-Gals from eucaryotic and prokaryotic sources. BLAST and ClustalW analyses show high homology of α-Gals in their core domain (Fig. 2). Probably this region makes part of the active site of the enzymes, but this has not yet been demonstrated. In contrast with plant and yeast α-Gals that belong to family 27 and that can occur as monomers, the bacterial α-Gals so far characterized occur as oligomers (35). Analysis of the *melA* gene product using PSORT software from ExPASy did not show the presence of sorting signals (characteristic signal peptide or transmembrane segment), suggesting that MelA occurs as a soluble enzyme in the cytoplasm of *L. plantarum* ATCC 8014. This is consistent with MelA extraction experiments that showed that MelA was present in cell lysates from *L. plantarum*. This cytoplasmic localization of MelA implies that the degradation of α-galactosides in *L. plantarum* relies on the presence of a galactoside transporter.

A putative raffinose transporter, RafP, was identified for *L. plantarum* ATCC 8014 that could transport both α- and β-galactosides in *L. plantarum* as suggested by its homology with the

unique galactoside transporter LacS of *S. thermophilus*. LacS has affinity not only for β-galactosides and galactose but also for melibiose and to a lesser extent for raffinose (17, 43, 44). LacS functions both as a lactose-galactose antiporter when sugars are in excess at either side of the membrane and as a proton symport system. It has an amino-terminal domain with homology to the melibiose carrier of various microorganisms and a carboxy-terminal domain with homology to domain I of enzyme EIIA of various phosphoenolypyruvate phosphotransferase systems (PEP-PTS) from gram-positive and -negative organisms (44). It will be of great interest to determine which type of α-galactosides can be transported by RafP and also if this is an active transporter or a PTS transporter. In particular, the transport of stachyose and raffinose, the two major α-galactosides of soy, should be assessed.

Expression and transcriptional studies demonstrate that both *melA* and *rafP* are expressed in *L. plantarum* ATCC 8014. The *melA* gene is transcribed from its promoter, yielding a monocistronic RNA. Therefore, the *melA* gene is not part of an operon, in contrast to *melA* genes in other microorganisms, e.g., *E. coli* (4), *Klebsiella pneumoniae* (23), *Enterobacter cloacae* (42), *Citrobacter freundii* (50), *P. pentosaceus* (GenBank sequence accession no L32093, unpublished data; inferred from the sequence data), *T. maritima* (35), and *Thermoanaerobacter ethanolicus* (GenBank accession no. Y08557, unpublished data; inferred from the sequence data). The *melA* gene is regulated at the transcriptional level, as shown by the fourfold increase of *melA* transcript in the presence of melibiose compared with that in the presence of glucose. However, *melA* is not totally repressed in the presence of glucose, and this feature together with the absence of a CRE site in the *melA* promoter region suggests that *melA* regulation is mediated by an induction mechanism rather than by catabolite repression (27).

The assessment of MelA activity in cultures of *L. plantarum* showed important differences according to the carbohydrate used. Interestingly, MelA activity in melibiose-containing cultures was more than 150-fold higher than that in glucose-containing cultures. This ratio is much higher than the ratio of 4 observed at the transcriptional level. These results suggest that posttranscriptional regulation could also occur. It could result from allosteric control mechanisms that have been shown to modulate the activity of glycolytic enzymes in LAB (20).

The 5′ RACE experiment showed that *rafP* is also expressed as a monocistronic mRNA, indicating the functionality of its promoter. However, due to the limitation of the 5′ RACE methodology at the PCR amplification step, this should be confirmed by Northern blot analysis, and we cannot rule out at the moment that *rafP* could also be part of a larger operon.

In view of future applications in the probiotic field, we decided to investigate the influence of different carbohydrates on the α-Gal activity at both 30°C (the optimal growth temperature for *L. plantarum*) and 37°C (the human body temperature). MelA activity assayed in cultures grown with various carbohydrates showed that lactose, melibiose, galactose, and raffinose increase MelA activity in *L. plantarum* ATCC 8014 (lactose and raffinose showed the lowest and highest increase, respectively). This suggests an activation at the enzyme level or an induction at the gene level. The same order in MelA activity

was observed in cultures assayed at 30°C and at 37°C, although the level of activity was lower at 37°C than at 30°C. Although we cannot rule out that α-Gal is thermosensitive at 37°C, this result together with the observation of Tamura and Matsushita (52), who showed a decrease of both lactose and melibiose uptake at 37°C from that at 30°C for *L. plantarum* ATCC 8014, suggests that two types of regulation occur: (i) one at the transporter level, and this regulation would be thermosensitive; and (ii) another at the α-Gal level in response to the carbon source that is similar at 30 and 37°C.

In the course of this study, *galM* and *lacLM* were identified. The presence of *galM* that specifies a mutarotase suggests that *rafP* is preceded by a galactose operon, since *galM* is the last 3′ gene of the *gal* operon in various lactobacilli (*Lactobacillus helveticus galKTM* [GenBank accession no. X57248] [39], *L. sakei galETM* [GenBank accession no. AF401040; unpublished data], and in *Lactobacillus casei* 64H *galKETRM* [GenBank accession no. AF005933] [5]) as well as in *S. thermophilus* (GenBank accession no. U61402) (55) and in *S. salivarius* (GenBank accession no. AF389474) (53) (*galR-galKTE-galM*). The *lacLM* genes identified in this study specify a heterodimeric β-galactosidase, the main enzyme involved in lactose utilization (29). Although *lacLM* genes have been detected both in plasmids (36) and on the chromosome (16) in *L. plantarum*, the sequence described here corresponds to a chromosomal region, since no amplification was obtained using plasmid DNA as a template (data not shown). Our observation confirms that of Fernandez et al. (16), and this establishes that *L. plantarum* ATCC 8014 *lacLM* genes are present on the chromosome. Altogether, these data show that the genes involved in galactosides utilization are clustered in the same locus in *L. plantarum* ATCC 8014.

This work is the first step in the characterization of a *melA* gene involved in α-galactoside catabolism in lactobacilli. Further studies should now focus on the biochemical characterization of MelA. It is also of interest to investigate RafP functionality, its regulation aspect, and its substrate specificity. Control of MelA activity in lactobacilli should open doors to interesting applications. One would be to develop starters for soymilk fermentation, since there is actually a huge demand for this kind of product devoid of undesirable side effects. Also, the development of probiotic bacteria is foreseen to deliver α-Gal in the digestive tract of humans and animals. The objective is to deliver α-Gal in the duodenum so that α-galactosides can be degraded at this level. First, this would address the problem of flatulence associated with the presence of α-galactosides in the lower intestine. Second, this could increase the metabolizable energy of-soy derived products since the microflora of the lower gut would then utilize other carbohydrate substrates, such as fibers (12).

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