

# Regulation of *Lactobacillus casei* Sorbitol Utilization Genes Requires DNA-Binding Transcriptional Activator GutR and the Conserved Protein GutM<sup>∇</sup>

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Sequence analysis of the five genes (*gutRM**CBA*) downstream from the previously described sorbitol-6-phosphate dehydrogenase-encoding *Lactobacillus casei* *gutF* gene revealed that they constitute a sorbitol (glucitol) utilization operon. The *gutRM* genes encode putative regulators, while the *gutCBA* genes encode the EIIC, EIIBC, and EIIA proteins of a phosphoenolpyruvate-dependent sorbitol phosphotransferase system (PTS<sup>Gut</sup>). The *gut* operon is transcribed as a polycistronic *gutFRMCBA* messenger, the expression of which is induced by sorbitol and repressed by glucose. *gutR* encodes a transcriptional regulator with two PTS-regulated domains, a galactitol-specific EIIB-like domain (EIIB<sup>Gat</sup> domain) and a mannitol/fructose-specific EIIA-like domain (EIIA<sup>Mtl</sup> domain). Its inactivation abolished *gut* operon transcription and sorbitol uptake, indicating that it acts as a transcriptional activator. In contrast, cells carrying a *gutB* mutation expressed the *gut* operon constitutively, but they failed to transport sorbitol, indicating that EIIBC<sup>Gut</sup> negatively regulates GutR. A footprint analysis showed that GutR binds to a 35-bp sequence upstream from the *gut* promoter. A sequence comparison with the presumed promoter region of *gut* operons from various firmicutes revealed a GutR consensus motif that includes an inverted repeat. The regulation mechanism of the *L. casei* *gut* operon is therefore likely to be operative in other firmicutes. Finally, *gutM* codes for a conserved protein of unknown function present in all sequenced *gut* operons. A *gutM* mutant, the first constructed in a firmicute, showed drastically reduced *gut* operon expression and sorbitol uptake, indicating a regulatory role also for GutM.

Sorbitol, also called glucitol, is found in many fruits in nature and is widely used in the food industry as a sweetener, humectant, texturizer, and softener (25). Besides, a probiotic effect has been demonstrated for this hexitol (22), which would be important for the development of functional foods. Utilization of sorbitol as a carbon source has been described for some bacteria, including *Escherichia coli* (30), *Erwinia amylovora* (1), *Clostridium beijerinckii* (27), *Streptococcus mutans* (2), and *Lactobacillus casei* (32). All these organisms rely on the phosphoenolpyruvate:sugar phosphotransferase system (PTS) for the transport of sorbitol into the cell. The PTS consists of the general phosphotransferase proteins enzyme I (EI) and HPr and carbohydrate-specific transporters (EII). The EI auto-phosphorylates at a histidine residue by using phosphoenolpyruvate and then transfers the phosphoryl group to HPr, which becomes phosphorylated at the conserved histidine-15 residue. P~His-HPr (where the P~ prefix stands for phospho) functions as a phosphoryl donor for the different PTS transporters, which usually consist of three different proteins or domains: the cytoplasmic domains EIIA and EIIB and the transmembrane transporter EIIC (20). The EIIs responsible for sorbitol uptake (EII<sup>Gut</sup>) have an unusual domain structure,

consisting of EIIC, EIIBC, and EIIA, where EIIC corresponds only to the amino-terminal part of the EIIC domain and the carboxy-terminal half is fused to the EIIB domain (1, 2, 27).

The enzymes responsible for sorbitol transport and metabolism are encoded by the *gut* operon. This operon includes the genes encoding the EII domains of the PTS involved in the transport and phosphorylation of sorbitol to sorbitol-6-phosphate, the gene encoding sorbitol-6-phosphate dehydrogenase, which converts sorbitol-6-phosphate to fructose-6-phosphate, and two genes encoding regulatory proteins (*gutM* and *gutR*). The role of these regulators in *E. coli* has been studied, and a complex but yet unknown regulatory mechanism, where GutM is an activator and GutR a repressor, has been suggested (30). In *E. coli*, *gutM* and *gutR* are located downstream from the EII and sorbitol-6-phosphate dehydrogenase-encoding genes. Constitutive transcription of *gutR* occurs from its own promoter (monocistronic mRNA), whereas inducible transcription starts at the *gut* promoter upstream from the EIIC-encoding gene (polycistronic mRNA). It has been suggested that the ratio of GutR and GutM might determine the extent of *gut* operon expression (30). In firmicutes, the *gut* operons analyzed so far also contain a *gutM* homologue, but the putative regulator GutR differs from *E. coli* GutR. The activity of *E. coli* GutR is controlled by a sorbitol-6-phosphate-binding domain, but GutR from firmicutes contains PTS regulated domains (PRDs) and a mannitol/fructose-specific EIIA-like domain (EIIA<sup>Mtl</sup> domain) (8, 26). GutRs of firmicutes were therefore thought to be controlled via PTS-mediated phosphorylation,

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an assumption which so far has not been experimentally confirmed (2, 27). An exception is the presumed *gut* operon of *Listeria*, which contains no *gutR* but which contains *gutM* (Imo0545/lin0549) that is neither of the *E. coli* nor of the firmicute type.

The *L. casei* *gutF* gene encoding the sorbitol-6-phosphate dehydrogenase involved in sorbitol metabolism has previously been cloned and characterized (32). Sequence analyses of the region downstream from *gutF* revealed the presence of five genes, *gutR*, *gutM*, *gutC*, *gutB*, and *gutA*. The genes *gutR*, *gutM*, and *gutB* were disrupted, and their role in *gut* operon transcriptional regulation and sorbitol uptake was studied. Additionally, GutR and GutM were overproduced and purified from *E. coli*, allowing for the first time the identification of the DNA-binding site of a PRD-containing GutR regulator within the *gut* promoter region.

#### MATERIALS AND METHODS

**Bacterial strains, culture conditions, and plasmids.** The strains and plasmids used in this study are listed in Table 1. The *L. casei* strains (ATCC 334 and BL23 and its various *gut* mutant derivatives) were grown at 37°C under static conditions on MRS medium (Oxoid) or MRS fermentation medium (Adsa-Micro) supplemented with 0.5% of the appropriate sugar, as indicated in the figure legends and text. *E. coli* and *Lactococcus lactis* strains, which were used as hosts in cloning experiments, were grown in Luria-Bertani medium at 37°C and M17 medium (Oxoid) plus 0.5% glucose at 30°C, respectively. The corresponding solid media were prepared by adding 1.5% agar. *E. coli* transformants were selected with ampicillin (50 µg/ml), and *L. lactis* and *L. casei* transformants were selected with erythromycin (5 µg/ml).

Vector pRV300 (15) was used for cloning experiments with *E. coli* and for insertional inactivation of genes in *L. casei*. Vector pTINX (23) was used for cloning experiments with *L. lactis* and for expression of GutM and GutR in *L. casei*. *E. coli* M15(pREP4) was used for the synthesis of His-tagged GutR and His-tagged GutM. *E. coli* and *L. lactis* strains were transformed by electroporation with a Gene Pulser apparatus (Bio-Rad Laboratories) as recommended by the manufacturer (*E. coli*) or as described earlier (*L. lactis*) (11). *L. casei* strains were transformed as previously described (19).

**DNA manipulation, oligonucleotides, and sequencing.** The oligonucleotides used in this study are listed in Table 2. Total DNA was isolated from *L. casei* BL23 as described before (19). Recombinant DNA techniques were performed by following standard procedures (21). All PCRs were performed with an Expand High Fidelity PCR system (Roche), which contains an enzyme mix of *Taq* and *Tgo* DNA polymerases. DNA sequencing was carried out by the Central Service of Research Support of the University of Valencia (Spain) by using the dideoxynucleotide DNA chain termination method. M13 universal and reverse primers or custom primers hybridizing within the appropriate DNA fragments were used for sequencing. Sequence analyses were carried out with DNAMAN 4.0 for Windows (Lynnon BioSoft), and sequence similarities were analyzed with the BLAST program.

**Construction of a *gutB* mutant strain.** A DNA fragment containing 253 bp of the 3' end of *gutC* and 314 bp of the 5' end of *gutB* was amplified by PCR using *L. casei* BL23 chromosomal DNA and primers *gutbc1* and *gutbc2*. Another DNA fragment, containing 321 bp of the 3' end of *gutB* and 310 bp of the 5' end of *gutA*, was amplified with primers *gutbc3* and *gutbc4*. The two DNA fragments with blunt ends were cloned successively in the appropriate orientation in the integrative vector pRV300. The resulting plasmid, pRVB1, was used for single integration into *L. casei* BL23. Subsequently, one of the integrants was selected and grown without erythromycin for approximately 200 generations to facilitate the second recombination. One of the mutants with a deleted *gutB* region was selected and named BL282. The deletion was confirmed by PCR analysis and DNA sequencing.

**Construction of *gutR* and *gutM* mutant strains.** DNA fragments containing part of *gutR* or *gutM* were obtained by PCR using *L. casei* BL23 chromosomal DNA. A *gutR* fragment was obtained with the primers *gutF1* and *gutR1*; the *gutM* fragment was obtained with primers *gutR2* and *gutC1*. These DNA fragments were cloned in the integrative vector pRV300 digested with *Sma*I and *Eco*RV, giving pRVR1 and pRVM4, respectively. These plasmids contain a unique *Pst*I restriction site present in the *gutR* and *gutM* coding regions. pRVR1 and pRVM4

TABLE 1. Strains and plasmids used in this study<sup>a</sup>

Strain or plasmid	Genotype or properties	Source or reference
<i>L. casei</i>		
BL23	Wild type	CECT 5275
BL282	BL23 <i>gutB</i> (480-bp deletion in <i>gutB</i> )	This work
BL285	BL23 <i>gutR</i> (frameshift at <i>Pst</i> I restriction site)	This work
BL286	BL23 <i>gutM</i> (frameshift at <i>Pst</i> I restriction site)	This work
BL291	BL286 containing pT1NgutM; <i>Erm</i> <sup>r</sup>	This work
BL307	BL285 containing pT1NgutR; <i>Erm</i> <sup>r</sup>	This work
ATCC 334	Wild type; carries an insertion element in <i>gutR</i>	ATCC
<i>L. lactis</i>		
MG1363	Plasmid- and phage-free derivative of NCDO712	9
<i>E. coli</i>		
DH5a	F <sup>-</sup> <i>endA1 hsdR17 gyrA96 thi-1 recA1 relA1 supE44 DlacU169</i> (F80 <i>lacZ</i> DM15)	Stratagene
M15(pREP4)	<i>Nal</i> <sup>s</sup> , <i>Str</i> <sup>s</sup> , <i>Rif</i> <sup>r</sup> , <i>Thi</i> <sup>-</sup> , <i>Lac</i> <sup>-</sup> , <i>Ara</i> <sup>+</sup> , <i>Gal</i> <sup>+</sup> , <i>Mtl</i> <sup>-</sup> , <i>F</i> <sup>-</sup> , <i>RecA</i> <sup>+</sup> , <i>Uvr</i> <sup>+</sup> , <i>Lon</i> <sup>+</sup>	Qiagen
Plasmids		
pRV300	Suicide vector carrying <i>Erm</i> <sup>r</sup> from pAMb1	15
pRVB1	pRV300 with a fragment carrying a deletion in <i>gutB</i>	This work
pRVR1	pRV300 with a frameshift at <i>Pst</i> I site in <i>gutR</i>	This work
pRVM4	pRV300 with a frameshift at <i>Pst</i> I site in <i>gutM</i>	This work
pTINX	Contains the P1 constitutive promoter; <i>Erm</i> <sup>r</sup>	23
pT1NgutM	pTINX containing <i>gutM</i> -encoding region	This work
pT1NgutR	pTINX containing <i>gutR</i> -encoding region	This work
pQE30	<i>Amp</i> <sup>r</sup>	Qiagen
pQEGutR	pQE30 containing <i>gutR</i> -encoding region	This work
pQEGutM	pQE30 containing <i>gutM</i> -encoding region	This work

<sup>a</sup> CECT, Colección Española de Cultivos Tipo; ATCC, American Type Culture Collection; *Amp*<sup>r</sup>, ampicillin resistant; *Erm*<sup>r</sup>, erythromycin resistant; *Nal*<sup>s</sup>, nalidixic acid sensitive; *Str*<sup>s</sup>, streptomycin sensitive; *Rif*<sup>r</sup>, rifampin sensitive.

were digested with *Pst*I, and the 3'-protruding ends were removed with the exonuclease activity of the Klenow fragment of DNA polymerase I. The plasmids were subsequently self-ligated to introduce a frameshift into each gene, which was confirmed by DNA sequencing. These constructs were used to transform *L. casei* BL23. One integrant of each gene was grown for 200 generations on medium without antibiotics to facilitate the second recombination, which would excise the vector. Two mutant strains were obtained, one with a frameshift in *gutR* (named BL285) and the other with a frameshift in *gutM* (named BL286). The two frameshifts were confirmed by PCR analysis, using as a template *L. casei* BL285 and BL286 chromosomal DNA, respectively, followed by DNA sequencing.

**Complementation of *gutR* and *gutM* mutants.** The coding regions of *gutR* and *gutM* were amplified by PCR using *L. casei* BL23 chromosomal DNA as a template. The *gutR* gene was obtained with the primers *gutRBgII* and *gutRSpeI*, and the *gutM* gene was obtained with primers *gutMBgII* and *gutMSpeI*. The amplified DNA fragments were digested with *Bgl*II and *Spe*I and cloned into the replicative vector pTINX previously digested with the same enzymes, giving

TABLE 2. Oligonucleotides used in this study

Function and name	Sequence <sup>a</sup>	Location <sup>b</sup>	Utilization
<b>Construction of mutants</b>			
gutbc1	CTGCCATACCAGCAATGGC	-285	Cloning <i>gutB</i> with an internal 480-bp deletion
gutbc2	TGCCAGCGGACCAGACTTC	+315	
gutbc3	TATCGTCATCGGCTTTATCTG	+796	
gutbc4	ATCCTGCGAGCTCAGGTG	+1455	
gutF1	GCAAGTAAAGGCATC	-350	Cloning part of <i>gutR</i> to create a frameshift at PstI restriction site (codon 48)
gutR1	TGGTGAATCGTCCATCAAG	+689	
gutR2	TGATGCTGGCAGAATCATC	-451	Cloning <i>gutM</i> to create a frameshift at PstI restriction site (codon 16)
gutC1	TGGAACAGCTTCATGAAGC	+574	
<b>Complementation of mutants</b>			
gutRBglII	CATAAGATCTGGGAGGTAACCTG	-24	Cloning <i>gutR</i> in pT1NX
gutRSpeI	TATCAACTAGTTCAITTTAAAGTCCCTC	+1884	
gutMBglII	CCTTAACAGATCTCACGGAGGGAC	-29	Cloning <i>gutM</i> in pT1NX
gutMSpeI	CATCTACTAGTTTAAATTGTATTC	+522	
<b>Transcription analysis</b>			
ribu1	TATTATGGGCAGGATAATGC	-302	DNA probe (the primer location is indicated with respect to <i>gutF</i> )
gutF3	GCCGTAAACAACCGTAGCG	+117	
gut3	GACTTTAAGGACCCTAA	+133	RT-PCR analysis (the primer location is indicated with respect to <i>gutF</i> )
gutbc4	ATCCTGCGAGCTCAGGTG	+5277	
gut5	TTTTCAACTTCGTCAGGATC	+197	Identification of the transcription start site of the <i>gut</i> operon (the primer location is indicated with respect to <i>gutF</i> )
gutF3	GCCGTAAACAACCGTAGCG	+117	
<b>Overexpression of genes</b>			
gutRBamHI	GGGAGGGATCCGATTGAATAG	-14	Cloning <i>gutR</i> in pQE30
gutRSalI	CAAAACGTCGACTTAAAGTCCC	+1881	
gutMBamHI	CGGAGGGATCCAAAATGAACG	-14	Cloning <i>gutM</i> in pQE30
gutMHindIII	CTCCTAAGCTTAAATGTATTC	+519	
<b>Gel mobility shift and DNase I footprinting</b>			
gut8D	GTCAAGCACCGCTAACC	-200	Amplified 179-bp DNA fragment of the <i>gut</i> promoter region (the primer location is indicated with respect to <i>gutF</i> )
gutF4	TTTCTGCTCTATGGTAGC	-22	

<sup>a</sup> Letters in italics indicate newly created restriction sites.

<sup>b</sup> The numbers indicate the position of the 5' end of the primer with respect to the start codon of the gene specified in the "Utilization" column.

pT1NgutR and pT1NgutM. These plasmids were used to transform *L. casei* strains BL285 and BL286, respectively. One transformant of each strain was selected, and the transformants were named BL307 (BL285[pT1NgutR]) and BL291 (BL286[pT1NgutM]).

**RNA isolation and Northern blot analysis.** RNA was isolated from *L. casei* cells grown to an optical density at 550 nm (OD<sub>550</sub>) of 0.8 in MRS fermentation medium containing 0.5% of the appropriate sugars. Cells extracts were prepared as previously described (31), and total RNA was isolated according to the protocol of the Trizol manufacturer. Sample preparation, denaturing agarose gel electrophoresis, and RNA transfer were performed by standard methods (21). The DNA probe for the *gut* operon was synthesized by PCR using *L. casei* BL23 chromosomal DNA as a template, primers ribu1 and gutF3, and digoxigenin DNA labeling mix (Roche).

**RT-PCR and RACE.** Reverse transcriptase PCR (RT-PCR) was carried out as previously described (31). RT reactions were performed with 2 µg total RNA isolated from *L. casei* BL23 cells grown on sorbitol, avian myeloblastosis virus RT (AMV-RT) (Sigma), and gutbc4 oligonucleotide. The subsequent PCR amplifications were performed with purified cDNA and primers gut3 and gutbc4. The transcription initiation of the *gut* operon was determined with a 5'/3' rapid amplification of cDNA ends (RACE) kit (Roche) by following the manufacturer's instructions. Total RNA isolated from *L. casei* BL23 cells grown on sorbitol and the primer gut5 were used for cDNA synthesis. The cDNA was dA tailed and then amplified by PCR using the primer oligonucleotide dT anchor supplied in the kit and the primer gutF3. The resulting PCR product was used in a second PCR with primer PCR anchor (supplied with the kit) and primer gutF3. The amplified DNA fragment of about 0.2 kb was purified and sequenced.

**Sorbitol uptake assays.** D-[<sup>14</sup>C]sorbitol uptake by whole cells of *L. casei* was performed according to the method described by Chassy and Thompson (5, 6).

BL23 (wild type), BL282 (*gutB*), BL285 (*gutR*), BL286 (*gutM*), BL291 (BL286[pT1NgutM]), and BL307 (BL285[pT1NgutR]) were grown in 10 ml of MRS fermentation medium supplemented with a mixture of 0.5% sorbitol and 0.5% ribose to an OD<sub>550</sub> of 0.8. Cells were prepared and incubated as previously described (31) and D-[<sup>14</sup>C]sorbitol (125 µM final concentration; 0.6 mCi/mmol) was added. The uptake rate was determined in the first 2 min of the reaction. One-milliliter aliquots were withdrawn and filtered through Millipore membranes (pore size, 0.45 µm). Filters were washed with cold buffer, and radioactivity was quantified by scintillation counting.

**Expression and purification of His-tagged GutR and GutM.** The coding regions of *gutR* and *gutM* were amplified by PCR using chromosomal DNA from *L. casei* BL23 as a template and appropriate primers (Table 2), which added restriction sites to the 5' and 3' ends. The PCR fragments of 1,881 bp and 509 bp, respectively, were cleaved with the restriction enzymes recognizing the sites added by the PCR and cloned into pQE30. The resulting plasmids, pQEGutR and pQEGutM, were used to transform *E. coli* M15(pREP4), and the correct sequence of the inserts was confirmed by DNA sequencing. One clone of each was grown in 0.5 liter of Luria-Bertani medium with ampicillin (100 µg/ml) and kanamycin (25 µg/ml) at 30°C with agitation. When the cultures reached an OD<sub>550</sub> of 0.5, isopropyl-β-D-thiogalactopyranoside (1 mM) was added and incubation was continued for 1 h. Cells were harvested by centrifugation and resuspended in ice-cold lysis buffer A (50 mM NaH<sub>2</sub>PO<sub>4</sub> [pH 7.5], 300 mM NaCl, and 10 mM imidazole) and buffer B (50 mM NaH<sub>2</sub>PO<sub>4</sub> [pH 7.5], 800 mM NaCl, 2.5 mM imidazole, 0.1% Tween 20, and 10 mM β-mercaptoethanol) for GutR and GutM purification, respectively. The suspensions were treated with 1 mg/ml lysozyme on ice for 10 min, quickly frozen in a dry ice/ethanol bath, thawed at 37°C, and then returned to ice. Cells were then sonicated, and the cell debris was removed by centrifugation at 12,000 × g for 20 min at 4°C. The cleared extracts

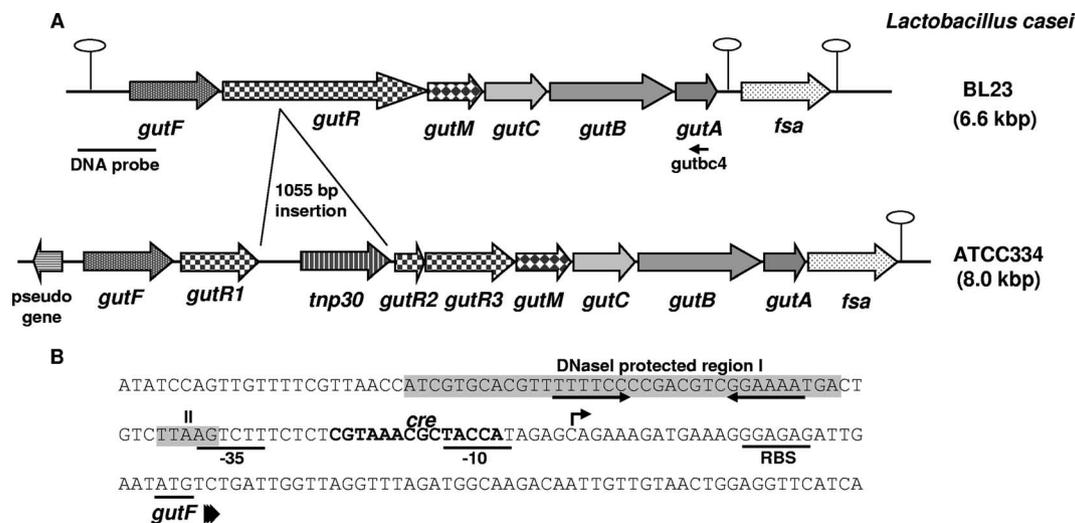


FIG. 1. (A) Genetic organization of the chromosomal locus containing the *gut* operon in *L. casei* strains BL23 and ATCC 334. Hairpin loops indicate putative *rho*-independent transcriptional terminators. *fsa* refers to fructose-6-phosphate aldolase. The positions of the DNA probe used in Northern blot experiments and primer *gutbc4* used in RT-PCR analysis are indicated. (B) DNA sequence of the *gut* promoter region and the 5' end of *gutF*. The *gutR*-protected regions (I and II) are shown in shaded boxes. The inverted repeat motif is shown by convergent arrows. The transcriptional start site of the *gut* operon is indicated by an arrow. The putative ribosome-binding site (RBS) and  $-35$  promoter sequences are underlined. A *cre*-like sequence is shown in boldface type.

were loaded onto Ni-nitrilotriacetic acid agarose columns (Qiagen). After the columns were washed with buffer A containing 20 mM imidazole and buffer B containing 15 mM imidazole for GutR and GutM purification, respectively, GutR was eluted with 250 mM imidazole and GutM was eluted with a 50 to 250 mM imidazole gradient. Fractions containing the proteins of interest were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, pooled, dialyzed against 10 mM  $\text{NaH}_2\text{PO}_4$  (pH 7.5) containing 45% glycerol and 10 mM  $\beta$ -mercaptoethanol, and kept frozen at  $-80^\circ\text{C}$ . Protein concentrations were determined with a Bio-Rad dye-binding assay.

**Gel mobility shift and DNase I footprinting assays.** The upstream region of the *gutF* gene was amplified by PCR with *L. casei* BL23 chromosomal DNA and primers *gutF4* and *gut8D*. The amplified 179-bp DNA fragment was used in electrophoretic mobility shift assays with purified His-tagged GutR or purified His-tagged GutM. The binding assay was carried out in binding buffer (20 mM Tris-HCl [pH 6.5], 40 mM KCl, 5 mM  $\text{MgCl}_2$ , 1 mM dithiothreitol, and 10% glycerol) with 0.1  $\mu\text{g}$  of target DNA and different amounts of His-tagged GutR or His-tagged GutM. The binding mixtures were incubated for 30 min at room temperature and separated on 5% nondenaturing polyacrylamide gels in 100 mM Tris-HCl [pH 7.5]–1 mM EDTA buffer at 60 V for 1 h. The DNA was stained with ethidium bromide. To perform footprinting assays, two single-end  $^{32}\text{P}$ -labeled DNA probes of the sorbitol promoter region were synthesized in two separated PCRs. The 179-bp DNA fragment described above was used as a template, and the oligonucleotides *gutF4* or *gut8D*, 5' labeled with T4 polynucleotide kinase and  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ , were used as primers with the second oligonucleotide *gut8D* or *gutF4* unlabeled. The reaction mixture contained binding buffer, 30,000 dpm of the labeled PCR fragments, 0.5  $\mu\text{g}$  of herring sperm DNA, and different amounts of His-tagged GutR, in a final volume of 25  $\mu\text{l}$ . After incubation for 30 min at room temperature, the DNA was digested with 0.0125 U of DNase I for 1 min at room temperature. The digestion was stopped by heating the samples for 10 min to  $75^\circ\text{C}$  in the presence of 5 mM EDTA. The reaction mixtures were passed through MicroSpin G-50 columns (GE Healthcare) to remove glycerol and subsequently separated on a 5% sequencing gel. A reference sequence ladder was generated using a Sequenase version 2.0 kit (GE Healthcare) with the same labeled oligonucleotide.

**Nucleotide sequence accession number.** The nucleotide sequence reported in this work has been deposited in GenBank under accession number EU315692.

## RESULTS

**Characterization of the *L. casei* sorbitol operon.** The gene encoding sorbitol-6-phosphate dehydrogenase (GutF), involved in sorbitol metabolism in *L. casei* BL23, has previously

been isolated (32). Analysis of the chromosomal region of this gene using the genomic sequence of *L. casei* BL23 (GenBank accession no. FM177140) (13), showed that, downstream from *gutF*, five genes (*gutRMCBA*) were present which probably constitute an operon (Fig. 1). *gutR* encodes a protein homologous to putative sorbitol operon transcriptional regulators, showing the highest similarity to GutR from *Lactobacillus salivarius* (28% identity, 49% similarity) (7). The *gutM* gene encodes a protein homologous to putative sorbitol operon activators, e.g., SrlM1 from *Lactobacillus plantarum* (45% identity, 72% similarity) (14). Following *gutM*, three genes (*gutCBA*) encode proteins with significant homology to membrane-bound (EIIC<sup>Gut</sup> and EIIBC<sup>Gut</sup>) and soluble (EIIA<sup>Gut</sup>) components of the sorbitol-specific PTS. This transport system has the singularity that the EIIC domain is divided into two parts; one corresponds only to the amino-terminal half (189 residues) and is encoded by *gutC*, whereas the carboxy-terminal end is fused to the EIIB domain and the fused protein (372 residues) is encoded by *gutB*. Two putative *rho*-independent terminators were identified downstream from *gutA*, one between *gutA* and the following gene (*fsa*) ( $\Delta G$ ,  $-19.4$  kcal/mol) and another located in the *fsa* 3' region ( $\Delta G$ ,  $-12.3$  kcal/mol). The deduced amino acid sequence of Fsa shows significant homology to transaldolase-like fructose-6-phosphate aldolases (24). Genes encoding transaldolase-like proteins have been found associated with catabolic operons in other organisms, for example, the *gut* operon from *C. beijerinckii* (27) and the sorbose operon from *L. casei* (33).

When comparing the *gut* operon of *L. casei* strain BL23 with the equivalent region in *L. casei* strain ATCC 334 (GenBank accession no. CP000423) (16), we observed the same gene organization, except that in the latter strain, *gutR* contains a 1,055-bp DNA insertion. The inserted DNA encodes a putative transposase of the IS30 family (Fig. 1). In addition, a frameshift was found in the 3' half of the ATCC 334 *gutR*

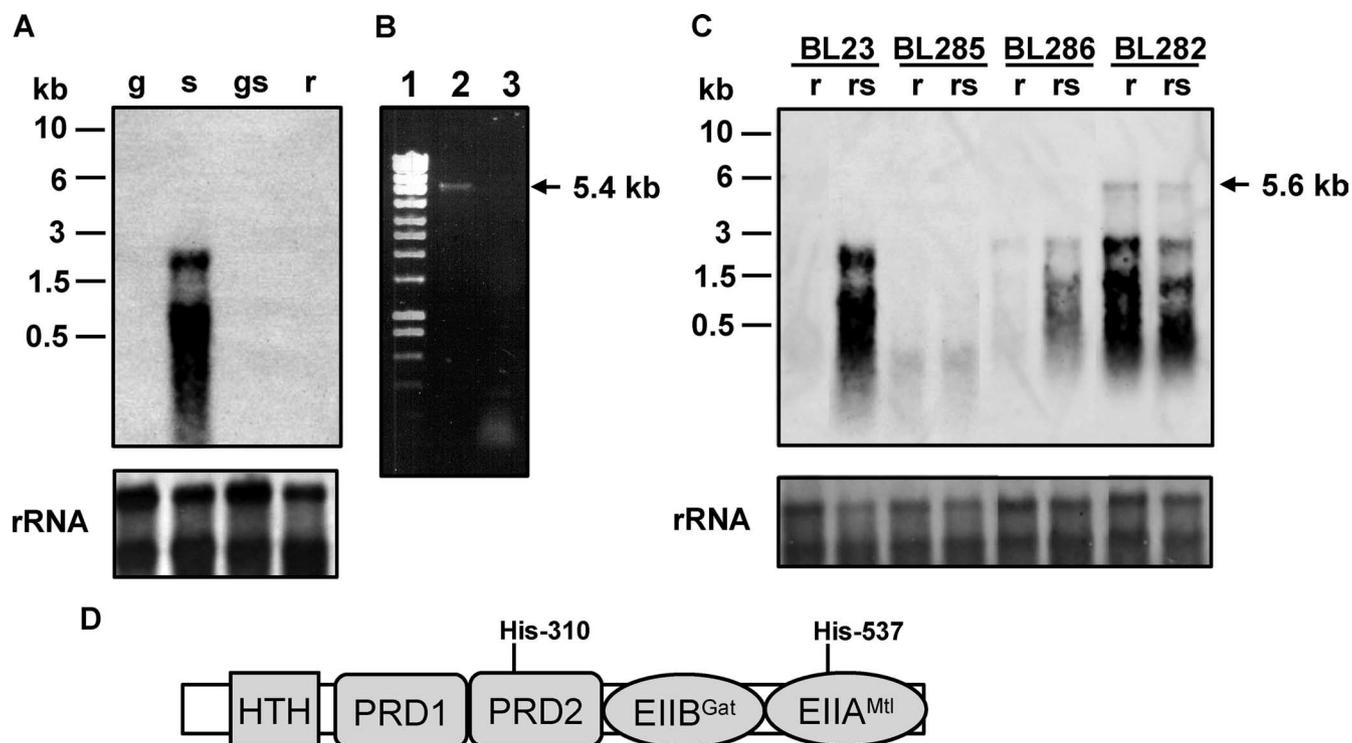


FIG. 2. (A) Northern blot analysis performed with RNA (1  $\mu$ g) isolated from *L. casei* BL23 (wild type) grown in MRS fermentation medium with 0.5% glucose (g), 0.5% sorbitol (s), 0.5% glucose plus 0.5% sorbitol (gs), or 0.5% ribose (r). The DNA probe spanned the promoter region of the *gut* operon and the 5' end of *gutF*. Positions of size standard markers are indicated on the left. Stained rRNAs (23S and 16S) are shown as loading controls. (B) Agarose gel showing a RT-PCR band obtained with RNA isolated from BL23 grown on 0.5% sorbitol. Total RNA was used in RT reactions with primer *gutbc4* and AMV-RT (lane 2) or without AMV-RT (lane 3). The cDNAs obtained were used in PCRs with primers *gut3* and *gutbc4*. Ecoladder 1 (Ecogen) was used as a size standard marker (lane 1). The size of the fragment obtained is marked on the right. (C) Northern blot analysis using RNA (1  $\mu$ g) isolated from *L. casei* BL23 (wild-type), BL282 (*gutB*), BL285 (*gutR*), and BL286 (*gutM*) strains. The conditions of cell cultures (0.5% ribose or 0.5% ribose plus 0.5% sorbitol [rs]) and the probe are the same as in panel A. A transcript of 5.6 kb is indicated with an arrow. Positions of size standard markers are indicated on the left. Stained rRNAs (23S and 16S) are shown as loading controls. (D) Domain organization of GutR. HTH, helix-turn-helix. Potential PTS phosphorylation sites (histidyl residues) are indicated.

homologue; therefore, the mutated *gutR* in the ATCC 334 genome is annotated as three different open reading frames: LSEI\_2728, LSEI\_2726, and LSEI\_2725 (*gutR1*, *gutR2*, and *gutR3* in Fig. 1).

**The *gut* genes are induced by sorbitol and repressed by glucose.** Northern blot experiments were performed with RNA isolated from *L. casei* BL23 (wild type) grown in media containing glucose, sorbitol, glucose plus sorbitol, or ribose (Fig. 2A). The results showed that the sorbitol operon was induced by sorbitol and repressed by glucose. This was in agreement with the presence of a DNA sequence matching the consensus motif of a *cre* (catabolite responsive element) gene (12), the target site for carbon catabolite repression in firmicutes, in the promoter region of the *gut* operon (Fig. 1B). The transcription signal was at the level of ribosomal RNAs (Fig. 2A), likely due to degradation or comigration of the RNA, a common problem in the analysis of long transcripts (33). However, RT-PCR analysis using total RNA isolated from strain BL23 grown on sorbitol showed a DNA band of about 5.4 kb, which confirmed that the *gutFRMCBA* genes are transcribed as a single mRNA (Fig. 2B). The transcription initiation site was determined by 5' RACE and shown to be the cytosine located 27 bp upstream from the *gutF* start codon (Fig. 1B).

***gutR* codes for a transcriptional activator of *gut* genes.** To determine the participation of GutR in the regulation of sorbitol utilization in *L. casei* BL23, a mutant with a frameshift in *gutR* was constructed (strain BL285). This mutant lost the ability to ferment sorbitol, and transcription of the *gut* operon in cells grown with a mixture of ribose (a sugar that does not trigger catabolite repression in *L. casei*) plus sorbitol was abolished (Fig. 2C). In addition, the sorbitol uptake rate in strain BL285 was 88-fold lower than in the wild type ( $30.76 \pm 0.89$  and  $0.35 \pm 0.21$  nmol mg of dry weight<sup>-1</sup> min<sup>-1</sup> for wild-type and BL285 strains, respectively). *gutR* precedes *gutM* and the genes coding for the putative transport proteins of PTS<sup>Gut</sup>. Although it is likely that the absence of GutR or GutM (see below) from strains BL285 and BL286, respectively, is responsible for the loss of sorbitol utilization capacity, we cannot exclude the possibility that the frameshift mutations might have a polar effect on the downstream *gut* genes. To test this possibility, BL285 was transformed with pT1NgutR (Table 1), which constitutively expresses *gutR* in *trans*. In the complemented strain (BL307), sorbitol uptake was restored ( $23.79 \pm 1.5$  nmol mg of dry weight<sup>-1</sup> min<sup>-1</sup>), and the strain was able to ferment sorbitol, demonstrating that the phenotype of BL285 was due to an impaired *gutR*. These results indicate that GutR

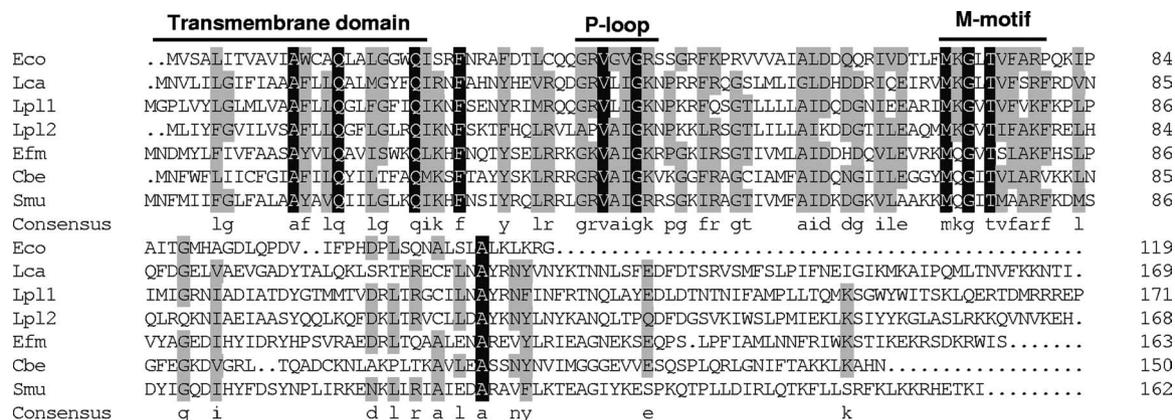


FIG. 3. Multiple amino acid sequence alignment of GutM from *L. casei* (Lca, this work), *E. coli* (Eco, GenBank accession no. NP\_417186), *L. plantarum* (Lpl1; GenBank accession no. NP\_786817), *L. plantarum* (Lpl2; GenBank accession no. NP\_786847), *Enterococcus faecium* (Efm; GenBank accession no. ZP\_00604866), *C. beijerinckii* (Cbe; GenBank accession no. YP\_001307479), and *S. mutans* (Smu, GenBank accession no. AAD33520). The residue number of each protein is indicated on the right. Residues conserved in all sequences are shown against a black background. Residues conserved among at least four of the seven sequences appear against a grey background. The consensus sequence (at least four residues conserved) is shown in lowercase letters. The predicted transmembrane domain and the P-loop motif are shown. M-motif indicates a highly conserved region of unknown function.

acts as a transcriptional activator and that no other sorbitol utilization system is present in BL23. In accordance with these data, *L. casei* ATCC 334, which contains a *gutR* inactivated by an insertion element, was also found to be unable to utilize sorbitol.

A sequence analysis of GutR showed a multidomain structure of the protein (Fig. 2D). In its N-terminal region, GutR contains a helix-turn-helix domain (type 11), located at positions 88 to 146. GutR exhibits significant similarity (42%) to LicR from *Bacillus subtilis*, which controls the expression of a cellobiose-specific PTS. The helix-turn-helix domain in LicR is followed by two PRDs, a galactitol-specific EIIB-like domain (EIIB<sup>Gat</sup> domain) and, finally, an EIIA domain of a mannitol/fructose (EIIA<sup>Mtl/Fru</sup>) class PTS (8). The four phosphorylatable histidyl residues in the two PRDs of LicR were identified as the sites of positive regulation by P~His-HPr, whereas the conserved histidine in the EIIA<sup>Mtl</sup> domain represents the site of negative control by P~EIIB<sup>Cel</sup>; where Cel is cellobiose (28). The regulation of *L. casei* GutR must be slightly different, as it contains only one of the four phosphorylatable histidyl residues in the two PRDs (His-310, the first conserved His in PRD2) (Fig. 2D). A phosphorylatable histidine in PRD1 is present only in GutR of *L. salivarius*, and PRD1 is generally poorly conserved among GutR proteins. Sequence comparisons between *L. casei* GutR and other members of this transcriptional regulator family showed that the *L. casei* protein also contains an EIIB<sup>Gat</sup> domain with a length of approximately 80 amino acids. The potentially phosphorylatable cysteyle residue in the EIIB<sup>Gat</sup> domain is lacking in GutR of *L. casei* and other lactobacilli, but it is present in GutR of, for example, clostridia, enterococci, and *Streptococcus mutans*. In contrast, the phosphorylatable histidyl residue in the EIIA<sup>Mtl</sup> domain is conserved in GutR of all known *gut* operon-containing firmicutes (His-537 in GutR of *L. casei*). In fact, the EIIA<sup>Mtl/Fru</sup> domain is characteristic of DeoR-type PRD-containing transcriptional activators in firmicutes (8). In analogy with *B. subtilis* LicR, it was likely that *L. casei* GutR

would be positively controlled by P~His-HPr-mediated phosphorylation at His-310 and negatively regulated by P~EIIB<sup>Gut</sup>-mediated phosphorylation at His-537 (Fig. 2D).

In order to demonstrate the presumed effect of the PTS<sup>Gut</sup> on the activity of GutR, we constructed a strain (BL282) with a mutated *gutB* gene, which encodes the sorbitol-specific EIIB<sup>Gut</sup>. As expected, this strain displayed a sorbitol-negative phenotype and showed a sorbitol uptake rate ( $0.54 \pm 0.20$  nmol mg of dry weight<sup>-1</sup> min<sup>-1</sup>) 57-fold lower than that of the wild type. However, in contrast to BL285, Northern blot analysis showed that transcription of the *gut* operon in BL282 occurred independently of the presence of sorbitol (Fig. 2C). This indicated that the product of *gutB* is necessary for sorbitol uptake and that it has a negative effect on the expression of the *gut* operon, which it probably exerts via GutR phosphorylation at His-537. A transcript of about 5.6 kb was observed for strain BL282, which would correspond to an mRNA starting at the *gutF* gene and extending to the first putative *rho*-independent terminator located downstream from *gutA*, thus supporting the above RT-PCR results.

**GutM is necessary for transcription of the *gut* genes.** Sequence analysis showed that GutM proteins encoded within different sorbitol operons in both proteobacteria and firmicutes share relatively high conservation in their N-terminal part, with less homology in the C terminus (Fig. 3). The TMHMM program (server version 2.0; CBS, Denmark) for the prediction of transmembrane helices in proteins detected a potential transmembrane segment of 20 or 23 amino acids at the N terminus of GutM (positions 2 to 4 to positions 21 to 26). The alignment of GutM proteins also showed a presumed Walker motif A (G-R-V-A-I-G-K-S/N/R/V), which differs in one residue from the P-loop pattern A/G-X(4)-G-K-S/T (Fig. 3). Some protein kinases that differ in one residue at the beginning or at the end of the sequence pattern without losing their functionality have also been described (3). Downstream from the P-loop domain, there is another amino acid sequence motif (M-K-G-hydrophobic-T-V-F-A-R-F; boldface indicates

conservation in all the GutM proteins) of unknown function but well conserved in all GutM proteins, which we dubbed M-motif (Fig. 3).

According to these data, GutM might be a membrane-bound protein with a nucleotide-binding activity, but no clear function has been reported for it. In order to shed some light on GutM function, a strain with a frameshift in this gene was constructed (BL286). This strain lost the ability to ferment sorbitol, indicating that the gene is essential for sorbitol metabolism. In addition, Northern blot experiments showed that transcription of the *gut* genes was strongly reduced in this strain (Fig. 2C) and that the sorbitol uptake rate was 70-fold lower than in the wild type ( $30.76 \pm 0.89$  and  $0.44 \pm 0.05$  nmol mg of dry weight<sup>-1</sup> min<sup>-1</sup> for wild-type and BL286 strains, respectively), suggesting that GutM plays an important regulatory role in *gut* operon expression. *gutM* precedes the genes coding for the putative transport proteins of the PTS<sup>Gut</sup>. To test the possibility if a frameshift in *gutM* leads to polar effects on the downstream genes, BL286 was transformed with pT1N<sub>gutM</sub> (Table 1), which constitutively expresses *gutM* in *trans*. In the complemented strain (BL291), sorbitol uptake was restored ( $9.41 \pm 0.23$  nmol mg of dry weight<sup>-1</sup> min<sup>-1</sup>), albeit to a lower rate than that of the wild-type, and the strain was able to ferment sorbitol, demonstrating that the phenotype of BL286 was due to an impaired *gutM*.

**Binding of GutR to the *gutFRMCBA* promoter.** To address the question of whether the regulatory effect of the products of the *gutR* and *gutM* genes required a direct interaction with the promoter region of the *gut* operon, His-tagged GutR and GutM were produced in *E. coli* and purified. Both proteins were used in gel mobility shift assays with a 179-bp DNA fragment from positions -173 to +6 relative to the translation initiation site. Under the tested conditions, His-tagged GutR retarded the DNA fragment, while His-tagged GutM did not (data not shown). The inclusion of GutM in binding assays containing GutR did not influence its binding activity under the tested conditions (data not shown). To identify the exact DNA-binding site within the *gut* promoter recognized by GutR, DNase I footprinting experiments with the purified His-tagged GutR were performed. A radiolabeled DNA fragment from position -173 to +6 was incubated with different amounts of GutR, and the footprint analysis showed a protected region (I) spanning 35 bases, from positions -73 to -39 in the coding strand. The equivalent region was protected on the noncoding strand (Fig. 4). Additionally, a short region (II) extending from positions -33 to -29 just downstream from region I was protected in both strands. Hypersensitivity to DNase I digestion, induced by the presence of GutR, was observed at positions -35 and -75 in the noncoding strand (Fig. 4A). The 35-bp protected sequence contains an inverted repeat that could be the recognition motif for GutR (Fig. 1B). An alignment of the DNA regions upstream from *gut* operons from various firmicutes with the DNA-binding sequence determined for *L. casei* GutR revealed for all bacteria a consensus inverted repeat containing a 3T/3A conserved core (Fig. 4B). This motif could be the putative *gut* transcriptional activator recognition sequence in firmicutes.

## DISCUSSION

The *L. casei gutFRMCBA* operon involved in sorbitol utilization has been characterized. The *gutF* gene encodes a sorbi-

tol-6-phosphate dehydrogenase enzyme that converts sorbitol-6-phosphate to fructose-6-phosphate with the associated reduction of NAD<sup>+</sup> to NADH (32). The *gutR* and *gutM* genes encode proteins involved in regulation of the *gut* operon, and *gutC*, *gutB*, and *gutA* encode the EII domains of a sorbitol PTS. The gene organization in the *L. casei gut* operon is also found in *L. plantarum* (14) and in *L. salivarius* (7). This organization is typical of firmicutes, with the exception of *C. beijerinckii*, in which the *gutF* gene is located downstream from *gutA* (27), and listeriae, which lack the *gutR* gene. Firmicutes therefore differ from proteobacteria, where the regulatory genes are located downstream from the transporter and enzyme genes, leading to the organization *gutCBAFMR* (1, 30). Curiously, one of the regulatory proteins (GutM) is conserved in both proteobacteria and firmicutes, whereas the other (GutR) belongs to different families of regulators. GutRs from proteobacteria are repressors of the DeoR family controlled by small metabolites, whereas GutRs from firmicutes are PRD-containing transcriptional activators controlled via phosphorylation (1, 2, 27, 30). Since the EII<sup>Gut</sup> are highly conserved in both bacterial groups, it is possible that *gutCBA* and *gutM* could have a common origin and the different *gutR* genes might have been acquired later.

An *L. casei gutB* mutant strain (BL282) constitutively expressed the *gut* operon but was impaired in sorbitol uptake. These results strongly suggest that EIIBC<sup>Gut</sup> participates in sorbitol transport and that this PTS probably represents the only route for sorbitol uptake in *L. casei*. Furthermore, a mutant defective in the general PTS protein EI failed to ferment sorbitol (29). The fact that transcription of the *gut* operon in BL282 occurs independently of the presence of sorbitol also indicates that the product of *gutB* has a negative effect on the expression of the operon. The target for this regulatory effect is most probably the GutR regulator, which is required for *gut* operon transcription. Previous results have shown that mutations in *levB*, encoding the EIIB<sup>Lev</sup> of a PTS<sup>Lev</sup>, abolished the uptake of fructose via this PTS in *B. subtilis* and led to constitutive expression of the *lev* operon in *B. subtilis* and *L. casei* (4, 18). For both bacteria, it was demonstrated that phosphorylation of the levanase operon regulator, LevR, by EIIB<sup>Lev</sup> at a histidine in PRD2 inhibits its transcriptional activator function (17, 18). Similarly, regulation of the *L. casei esu* operon depended on a LevR-like activator (EsuR) that was negatively regulated by EIIB<sup>Esu</sup> (34). However, GutR of *L. casei* more resembles LicR- and MtlR-like regulators, which are controlled via P~EIIB-mediated phosphorylation at the C-terminal EIIA<sup>Mtl</sup>-like domain (8). Because *L. casei* GutR also contains an EIIA<sup>Mtl</sup> domain, its transcriptional activator function might be inhibited by EIIBC<sup>Gut</sup>-mediated phosphorylation at the conserved His-537. In accordance with LicR and MtlR, the conserved His-310 in PRD2 of GutR might become phosphorylated via P~His-HPr. In the case of *B. stearothermophilus* MtlR, phosphorylation at PRD2 by P~His-HPr increases its affinity for its DNA-binding site (10). The absence of P~His-HPr-mediated phosphorylation during the uptake of a rapidly metabolizable PTS sugar serves as a secondary carbon catabolite repression mechanism (8). However, other possibilities cannot be excluded at present, and an understanding of the detailed regulation mechanism of GutR will require additional studies.

We have shown that GutR from *L. casei* binds to a region of the *gut* promoter, which is conserved within firmicutes, suggesting

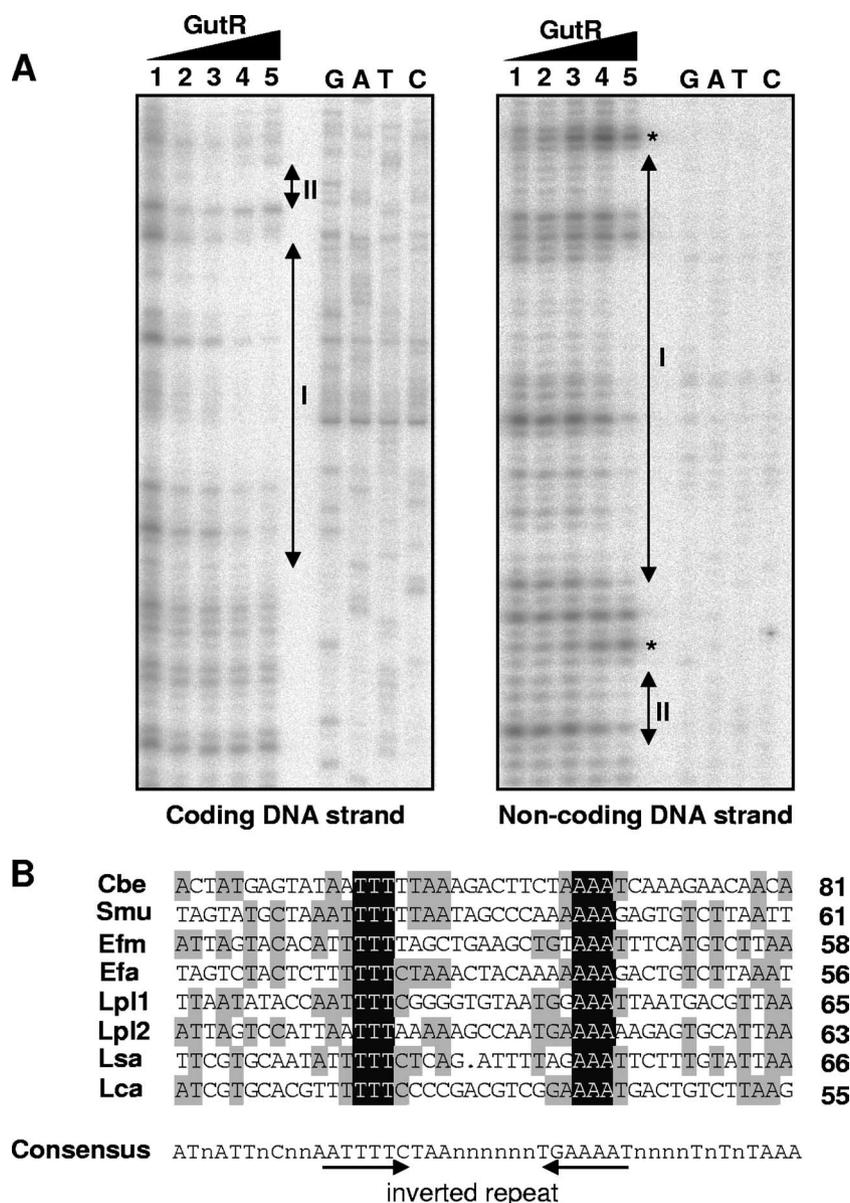


FIG. 4. (A) DNase I footprinting analysis of GutR binding to the sorbitol promoter region. Protection of the coding and noncoding DNA strands in the presence of 0.00, 0.02, 0.17, 0.68, and 1.36  $\mu\text{g}$  of His-tagged GutR (lanes 1 to 5, respectively). The GutR-protected regions (I and II) in the coding and noncoding DNA strands are indicated by double-arrowhead vertical bars. Asterisks indicate the DNA positions with hypersensitivity to DNase I digestion. Binding positions were determined by the sequence ladder (GATC). (B) Alignment of the GutR-protected region of *L. casei* (Lca; see panel A) with the hypothetical promoter regions (45 bases) of *gut* operons from *C. beijerinckii* (Cbe; GenBank accession no. NC\_009617.1), *S. mutans* (Smu; accession no. AF132127.1), *E. faecium* (Efm; accession no. NZ\_AAAK03000093.1), *E. faecalis* (Dfa; accession no. NC\_004668.1), (Lpl) *L. plantarum* (accession no. NC\_004567.1.1), and *L. salivarius* (Lsa; accession no. NC\_007930.1). At the right end of each sequence lane, we indicated the distance (in bases) to the start codon of the first gene of the corresponding *gut* operon. Bases conserved in all sequences are shown against a dark background. Bases conserved in at least four of the eight sequences appear against a shaded background. A consensus sequence (50% conservation) is shown below the alignment and contains an inverted repeat marked with arrows.

that this group shares a common mechanism of regulation. Unlike MtlR, which protected five small and discontinuous DNA regions, GutR binds upstream from the promoter and protects a 35-base continuous DNA stretch, which contains an inverted repeat with a 3T/3A conserved core and thus resembles the DNA-binding site determined for LicR of *B. subtilis* (28). Additionally, a short region of five bases that partially overlaps the  $-35$  box was also protected.

Transcription analysis of the *gut* operon indicated that it is transcribed as a polycistronic mRNA comprising the six *gut* genes, their expression being induced by sorbitol. This result supports the earlier biochemical studies that indicated that sorbitol-fermenting *L. casei* possess an inducible sorbitol-6-phosphate dehydrogenase (32). Sorbitol availability must be sensed by the phosphorylation state of EII<sup>Gut</sup> proteins which in turn leads to changes in the phosphorylation state of the tran-

scriptional activator GutR. The fact that *L. casei* *gutR* was transcribed from the same GutR-regulated promoter adds an autoregulation mechanism to the system. This regulation differs from that described for *E. coli* (30), although both *E. coli* and firmicutes share a common regulator (GutM). An *L. casei* *gutM* mutant (BL286) showed lower levels of *gutFRMCBA* transcription than the wild-type strain, denoting that GutM also exhibits an activation effect on *gut* operon expression. Despite the different nature of *E. coli* GutR, transcription of the *gut* genes in this organism is also activated by GutM (30). Compared to GutM from *E. coli*, the *L. casei* protein contains 50 additional residues at the carboxy-terminal end. Nevertheless, the two proteins exhibit significant similarity (22% identity). GutM from *E. coli* was postulated to be a DNA-binding protein. However, this has never been proven, and *L. casei* GutM did not bind to the promoter region of the *gut* operon or a DNA fragment comprising the intergenic region between *gutF* and *gutR* (data not shown). A sequence analysis of GutM proteins revealed that they contain a potential transmembrane domain at the N-terminal end and a presumed nucleotide-binding domain (Walker motif A), suggesting that they might be membrane-located sensors. The presence of a *gutM* gene in the PTS<sup>Gut</sup>-encoding operons and the results obtained with *E. coli* and *L. casei* favor the hypothesis that GutM plays a role in the transcriptional regulation of the *gut* operon.

In this study, functional and regulatory molecular and genetic analyses of the sorbitol operon from *L. casei* have been carried out. Although several sorbitol utilization operons have been described, information about them is very limited. We have characterized for the first time the binding site for a PRD-containing GutR regulator, appearing to be a PTS-controlled transcriptional activator, which binds to a DNA site conserved in all sorbitol operons of firmicutes. We began to study the function of GutM in this group of bacteria and showed that it probably plays a regulatory role. Further work will be necessary to elucidate the exact function of GutM in the regulation of the *gut* operon.

#### ACKNOWLEDGMENTS

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