

# Regulation and Adaptive Evolution of Lactose Operon Expression in *Lactobacillus delbrueckii*

Luciane Lapierre, Beat Mollet, and Jacques-Edouard Germond\*

Nestlé Research Center, Nestlé, Ltd., CH-1000 Lausanne 26, Switzerland

Received 21 August 2001/Accepted 9 November 2001

*Lactobacillus delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactis* are both used in the dairy industry as homofermentative lactic acid bacteria in the production of fermented milk products. After selective pressure for the fast fermentation of milk in the manufacture of yogurts, *L. delbrueckii* subsp. *bulgaricus* loses its ability to regulate *lac* operon expression. A series of mutations led to the constitutive expression of the *lac* genes. A complex of insertion sequence (IS) elements (ISL4 inside ISL5), inserted at the border of the *lac* promoter, induced the loss of the palindromic structure of one of the operators likely involved in the binding of regulatory factors. A *lac* repressor gene was discovered downstream of the  $\beta$ -galactosidase gene of *L. delbrueckii* subsp. *lactis* and was shown to be inactivated by several mutations in *L. delbrueckii* subsp. *bulgaricus*. Regulatory mechanisms of the *lac* gene expression of *L. delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactis* were compared by heterologous expression in *Lactococcus lactis* of the two *lac* promoters in front of a reporter gene ( $\beta$ -glucuronidase) in the presence or absence of the *lac* repressor gene. Insertion of the complex of IS elements in the *lac* promoter of *L. delbrueckii* subsp. *bulgaricus* increased the promoter's activity but did not prevent repressor binding; rather, it increased the affinity of the repressor for the promoter. Inactivation of the *lac* repressor by mutations was then necessary to induce the constitutive expression of the *lac* genes in *L. delbrueckii* subsp. *bulgaricus*.

The two phenotypically related subspecies of *Lactobacillus delbrueckii*, *L. delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactis*, are gram-positive, facultative anaerobes, and homofermentative lactic acid bacteria. They are economically very important because of their use in milk fermentation to convert lactose to lactic acid for food product preservation. Since the domestication of milk-producing animals by humans, microorganisms that can acidify milk in a reasonable time and yield tasty products were subjected to high selective pressure for an efficient utilization of lactose, the unique sugar found in milk. *L. delbrueckii* subsp. *bulgaricus* was probably selected, together with *Streptococcus thermophilus*, as the typical dairy starter strain for yogurt production. This selection may be the result of the fast and reliable fermentation capacity of *L. delbrueckii* subsp. *bulgaricus* versus *L. delbrueckii* subsp. *lactis*, as well as its robust resistance at low pH values. In many countries, it is now even required by law to use *L. delbrueckii* subsp. *bulgaricus* for the production of yogurt. *L. delbrueckii* subsp. *lactis* is found in a variety of cheese products, where it contributes to the fermentation process in combination with a diversity of other lactic acid bacteria.

The two enzymes involved in the metabolism of lactose were isolated and characterized from *L. delbrueckii* subsp. *bulgaricus* N299, a variant of the type strain ATCC 11842. They comprise a lactose antiport permease (*lacS*) involved in the uptake of the sugar and a  $\beta$ -galactosidase (*lacZ*) for the hydrolysis of lactose into glucose and galactose (13, 23). The presence of a phosphoenolpyruvate lactose phosphotransferase system with a phospho- $\beta$ -galactosidase regulating sugar uptake and hydroly-

sis, as found in many other dairy lactic acid bacteria, has never been identified in an *L. delbrueckii* strain. On the contrary, expression of  $\beta$ -galactosidase in *L. delbrueckii* subsp. *bulgaricus* N299 was shown to be constitutive (13). The lactose operon itself was found to present genetic instabilities, resulting in large deletions at the level of the *lac* operon locus (16). Characterization of such deletions resulted in the identification of an active nonreplicative IS element, ISL3, located 1.2 kb downstream of the end of the *lacZ* gene (4). Two genes coding for enzymes involved in the metabolism of tRNA, an asparagine tRNA ligase (*asnS1*) and an aspartate-ammonia ligase (*asnA*), were localized just downstream of this IS element (10, 11). Next to these RNA genes is a gene coding for the cell wall-anchored protease (*prtB*) (5) essential for digestion of casein during growth in milk.

In this study, we extended sequence analysis of the lactose operon in different strains of *L. delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactis*. We show here how unregulated expression of the *lac* operon in *L. delbrueckii* subsp. *bulgaricus* evolved from an intact regulatory gene expression system discovered in *L. delbrueckii* subsp. *lactis*.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** All bacterial strains and plasmids used in this study are listed in Table 1. Lactobacilli were grown in MRS broth (Difco Laboratories) or in BHI broth (Oxoid Unipath, Basingstoke, England) at 42°C. *Lactococcus lactis* was cultivated in M17 broth (Difco) supplemented with 0.5% glucose (GM17). Plasmids pNZ272 and pNZ273 (19) were maintained in *Lactococcus lactis* MG1363 (3) by the addition of 10  $\mu$ g of chloramphenicol/ml. Histochemical screening for *gusA*-positive clones was performed with 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronic acid (X-Gluc; Sigma) at a final concentration of 50  $\mu$ g/ml.

**DNA manipulations.** Total lactobacillus DNA was prepared by a modification of the spooling method (2). Bacteria grown in MRS broth were collected by centrifugation, washed once with 1 M NaCl, and incubated for 1 h at 37°C in the presence of proteinase K (250  $\mu$ g/ml) and pronase E (500  $\mu$ g/ml). Cells were

\* Corresponding author. Mailing address: Nestlé Research Center, Nestlé, Ltd., Vers-chez-les-Blanc, P.O. Box 44, CH-1000 Lausanne 26, Switzerland. Phone: 41-21-785-88-35. Fax: 41-21-785-89-25. E-mail: jacques-edouard.germond@rdls.nestle.com.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype <sup>a</sup>	Source or reference
<i>Lactobacillus delbrueckii</i> subsp. <i>lactis</i>		
LL44 (NCC936)	Inducible <i>lac</i> operon	Our collection
LL44/6 (NCC2727)	$\Delta$ <i>lacR</i> , constitutive <i>lac</i> operon	NCC936; this work
N62 (NCC181)	Inducible <i>lac</i> operon	NCDO270
N141 (NCC2506)	Inducible <i>lac</i> operon	Our collection
LB68 (NCC653)	Inducible <i>lac</i> operon	ATCC 21815
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>		
N299 (NCC2515)	Constitutive <i>lac</i> operon	ATCC 11842 <sup>T</sup>
YL5 (NCC408)	Constitutive <i>lac</i> operon	Our collection
<i>Lactococcus lactis</i>		
MG1363	Plasmid-free derivative of NCDO 712, <i>lac</i> mutant	3
Plasmids		
pNZ272	Cam <sup>r</sup> , promoterless <i>gusA</i> gene, shuttle <i>E. coli</i> ; gram positive	20
pNZ273	Cam <sup>r</sup> , pNZ272 derivative	20
pLL110	pNZ272 with LL44 <i>lac</i> promoter	This work
pLL112	pNZ272 with LL44 <i>lac</i> promoter, LL44 <i>lacR</i> gene	This work
pLL113	pNZ273 with N299 <i>lac</i> promoter	This work
pLL116	pNZ273 with N299 <i>lac</i> promoter, LL44 <i>lacR</i> gene	This work

<sup>a</sup> Cam<sup>r</sup>, chloramphenicol resistant.

washed twice in TE10 (10 mM Tris-HCl, pH 8.0; 1 mM EDTA), resuspended in TE50 (50 mM Tris, pH 8.0; 10 mM EDTA) containing mutanolysin (125 µg/ml) and lysozyme (1 mg/ml), and incubated for 1 h at 37°C. One volume of 0.5% sodium dodecyl sulfate was added to lyse the cells. Finally, proteinase K was added to 200 µg/ml, and the solution was further incubated for 30 min at 65°C. The DNA was extracted with phenol, precipitated with ethanol, spooled out on a sterile toothpick, washed in 70% ethanol, and drained. The DNA was then dissolved in TE50 in the presence of 200 µg of RNase A/ml, chloroform extracted, reprecipitated in ethanol, and spooled again. The purified DNA was dissolved in TE10 to give a final concentration of 500 to 1,000 µg/ml. Plasmid DNA was isolated from *Lactococcus lactis* with the QiaPrep 8 Miniprep Kit (Qiagen) with a first incubation step in 10 mM Tris-HCl (pH 8.0)–1 mM EDTA containing 1 mg of lysozyme/ml for 30 min at 37°C. DNA fragments were isolated from agarose gels with the Wizard PCR Preps DNA Purification System (Promega). DNA amplifications were performed in a total volume of 50 µl containing 0.2 mM concentrations of each deoxynucleotide triphosphate (Pharmacia Biotech), 1 µM concentrations of each primer (Microsynth GmbH, Balgach, Switzerland), standard buffers, and polymerase from the Expand Long Template PCR System (Boehringer, Mannheim, Germany) or 0.5 U of SuperTaq polymerase (Endotel, Allschwil, Switzerland). DNA amplifications (by PCR) were performed for 35 cycles of 1 min at 95°C, 2 min at 55°C, and 1 min/kb at 72°C with a DNA thermocycler (Perkin-Elmer). DNA sequences were determined directly by PCR by using the VISTRA Thermo Sequenase Sequencing kit (Amersham) on a DNA LICOR sequencer (model 4000; MWG-Biotech). DNA sequences were analyzed with the help of the Wisconsin Sequence Analysis Package from the Genetics Computer Group (University of Wisconsin).

**Amplification of the *lac* promoter and the *lacR* gene.** The region upstream of the *lac* operon of different *L. delbrueckii* subsp. *lactis* and *L. delbrueckii* subsp. *bulgaricus* strains was amplified with the forward primer 2005 (5'-ACAGTGACTTAAACTGG), located close to the stop codon of the *lacA* gene, and the reverse primer 1994 (5'-ACGTCGTTGCCAAAGGC), located close to the start codon of the *lacS* gene. The region downstream of the *lac* operon was amplified with the primer 2030 (5'-CGCCTGGTGATTCAGCC), located close to the stop codon of the *lacZ* gene, and the reverse primer 946 (5'-AGCTTTACGGGGAAGTCGGG), located close to the stop codon of the *asnS1* gene, which is in the reverse orientation compared to *lacZ*.

**Construction of the expression vectors.** The *lac* promoters of *L. delbrueckii* subsp. *lactis* LL44 and *L. delbrueckii* subsp. *bulgaricus* N299 were amplified with forward primers containing an *EcoRI* site (underlined), i.e., 2409 (5'-ATATTA GAATTCAGTGACTTAAACTGG), located close to the stop codon of *lacA* gene, and 2410 (5'-ATATTAGAATTCAGAGGCTATATCGC), located close to the end of ISL5, and the reverse primer 250 (5'-GGTTAATGCCGCAAAA GT), located at position 325 of the *lacS* gene. The amplification products were cleaved with *EcoRI* and *BspEI* (blunt) and cloned in pNZ272 or in pNZ273 restricted with *EcoRI* and *AvaII* (blunt) in front of the promoterless *gusA* gene

(plasmids pLL110 and 113). The *lacR* gene was amplified from *L. delbrueckii* subsp. *lactis* LL44 with primers containing an *XhoI* site (underlined): forward primer 3174 (5'-ATAAATCTCGAGTGGTGATTCAGCC), located close to the stop codon of the *lacZ* gene, and reverse primer 3033b (5'-ATATTACTCGAG ACAGAATGCAGCC), located close to the stop codon of *asnS1* gene. The amplified product and the plasmids pLL110 and pLL113 were digested with *XhoI* and ligated (plasmids pLL112 and pLL116). All of the plasmids were used to transform directly *Lactococcus lactis* MG1363 by electroporation (7).

**Isolation of spontaneous *lacR* mutants.** *L. delbrueckii* subsp. *lactis* LL44 (NCC936) was incubated overnight at 42°C in MRS broth containing 10% lactose. The culture was stored refrigerated for 24 h and subcultured in MRS broth containing 10% lactose for 7 h at 42°C. A dilution of the culture was plated on Reinforced Clostridial Medium (Oxoid) containing glucose and 200 µg of X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside; Sigma)/ml. Plates were incubated for 72 h at 40°C under microaerophilic conditions (BBL).

**$\beta$ -Galactosidase assays.** *L. delbrueckii* subsp. *lactis* and *L. delbrueckii* subsp. *bulgaricus* were grown overnight in MRS broth and used to inoculate BHI medium containing 0.5% lactose, 0.5% glucose, or 0.5% glucose plus 0.5% lactose. Cells were grown at 42°C to an optical density at 600 nm (OD<sub>600</sub>) of ca. 1.0, harvested, and resuspended in 0.2 ml of distilled water containing 0.1% Triton X-100. Cells were incubated for 30 min at room temperature, centrifuged, and resuspended at different concentrations in 500 µl of potassium phosphate (pH 7.5) containing 50 µg of ONPG (*o*-nitrophenyl- $\beta$ -D-galactopyranoside; Sigma)/ml. After 20 min of incubation at 42°C, 200-µl aliquots were mixed with 0.8 ml of 400 mM Na<sub>2</sub>CO<sub>3</sub>–50 mM EDTA to stop the reaction. The OD of the mixture was measured after centrifugation at 420 nm, and the  $\beta$ -galactosidase activity in units (U) was calculated as follows:  $U = (OD_{420} \times d) / (3.5 \times t \times OD_{600})$ , where  $d$  is the dilution factor, 3.5 is the millimolar extinction coefficient of the *ortho*-nitrophenol, and  $t$  is the reaction time.

**$\beta$ -Glucuronidase assays.** Transformed *Lactococcus lactis* cells were grown overnight in M17 broth containing 0.5% mannose and 10 µg of chloramphenicol/ml and used to inoculate M17 containing 0.5% mannose, 0.5% lactose, 0.5% glucose, or 0.5% glucose plus 0.5% lactose. Cells were grown at 30°C to an OD<sub>660</sub> of ca. 1.0, harvested, and resuspended in 2 ml of distilled water containing 0.1% Triton X-100. Cells were incubated for 30 min at room temperature, centrifuged, and resuspended in 2 ml of 50 mM NaHPO<sub>4</sub> (pH 7.0)–10 mM  $\beta$ -mercaptoethanol–1 mM EDTA–0.1% Triton X-100–50 µg of *para*-nitro- $\beta$ -D-glucuronic acid (Clontech)/ml. After 1 h of incubation at 37°C, 500-µl aliquots were drawn, and the reaction was stopped with 0.5 ml of 2 mM Na<sub>2</sub>CO<sub>3</sub>. The OD of the reaction mixture was measured after centrifugation at 415 nm, and the  $\beta$ -glucuronidase activity (in units) was calculated as follows:  $U = (OD_{415} \times d) / (18 \times t)$ , where  $t$  is the reaction time in minutes, 18 is the millimolar extinction coefficient of the *para*-nitrophenol, and  $d$  is the dilution factor. The protein content of the culture was determined by using a Bio-Rad protein assay with bovine serum albumin as the standard.

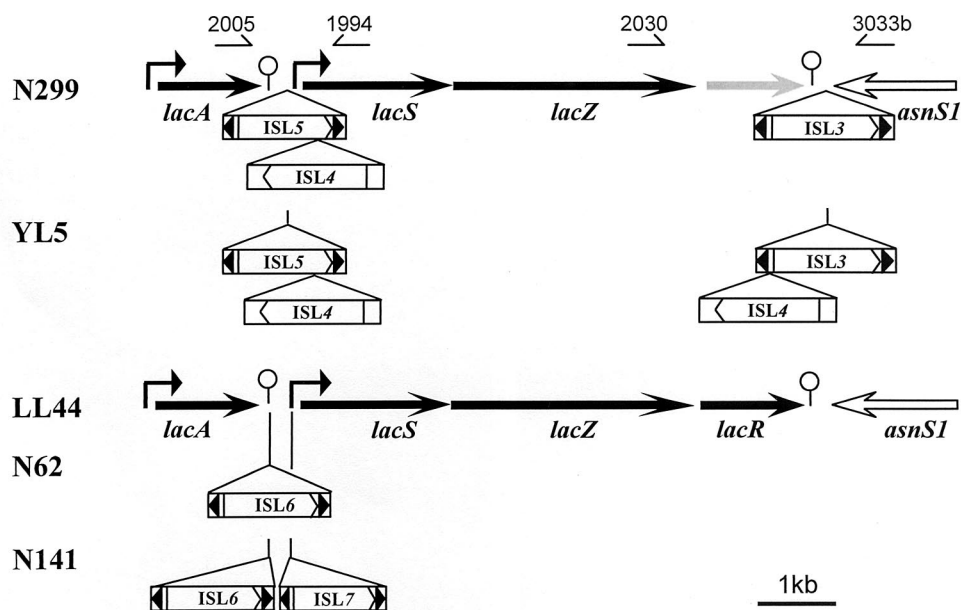


FIG. 1. Schematic representation of the *lac* operon in different *L. delbrueckii* subsp. *bulgaricus* (N299 and YL5) and *L. delbrueckii* subsp. *lactis* (LL44, N62, and N141) strains. The different ORFs are represented by arrows, and the IS elements are represented by boxes. The broken arrows indicate the promoters, the arrowheads inside the IS elements show the terminal inverted repeats, and small arrows point to primers used for DNA amplification.

## RESULTS

**Structural analysis of the *L. delbrueckii* subsp. *bulgaricus* *lac* operon.** Existing information about the sequence of the lactose operon (*lacS* and *lacZ*) in *L. delbrueckii* subsp. *bulgaricus* N299 was extended by chromosomal crawling by using DNA amplification (21) and sequencing of the regions located upstream and downstream of the operon. Thereby, several open reading frames (ORFs) were identified (Fig. 1). Upstream, a gene coding for a peptide of 189 amino acids, showed 50% sequence identity to the thiogalactosyl-transacetylase (*lacA*) gene of *Escherichia coli* (6) (Table 2) and is in the same orientation as the lactose genes. The presence of a putative promoter and a potential rho-independent terminator suggests that this gene is independently transcribed from the *lac* operon. Between this gene and the promoter of *lacS* appeared ORFs in both orientations showing low homologies to transposases. This finding suggests the presence of at least two putative new insertion sequences (IS elements), *ISL4* and *ISL5*. At this stage, how-

ever, it was not possible to determine the delineations of these newly identified IS elements.

The region downstream of the *lac* operon was amplified with primers designed from *lacZ* and *ISL3* and sequenced, but only stretches of putative ORFs in the different reading frames showed significant homologies to repressor molecules. This indicated the presence of remnants of the *lacR* gene, which were oriented in the same direction as the lactose genes. Thus, the presence of several IS elements close to the *lac* promoter and remnants of a potential repressor gene suggested that expression of the *lac* operon in *L. delbrueckii* was once regulated. This prompted us to investigate this genetic locus in related *L. delbrueckii* strains.

**Structural analysis of the *lac* operon in other strains of *L. delbrueckii*.** The structure of the *lac* operon was analyzed in different strains of *L. delbrueckii* in order to identify the functional structures responsible for the control of *lac* gene expression. Five strains were chosen from among sixty-four, which

TABLE 2. Characteristics and GenBank accession numbers of the different sequences involved in this study<sup>a</sup>

Name	GenBank no.	Size (bp)	ORF (aa)	Length of IRs (bp)	Homology (gene, organism, % identity, GenBank no.) <sup>b</sup>
<i>lacSZ</i>	M55068	5,015	627, 1008		Permease and $\beta$ -galactosidase ( <i>lacS</i> and <i>lacZ</i> ) (13)
<i>asnS1</i>	X89438	1,298	432		Asn tRNA ligase (10)
<i>ISL3</i>	X79114	1,492	433	38	Transposase (4)
<i>lacA</i>	AY040211	570	189		Acetyltransferase, yeast, 54% in 188 aa, P40892
<i>lacR</i>	AY040212	1,121	332		Gal repressor, <i>Streptococcus mutans</i> , 36% in 332 aa, JC5310
<i>ISL4</i>	AY040213	1,658	386		ATP-binding cassette, <i>Streptococcus crista</i> , 44% in 265 aa, U96166
<i>ISL5</i>	AY040214	1,590	240	65	Transposase, <i>Bacteroides fragilis</i> , 28% in 240 aa, U75371
<i>ISL4/5</i>	AY040218	3,264	386	65	Mutated <i>ISL5</i> in the promoter of the <i>L. delbrueckii</i> subsp. <i>bulgaricus</i> <i>lac</i> operon
<i>ISL6</i>	AY040215	1,458	420	24	Transposase, <i>Streptococcus agalactiae</i> , 49% in 263 aa, M22449
<i>ISL7</i>	AY040216	1,239	349	28	Transposase, <i>Clostridium perfringens</i> , 48% in 352 aa, X71844

<sup>a</sup> IR, inverted repeat; aa, amino acids.

<sup>b</sup> The source or reference is indicated in parentheses.

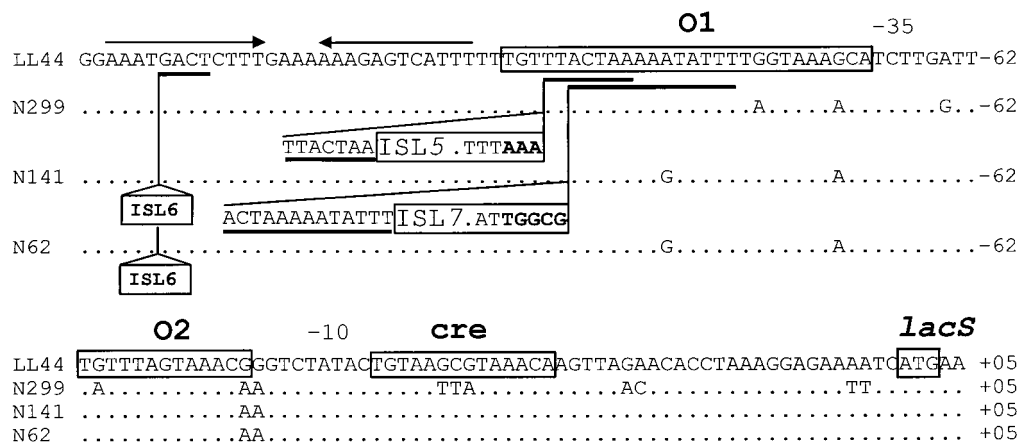


FIG. 2. Alignment of the sequence of the *lac* promoters with their operators (O1 and O2) and the catabolite responsive element (*cre*) in different strains of *L. delbrueckii* subsp. *lactis* (LL44, N141, and N62) and *L. delbrueckii* subsp. *bulgaricus* (N299). The dots are nucleotides that are the same as in strain LL44, nucleotides in boldface indicate changes in O1 due to IS element insertions, direct repeats of the insertion sites are underlined, and arrows show the rho independent terminator of *lacA*. Sequences are numbered from the start codon of *lacS*.

had been studied in detail in the scope of a taxonomic study of the *L. delbrueckii* group (J. E. Germond, L. Lapiere, M. Delley, and B. Mollet, unpublished data). Two regions were amplified with primers designed from *lacA* and *lacS* for the region upstream and from *lacZ* and *asnS1* for the region downstream of the *lac* operon (Fig. 1). In both cases, the size of the amplification products differed from strain to strain and revealed several different structures.

The amplification product obtained from the region upstream of the *lac* operon in *L. delbrueckii* subsp. *lactis* LL44 was the shortest with 200 bp. Its sequence revealed, upstream of the ribosome-binding site of the *lacS* gene, the presence of three palindromes (Fig. 2 and Fig. 3). Two are homologous to operators (O1 and O2) involved in the binding of specific regulatory factors such as repressors (12, 17). The third is homologous to the catabolite regulatory element (*cre*) involved in nonspecific carbon catabolite regulation (8, 14, 15). The operator O1 is directly preceded by the stem-loop structure of a rho-independent terminator for the newly identified *lacA* gene.

In other strains, the amplification products were longer, and their sequences revealed the presence of several new putative IS elements. In *L. delbrueckii* subsp. *lactis* strains N62 and N141, a new IS element, ISL6, was localized exactly in the rho-independent terminator of *lacA*. It contains 24-bp long-terminal inverted repeats and duplicated the sequence GACT at its integration site (Fig. 2 and Table 2). This feature of inverted terminal repeats is characteristic of IS elements and is involved in the process of excision and insertion of the element. Next to ISL6, another new IS element, ISL7, was localized at the border of the operator O1 in *L. delbrueckii* subsp. *lactis* N141. It contains 28-bp long-terminal inverted repeats and duplicated the sequence ACTAAAATATTT at its integration site (Fig. 2 and Table 2). Finally, the IS element free sequence of the *lac* promoter allowed us to localize exactly the complex structure of the two IS elements detected upstream of the *lac* operon in the *L. delbrueckii* subsp. *bulgaricus* N299. This structure is integrated at the border of the operator O1 by duplication of the sequence TTACTAA and has 65-bp long-terminal inverted repeats (Fig. 2 and Table 2). Thus, it should

Strains	Operator O1	Operator O2	<i>cre</i>
LL44	T G T T T A C T A A A A A T T G C T T T A C C A A A A T	T G T T T A G C G T T T A	T G T A A G C T G T T T A C
N141	<b>T G G C G</b> A C T A A A A G T T G T T T T A C C A A A A T	T G T T T A G T G T T T A	T G T A A G C T G T T T A C
N299	<b>A A A</b> T T A C T A A A A A T T G T T T T A C T A A A A T	T A T T T A G T G T T T A	T G T A A T T T G T T T A T
*	T <sub>5</sub> G <sub>5</sub> T <sub>3</sub> T <sub>5</sub> T <sub>5</sub> A <sub>3</sub> C <sub>3</sub> T <sub>3</sub> A <sub>3</sub> A <sub>6</sub> A <sub>6</sub> A <sub>6</sub> A <sub>5</sub> T <sub>6</sub> A <sub>1</sub> A <sub>1</sub> A <sub>1</sub> C <sub>1</sub> G <sub>1</sub> T <sub>3</sub> A <sub>3</sub> C <sub>3</sub> C <sub>2</sub> G <sub>1</sub> C <sub>1</sub> T <sub>1</sub> G <sub>1</sub>	T <sub>5</sub> G <sub>5</sub> T <sub>6</sub> T <sub>6</sub> T <sub>6</sub> A <sub>6</sub> G <sub>3</sub> C <sub>1</sub> A <sub>1</sub>	T <sub>6</sub> G <sub>6</sub> T <sub>6</sub> T <sub>3</sub> T <sub>3</sub> A <sub>3</sub> C <sub>4</sub> A <sub>3</sub> A <sub>3</sub> G <sub>2</sub> T <sub>2</sub> T <sub>1</sub>

FIG. 3. Alignment of the half-site sequences of the operators (O1 and O2) and the catabolite responsive element (*cre*) of different *L. dubrueckii* strains. Nucleotide changes due to IS element insertions are indicated in boldface. \*, consensus sequences.

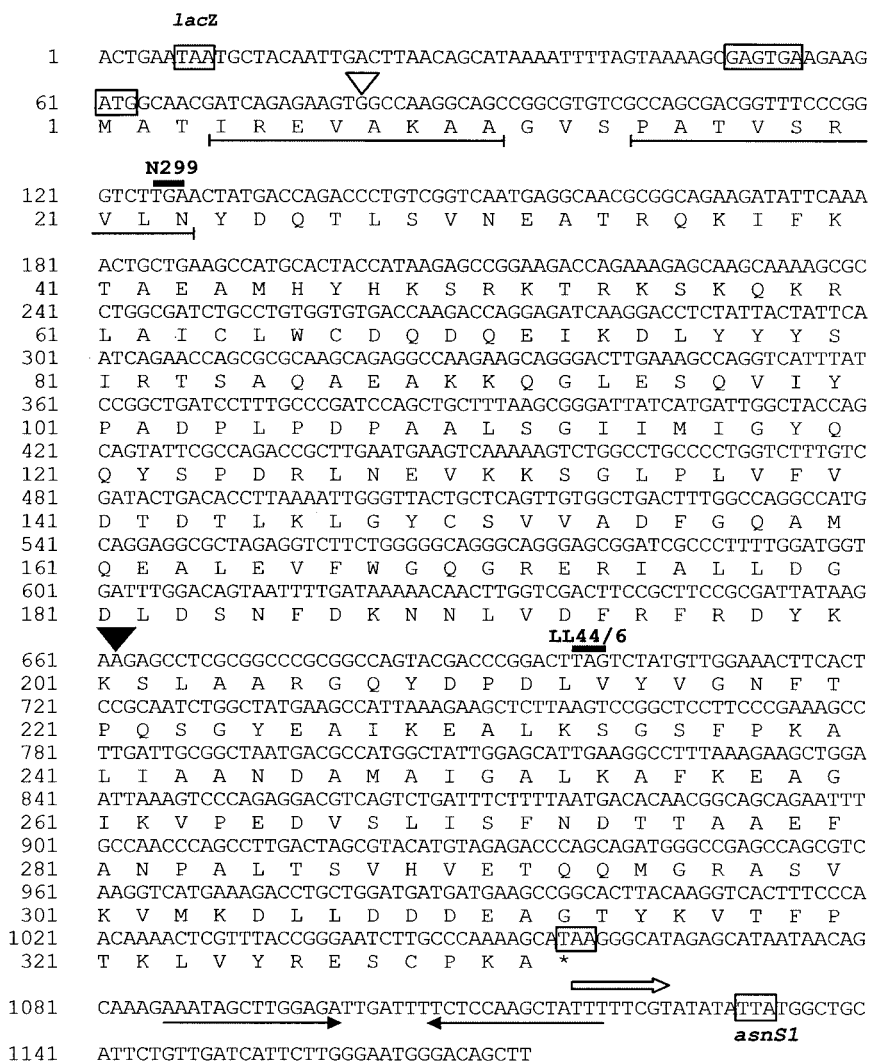


FIG. 4. Nucleotide and amino acid sequences of the *L. delbrueckii* subsp. *lactis* LL44 *lacR* gene. Start and stop codons and the ribosome-binding site are boxed. Arrows show the putative rho-independent terminators. The large open arrow indicates the insertion site of ISL3, and the large bars indicate the helix-turn-helix motif. Single nucleotide deletions in N299 and LL44/6 are indicated by open and filled arrowheads, respectively, and the small bars point out premature stop codons in these mutated *lacR* genes.

be constituted of one IS element, ISL5, in which another IS element, ISL4, is integrated. At this stage, however, it was not possible to determine the delineations of ISL4.

The amplification product, obtained from the region downstream of the *lac* operon, was the same for all strains of *L. delbrueckii* subsp. *lactis* analyzed. Sequencing of the product obtained from strain LL44 revealed the presence of only one ORF coding for a peptide of 332 amino acids located 52 bp downstream and in the same orientation as the *lacZ* gene (Fig. 1). The peptide showed 32% sequence identity to the *ebgR* gene of *E. coli* (24); this gene codes for a repressor belonging to the *lacI* family of regulators and was called *lacR* (Fig. 4). Its sequence predicts the helix-turn-helix characteristic of repressor molecules (1, 18), and its gene is likely transcribed as part of the *lac* operon since no promoter sequence could be identified. This structure is unusual, since *lacR* genes are generally found outside the *lac* operon they control and under their own promoter, as in *Lactococcus lactis* (25). The amplification

products were longer in *L. delbrueckii* subsp. *bulgaricus* strains. Sequencing of these products revealed that the *lacR* gene was inactivated by a series of deletion or insertion events, which are described elsewhere (Germond et al., unpublished). These mutations led to the fragmentation of the gene in several ORFs in the different reading frames. The first of these events, the deletion of a single nucleotide 18 bp downstream of the start codon, led to a premature stop before the end of the helix-turn-helix motif (Fig. 4). The previously described IS element, ISL3, was found downstream of this pseudo *lacR* gene (4) (Fig. 1 and Table 2). In *L. delbrueckii* subsp. *bulgaricus* YL5, in addition, the newly identified ISL4 was found inserted into an A-rich region of the promoter of the ISL3 transposase gene (Fig. 1). ISL4 has no terminal inverted repeats and had been inserted without duplication of its integration site (Table 2). Thus, the delineation of ISL4 present in the complex structure of two IS elements upstream of the *lac* operon in the *L. delbrueckii* subsp. *bulgaricus* N299 was exactly localized. ISL4 was

TABLE 3.  $\beta$ -Galactosidase activities in different strains of *L. delbrueckii* grown in MRS broth

Strain	Presence (+) or absence (-) of activity	
	Lactose	Glucose
N299	+	+
YL5	+	+
LL44	+	-
N62	+	-
N141	+	-

inserted close to the end of the transposase coding sequence of ISL5, leading to inactivation of ILS5. Sequencing of an ISL4-free ISL5, found in another strain of *L. delbrueckii* subsp. *bulgaricus* (LB68) revealed that the ISL5 containing ISL4 acquired a series of deletions and/or insertions. These mutations prevented ISL5 from being transposed and stabilized its presence at this locus. All strains of *L. delbrueckii* subsp. *bulgaricus* available from the Nestlé Culture Collection were shown to have this complex of ISL4 and ISL5 in the *lac* promoter and to demonstrate constitutive expression of the *lac* genes (Germont et al., unpublished). All of the sequences described here have been deposited in the GenBank database (Table 2).

**Regulation of  $\beta$ -galactosidase expression in vivo.** The different strains of *L. delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactis* were grown in MRS broth in the presence of either lactose or glucose, and the  $\beta$ -galactosidase activity was measured (Table 3).  $\beta$ -Galactosidase activity was observed in the presence of lactose and glucose with both *L. delbrueckii* subsp. *bulgaricus* N299 and YL5, indicating that the regulation of the *lac* gene expression was lost and that  $\beta$ -galactosidase production is constitutive, whereas with *L. delbrueckii* subsp. *lactis* LL44, N62, and N141  $\beta$ -galactosidase activity was detected only in the presence of lactose, indicating that the production of  $\beta$ -galactosidase was repressed in the absence of lactose.

In order to determine the functionality of the identified *lac* repressor and its involvement in the regulation of the *lac* gene expression, spontaneous constitutive mutants of the *L. delbrueckii* subsp. *lactis* LL44 were screened on agar plates containing glucose and X-Gal. One colony appeared blue ( $>10^4$  screened), indicating production of  $\beta$ -galactosidase in the presence of glucose and thereby constitutive expression of the *lac* genes. This mutant (LL44/6), its parent strain (LL44), and *L. delbrueckii* subsp. *bulgaricus* N299 were grown in MRS broth in the presence of glucose, lactose, or both sugars, and the  $\beta$ -galactosidase activity was measured (Table 4).  $\beta$ -Galactosidase activity was observed only in the presence of lactose for LL44, whereas for the mutant LL44/6 and for N299,  $\beta$ -galactosidase activity was recorded in the presence of both sugars, indicating that  $\beta$ -galactosidase production can no longer be repressed in the absence of lactose. Sequencing of the *lacR* gene of the LL44/6 mutant revealed a single nucleotide deletion 722 bp downstream of the start codon, leading to a premature stop after 212 amino acids (Fig. 4). These results confirmed that the isolated repressor gene is responsible for the regulation of *lac* gene expression.

**Functionality of the *lac* promoter.** The three palindromes found in the different *lac* promoters analyzed were aligned as half-site sequences (Fig. 3) and were shown to be conserved in

the different strains analyzed. These structures involved in the binding of transcription regulatory factors have in general the conserved dinucleotides TG/AC at their extremities (12, 17). In *L. delbrueckii* several IS elements were found integrated at the 5' end of the O1 operator. For *L. delbrueckii* subsp. *lactis* N141, insertion of ISL7 at the border of the first operator (O1) has modified the sequence of the palindrome but has kept the two important terminal nucleotides, whereas in *L. delbrueckii* subsp. *bulgaricus* N299 and YL5 the insertion of ISL5 in the same region modified the sequence of the 5' end of the palindrome (Fig. 3). Loss of the conserved TG motif of the O1 operator could induce a constitutive production of the enzymes coded by the *lac* operon. Beside insertion of IS elements, several base changes occurred in the *lac* promoter sequence of the different strains analyzed. In *L. delbrueckii* subsp. *bulgaricus* N299, three of these mutations modified the important central CG motif of the catabolite regulatory element (*cre*) (Fig. 2 and 3) (8). This modification of the *cre* sequence could prevent binding of the regulatory factor (CcpA complex) in the presence of glucose. The result will be a loss of glucose catabolite repression and an unrestrained production of  $\beta$ -galactosidase.

In order to determine the importance of the different nucleotide changes in their *lac* operon on the regulation of the *lac* gene expression, the *lac* promoters of *L. delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactis* were cloned in front of a reporter gene in the presence or absence of the *lac* repressor. The *lac* promoters of *L. delbrueckii* subsp. *bulgaricus* N299 and *L. delbrueckii* subsp. *lactis* LL44 were amplified with primers in ISL5 and *lacA*, respectively, and *lacS* (Fig. 1). The amplification products were then cloned in the pNZ272 (20) in front of the promoterless  $\beta$ -glucuronidase (*gusA*) gene (Fig. 5). The resulting plasmids, pLL110 and pLL113, respectively, were transformed in a plasmid free strain of *Lactococcus lactis* MG1363. The transformed cells were grown in the presence of different sugars, and the  $\beta$ -glucuronidase activity was measured (Table 5). The results showed that both *L. delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactis* *lac* promoters were able to induce the production of  $\beta$ -glucuronidase in *Lactococcus lactis*. The same activity was observed in the presence of mannose and lactose for each promoter but was two times higher for the *L. delbrueckii* subsp. *bulgaricus* promoter. It can be argued that the mutation induced by ISL5 insertion likely led to a more active promoter. The presence of glucose led only to a strong reduction of the  $\beta$ -glucuronidase activity with the *L. delbrueckii* subsp. *lactis* LL44 promoter. This observation indicates that the LL44 promoter is subjected to glucose catabolite regulation in *Lactococcus lactis*, whereas no such

TABLE 4. Activities of  $\beta$ -galactosidase in different strains of *L. delbrueckii* grown in MRS broth

Sugar(s) added <sup>a</sup>	Mean activity $\pm$ SD (mU/OD <sub>600</sub> ) <sup>b</sup> in:		
	LL44	LL44/6	N299
0.5% Lac	117.1 $\pm$ 26.9	237.1 $\pm$ 14.3	142.6 $\pm$ 9.7
0.5% Lac + 0.5% Glu	126.8 $\pm$ 10.3	240.6 $\pm$ 25.1	357.2 $\pm$ 86.2
0.5% Glu	4.0 $\pm$ 0.9	342.2 $\pm$ 22.2	343.4 $\pm$ 80.5

<sup>a</sup> Lac, lactose; Glu, glucose.

<sup>b</sup> Mean of five experiments.

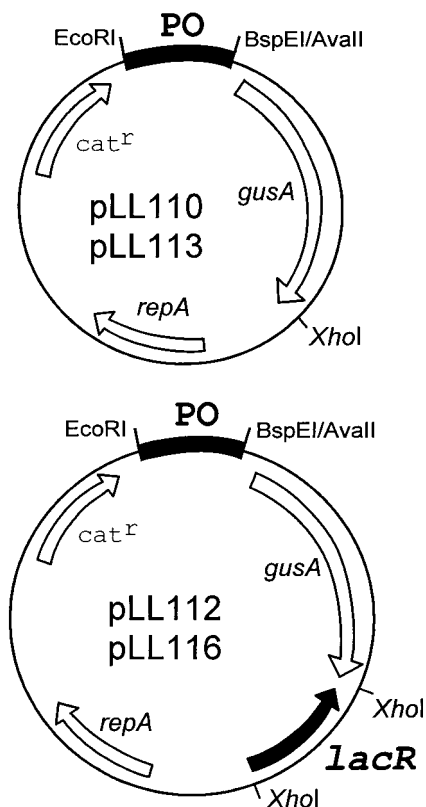


FIG. 5. Physical map of the plasmids used for the study on the regulation of the *L. delbrueckii* *lac* promoters.

reduction of  $\beta$ -glucuronidase was observed with the N299 promoter in which the *cre* sequence was shown to be mutated (Fig. 2).

The next step was to analyze the activity of the newly identified LL44 *lac* repressor on both promoters. The *lacR* gene was amplified from *L. delbrueckii* subsp. *lactis* LL44 with primers in *lacZ* and *asnS1* (Fig. 1). The product of amplification was cloned in both plasmids containing the promoters of LL44 and N299 in the *XhoI* site located downstream of the *gusA* gene, yielding pLL112 and pLL116, respectively (Fig. 5). In both cases, the *lacR* gene was inserted in the reverse orientation compared to the *gusA* gene. Both were then cloned in *Lactococcus lactis*, and the  $\beta$ -glucuronidase activity was measured in the presence of various sugars (Table 5). With both promoters, the  $\beta$ -glucuronidase activity was produced only when bacteria were grown in the presence of lactose and was repressed with the other sugars. The LL44 *lac* repressor is thus able to repress both promoters. However, induction of  $\beta$ -glucuronidase activity by lactose was different for each promoter. For the LL44 promoter,  $\beta$ -glucuronidase activity measured in the presence of lactose is similar in the presence or absence of repressor, whereas for the N299 promoter, it is three times lower in the presence of the repressor. These results indicate that the *lac* repressor probably controls the *lac* promoter of *L. delbrueckii* subsp. *bulgaricus* N299 more strongly than the *lac* promoter of *L. delbrueckii* subsp. *lactis* LL44. The *lacR* gene was also cloned in the same orientation as the *gusA* gene expressed from the N299 promoter, likely inducing a higher production of repres-

or. In this case,  $\beta$ -glucuronidase activity could hardly be induced, even in the presence of 1% lactose (data not shown). Thus, the mutation induced by integration of the complex of two IS elements in the operator O1 does not prevent the regulation of the promoter by the repressor but induces an even tighter repression of its activity. The conserved TG motif of the palindrome seems to not be essential for the control of the promoter by the repressor in *L. delbrueckii*.

## DISCUSSION

In *L. delbrueckii* subsp. *bulgaricus* N299, the presence of numerous IS-like structures (IS elements) in the *lac* promoter and remnants of a repressor gene downstream of the *lacZ* gene strongly indicated that the *lac* operon could be subjected to genetic instability and was once regulated. In order to identify the original regulatory elements, the regions upstream and downstream of the *lac* operon were analyzed in different strains of *L. delbrueckii*. A gene coding for a repressor (*lacR*) was found downstream of the *lacZ* gene in *L. delbrueckii* subsp. *lactis*. Its sequence allowed us to define the different insertion or deletion events which led to its inactivation in *L. delbrueckii* subsp. *bulgaricus*. Loss of *lac* repressor production would thus be sufficient to induce the constitutive expression of the *lac* genes in *L. delbrueckii* subsp. *bulgaricus*. However, other genetic events occurred in the promoter region, which could be responsible for the constitutive expression of the *lac* genes. Several IS elements were found inserted at the 5' end of the first operator (O1). In some *L. delbrueckii* subsp. *lactis* strains, ISL7, was inserted with minor modifications of the palindromic structure of the O1 operator. Whereas in all *L. delbrueckii* subsp. *bulgaricus*, a complex of two IS elements inserted into each other, ISL4/5, induced a major modification of the palindromic structure of the O1 operator. Loss of the palindromic structure of the O1 operator in *L. delbrueckii* subsp. *bulgaricus* could have been the prerequisite for a more efficient and constitutive activity of the promoter, by modifying the binding of regulatory factors such as repressors as well as enhancers.

To rule out these possibilities, both *lac* promoters were cloned in front of the  $\beta$ -glucuronidase gene (*gusA*), transformed in *Lactococcus lactis* since no transformation system exists for *L. delbrueckii*. Both promoters were shown to be

TABLE 5. Activities of the  $\beta$ -glucuronidase gene (*gusA*)<sup>a</sup>

Sugar(s) added <sup>b</sup>	Mean activity $\pm$ SD (mU/mg of protein) <sup>c</sup> with:			
	No <i>lacR</i>		LL44 <i>lacR</i>	
	pLL110 (LL44)	pLL113 (N299)	pLL112 (LL44)	pLL116 (N299)
0.5% Man	2.01 $\pm$ 0.80	3.58 $\pm$ 0.05	0.05 $\pm$ 0.05	0.07 $\pm$ 0.07
0.05% Lac	1.56 $\pm$ 0.44	3.20 $\pm$ 0.41	0.34 $\pm$ 0.11	0.07 $\pm$ 0.08
0.5% Lac	2.01 $\pm$ 1.26	3.41 $\pm$ 0.94	2.71 $\pm$ 0.75	1.11 $\pm$ 0.13
0.5% Glu	0.51 $\pm$ 0.37	2.23 $\pm$ 0.20	0.05 $\pm$ 0.02	0.06 $\pm$ 0.06
0.5% Glu + 0.5% Lac	0.35 $\pm$ 0.14	2.80 $\pm$ 0.76	0.33 $\pm$ 0.16	0.20 $\pm$ 0.07

<sup>a</sup> The gene was fused to different *L. delbrueckii* *lac* promoters in the absence or presence of *lacR* genes and cloned in *Lactococcus lactis* MG1363 grown in M17.

<sup>b</sup> Man, mannose; Lac, lactose; Glu, glucose.

<sup>c</sup> Mean of three experiments.

active in *Lactococcus lactis*. Cloning of the *lacR* gene on the same plasmid resulted in repression in *Lactococcus lactis* of the activity of both promoters. The activity of the *L. delbrueckii* subsp. *bulgaricus* *lac* promoter, which acquired several mutations, was even strongly repressed. One can speculate regarding which mutations occurred first. One possibility is that the promoter mutated, providing better access to enhancer factors of RNA polymerase, but with a stronger affinity also for the repressor. In this case, a *lacR* mutant would have been rapidly selected. It has been shown that a single nucleotide deletion at the beginning of this gene is present in all *L. delbrueckii* subsp. *bulgaricus* strains. Other mutations are observed only in some strains and could have appeared during the speciation of *L. delbrueckii* subsp. *bulgaricus* (Germond et al., unpublished). Another possibility is that two independent mutations occurred first in the repressor gene to induce a constitutive production of the *lac* genes and second in the promoter to induce a higher activity.

Another interesting selective advantage for higher expression of *lac* genes in *L. delbrueckii* subsp. *bulgaricus* is the accumulation of mutations in the catabolite responsive element responsible for nonspecific carbon catabolite regulation induced by the CcpA phosphorylated Hpr-like complex (9). In *Lactococcus lactis*, the cloned *L. delbrueckii* subsp. *bulgaricus* promoter was effectively shown not to be under carbon catabolite repression in the presence of glucose, whereas in the same conditions the activity of the *L. delbrueckii* subsp. *lactis* promoter was reduced by a factor of 4 or 5. However, this *cre* element should not play a role, because it was shown recently that even though a protein homologous to the CcpA factor was found in *L. delbrueckii* subsp. *lactis*, no significant carbon catabolite regulation could be observed in this strain (22). This indicates that a factor that allows glucose catabolite regulation to be active in *L. delbrueckii* is missing. Indeed, this repression activity was not observed, since the  $\beta$ -galactosidase activity in the *L. delbrueckii* subsp. *lactis* *lacR* mutant LL44/6 was the same in the presence of glucose and lactose. The absence of repressor in this mutant would have left an active glucose catabolite regulation.

From these results we conclude that, due to its selection for fast growth in milk for yogurt production, *L. delbrueckii* subsp. *bulgaricus* optimized its metabolism for an efficient utilization of the milk sugar. *L. delbrueckii* subsp. *lactis*, however, was not primarily selected for fast growth in milk but for more varied metabolic capabilities for the production of specific metabolites such as flavors and proteolytic enzymes for the maturation of cheese products.

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