

## Transcriptional Regulation and Evolution of Lactose Genes in the Galactose-Lactose Operon of *Lactococcus lactis* NCDO2054

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**The genetics of lactose utilization within the slow-lactose-fermenting *Lactococcus lactis* strain NCDO2054 was studied with respect to the organization, expression, and evolution of the *lac* genes. Initially the  $\beta$ -galactosidase gene (*lacZ*) was cloned by complementation of an *Escherichia coli* mutant on a 7-kb *HpaI* fragment. Nucleotide sequence analysis of the complete fragment revealed part of a *gal-lac* operon, and the genes were characterized by inactivation and complementation analyses and in vitro enzyme activity measurements. The gene order is *galK-galT-lacA-lacZ-galE*; the *gal* genes encode enzymes of the Leloir pathway for galactose metabolism, and *lacA* encodes a galactoside acetyltransferase. The *galT* and *galE* genes of *L. lactis* LM0230 (a lactose plasmid-cured derivative of the fast-lactose-fermenting *L. lactis* C2) were highly similar at the nucleotide sequence level to their counterparts in strain NCDO2054 and, furthermore, had the same gene order except for the presence of the intervening *lacA-lacZ* strain NCDO2054. Analysis of mRNA for the *gal* and *lac* genes revealed an unusual transcriptional organization for the operon, with a surprisingly large number of transcriptional units. The regulation of the *lac* genes was further investigated by using fusions consisting of putative promoter fragments and the promoterless  $\beta$ -glucuronidase gene (*gusA*) from *E. coli*, which identified three lactose-inducible intergenic promoters in the *gal-lac* operon. The greater similarity of the *lacA* and *lacZ* genes to homologs in gram-negative organisms than to those of gram-positive bacteria, in contrast to the homologies of the *gal* genes, suggests that the genes within the *gal* operon of *L. lactis* NCDO2054 have been recently acquired. Thus, the *lacA-lacZ* genes appear to have engaged the promoters of the *gal* operon in order to direct and control their expression.**

Lactococci play a vital role in commercial milk fermentations, in which their primary function is to convert lactose to lactic acid. Typical *Lactococcus lactis* strains were selected as starter cultures because of their rapid fermentation of lactose. They transport lactose via a phosphoenolpyruvate-dependent phosphotransferase system (PTS), and it is subsequently hydrolyzed by phospho- $\beta$ -galactosidase, giving glucose and galactose-6-phosphate. The galactose-6-phosphate is further metabolized to triose phosphates by the enzymes of the D-tagatose-6-phosphate pathway. It has been suggested that the lactose PTS is essential for rapid homolactic fermentation by starter cultures (29) because the affinity of the lactose PTS for lactose is very high (46) and one lactose molecule is translocated and simultaneously phosphorylated at the expense of one ATP equivalent. The fact that the genes determining these functions are plasmid encoded in many *L. lactis* strains has aided their characterization with respect to genetic organization and regulation of expression (11, 48).

In contrast to the industrial starter strains, some lactococci ferment lactose slowly and produce a variety of end products, such as acetate, formate, and ethanol, in addition to lactate (13). Although starter lactococci may also convert pyruvate to a variety of end products, for example, by fermentation of galactose alone via the D-tagatose-6-phosphate or Leloir pathway, these pathways are not expressed during lactose fermentation, which is homolactic in most strains (45). Recently a galactose gene cluster which has the gene order *galA-galM-galK-galT-galE*, encoding a galactose permease, mutarotase,

galactokinase, galactose-1-phosphate uridylyltransferase, and UDPglucose 4-epimerase, respectively, has been reported for *L. lactis* MG1363 (20). *L. lactis* NCDO2054, which is the best-studied slow lactose fermenter, hydrolyzes lactose via a  $\beta$ -galactosidase and, therefore, metabolizes the galactose moiety of lactose through the Leloir pathway (3). The properties and evolution of the defective nature of lactose metabolism in strain NCDO2054 have intrigued researchers (7, 44). This strain, which can accumulate lactose-6-phosphate to a high intracellular concentration by using an efficient lactose PTS system, contains enzymes of the D-tagatose-6-phosphate pathway (3) but possesses low-level phospho- $\beta$ -galactosidase activity. Thus, the slow fermentation of lactose has been attributed to this rate-limiting phospho- $\beta$ -galactosidase activity (7), and it has also been suggested that the high levels of accumulated lactose-6-phosphate might be deleterious to growth of the strain (10). A proton-coupled  $\beta$ -galactoside transport system which has a much higher affinity for galactose and its analogs than for lactose was discovered in NCDO2054 (26); it is probably the same galactose permease described for *L. lactis* MG1363. However, the precise manner by which lactose is translocated into the cell and made available for hydrolysis by  $\beta$ -galactosidase in order to achieve sufficient metabolism of lactose for growth is still essentially unknown. The presence of an inducible  $\beta$ -galactosidase gene (*lacZ*) within this *Lactococcus* strain has attracted additional attention (6, 18) because of the biotechnological potential of the gene and its promoter(s) for the construction of selection and expression vectors.

The present study was undertaken to further our knowledge of the genetics and evolution of lactose metabolism in the unusual *L. lactis* NCDO2054 strain. Here we describe the location of the *lacZ* and *lacA* (galactoside acetyltransferase) genes, associated together with genes encoding enzymes for

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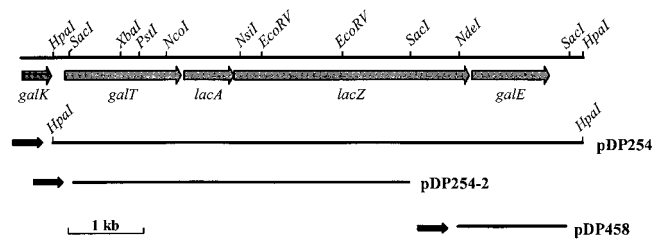


FIG. 1. The genetic organization of genes within the *gal-lac* operon on the chromosome of *L. lactis* NCDO2054. Relevant restriction sites used in cloning are indicated, and the extent and direction of transcription of the genes are illustrated with shaded arrows. DNA inserts in plasmid constructions pDP254, pDP254-2, and pDP458, which were used for the isolation and characterization of the genes, and the direction of the *lacZ* promoter of pUC19 within the plasmids are indicated below.

the Leloir pathway of galactose metabolism. The transcriptional organization and regulation of the *lac* genes within the *gal-lac* operon were studied by analysis of mRNA and by using gene fusions consisting of putative promoter fragments and the promoterless  $\beta$ -glucuronidase gene (*gusA*) from *Escherichia coli*. The evidence presented suggests that the *lacA* and *lacZ* genes have recruited the promoters of the *gal* operon not simply to drive their expression but to do so in a regulated manner.

#### MATERIALS AND METHODS

**Bacterial strains and culture conditions.** *L. lactis* NCDO2054 (also known as ATCC 7962) and LM0230 were originally obtained from the American Type Culture Collection, Rockville, Md. *E. coli* JM109#8, a derivative of JM109 cured of its F' episome and therefore no longer capable of  $\alpha$ -complementation (34), was used for isolation of the  $\beta$ -galactosidase gene. *E. coli* BZ234, a derivative of C600 containing the F' episome, was a gift from Tom Bickle, Biozentrum, University of Basel, Switzerland, and was used for routine isolation of plasmid DNA. *E. coli* MK30-3 was purchased from New England Biolabs, Inc.

*L. lactis* strains were grown at 30°C in M17 broth (Oxoid, Hampshire, England) containing either glucose, lactose, or galactose at 1% as required. *E. coli* was routinely grown in Luria-Bertani medium with aeration at 37°C. Antibiotics were added at the following concentrations: ampicillin (Ap) at 100  $\mu\text{g ml}^{-1}$ , chloramphenicol (Cm) at 20  $\mu\text{g ml}^{-1}$ , and erythromycin (Em) at 100  $\mu\text{g ml}^{-1}$  for *E. coli*; and Cm at 4  $\mu\text{g ml}^{-1}$  and Em at 5  $\mu\text{g ml}^{-1}$  for lactococci. Functional  $\beta$ -galactosidase was detected by the addition of 40  $\mu\text{g}$  of 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal)  $\text{ml}^{-1}$  to Luria-Bertani solid medium, and isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG), when required, was added at a final concentration of 1 mM. Histochemical screening for *gusA*-positive clones was performed with 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronic acid (X-Gluc; Amresco, Solon, Ohio) at a final concentration of 0.5 mM.

**Molecular techniques and transformation.** General molecular cloning techniques, restriction enzyme analysis, and transformation of *E. coli* by  $\text{CaCl}_2$ -induced competence were performed as described by Sambrook et al. (42). Plasmid DNA was isolated from *E. coli* and *L. lactis* by using Qiagen columns (Kontron Instruments, Basel, Switzerland); 5 mg of lysozyme  $\text{ml}^{-1}$  was added to buffer P1 to aid lysis of the lactococci. Electroporation of *L. lactis* was performed essentially as described by Wells et al. (52). Total DNA from *L. lactis* was isolated as described by Hayes et al. (22). Restriction endonucleases, calf intestinal alkaline phosphatase, and T4 DNA ligase were purchased from Boehringer Mannheim and New England Biolabs, Inc., and were used as recommended by the manufacturer. PCR amplification was performed as previously reported (41). DNA sequences of plasmid inserts were determined by the dideoxy-chain termination method (43), using pUC19-derived plasmid DNA as the template, with the M13 universal primer or by primer walking. Custom-made primers were purchased from Microsynth (Balgach, Switzerland). The sequence data were assembled and analyzed by using the Wisconsin package, version 8.0 (Genetics Computer Group [GCG], Madison, Wis.), and amino acid comparisons were performed with the FASTA (36) and GAP (35) programs.

**Cloning of the  $\beta$ -galactosidase gene of *L. lactis* NCDO2054.** Chromosomal DNA, digested to completion with *HpaI*, was ligated to pUC19 (54) that had been linearized with *SmaI* and treated with calf intestinal alkaline phosphatase. The ligation mixture was transformed into *E. coli* JM109#8, and clones expressing  $\beta$ -galactosidase activity, as evidenced by the formation of blue colonies on Luria-Bertani agar plates supplemented with Ap, X-Gal, and IPTG, were selected. Restriction enzyme analysis of plasmid DNA from several blue colonies revealed common 7-kb *HpaI* inserts which were in the same orientation relative to the *lacZ* promoter of pUC19 (Fig. 1). The restriction map constructed for a

representative of these plasmids, designated pDP254, was used to locate sites for cloning the DNA upstream of the 5' *HpaI* site for nucleotide sequence analysis (Fig. 1).

**Directed gene disruption of *lacZ* and *galT*.** To disrupt *lacZ*, the 4.3-kb *SacI* fragment of pDP254 was cloned into *SacI*-digested vector pJDC9 (5). The resulting construct, pDP254-2 (Fig. 1), was digested with *EcoRV*, which removed a 1,007-bp fragment internal to the *lacZ* gene, and self-religation produced pDP418, which contains an in-frame deletion in the gene ( $\Delta\text{lacZ}$ ). This pJDC9-derived plasmid, which cannot replicate in lactococci, was used to transform *L. lactis* NCDO2054. Em-resistant transformants which harbored a chromosomal copy of pDP418 were obtained that were either blue or white (50:50 ratio) on X-Gal plates. A  $\beta$ -galactosidase-positive and a  $\beta$ -galactosidase-negative transformant were cultivated for approximately 100 generations in the absence of Em. Only the  $\beta$ -galactosidase-negative transformant resulted in Em-sensitive, white colonies when the cultures were screened for the loss of Em resistance and for  $\beta$ -galactosidase activity on X-Gal plates. Several white colonies were examined by PCR amplification of the *lacZ* region and by Southern hybridization, which confirmed the deletion. Following these experiments, a single strain, designated LL139, that contained a single copy of the  $\Delta\text{lacZ}$  gene was selected.

To inactivate the *galT* gene, a strategy involving the site-specific recombinase (FLP) encoded by the *Saccharomyces cerevisiae* 2 $\mu$  circle plasmid, which catalyzes recombination between two target sequences (FRTs), was employed (21). When the FRTs are present as direct copies, FLP-mediated recombination leads to deletion of the intervening DNA, leaving a single copy of an FRT. The disruption of the *galT* gene was achieved by inserting the *erm* gene of pAM $\beta$ 1, which was flanked by direct copies of the *S. cerevisiae* FRT, into a plasmid-encoded  $\Delta\text{galT}$  gene. Following integration of this linear structure into the *L. lactis* NCDO2054 genome via a double crossover, the *erm* gene was removed by the *S. cerevisiae* recombinase, resulting in a 580-bp deletion in *galT* and a single copy of an FRT in the resultant strain, designated *L. lactis* LL144. The construction of the deletion within the *galT* gene is described in detail in the legend to Fig. 2.

**Enzyme assays.** In preparation for spectrophotometric assays, cells growing exponentially were harvested at an optical density at 600 nm ( $\text{OD}_{600}$ ) of 1.0 and washed in 20 mM phosphate buffer (pH 6.5) containing 10 mM  $\text{MgCl}_2$  and 1 mM dithioerythritol. The cells were disrupted by shaking at 4°C with glass beads (105  $\mu\text{m}$ -diameter; Sigma) in a Bead Beater (Biospec Products) for three cycles of 3-min treatments, with 1-min intervals in between on ice. Cellular debris was removed by a 15-min centrifugation at 13,000  $\times g$  at 4°C, the extracts were stored on ice, and all enzyme assays were completed within 4 h after preparation of the extracts.  $\beta$ -Galactosidase and galactose-1-phosphate uridylyltransferase were assayed at 30 and 25°C, respectively, by standard protocols (24, 32). UDPglucose 4-epimerase activity was determined by the method of Wilson and Hogness (53) with the modifications of Poolman et al. (38). Toluene-treated cells were prepared and assayed for  $\beta$ -galactosidase activity by the method of Miller (32). For the determination of  $\beta$ -glucuronidase activity, cell extracts were prepared as described for spectrophotometric assays, except that GUS buffer (50 mM  $\text{NaH}_2\text{PO}_4$ , pH 7.0; 10 mM  $\beta$ -mercaptoethanol; 1 mM EDTA; 0.1% Triton X-100) was used, and the assay was performed as previously described (8). Protein concentrations were estimated by using the Bio-Rad protein assay reagent (4).

**RNA isolation, Northern hybridization, and primer extension analysis.** *L. lactis* NCDO2054 was grown in glucose-, lactose-, or galactose-containing M17 medium to an  $\text{OD}_{600}$  of 0.7. The cells were immediately harvested, and their RNA was extracted by using Macaloid (Rheox) and glass beads (<100  $\mu\text{m}$  diameter; Sigma) as previously described (27). The RNA was resuspended in diethylpyrocarbonate-treated sterile water and stored at -80°C. The RNA obtained was of high purity ( $A_{260}/A_{280} > 2.0$ ) and showed no degradation of the major 23S and 16S rRNA species. For analysis of transcripts, equal quantities of RNA were glyoxalated and fractionated by using a 1% agarose gel (49). A part of the gel, containing the RNA ladder (0.24 to 9.5 kb; Gibco BRL), was stained with ethidium bromide as recommended by the manufacturer. Transfer of the RNA to a nylon membrane (GeneScreen Plus; DuPont), hybridization, and analysis were carried out according to the manufacturer's instructions.

The positions of the DNA probes used in the Northern hybridizations are shown in Fig. 3E. The probes (350 bp for *galK* and 600 bp for the others) for the *galK*, *galT*, *lacZ*, and *galE* genes were made by PCR with primer pairs GALT5-GALT10, GALT2-GALT3, LACZ1-LACZ2, and GALE2-GALE3, respectively (Table 1). The PCR fragments were labeled with [ $\alpha$ - $^{32}\text{P}$ ]dCTP by using a Random Primed DNA labeling kit (Boehringer Mannheim) and purified on NICK columns (Pharmacia) before use.

Primer extension of the *galE* promoter was performed by annealing 1 pmol of primer GALE.PE or GUS2-AS (Table 1), which are complementary to the *galE* mRNA and *gus* mRNA of pCRN20, respectively, to 20  $\mu\text{g}$  of RNA followed by cDNA synthesis as previously described (49). Primer-extended products were electrophoresed on a sequencing gel together with the products of plasmid sequencing reactions obtained with the same primers.

**Construction of plasmids for promoter analysis.** The regions preceding the *galT*, *lacA*, and *galE* genes were amplified by PCR with primers GALT4 and GALT6, LACA1 and LACA2, and GALE3 and GALE4, respectively (Table 1). The *Bam*HI and *Eco*RI sites incorporated in the primer sequences allowed the fragments to be cloned in the correct orientation in front of the  $\beta$ -glucuronidase gene (*gusA*) of pNZ273 (37). The ligation products were electrotransformed into

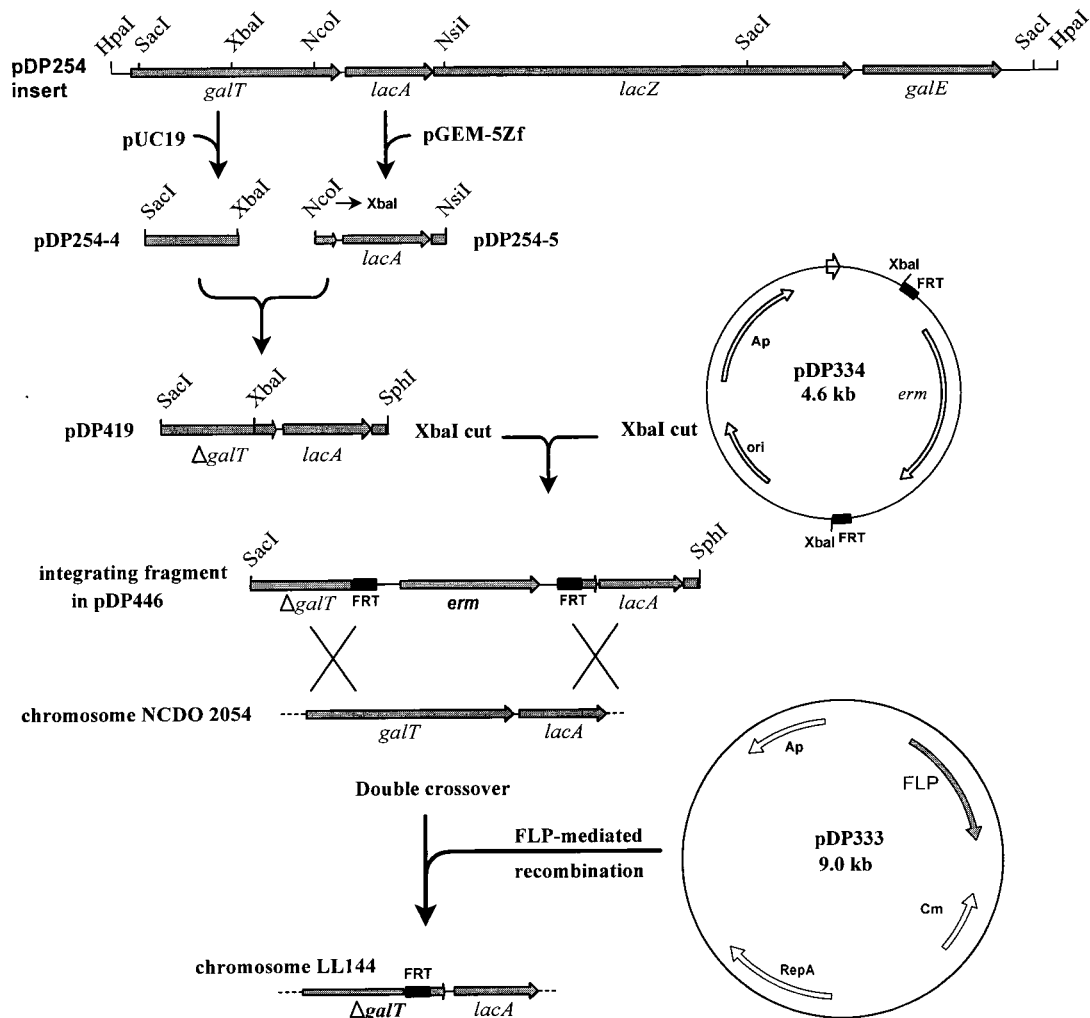


FIG. 2. Schematic representation of the construction of the deletion in the *galT* gene in the *gal-lac* operon of *L. lactis* NCDO2054. The 650-bp *SacI-XbaI* fragment from pDP254, which contains the 5' portion of *galT*, was cloned into pUC19, resulting in pDP254-4. The 931-bp *NcoI-NsiI* fragment, containing the 3' part of *galT* from pDP254, was cloned into pGEM-5Zf (Promega), and the *NcoI* site in the construct was converted to an *XbaI* site by using the oligonucleotide 5'-CATGAATCTA GATT-3', generating pDP254-5. The *XbaI-NsiI* fragment from pDP254-5 was then combined with the *SacI-XbaI* fragment by ligation into *XbaI*- and *NsiI*-digested pDP254-4, which produced a 580-bp deletion in the *galT* gene ( $\Delta galT$ ) in plasmid pDP419. Plasmid pUC-EM was constructed by cloning the erythromycin resistance gene (*erm*) of pAM $\beta$ 1, obtained as a 1.7-kb *AvaI-HindIII* fragment from pVA838 (31), into pUC19. The FRT of the site-specific FLP produced by *S. cerevisiae* was synthesized by using oligonucleotides based on the published DNA sequence (1). The two oligonucleotides FRT1 and FRT2 (Table 1), which were equipped with 3' overhangs of appropriate recognition sites, were annealed together and ligated into pGEM-5Zf previously digested with the same enzymes, generating the plasmid pGEM-FRT. The restriction sites flanking the FRTs in pGEM-FRT were converted by using a series of linkers to sites which were suitable for cloning on either side of the *erm* gene in pUC-EM, resulting in plasmid pDP334 (39). Plasmid pDP446 was constructed by cloning the *erm* gene, flanked by the FRTs from pDP334, into the *XbaI* site of pDP419. The linear *SacI-SphI* fragment of pDP446 was transformed into *L. lactis* NCDO2054, and Em-resistant transformants were obtained. Plasmid pDP333, from which the *S. cerevisiae* 2 $\mu$  circle FLP is expressed (40), was subsequently transformed into the latter Em-resistant strains, and the transformants were selected on plates containing Cm. When these transformants were replica plated onto media with and without Em, approximately 90% of the colonies were found to have lost their Em resistance, indicating FLP-mediated excision of the *erm* gene. A strain designated LL144 was characterized by PCR and Southern hybridization to confirm the presence of the  $\Delta galT$  gene and a single copy of an FRT. The temperature-sensitive pDP333 plasmid was cured from LL144 by passing the culture at 35°C and screening for the loss of Cm resistance.

*L. lactis* NCDO2054, generating pCRN24, pCRN21, and pCRN20, harboring the *galT*, *lacA*, and *galE* potential promoter regions, respectively. The sequences of the inserts in the plasmids were confirmed by nucleotide sequence analysis with the GUS-AS primer (Table 1).

**Nucleotide sequence accession number.** The GenBank accession numbers for the nucleotide sequences reported for *L. lactis* NCDO2054 and LM0230 are AF082008 and AF082009, respectively.

## RESULTS

**Nucleotide sequence and organization of the *L. lactis* NCDO 2054 *gal-lac* region.** Initially the *lacZ* gene of *L. lactis* NCDO 2054 was cloned on an estimated 7-kb *HpaI* fragment in

pUC19 (Fig. 1) by  $\alpha$ -complementation of *E. coli* JM109#8 in the presence of IPTG, a gratuitous inducer of the *E. coli lacZ* promoter. In the absence of IPTG,  $\alpha$ -complementation was no longer observed, suggesting that a functional promoter of *L. lactis lacZ* might not be present in the insert. The nucleotide sequence of a 7,172-bp region within the *HpaI* fragment was determined, revealing the presence of four complete open reading frames (ORFs) and one partial ORF, which are initially designated here based on their homology to previously characterized genes (Fig. 1). The *lacZ* gene was 3,071 bp in length, and although there were four possible translational initiation

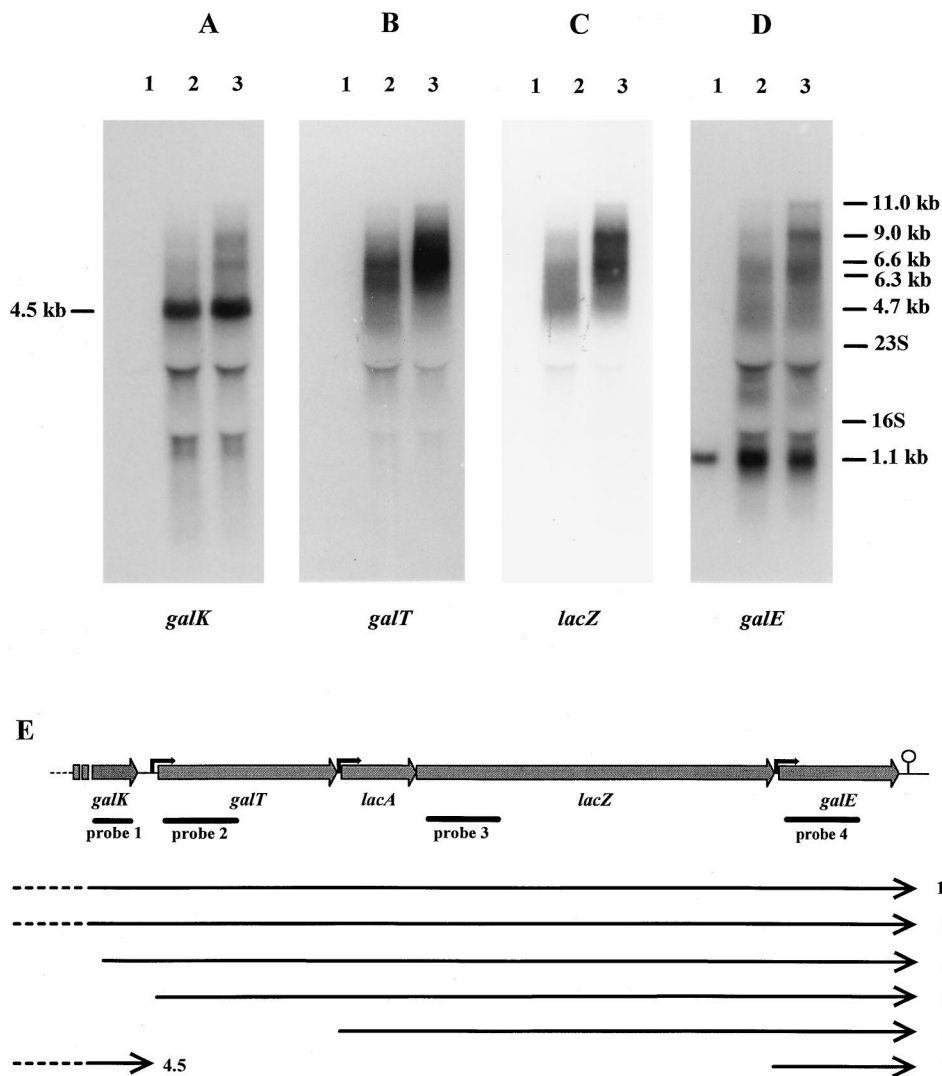


FIG. 3. Northern blot analysis of *gal-lac* operon expression from *L. lactis* NCDO2054 grown on medium containing glucose (lane 1), lactose (lane 2), or galactose (lane 3) and hybridized with probes specific for *galK* (A), *galT* (B), *lacZ* (C), and *galE* (D). The positions of the 23S (2.9-kb) and 16S (1.5-kb) rRNAs and the estimated sizes of the *gal-lac*-specific transcripts are indicated. (E) Illustration of the transcriptional units of the *gal-lac* operon of *L. lactis* NCDO2054. The positions of potential promoters (black arrows) and the transcriptional terminator ( $\odot$ ) are indicated. The transcripts are represented as lines with arrows, and their estimated lengths (in kilobases) are indicated at the right. Lines labeled probe 1, probe 2, probe 3, and probe 4 show the extents of the DNA probes used in Northern analysis of the mRNA of the operon.

sites in frame, the most likely was that positioned 6 bp downstream of a putative ribosome-binding site (RBS). Just 11 bp upstream of *lacZ* and oriented in the same direction was a 784-bp *lacA* gene encoding a galactoside acetyltransferase. A putative RBS preceded the start codon of *lacA* by 8 bp. Upstream of *lacA*, separated by 40 bp, was a 1,479-bp galactose-1-phosphate uridylyltransferase gene (*galT*) with a potential RBS 6 bp upstream. The short intergenic regions between *galT*, *lacA*, and *lacZ* suggested that these genes might be co-transcribed. The partial sequence (373 bp) of an ORF encoding the galactokinase gene (*galK*) was found 173 bp upstream of *galT*. Downstream of *lacZ*, separated by 51 bp, was a 981-bp UDPgalactose 4-epimerase gene (*galE*) gene, and this was followed by a stem-loop structure, with a free energy value of  $-12.7 \text{ kcal mol}^{-1}$ , which could function as a transcriptional terminator (Fig. 4D).

Homology values for the predicted amino acid sequences of the genes are presented in Table 2. The deduced sequences of

*galT* and *galE* and the partial sequence of *galK* showed the highest degrees of similarity (56 to 71%) to sequences from gram-positive microorganisms (33, 38), especially other lactic acid bacteria. In contrast, the predicted amino acid sequence of the intervening *lacZ* gene exhibited the greatest homology to gram-negative homologs. A similarity of 60% to the  $\beta$ -galactosidase of *E. coli* (25) was found, while approximately 40% homology to  $\beta$ -galactosidases of lactic acid bacteria was exhibited. The *lacA* gene, when compared to related gram-negative genes, showed 54% homology to the galactoside acetyltransferase of *E. coli* (23) and 46% homology to *lacA* of *Lactobacillus delbrueckii* subsp. *bulgaricus* (28). Although another *lacA* gene has been discovered in the gram-positive bacterium *Streptococcus bovis*, its nucleotide sequence has not been reported (15).

The GC contents of the *galT*, *lacA*, *lacZ*, and *galE* genes were all in the range 34 to 38%, which is similar to the average 38% GC content of *L. lactis*. The codon usages of the *gal* and





TABLE 2. Homologies of the predicted proteins of the *lac-gal* genes of *L. lactis* NCDO2054

<i>L. lactis</i> gene	Homologous protein from microorganism:	% Identity	% Similarity
<i>galK</i> <sup>a</sup>	<i>Lactobacillus helveticus</i>	58	64
	<i>S. thermophilus</i>	59	64
	<i>E. coli</i>	41	52
<i>galT</i>	<i>Lactobacillus helveticus</i>	50	57
	<i>Bacillus subtilis</i>	47	56
	<i>E. coli</i>	— <sup>b</sup>	—
<i>lacA</i>	<i>E. coli</i>	44	54
	<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	39	46
<i>lacZ</i>	<i>E. coli</i>	52	60
	<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	32	40
	<i>S. thermophilus</i>	32	42
<i>galE</i>	<i>S. thermophilus</i>	61	71
	<i>E. coli</i>	39	57

<sup>a</sup> Note that homology values are for the partial sequence of *galK*.

<sup>b</sup> —, no significant homology evident.

**Transcriptional analysis of the *gal-lac* genes.** To determine the transcriptional organization and the nature of induction of the *gal-lac* genes, total RNA from glucose-, lactose-, and galactose-grown *L. lactis* NCDO2054 cells was analyzed by Northern hybridization (Fig. 3). When probes for the *galT* and *lacZ* genes were used, surprisingly, several large transcripts whose synthesis was induced during growth on lactose and galactose were visible (Fig. 3B and C). The transcripts which were more intense and sharper in the galactose-grown than in the lactose-grown cultures were estimated to be 11.0, 9.0, 6.6, and 6.3 kb. In addition, a weak signal of approximately 4.7 kb appeared to be present with the *lacZ* probe for lactose-grown cultures (Fig. 3C). An identical pattern of four large, weak transcripts was observed with the *galE* probe, but there was also a small band of 1.1 kb present under all growth conditions (Fig. 3D). This 1.1-kb mRNA corresponded to the size of the *galE* gene, indicating that *galE* was also transcribed alone from its own promoter and, furthermore, providing evidence of the functionality of the transcriptional terminator downstream of *galE*. Hybridization with the *galK*-specific probe revealed three long, weak transcripts of approximately 11.0, 9.0, and 6.6 kb in size which were also detected with the *galT*, *lacZ*, and *galE* probes, but the smaller, 6.3-kb mRNA was not detected (Fig. 3A). An extra, strong transcript, estimated to be 4.5 kb, was observed only with the *galK* probe in the lactose- and galactose-grown cultures.

When one assumes that the potential terminator following *galE* is functional, and since the mRNA required for a single transcript for the *galT*, *lacA*, *lacZ*, and *galE* genes must be at least 6.3 kb, the presence of identically sized transcripts for *galT*, *lacZ*, and *galE*, four of which were estimated to be larger than or equal to 6.3 kb, indicated either that these genes were cotranscribed from several promoters located upstream of *galT* or that mRNA processing of one or more of the larger transcripts might be occurring. It is noteworthy that the 9.0- and 11.0-kb transcripts containing the entire *galKT-lacAZ-galE* operon originate upstream of *galK*.

**Characterization of *galT*, *lacA*, and *galE* putative promoters regions.** The regions preceding the *galT* (Fig. 4A), *lacA* (Fig. 4B), and *galE* (Fig. 4C) genes were cloned in front of the *gusA* reporter gene (resulting in plasmids pCRN24, pCRN21, and pCRN20, respectively) in order to locate and investigate the inducibility of promoters which might be responsible for the smaller transcripts of the *gal-lac* operon. The extents of the cloned fragments in the plasmids are indicated in Fig. 4, and the results of the study are presented in Table 4. On medium containing glucose, lactose, or galactose, colonies of *L. lactis* NCDO2054 harboring pCRN20 developed a strong blue color following overnight incubation, indicating the presence of a functional promoter preceding the *galE* gene. Growth of *L. lactis* NCDO2054(pCRN24) transformants resulted in blue colonies on X-Gluc plates supplemented with lactose after 48 h of incubation and in pale blue colonies on galactose medium after 72 h, while no color development was observed with glucose. Thus, the region immediately upstream of the *galT* gene contains a weak promoter which is induced by lactose and, to a much lesser extent, by galactose. The *L. lactis* NCDO 2054(pCRN21) colonies developed a pale blue color on X-Gluc plates containing lactose after 72 h of incubation, but no reaction was detected for glucose- or galactose-containing medium, indicating the presence of a very weak lactose-inducible promoter upstream of the *lacA* gene. The *galT* and *lacA* promoters were both repressed by glucose, since colonies of *L. lactis* harboring either pCRN24 or pCRN21 failed to develop a blue color when grown in the presence of lactose plus glucose.

$\beta$ -Glucuronidase activity was approximately twofold higher for *L. lactis* NCDO2054(pCRN20) grown on lactose than the level achieved in medium with glucose or galactose. Therefore, although *galE* is constitutively expressed from the *galE* promoter on glucose- and galactose-containing-media, this promoter is weakly induced by lactose. Since X-Gluc is an extremely sensitive substrate for  $\beta$ -glucuronidase activity, the

TABLE 3. Enzyme activity measurements

Strain	Carbohydrate in growth medium	Induction with galactose <sup>a</sup>	Activity of <sup>b</sup> :		
			Galactose-1-phosphate uridylyltransferase	$\beta$ -Galactosidase	UDPgalactose 4-epimerase
NCDO2054	Glucose	No	— <sup>c</sup>	3.8 $\pm$ 0.2	788.5
	Lactose	No	225.9 $\pm$ 9.5	1,305.4 $\pm$ 122.6	8,735.2
	Galactose	No	350.2 $\pm$ 34.9	1,008.2 $\pm$ 80.5	7,801.1
	Glucose	Yes	178.8	441.5	ND <sup>d</sup>
LL144 $\Delta$ <i>galT</i>	Glucose	No	—	69.3 $\pm$ 5.1	ND
	Glucose	Yes	—	283.0	ND
LL139 $\Delta$ <i>lacZ</i>	Glucose	No	—	—	ND
	Galactose	No	317.8 $\pm$ 37.9	—	ND

<sup>a</sup> To check for induction of enzyme activity, 20-ml cultures grown for 16 h in M17-glucose were washed and resuspended in 50 ml of fresh M17 broth containing 1% galactose and incubated at 30°C. Aliquots were removed after 3 h and assayed for galactose-1-phosphate uridylyltransferase and  $\beta$ -galactosidase activities.

<sup>b</sup> In nanomoles per minute per milligram of total protein.

<sup>c</sup> —, no measurable enzyme activity; i.e. <0.2 nmol per min per mg.

<sup>d</sup> ND, not determined.

TABLE 4. Development of blue color on plates containing X-Gluc, and  $\beta$ -glucuronidase activity detected for *L. lactis* strains harboring *gal-lac* promoter fusion plasmids

Plasmid	Putative promoter	Growth medium carbohydrate	Color development at <sup>a</sup> :		$\beta$ -Glucuronidase activity <sup>b</sup>
			48 h	72 h	
pCRN24	<i>galT</i>	Glucose	-	-	ND <sup>c</sup>
		Lactose	+	++	
		Galactose	-	+	
		Glucose-lactose	-	-	
pCRN21	<i>lacA</i>	Glucose	-	-	ND
		Lactose	-	+	
		Galactose	-	-	
		Glucose-lactose	-	-	
pCRN20	<i>galE</i>	Glucose	++++		459
		Lactose	++++		935
		Galactose	++++		492

<sup>a</sup> -, no blue color; increasing numbers of + signs indicate more-intense blue color.

<sup>b</sup> In micromoles per minute per milligram of protein.

<sup>c</sup> ND, not detectable.

functional *galT* and *lacA* promoters are obviously very weak. Thus, despite the use of very concentrated cell extracts, the spectrophotometric-assay substrate (*para*-nitro- $\beta$ -D-glucuronic acid) for  $\beta$ -glucuronidase activity was not sensitive enough to measure the strengths of these promoters.

To map the transcriptional start sites of these three putative promoters, primer extension was performed with total RNA from NCDO2054. A primer-extended product, 5 bp downstream of the *galE* gene -10 region, was reproducibly obtained from mRNA of glucose-grown cells (Fig. 4C). The same start point was obtained for NCDO2054 containing the promoter cloned in pCRN20. The *galT* and *lacA* regions also contain functional promoters, but no reproducible extended products were detected with the mRNA of lactose- or galactose-grown cells. The fact that the *galT* and *lacA* genes are present on several transcripts, of which the larger mRNAs are less abundant, could explain the difficulties of obtaining a clearcut result. The experiments were repeated with NCDO2054 harbor-

ing the recombinant pCRN21 or pCRN24, but still no signals were detected by primer extension.

**Analysis of the insertion or deletion point of *lacA-lacZ* in the *gal-lac* operon.** The *lacA* and *lacZ* genes of *L. lactis* NCDO2054 exhibit substantial homology to homologs from gram-negative organisms, while the flanking *galT* and *galE* genes are most similar to related gram-positive genes. This suggests that the *lacA* and *lacZ* genes may have been recently acquired by the *gal-lac* operon of strain NCDO2054. Since dairy starter *L. lactis* strains do not possess a  $\beta$ -galactosidase gene but have the enzymatic potential to ferment galactose via the Leloir pathway, it was interesting to determine if a related *gal* operon was present in a rapid-lactose-fermenting *L. lactis* strain and, if so, to determine the putative insertion point of the *lacA* and *lacZ* genes. *L. lactis* LM0230 is a strain derived from the fast lactose fermenter *L. lactis* C2 which has been cured of its lactose plasmid (12). Using a set of primers, 4701 and 4702 (Table 4), which were homologous to the *galT* and *galE* genes of strain NCDO 2054, an 848-bp fragment was amplified from the chromosome of *L. lactis* LM0230. Sequence analysis indeed revealed that the 3' and 5' ends of ORFs were very similar at the nucleotide sequence level to the *galT* and *galE* genes, respectively, of strain NCDO2054 (Fig. 5A). The interspersed *lacA* and *lacZ* genes of NCDO2054 were missing in strain LM0230. Instead, LM0230 contained a 110-bp intergenic region which, upon introduction of gaps, exhibited homology to the 3' end of the *lacZ* gene of strain NCDO2054. Nevertheless, the promoter regions preceding the *galE* gene of both strains were conserved and highly homologous (Fig. 5B).

DISCUSSION

The present study describes the location and characterization of the *lacA* and *lacZ* genes of *L. lactis* NCDO2054 as a functional part of an operon also encoding the *galK*, *galT*, and *galE* genes and confirms the functions of these genes, which are involved in the metabolism of galactose and lactose. Furthermore, evidence for the recent acquisition within this *gal-lac* operon of the *lac* genes, which have engaged the promoters of the original *gal* operon in order to direct and control their transcription, is provided.

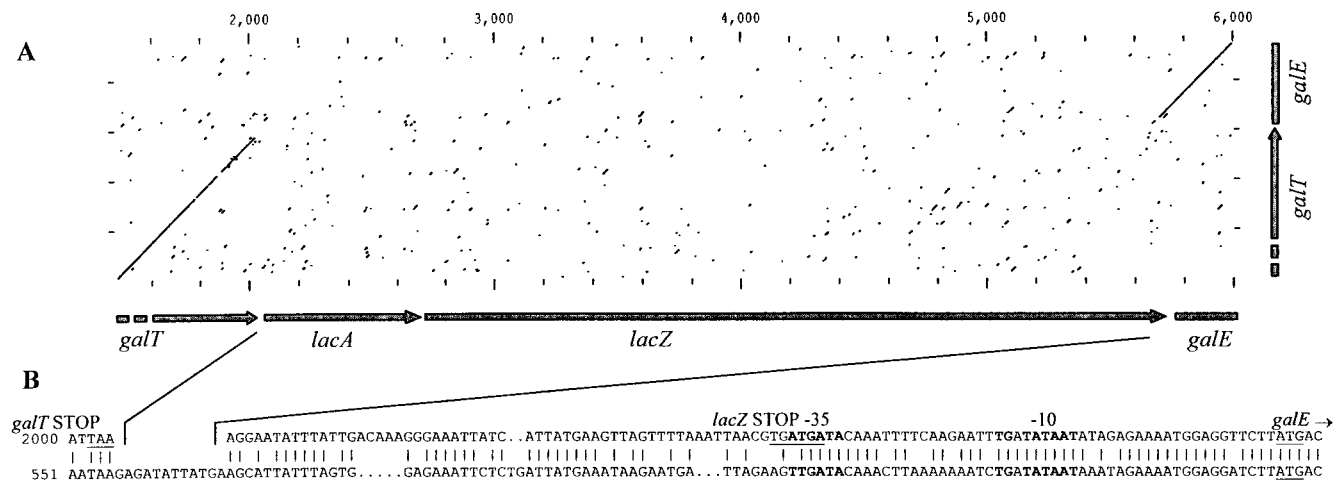


FIG. 5. (A) Dot plot presentation of nucleotide sequence comparisons between the *galT-lacA-lacZ-galE* of *L. lactis* NCDO2054 (horizontal axis) and the 5' end of *galT* and 3' end of *galE* of *L. lactis* LM0230 (vertical axis). Numbers above the dot plot indicate nucleotide positions. (B) Nucleotide sequences of the *galT-lacA* and *lacZ-galE* intergenic regions of *L. lactis* NCDO2054 (upper sequence) aligned with the *galT-galE* intergenic region of *L. lactis* LM0230 (lower sequence). Identical nucleotides (|) are indicated. The genes encoded by the DNA are shown above the sequence. The translational start and stop sequences are underlined, and potential promoter sequences for *galE* are indicated in boldface.



**Organization of the *gal-lac* region.** In many organisms, combinations of the *galK*, *galT*, *galE*, and *galM* (mutarotase gene) genes, encoding enzymes for the Leloir pathway of galactose metabolism, are clustered or organized in a single operon (11, 20). In *E. coli*, the *gal* operon contains *galE-galT-galK-galM*, in that order, and the genes encoding galactose transport are located elsewhere (50). Recently a galactose gene cluster which has the gene order *galA-galM-galK-galT-galE*, with *galA* encoding a galactose permease, was reported for *L. lactis* MG1363 (20). Similarly, the genes for lactose utilization are usually grouped together; e.g., in *E. coli* the *lacZYA* genes constitute an operon, with *lacY* encoding a lactose permease (2). In the gram-positive organism *Streptococcus thermophilus*, the *lacZ* gene is cotranscribed with the gene responsible for lactose transport, *lacS*, and although the genes encoding enzymes of the Leloir pathway are located just upstream of *lacSZ*, they are not part of the same operon (9, 38). The nucleotide sequences of the *lacA* and *lacZ* genes of *L. lactis* NCDO2054 have been reported (17, 18), but their proximity to each other and their location within a *gal-lac* operon have not been described. So far, the organization *galKT-lacAZ-galE* is unique since it is the only occurrence of *lac* genes interspersed in an operon with genes encoding enzymes for the Leloir pathway. Despite the considerable similarities of the *L. lactis lacA* and *lacZ* genes to their counterparts in *E. coli*, the *lac* gene orders of these two bacteria are different.

**Functions of *galT*, *lacZ*, and *galE*.** The functions of the *galT* and *lacZ* genes were confirmed by deleting internal portions of the genes (Table 3). Surprisingly, the  $\Delta galT$  mutation in strain LL144 resulted in the inability to grow on lactose or galactose. In *E. coli*, the growth of mutants lacking galactose-1-phosphate uridylyltransferase is arrested by accumulation of galactose-1-phosphate (30). Similarly, when LL144 is grown in the presence of galactose or lactose, this same intermediate may be responsible for the observed growth inhibition. There was a slight increase in expression of the downstream *lacZ* gene in LL144 grown in glucose-containing medium. The remaining copy of the recombinase target site (FRT) in the  $\Delta galT$  gene may have created a weak promoter which is not regulated, or, alternatively, the 600-bp deletion within *galT* may have resulted in a more stable transcript. Either of these possibilities could have enhanced expression of the genes downstream of *galT*.  $\beta$ -Galactosidase activity could still be induced in LL144 in the presence of galactose, which suggests that alterations in transcription of *lacZ* are not the obstacle to growth on lactose.

Attempts to confirm the function of *galE* by gene disruption were unsuccessful, which suggests that the UDPgalactose 4-epimerase enzyme possesses a function(s) other than the metabolism of galactose. Since the *galE* gene was expressed at a substantial level from its own promoter even in the absence of lactose or galactose, it is not unreasonable to assume that this other function(s) must be essential for viability of *L. lactis* cells. UDPgalactose 4-epimerase has been demonstrated to be involved in the preparation of carbohydrate residues for incorporation into complex polymers such as exopolysaccharide(s) or lipopolysaccharide(s) for cell wall synthesis (14).

**Transcriptional regulation within the *gal-lac* operon.** Northern analysis of the *gal* and *lac* genes revealed an unusual transcriptional organization for the operon, with a surprisingly large number of transcriptional units, as illustrated in Fig. 3E. The *galKT-lacAZ-galE* genes were transcribed together as part of the two longest mRNAs, with sizes of 11.0 and 9.0 kb, which both originated well upstream of the *galK* gene. A communication by Grossiord et al. (19) described a 7.5-kb mRNA transcript of the *gal* operon of *L. lactis* MG1363 and determined by primer extension that its 5' end was just upstream of the *galA*

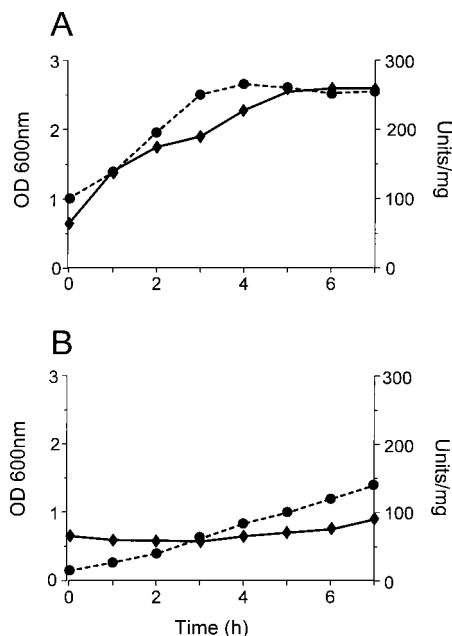


FIG. 6. Growth and  $\beta$ -galactosidase activity of *L. lactis* NCDO2054 in M17 medium containing galactose (A) or lactose (B). A 16-h culture of strain NCDO 2054 grown in M17-glucose was pelleted, washed, and resuspended (1:10) in fresh M17 broth supplemented with 0.5% galactose or lactose. At hourly time intervals, the OD<sub>600</sub> (—◆—) was measured and a 10-ml sample was removed to assay for  $\beta$ -galactosidase activity. One unit of  $\beta$ -galactosidase enzyme is equivalent to 1  $\mu$ mol of *o*-nitrophenol released from *o*-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG) per minute. The  $\beta$ -galactosidase activity, in units per milligram of total protein, is indicated (---●---).

gene. The occurrence of several smaller transcripts was reported as well and was explained as being due to mRNA processing at the *galM-galK* and *galK-galT* junctions. Since the MG1363 *gal* operon lacks the ca. 3.6-kb *lacAZ* cistrons, this major 7.5-kb transcript is the equivalent of the 11-kb transcript of NCDO 2054. The smaller, 9.0- and 6.6-kb transcripts of NCDO2054 correspond to the respective smaller processed mRNA fragments of MG1363. As observed by Grossiord et al. (19) and ourselves, these transcripts are all induced by galactose and are responsible for the major expression of the operon.

Interestingly, the level of induction of the  $\beta$ -galactosidase activity of NCDO2054 was approximately 30% higher in the lactose-grown cultures than in those grown on galactose (Table 3) (6). Furthermore, Northern blot analysis with the *lacZ* probe revealed the appearance of a weak signal at approximately 4.7 kb in the lactose-grown cultures only (Fig. 3C). These results strongly suggest the presence of an additional lactose-inducible promoter activity following *galT* and preceding *lacA* or *lacZ*.

In fact, DNA sequence analysis identified within the *gal-lac* operon three putative promoter sequences that preceded the *galT*, *lacA*, and *galE* genes. These were investigated by transcriptional fusion to the promoterless  $\beta$ -glucuronidase reporter gene (Table 4). All three promoters were induced by lactose, and the *galT* promoter was also induced by galactose, although to a lesser extent. Both the *galT* and *lacA* promoters were repressed by glucose, while the *galE* promoter was constitutively active on glucose and galactose. A search within the *galT* and *lacA* promoter regions located sequences that were homologous to the 14-bp palindromic catabolite-responsive element sequences of catabolite-repressed promoters in gram-positive bacteria (51). Comparison with the consensus sequence (5'-TGWAANCGNTNWCA-3') showed 12 of 14 and



9 of 14 matches with the potential sequences within the *galT* and *lacA* promoter regions, respectively (Fig. 4A and B).

Despite these observations, NCDO2054 has a long lag phase, prior to growth on lactose, during which time  $\beta$ -galactosidase activity is induced far more rapidly during growth on galactose than on lactose and the strain grows substantially slower on lactose (Fig. 6). Therefore, the overall driving force for the transcriptional expression of this *gal-lac* operon is dependent on induction by galactose rather than lactose. It is noteworthy that the manner by which lactose becomes available for hydrolysis by  $\beta$ -galactosidase within strain NCDO2054 is presently unknown. Thus, it is possible that the form of lactose required for induction of the *gal-lac* transcripts is a limiting factor within the cell if the growth medium contains lactose as the unique carbon source (e.g., milk).

**Evolution of *lacA-lacZ* within the *gal-lac* operon.** The *galT* and *galE* genes of fast-lactose-fermenting *L. lactis* LM0230 were highly similar to their counterparts in strain NCDO2054 at the nucleotide sequence level, and they have the same gene order except for the presence of the intervening *lacA-lacZ* within strain NCDO2054. The greater degree of similarity of the *lacA* and *lacZ* genes to homologs of gram-negative organisms than to those of gram-positive bacteria implies that the genes have likely been recently acquired within the *gal* operon of NCDO2054 rather than being lost via the generation of a *lac* gene-free *gal* operon as in LM0230. This supports the theory that this slow-fermenting strain has arisen from a fast lactose fermenter as opposed to the reverse situation (7). Horizontal gene transfer or insertion sequence-mediated events may have played a role in procurement of these genes by NCDO2054. However, the *galT-lacA* and *lacZ-galE* intergenic regions evolved into very compact sequences, leaving behind insufficient traces with which to ascertain the mode of acquisition of the *lac* genes within the operon.

It is interesting that several promoters are involved in the transcription of the *gal-lac* genes. The lactose-inducible *lacA* promoter activity may have resulted from a remnant promoter structure which was linked to the *lac* genes from their original source. The other promoter activities directing the transcription of the *lac* genes were probably already present in the original *gal* operon. However, some of these promoters, especially those preceding the *galT* gene, may have evolved further to allow *lacA-lacZ* gene expression levels adequate for the efficient metabolism of lactose in this strain.

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