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Characterization of the *Lactococcus lactis* Lactose Operon Promoter: Contribution of Flanking Sequences and LacR Repressor to Promoter Activity

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We determined the location, activity, and regulation of the promoter of the *Lactococcus lactis* 8-kb lactose operon (*lacABCDFEGX*), which encodes the enzymes of the lactose phosphotransferase system and the tagatose 6-phosphate pathway. The *lac* promoter sequence corresponds closely to the consensus promoter described for gram-positive bacteria and is located in a back-to-back configuration with the promoter of the divergently transcribed *lacR* gene, which encodes the LacR repressor. The transcription start sites used under induced (lactose) and noninduced (glucose) conditions were determined. The minimal promoter region that could be isolated on a single restriction fragment included sequences ranging from −75 to +42. The effect of the presence of flanking sequences and the *lacR* gene on promoter activity and regulation was studied in *Escherichia coli* and *L. lactis* strains by using transcriptional fusions with promoterless chloramphenicol acetyltransferase reporter genes. The results showed that transcriptional regulation of the *lac* operon is mediated by the interaction between the LacR repressor, the *lac* promoter, and sequences in the noncoding region between the *lacR* and *lacA* genes. Sequences flanking the minimal promoter region appeared to enhance *lac* promoter activity much more in *L. lactis* (5- to 38-fold) than in *E. coli* (1.3- to 5-fold).

The last decade has shown considerable progress in the molecular genetics of lactic acid bacteria. Several genes that encode the key enzymes of catabolic pathways have been cloned and characterized, including those involved in sugar (15, 17, 37, 50), citrate (12), and casein utilization (25, 52). Relatively little is known about the regulation of expression of these and other genes in lactic acid bacteria. A consensus for *Lactococcus lactis* promoter sequences that conformed to the consensus for *Bacillus subtilis* (22) was postulated (14). However, significant differences in the activities of identical promoter DNA sequences were observed between these two gram-positive species (47), indicating that different cellular factors are involved in determining promoter activity and/or mRNA stability. Two regulatory proteins, both from *L. lactis*, have been identified: the MleR activator, which is homologous to the LysR family of positive regulators from gram-negative bacteria (38), and the LacR repressor, which belongs to the DeoR family of repressors (48). However, the molecular targets of these regulatory proteins have not been identified.

Regulation of expression of the *Escherichia coli* lactose-catabolic genes (*lacZYA*) has been studied in great detail (27, 34) and has been a paradigm for studying gene regulation in other bacteria. In *L. lactis* strains used in industrial dairy fermentations, lactose is metabolized via a phosphoenolpyruvate-dependent phosphotransferase system (PEP-PTS^{lac}; 15). The resulting intracellular lactose 6-phosphate is cleaved into galactose 6-phosphate and glucose, which are subsequently metabolized via the tagatose 6-phosphate (4) and glycolytic pathways, respectively. The genes that encode the PEP-PTS^{lac} and tagatose 6-phosphate pathway enzymes are organized in the 8-kb *lac* operon comprising the *lacABCDFEGX* genes (see Fig. 1; 15, 50). Expression of the *lac* operon is repressed 10-fold during growth on glucose and is regulated at the transcriptional level (15, 48) by the LacR repressor, the product of the divergently transcribed *lacR* gene (48). The *L. lactis* and *Staphylococcus aureus lac* operons and *lacR* genes appear to be highly homologous (15, 36, 48, 50). The main differences in their genetic organizations are that the *S. aureus lacR* gene has the same orientation as the structural genes and that the distal *L. lactis lacX* gene is not present in the *S. aureus lac* operon (15, 35). In this report, we present the molecular characterization of the promoter of the *L. lactis lac* operon. DNA sequences flanking the *lac* promoter appear to be involved in transcription activity, regulation, and/or stability of the transcript produced. Furthermore, the presence of the *lacR* gene results in decreased activity of the *lac* promoter.

MATERIALS AND METHODS

Bacterial strains, media, and plasmids. *E. coli* MC1061 (8), HB101 (39), and JM83 (51) were used as recipients in the cloning experiments. The *L. lactis* subsp. *lactis* strains used were MG1363 (plasmid-free strain, Lac$^-$; 19) and its Lac$^+$ derivatives MG1820, containing lactose miniplasmid pMG820 (29), and MG5267, containing a single chromosomally integrated copy of the *lac* operon (20). Media based on M17 broth (Difco Laboratories, Detroit, Mich.) containing 0.5% (wt/vol) glucose or lactose (43) and L-broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) were used for growth of *L. lactis* and *E. coli*, respectively. If appropriate, media were supplemented with ampicillin (50 μg/ml), erythromycin (5 μg/ml), and chloramphenicol (Cm; 10 μg/ml for *E. coli* and 5 μg/ml for *L. lactis*). Plasmid vectors used in the cloning experiments were pACYC184 (9), pKK232-8 (7), and pUC18 (54) for *E. coli* and pGKV210 (47) for *E. coli* and *L.*

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lactis. Plasmids pNZ337 (41), pNZ390 (50), and pNZ380 (48) were used as a source for the *prtP* promoter (52), the *lacRABC* genes, and the *lacR* gene, including its expression signals, respectively. Plasmid copy numbers in *E. coli* were determined by using part of plasmid pBR327::*recA* (16) as a probe for the chromosomal *recA* gene (23).

Molecular cloning, reagents, and enzymes. Isolation of DNA from *E. coli* and *L. lactis* was performed by the alkaline-lysis method (3) and a modified alkaline-lysis method (17), respectively. All manipulations in vitro and with *E. coli* were performed as previously described (39). DNA was transformed into *L. lactis* by electroporation as previously described (52). All enzymes and butyryl-coenzyme A were purchased from Bethesda Research Laboratories (Gaithersburg, Md.), New England BioLabs Inc. (Beverly, Mass.), or Promega Corporation (Madison, Wis.) and used in accordance with the instructions of the manufacturers. Sequenase and *o*-nitrophenyl-β-D-galactopyranoside 6-phosphate were purchased from U.S. Biochemical Corp. (Cleveland, Ohio) and Sigma Chemical Company (St. Louis, Mo.), respectively. [^{14}C]Cm, [α-^{32}P]dATP, and [γ-^{32}P]ATP were supplied by Amersham International plc (Amersham, United Kingdom). Oligonucleotides were synthesized on a Cyclone DNA synthesizer (Biosearch, San Rafael, Calif.).

RNA isolation and primer extension analysis. Total RNA was isolated from protoplasts obtained from glucose- or lactose-grown *L. lactis* MG1820 cells as described previously (48). Primer extension was performed by annealing 1 pmol of an oligonucleotide (5′-GCCATTTGGACTACCT-3′; complementary to the *lac* operon mRNA, positions 83 to 99) to 15 μg of RNA, followed by cDNA synthesis as previously described (48). Primer-extended products were separated on a 6% polyacrylamide–8 M urea sequencing gel together with the products of a double-stranded sequence reaction (10) obtained with the same primer and pMG820 DNA.

Enzyme assays. *L. lactis* and *E. coli* cultures were grown to the late-exponential phase (optical density at 600 nm, 0.7) and induced with 5 μg of Cm per ml for 30 min prior to harvesting (28). Cells were washed and suspended in 0.25 M Tris-hydrochloride (pH 8.0) and, for *L. lactis*, supplemented with 0.5 g of zirconium glass beads (0.1-mm diameter; Biospec Products, Bartlesville, Okla.) per ml. *L. lactis* and *E. coli* cells were disrupted by high-speed vortexing (2 min, three cycles; Biospec Mini BeadBeater) and sonication (15 s, two cycles; Heat Systems Sonicator), respectively. After disruption, cell extracts were isolated by centrifugation. Cm acetyltransferase (CAT) activities were determined at 37°C by measuring ^{14}C-labelled butyryl-coenzyme A in the phase extraction assay (40). Phospho-β-galactosidase (P-β-gal) activities were determined at 37°C by using the chromogenic substrate *o*-nitrophenyl-β-D-galactopyranoside 6-phosphate (29). Protein concentrations were measured as described by Bradford (5), with bovine serum albumin as the standard.

Determination of plasmid copy numbers. Cells were grown and harvested as described in the previous section, and total DNA was isolated after lysis of protoplasts as previously described (26, 39). Total DNA isolated from plasmid-containing *L. lactis* MG5267 or MG1363 or *E. coli* MC1061 cells was digested with *Eco*RV, *Hin*dIII, and *Hin*fI, respectively; subsequently electrophoresed on a 1% agarose gel (39); and transferred to a GeneScreen Plus (New England Nuclear) membrane as recommended by the manufacturer. Since a single chromosomal copy of the *lac* promoter is present in MG5267, the membrane containing DNA from MG5267 cells was hybridized only with a [γ-^{32}P]ATP end-labelled (39) primer specific for the *lac* promoter region (positions −40 to +5). This resulted in hybridization of linearized plasmid DNA (4.6 to 5.9 kb) and a 1.3-kb chromosomal restriction fragment. The membrane containing DNA from MG1363 cells was hybridized simultaneously with two primers, the *lac*-specific probe and a probe for the chromosomally located single copy of the *usp* gene (45). This resulted in hybridization of linearized plasmids (*lac*-specific probe; 4.6 to 5.9 kb) and a 2.9-kb chromosomal restriction fragment (*usp*-specific probe). The membrane containing DNA from *E. coli* MC1061 cells was hybridized with the *lac*-specific probe and a nick-translated (39) 0.45-kb *Eco*RV-*Eco*RI restriction fragment from pBR327::*recA* that contains part of the *E. coli recA* gene (16, 23). This resulted in hybridization of a 1.0-kb fragment (*recA*-specific probe) and plasmid-derived fragments that varied in size between 0.5 and 1.5 kb (*lac*-specific probe). Following autoradiography, the hybridizing restriction fragments were cut out and total radioactivity was determined by using a liquid scintillation counter (Beckman LS7500). The number of plasmid copies per chromosome in MG5267 was calculated on the basis of the ratio between the plasmid- and chromosome-derived hybridization signals. Since the specific activities of the *lac* and *usp* probes used appeared to be identical, the ratio between the signals of the plasmid-located *lac* and chromosomal *usp* genes was used to calculate the plasmid copy number per chromosome in MG1363. We did not determine whether the specific activity of the *recA* probe was identical to that of the *lac* probe. Therefore, in *E. coli* MC1061 only the relative plasmid copy numbers were calculated.

Construction of plasmids. Plasmids pNZ398 and pNZ399 contain the 0.5-kb *Xmn*I-*Bgl*II and 0.35-kb *Ssp*I restriction fragments of pNZ390 cloned into the *Sma*I-*Bam*HI and *Sma*I sites of pUC18, respectively. Both plasmids contain the *L. lactis lac* promoter in the same orientation as the vector-localized *E. coli lacZ*α gene. Plasmids pNZ3000 and pNZ3003 contain the 0.12-kb *Dra*I-*Bam*HI and 0.35-kb *Eco*RI-*Bam*HI fragments of pNZ399 cloned into the *Sma*I-*Bam*HI and *Eco*RI-*Bam*HI sites of pGKV210, respectively. Plasmids pNZ3001, pNZ3002, and pNZ3004 contain the 0.2-kb *Dra*I-*Sal*I, 0.32-kb *Ava*II (filled in with Klenow DNA polymerase)-*Sal*I, and 0.5-kb *Eco*RI-*Sal*I fragments of pNZ398 cloned into the *Sma*I-*Sal*I, *Sma*I-*Sal*I, and *Eco*RI-*Sal*I sites of pGKV210, respectively. Plasmid pNZ3005 contains the 1.45-kb *Eco*RV-*Bgl*II fragment of pNZ390 cloned into the *Sma*I-*Bam*HI site of pGKV210. Plasmids pNZ3006, pNZ3007, and pNZ3008 contain the 0.5-kb *Xmn*I-*Bgl*II and 0.4-kb *Xmn*I-*Eco*RV fragments of pNZ390 and the 0.35-kb *Eco*RI (filled in with Klenow DNA polymerase)-*Bam*HI fragment of pNZ399 cloned into the *Sma*I-*Bam*HI, *Sma*I, and *Sma*I-*Bam*HI sites of pKK232-8, respectively. Plasmids pNZ3009 and pNZ3010 were obtained by cloning the blunt-end 1.3-kb *Eco*RI-*Bam*HI (*lacR* gene) and 0.3-kb *Hpa*I-*Bam*HI (*prtP* promoter) fragments of pNZ380 and pNZ337 into the *Eco*RI (filled in with Klenow DNA polymerase) and *Sma*I-*Bam*HI sites of pACYC184 and pKK232-8, respectively. As a control in the *E. coli* complementation studies, *Eco*RI-linearized pACYC184 was made blunt ended, ligated, and transformed into *E. coli* HB101 harboring either pNZ3006 or pNZ3010. The resulting pACYC184-derived plasmid, designated pACYC184Cm^s, contains a mutated *cat* gene. As a consequence, Cm resistance in the multiplasmid strains obtained is derived solely from pNZ3006 and pNZ3010.

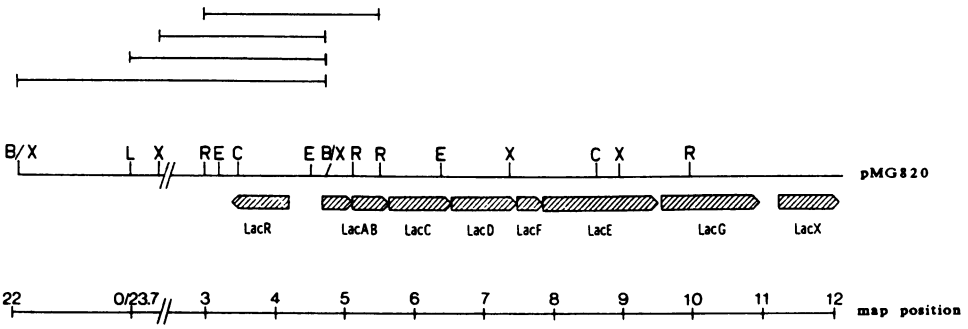

FIG. 1. Physical and genetic map of the *L. lactis* pMG820 *lac* regulon. The locations of the recognition sites for restriction endonucleases *Bcl*I (L), *Bgl*II (B), *Cla*I (C), *Eco*RI (R), *Eco*RV (E), and *Xho*II (X), as determined by physical mapping (29) and deduced from sequence analysis (48), are shown. The locations and orientations of the *lac* genes are shown; the *lacR*, *lacAB*, *lacC*, *lacD*, *lacF*, *lacE*, *lacG*, and *lacX* genes encode the LacR repressor, galactose 6-phosphate isomerase, tagatose 6-phosphate kinase, tagatose 1,6-diphosphate aldolase, enzyme III^{lac}, enzyme II^{lac}, P-β-gal, and a protein with unknown function, respectively. The map positions (in kilobases) (29) and the sizes of the DNA fragments used in localization of the *lac* promoter are indicated at the bottom and top, respectively.

RESULTS

Location of the *lac* operon promoter. The *L. lactis lac* operon that is located on lactose miniplasmid pMG820 (29) encodes the *L. lactis* PEP-PTS^{lac} and tagatose 6-phosphate pathway enzymes (15, 17, 50), which are essential for rapid lactose fermentation (Fig. 1). In initial attempts to localize the *lac* promoter, pMG820 DNA was digested with *Bgl*II, *Xho*II, and *Bcl*I-*Bgl*II and the resulting restriction fragments were shotgun cloned upstream of the promoterless *cat* gene of *E. coli* promoter-probe vector pKK232-8 (7), which was digested with *Bam*HI. Resistance to more than 200 μg of Cm per ml was obtained in *E. coli* MC1061 with only one orientation of the 3.5-kb *Xho*II (six clones were analyzed), 3.8-kb *Bcl*I-*Bgl*II (one clone was analyzed), or 5.8-kb *Bgl*II (nine clones were analyzed) fragment, as shown in Fig. 1. In all of the plasmids, the *Bgl*II-*Xho*II site at map position 4.8 kb (29; Fig. 1) appeared to be located immediately preceding the *cat* gene, indicating that the orientation of the promoter is towards the *lac* genes. To locate the presumed *lac* promoter further, the 2.0-kb *Eco*RI fragment that includes this site was made blunt ended and cloned into *Sma*I-linearized pKK232-8. Since the resulting plasmid containing the expected orientation of the *Eco*RI fragment yielded high Cm resistance in *E. coli*, we assumed that the *lac* promoter was located in the intercistronic region between the *lac* operon and the divergently transcribed *lacR* gene (Fig. 1). Further subcloning and deletion analysis allowed construction of plasmids containing small inserts of this region (Fig. 2) that were tested for *cat* gene expression. *E. coli* MC1061 cells harboring plasmid pNZ3006 or pNZ3008 were resistant up to a concentration of 700 μg of Cm per ml, whereas *E. coli* cells harboring vector pKK232-8 were sensitive to less than 1 μg of Cm per ml. However, deletion of DNA sequences downstream of the *Eco*RV restriction site (Fig. 2, pNZ3007) dramatically decreased Cm resistance to less than 10 μg/ml. Inspection of the nucleotide sequence of this region (48, 50) showed the presence of a putative promoter that contains an *Eco*RV site between the −35 and −10 sequences (Fig. 2). Since further analysis showed that these sequences constitute the *lac* promoter (see below), these results indicate that the *L. lactis lac* promoter is efficiently utilized in *E. coli*.

Primer extension mapping of the *lac* operon promoter. To determine the transcription initiation site of the *L. lactis lac* operon, total RNA was isolated from glucose- and lactose-grown *L. lactis* MG1820 cells and primer extension was performed by using an oligonucleotide primer complementary to the coding strand of the *lacA* gene. In both cultures, three similar-size primer-extended products that were more abundant (5 to 10 times) in the lactose-grown cells were detected (Fig. 3). These results confirm that the *lac* operon is regulated at the transcriptional level, as shown previously (15, 50). Assuming that the middle, most intense, band is the main primer extension product, transcription of the *lac* operon during growth on lactose initiates at the G residue at position 1 (Fig. 3). As a consequence, there is a 94-bp noncoding region upstream of the start codon of *lacA*, the first gene of the *lac* operon (Fig. 2). In addition to the three primer extension products around +1, two minor, similarly labelled, products were found on glucose and lactose (Fig. 3). Therefore, we cannot exclude the possibility that besides the inducible transcription initiation at position +1, minor constitutive transcription initiation occurs at positions +5 and +8.

Flanking regions enhance *lac* promoter activity much more in *L. lactis* than in *E. coli*. Fragments containing the promoter and flanking DNA sequences were fused to the promoterless *cat-86* gene of *L. lactis* promoter-probe vector pGKV210, which is also capable of replication in *E. coli* (47). Constructs pNZ3000 to pNZ3004 contain different fragments of the noncoding region between the *lacR* and *lacA* genes (Fig. 2). To determine the activity of the promoter in a Lac^- host, these constructs were used to transform *L. lactis* MG1363 and subsequently CAT activities were measured (Table 1). The plasmid copy numbers of the constructs were determined in all strains, and they varied between 2.2 and 6.2 copies per chromosome (Table 1). Cells harboring plasmid pNZ3000 showed very low CAT activity and were not able to grow on media containing more than 3 μg of Cm per ml. Cells of MG1363 harboring pNZ3001, pNZ3002, pNZ3003, or pNZ3004 showed considerably higher (5 to 16-fold) CAT activities. These results indicate that sequences downstream (positions +43 to +114; pNZ3001) and upstream (positions −76 to −322; pNZ3003) of the *lac* promoter contribute significantly to the promoter activity.

To investigate whether the role of these flanking regions is host specific, CAT activities were determined in *E. coli* (Table 2) and corrected for the copy number of the *lac* promoter plasmids. The presence of sequences from positions +43 to +114 (pNZ3001) or −322 to −76 (pNZ3003)

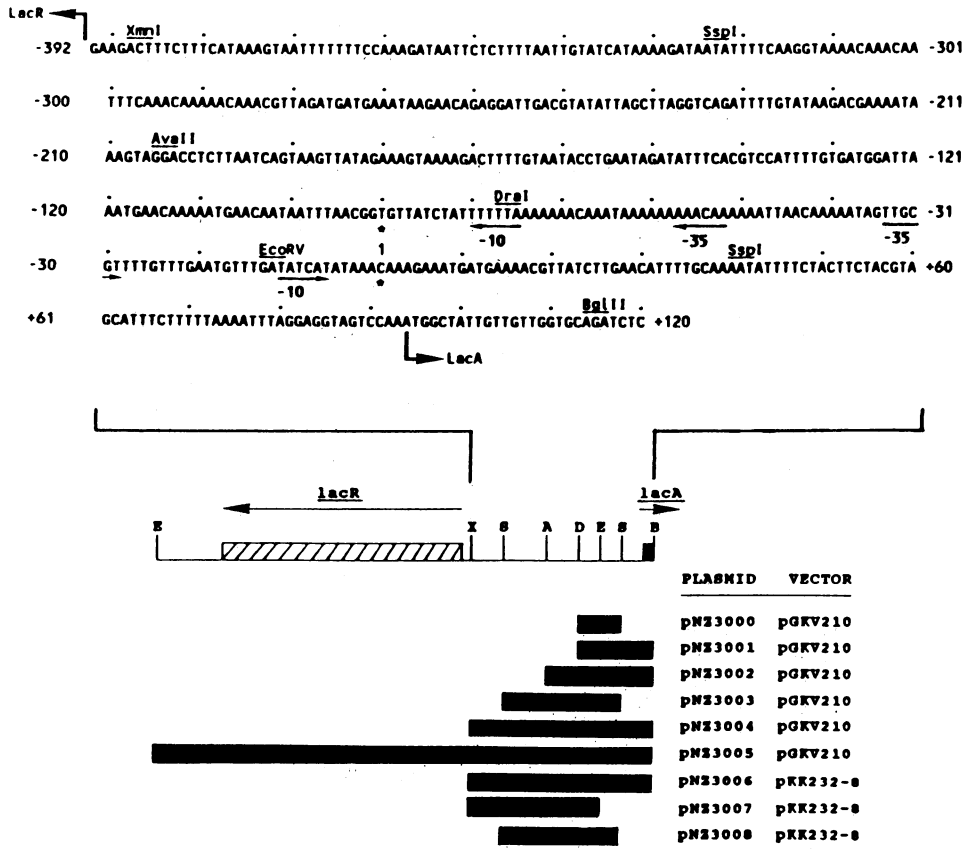

FIG. 2. Cloning, nucleotide sequence, and location of the *lac* operon and *lacR* promoters. A physical map and part of its nucleotide sequence (48, 50) are shown. Solid bars represent the DNA fragments used in construction of the plasmids by using the vectors indicated. The hatched and black bars indicate the coding regions of the *lacR* gene and part of the *lacA* gene, respectively. The positions of the restriction enzyme cleavage sites used in the cloning experiments are indicated on the physical map and in the sequence as follows: A, *Ava*II; B, *Bgl*II; D, *Dra*I; E, *Eco*RV; S, *Ssp*I; X, *Xmn*I. Transcription start sites (*) and the locations and directions of the canonical sequences of the *lac* promoter (this study) and *lacR* promoter, which is located on the opposite strand (48), are indicated. The hooked arrows in the sequence indicate the putative translational start sites of the *lacR* and *lacA* genes.

flanking the minimal promoter fragment (pNZ3000) resulted in 2.5- and 4-fold increases of CAT activity, respectively. The highest increase (fivefold) was observed when sequences from positions −387 to −76 and +43 to +114 (pNZ3004) were present. These results indicate that activity of the *L. lactis lac* promoter in *E. coli* is also enhanced by its upstream and downstream regions, but to a much lesser extent than in *L. lactis*.

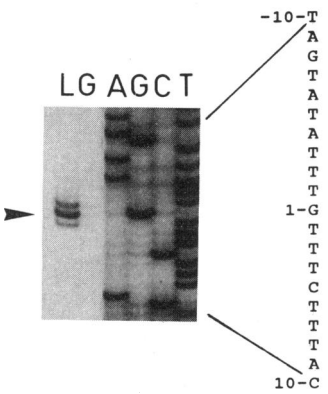

FIG. 3. Primer extension mapping of the *L. lactis lac* operon transcript. The plasmid pMG820 sequencing ladder obtained with the same primer is shown on the right. L, lactose-grown cells; G, glucose-grown cells. The arrowhead indicates a major transcription initiation site.

Presence of the *lacR* gene represses *lac* promoter activity in *L. lactis* and *E. coli*. To examine the role of the LacR repressor in determining *lac* promoter activity, pNZ3005 was used to transform *E. coli* MC1061 and *L. lactis* MG1363. Plasmid pNZ3005 contains the complete *lacR* gene and the *lac* promoter fused to the *cat-86* gene (Fig. 2). Both *E. coli* and *L. lactis* cells harboring pNZ3005 showed decreased CAT activities compared with pNZ3004-containing cells (Tables 1 and 2). Therefore, we conclude that the presence of the *lacR* gene decreases the activity of the *lac* promoter. Previously we found that in *L. lactis* LacR represses *lac* promoter activity in *trans* (48). To study the effect of *lacR* on *lac* promoter activity in *E. coli*, pACYC184 derivative pNZ3009, containing the *lacR* gene under control of its own expression signals, was used to transform *E. coli* HB101 carrying pNZ3006 (Fig. 2). Whereas HB101 harboring pNZ3006 and control plasmid pACYC184Cm^s was resistant up to 700 μg/ml, cells of HB101 harboring pNZ3006 and pNZ3009 were only resistant up to 200 μg of Cm per ml. The specificity of the *lacR* gene product in inhibiting only *lac* promoter activity was examined by introducing pNZ3009

TABLE 1. CAT activities and plasmid copy numbers of *L. lactis* MG5267 and MG1363 harboring the indicated plasmids[a]

Plasmid	CAT activity (U · mg^{-1})[b]			Plasmid copy no.[c]	
	MG1363 (glucose)	MG5267 (lactose)	MG5267 (glucose)	MG1363 (glucose)	MG5267 (glucose-lactose)
pNZ3000	0.1 (2.8)	0.1 (2.6)	0.1 (2.6)	2.8	3.0
pNZ3001	0.7 (14)	1.0 (31)	0.9 (28)	4.0	2.5
pNZ3002	1.0 (23)	2.7 (100)	1.7 (63)	3.4	2.1
pNZ3003	1.6 (28)	2.6 (23)	2.2 (19)	4.4	8.9
pNZ3004	3.7 (46)	5.5 (80)	1.4 (21)	6.2	5.3
pNZ3005	0.2 (7.1)	1.7 (73)	0.3 (13)	2.2	1.8
pGKV210	<0.01	<0.01	<0.01	ND	ND

[a] Average CAT activities of two independent determinations are given (the standard deviation was less than 15%). Plasmid copy numbers were determined in the same cultures that were used for determination of CAT and P-β-gal activities. The energy sources used in the growth medium are indicated.
[b] The numbers in parentheses indicate relative CAT activities (in percentages of the maximal value) that were corrected for plasmid copy number.
[c] Number of plasmid copies per chromosome. ND, not determined.

into strain HB101 harboring pNZ3010, which contains the unrelated *prtP* promoter upstream of the pKK232-8 *cat* gene. Similar Cm resistances (180 μg/ml) were observed in pNZ3010-containing HB101 cells harboring pNZ3009 or pACYC184Cm^s. From these and previous results (48), we conclude that the *lacR* gene product represses *lac* promoter activity in *trans* in both *E. coli* and *L. lactis*.

Regulation of the *lac* operon is mediated by interaction between LacR and the *lac* promoter region. To study the influence of flanking DNA sequences and the LacR repressor on regulation of *lac* promoter activity, constructs pNZ3000 to pNZ3005 were used to transform *L. lactis* MG5267, which contains a single chromosomal copy of the *lac* operon (20). Use of strain MG5267 has the additional advantage that the effect of the extrachromosomal *lac* promoter fragments on *lac* operon expression can be determined via measurement of the activity of P-β-gal (Fig. 1, LacG). Like *L. lactis* MG1820, which harbors pMG820, MG5267 shows a 5- to 10-fold induction of *lac* operon expression during growth on lactose (48). CAT and P-β-gal activities were measured to determine plasmid- and chromosome-derived *lac* promoter activities, respectively. The plasmid copy numbers were determined (Fig. 4 and Table 1) and found to be identical in glucose- and lactose-grown cultures (data not shown). The results (Tables 1 and 3) indicate that MG5267 cells harboring pNZ3000 show only very low CAT activity under both induced and noninduced conditions, similar to MG1363 cells harboring this plasmid. In contrast, pNZ3004- and pNZ3005-containing MG5267 cells showed lactose-inducible CAT activity. Cells of MG5267 harboring pNZ3001, pNZ3002, or pNZ3003 showed no significant induction of CAT activity during growth on lactose. The increased P-β-gal activities of MG5267 cells harboring pNZ3003 or pNZ3004 (Table 3) when grown on glucose suggest that the chromosomally encoded LacR repressor is titrated by the plasmid-derived copies of the *lac* promoter, resulting in a derepressed *lac* operon. Cells harboring pNZ3005 show super-repressed and lower P-β-gal activities during growth on glucose and lactose, respectively. This may be attributed to the excess of plasmid-encoded LacR, resulting in additional repression of the chromosomal copy of the *lac* promoter.

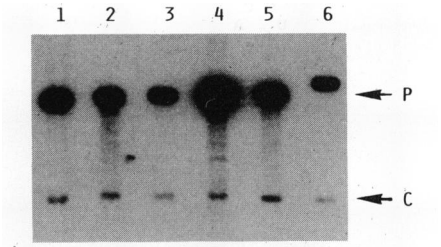

FIG. 4. Determination of plasmid copy number in lactose-grown *L. lactis* MG5267 cells. *Eco*RV-digested total DNA was separated on a 1% agarose gel, blotted onto a GeneScreen Plus membrane, and subsequently hybridized with an end-labelled *lac* promoter-specific probe. Lanes 1 to 6 contained MG5267 harboring pNZ3000, pNZ3001, pNZ3002, pNZ3003, pNZ3004, and pNZ3005, respectively. The positions of plasmid (P)- and chromosome (C)-derived copies of the *lac* promoter are indicated.

TABLE 2. CAT activities of *E. coli* MC1061 strains containing various constructs

Plasmid	CAT activity (U · mg^{-1})[a]	Plasmid copy no.[b]
pNZ3000	4.5 (20)	9.2
pNZ3001	3.1 (52)	2.4
pNZ3002	3.5 (47)	3.0
pNZ3003	6.8 (78)	3.5
pNZ3004	6.0 (100)	2.4
pNZ3005	1.1 (19)	2.3
pGKV210	<0.1	ND

[a] The numbers in parentheses indicate relative CAT activities (in percentages of the maximal value) that were corrected for plasmid copy number.
[b] Relative plasmid copy numbers are shown. ND, not determined.

DISCUSSION

Function of flanking sequences in determining *lac* promoter activity. We determined the transcription initiation site of the promoter of the *L. lactis lac* operon (Fig. 3). The canonical −35 and −10 sequences and their spacing correspond closely to the extended promoter consensus sequence for gram-positive bacteria postulated by Graves and Rabinowitz (22), in which the TG dinucleotide around position −13 and an AT-rich stretch upstream of −35 are also conserved. The smallest restriction fragment (positions −75 to +42) that contains these consensus sequences (minimal promoter fragment) was fused to a promoterless *cat-86* gene in pNZ3000. Unexpectedly, the presence of pNZ3000 in *L. lactis* and *E. coli* resulted in relatively low CAT activities, which were

TABLE 3. P-β-gal activities of *L. lactis* MG5267 cells containing the indicated plasmids

Plasmid	P-β-gal activity (μmol · min^{-1} · mg^{-1})	
	Lactose[a]	Glucose[a]
pNZ3000	1.8	0.4
pNZ3001	1.8	0.4
pNZ3002	1.9	0.4
pNZ3003	2.0	1.0
pNZ3004	1.9	1.0
pNZ3005	1.3	0.2
pGKV210	1.9	0.4

[a] Energy source used in the growth medium.

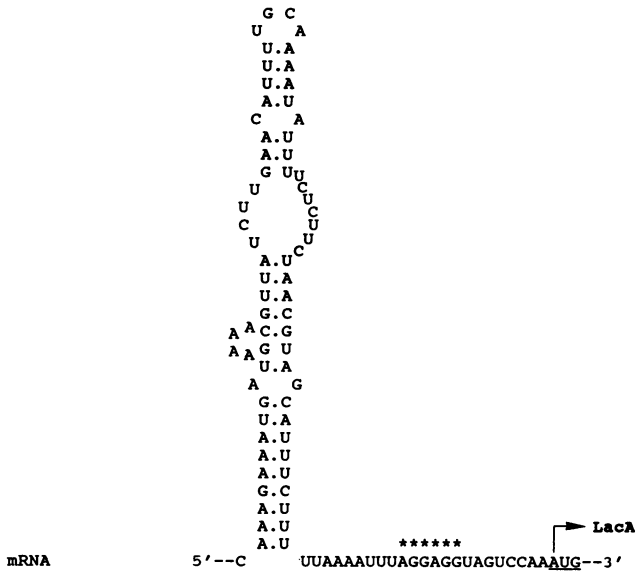

FIG. 5. Possible secondary structure of the 5′ noncoding region of the *lac* operon transcript. The free energy was calculated, as described by Tinoco et al. (44), as −5.8 kcal (1 cal = 4.184 J) · mol^{-1}. The positions of the LacA translation start (underlined) and putative ribosome-binding site (******) are indicated.

decreased 38- and 5-fold, respectively, compared with the highest activities (Table 1, pNZ3002 in MG5267 grown on lactose; Table 2, pNZ3005). The presence of DNA sequences downstream (positions +43 to +114) of the minimal promoter fragment increased CAT activities 5 (MG1363)- to 11 (MG5267)-fold in *L. lactis* (Table 1) and 2.5-fold in *E. coli* (Table 2). Sequences at positions +43 to +114 (pNZ3001) could be involved in the stability of the *lac* operon transcript by participating in the formation of a stem-loop structure (Fig. 5). In the absence of this stem-loop structure (pNZ3000), the transcript might be less stable, resulting in significantly decreased CAT activities. It has been shown that the 5′ leader sequences, which may contain stable stem-loop structures, of the *E. coli ompA* and *bla* (2), bacteriophage T4 gene 32 (21), and *B. subtilis sdh* transcripts (32) contribute to mRNA stabilization and protection against degradation. The observation that cells harboring pNZ3003 show considerably higher CAT activity than cells harboring pNZ3000 indicates that the presence of the sequences between +43 and +114 is not the only factor involved in the efficiency of the *lac* promoter. Since it is unlikely that the upstream sequences from −322 to −76 present in pNZ3003 affect the stability of the transcript produced, the 10-fold increase in CAT activity of cells harboring this plasmid compared with cells harboring pNZ3000 may be attributed to enhanced *lac* promoter activity. In *E. coli*, upstream activating sequences have been shown to activate the expression of some genes in part by intrinsic bending (24); for other genes, these sequences are targets for activator proteins (33). In *B. subtilis*, DNA curvature of upstream regions appears to stimulate gene expression, as has been shown for the Alu156 bacteriophage SP82 promoters (30, 31). A common feature of these sequences is the high level of AT residues. Since the −322 to −76 region of the *L. lactis lac* promoter is highly (74%) AT rich, it is tempting to speculate that activity of the *lac* promoter could be stimulated similarly. Alternatively, this region could be a target for a protein that stimulates transcription, comparable to the catabolite-activating protein of *E. coli* (13). In *E. coli*, the absence of sequences from +43 to +114 or −322 to −76 resulted in a less severe decrease of CAT activity than in *L. lactis*, indicating that different mechanisms or cellular factors might be involved in mRNA decay and enhancement of transcription initiation in *L. lactis* and *E. coli*.

Transcriptional regulation of the *L. lactis lac* operon. Expression of the *L. lactis lac* operon is repressed during growth on glucose and is mediated by the LacR repressor (48). Expression of the *lacR* gene, which is divergently transcribed from the *lac* operon (Fig. 1), is repressed during growth on lactose (48). Overproduction of LacR in lactose-grown cells resulted in substantial growth rate and PEP-PTS^{lac} enzyme activity decreases (48). Here we provide evidence that regulation is effected by the interaction between the LacR repressor and the *lac* promoter region. The involvement of *lacR* in repression of promoter activity is evident from the low CAT activities in *L. lactis* MG1363 and *E. coli* MC1061 cells harboring pNZ3005 (Table 1), whereas deletion of *lacR* resulted in a significant increase of activities (Tables 1 and 2, pNZ3004). In *E. coli*, the presence of the *lacR* gene in *trans* resulted in decreased Cm resistance of pNZ3006-containing cells, in which the *cat* gene is under control of the *lac* promoter. No decrease was observed when the *cat* gene was under control of the unrelated *prtP* promoter. These and earlier (48) results indicate that the promoter region in pNZ3006, which includes positions −387 to +114, is a target for the LacR repressor. As a consequence, introduction of pNZ3004 (containing the same promoter region) into Lac^+ *L. lactis* MG5267, which contains a chromosomal copy of all *lac* genes, results in inducible Cm resistance with CAT activities that are fourfold higher on lactose than on glucose (Table 1). The presence of additional copies of the *lacR* gene (pNZ3005) results in even higher (sixfold) CAT induction in MG5267. Comparison of the P-β-gal (LacG) activities, which reflect the activity of the chromosomal copy of the *lac* promoter, may register the distribution of LacR between the chromosome- and plasmid-derived copies of the *lac* promoter region. Cells of MG5267 harboring pNZ3004 show increased P-β-gal activity when grown on glucose, indicating that the chromosomally encoded LacR molecules are titrated by the excess of plasmid-located *lac* promoter regions. The presence of additional plasmid-encoded LacR, as in MG5267 harboring pNZ3005, results in a relatively lower activity of the chromosomally located *lac* promoter, as shown by the decreased P-β-gal activities on glucose and lactose (Table 3). The ability of pNZ3003 to titrate LacR, as indicated by the increased P-β-gal activities of MG5267 cells harboring this plasmid, did not result in induced CAT activities on lactose, in contrast to cells of MG5267 harboring pNZ3004. This may be a consequence of the absence in pNZ3003 of the DNA regions from −387 to −322 and from +43 to +114, which suggests that additional LacR-binding sites are located within this region. Alternatively, as a consequence of the absence of the putative stem-loop structure in pNZ3003, the higher promoter activity on lactose could be diminished by a higher turnover of mRNA. Although the mechanism of catabolite repression in gram-positive bacteria is poorly understood, there are reports of catabolite repression of amylase production and aconitase synthesis in *B. subtilis* (18, 53). Therefore, we cannot exclude the possibility that a similar control system operates in *L. lactis*, in addition to the LacR control circuit.

Previously we have shown that the LacR repressor is

FIG. 6. Homology between the *E. coli deoO1* and putative *L. lactis lacO* operators. Identical residues (*), axis of symmetry (·), inverted repeats (arrows), and transcription initiation sites (hooked arrows) are indicated.

homologous to *E. coli* DeoR, FucR, and GutR and *S. aureus* LacR (48) and contains a helix-turn-helix motif which is characteristic for DNA-binding proteins (6). Because the homology between these proteins is most significant in the helix-turn-helix motif, the DNA regions that are involved in binding of these proteins might also be homologous. To identify such a sequence, we searched for homologies between the characterized *deo* operator (11) and the *L. lactis lac* promoter region. Operators involved in binding of the regulatory proteins of the *E. coli fuc* and *gut* and *S. aureus lac* operons have not been characterized. Figure 6 shows the homology between the *deoO1* operator and an imperfect inverted repeat at positions −18 to +2 of the *L. lactis* promoter region. In analogy with the *deo* operon, LacR could bind to this sequence, resulting in inhibition of transcription initiation of the *lac* promoter. The sole presence of this putative operator on multicopy plasmids does not affect the P-β-gal activities of MG5267 cells harboring pNZ3000, pNZ3001, pNZ3002, or pGKV210 (control), indicating that no titration of chromosomally encoded LacR occurs. Apparently, the excess plasmid-located operators do not compete efficiently with those present at the chromosome. Cooperative LacR binding may take place, since *lac* promoter fragments containing the region from −322 to −205 (such as those present in pNZ3003 and pNZ3004) are able to compete with the chromosomal copy of the *lac* promoter. This is supported by preliminary footprinting studies that have showed the presence of multiple operators in this region (49).

Comparison of the specific CAT activities obtained with the various *lac* promoter plasmids in *L. lactis* (Table 1) shows that the highest CAT activities are found in MG5267 grown on lactose and not in MG1363. This was not to be expected, since MG1363 lacks the *lacR* gene, and hence, no repression of the *lac* promoter by LacR occurs. The most likely explanation for the lower-than-expected CAT expression in MG1363 is the absence of a lactose-inducible activating factor that is encoded by the *lac* operon. It is tempting to speculate that the product of the *lacX* gene is involved in this activation, as has previously been suggested (15).

The results described here and in previous work (48) indicate that the promoters of the *lac* operon and the *lacR* regulator gene are organized in a back-to-back arrangement with a regulator-structural type of regulation, as has been described for a variety of organisms (1). In this type of regulation, the regulatory molecule acts within the divergent transcription unit to control transcription of the structural genes, and often it also regulates its own synthesis (1). To the highly mobile *L. lactis lac* operon (42, 46), this has the advantage that it may be translocated to new locations without loss of autonomous regulation.

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