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Cloning and Partial Characterization of Regulated Promoters from *Lactococcus lactis* Tn*917-lacZ* Integrants with the New Promoter Probe Vector, pAK80

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Transposon Tn*917*-LTV1 was used to produce a collection of *Lactococcus lactis* strains with fusion of a promoterless *lacZ* gene to chromosomal loci. Screening 2,500 Tn*917*-LTV1 integrants revealed 222 that express β-galactosidase on plates at 30°C. Pulsed-field gel electrophoresis revealed Tn*917*-LTV1 insertions in at least 13 loci in 15 strains analyzed. Integrants in which β-galactosidase expression was regulated by temperature or pH and/or arginine concentration were isolated. In most cases, the regulation observed on plates was reproducible in liquid medium. One integrant, PA170, produces β-galactosidase at pH 5.2 but not at pH 7.0, produces more β-galactosidase at 15°C than at 30°C, and has increased β-galactosidase activity in the stationary phase. DNA fragments potentially carrying promoters from selected *Lactococcus lactis* integrants were cloned in *Escherichia coli*. A new promoter probe vector, pAK80, containing promoterless β-galactosidase genes from *Leuconostoc mesenteroides* subsp. *cremoris* and the *Lactococcus lactis* subsp. *lactis* biovar diacetylactis citrate plasmid replication region was constructed, and the lactococcal fragments were inserted. Plasmid pAK80 was capable of detecting and discriminating even weak promoters in *Lactococcus lactis*. When inserted in pAK80, the promoter cloned from PA170 displayed a regulated expression of β-galactosidase analogous to the regulation observed in PA170.

Lactococcus lactis is an important industrial microorganism used to produce a variety of cheeses and cultured milk products such as buttermilk. Genetic modification of *Lactococcus lactis* may be desirable, for example, to improve the acid production, bacteriophage resistance, or production of flavor compounds by industrially important strains. A better understanding of the regulation of gene expression in this organism and a collection of regulated promoters would facilitate the desired modifications.

Regulation of gene expression in *Lactococcus lactis* has been the subject of several studies (recently reviewed in references 11 and 36). A variety of promoter selection vectors have been developed; these vectors allow detection of promoters in *Lactococcus lactis* following insertion of DNA fragments into a polylinker preceding a promoterless reporter gene (1, 6, 22, 31, 33, 38). The function and regulation of these promoters were, in most cases, not elucidated. Several promoters from defined lactococcal genes have been shown to be regulated (9, 12, 15, 35, 39).

Accurate analysis of the regulation of gene expression is not always possible with multicopy systems. Several transposons carrying a promoterless reporter gene have been constructed and used as promoter probes in enterobacteria (3), allowing analysis of gene expression with one copy per chromosome. Youngman et al. (41) used the gram-positive *Enterococcus faecalis* transposon Tn*917* for studying the sporulation genes in *Bacillus subtilis*. Recently, we reported that Tn*917-lacZ* derivatives, including Tn*917*-LTV1 (8) (Fig. 1) and Tn*917*-TV32 (40), transpose in *Lactococcus lactis* and that the chromosome of this bacterium does not contain hot spots for Tn*917* insertions (18). Ten *Lactococcus lactis* Tn*917*-TV32 integrants that express β-galactosidase, presumably as a result of fusion of chromosomal promoters to the *lacZ* gene, were isolated (18).

In this paper, we describe the production of a collection of 222 *Lactococcus lactis* Tn*917*-LTV1 integrants that express β-galactosidase (β-Gal$^+$). Screening of integrants on plates for regulated expression of β-galactosidase was used as an initial step in identification of sites on the *Lactococcus lactis* chromosome where regulated gene expression occurs upon gene insertion. *Lactococcus* DNA adjacent to the *lacZ* end of the transposon from five Tn*917-lacZ* integrants was cloned in *Escherichia coli*. These integrants were chosen because β-galactosidase expression responds to environmental factors relevant to production processes used in dairies. A new promoter probe vector, pAK80, was constructed and shown to be more sensitive than pGKV210 (37) for promoter detection. Finally, we show that, when one of the promoters is inserted in pAK80, regulation of gene expression is analogous to the regulation observed in the original Tn*917*-LTV1 integrant.

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MATERIALS AND METHODS

Bacterial strains, culture media, reagents, and plasmids. *Lactococcus lactis* MG1363, MG1614 (14), and derivatives were routinely grown in M17 (34) containing 0.5% glucose instead of lactose at 30°C, unless otherwise specified. *E. coli* DH5α (Life Technologies, Gaithersburg, Md.) was cultured at 37°C in Luria-Bertani (LB) broth or LB agar (4). Erythromycin (ERY), chloramphenicol (CAM), ampicillin (AMP), *o*-nitrophenyl-β-D-galactopyranoside (ONPG), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were purchased from Sigma Chemical Co. (St. Louis, Mo.). ERY was used for selection of pGKV210 and pAK80 derivatives in *Lactococcus lactis* at 5.0 and 1.0 μg/ml,

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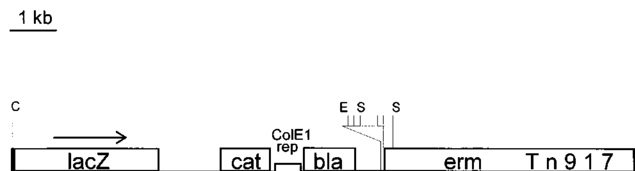

FIG. 1. Physical map of Tn*917*-LTV1 (redrawn from reference 8). The restriction enzyme sites used for cloning and mapping are abbreviated as follows: E, *Eco*RI; C, *Cla*I; S, *Sma*I. The heavy lines at each end of Tn*917*-LTV1 are the inverted repeats of Tn*917*.

respectively. The concentrations of CAM used for screening promoter activity on DNA fragments inserted into pGKV210 were 4, 8, 12, 16, and 20 μg/ml. ERY and AMP were used for selection in *E. coli* at 250 and 100 μg/ml, respectively. X-Gal was used at 160 μg/ml for *Lactococcus lactis*. Restriction enzymes were used as recommended by the supplier. Plasmids are listed in Table 1.

Production and screening of promoter fusions. A collection of about 2,500 Tn*917*-LTV1 insertions in the chromosome of *Lactococcus lactis* MG1363 was produced by the replica-plating technique described previously (18). Integrants were plated on M17 containing ERY and X-Gal and incubated at 30°C for 40 h and then at 20°C for 100 h. Increasing numbers of β-Gal$^+$ integrants were detected with increasing concentrations of X-Gal, with a maximum at 320 μg/ml. Blue colonies were picked and restreaked once on M17 containing ERY and X-Gal. The restreaked colonies were named PA1 to PA242. Twenty-one integrants died during the restreaking process. PA243, which expresses β-galactosidase, was isolated from a collection of 10 Tn*917*-LTV1 insertions in the chromosome of MG1614.

Tn*917*-LTV1 integrants showing pH-regulated β-galactosidase expression were identified with M17 (which contained 0.5% glucose) and M17arg (which contained 0.1% glucose and 0.1% arginine) plates, both supplemented with X-Gal. Following growth of *Lactococcus lactis* for 20 to 40 h at 30°C, the pH was about 5.3 on M17 and 6.9 on M17arg. The pH was estimated by use of pH indicator strips on a suspension of a colony in 250 μl of double-distilled water. The pH effect is a consequence of the arginine catabolism with concomitant formation of ammonia. Plates for screening were incubated at 30°C for 24 h.

Identification of integrants showing temperature-regulated β-galactosidase expression was by streaking onto a duplicate set of plates with X-Gal and incubating at 15 and 30°C. To obtain comparable bacterial growth in the streaks, the plates were incubated for 24 h at 30°C and 190 h at 15°C.

Inspection of single colonies was not suitable for screening because differences in colony size strongly influenced color development. Instead, reproducible results were obtained when integrants were patched onto agar plates in short streaks.

DNA preparation and manipulation. Plasmid extractions were done by published techniques (for *Lactococcus lactis* [29, 30] and *E. coli* [5]). Genomic *Lactococcus lactis* DNA was purified as described by Johansen and Kibenich (19). Southern hybridizations on *Eco*RI-digested genomic DNA were done as described previously (18). ^{32}P-labelled probes were either pLTV1 or a 4.0-kb *Eco*RI fragment from pLTV1 containing the pE194 replication region. Preparation of *Lactococcus lactis* genomic DNA, restriction enzyme digestion in agarose blocks, and pulsed-field gel electrophoresis were performed as described previously (18).

PCRs were done under the conditions described by Pedersen et al. (30).

TABLE 1. Plasmids used

Plasmid	Relevant characteristics	Source or reference
pLTV1	Delivery plasmid for Tn*917*-LTV1	8
p143	Plasmid containing rescued 1.7-kb chromosomal DNA fragment from *Lactococcus lactis* PA143	This work
p162	Plasmid containing rescued 1.8-kb chromosomal DNA fragment from *Lactococcus lactis* PA162	This work
p163	Plasmid containing rescued 1.0-kb chromosomal DNA fragment from *Lactococcus lactis* PA163	This work
p170	Plasmid containing rescued 9.7-kb chromosomal DNA fragment from *Lactococcus lactis* PA170	This work
p243	Plasmid containing rescued 1.8-kb chromosomal DNA fragment from *Lactococcus lactis* PA243	This work
pGKV210	Promoter selection vector containing a promoterless *cat86* gene	38
pGKV210/C	pGKV210 derivative containing a *Cla*I linker inserted into the multiple cloning site	This work
pGKV210/C::143	pGKV210/C containing the 1.7-kb chromosomal DNA fragment from p143	This work
pGKV210/C::162d	pGKV210/C containing 0.7 kb of the 1.8-kb chromosomal DNA fragment from p162	This work
pGKV210/C::162u	pGKV210/C containing 1.1 kb of the 1.8-kb chromosomal DNA fragment from p162	This work
pGKV210/C::163	pGKV210/C containing the 1.0-kb chromosomal DNA fragment from p163	This work
pGKV210/C::170	pGKV210/C containing the 9.7-kb chromosomal DNA fragment from p170	This work
pGKV210/C::243	pGKV210/C containing the 1.8-kb chromosomal DNA fragment from p243	This work
pVA891	Vector suitable for cloning replication regions from plasmids of gram-positive bacteria; Em^r	24
pIC19H	*E. coli* cloning vector	25
pKR41	pIC19H containing the citrate plasmid replication region	13
pAK66	*E. coli-Lactococcus* shuttle vector; Em^r	This work
pSB1	pIC19H containing the *Leuconostoc mesenteroides* subsp. *cremoris* β-galactosidase genes (*lacL lacM*)	20
pAK67	pSB1 from which regulatory region of β-galactosidase genes has been replaced with a polylinker	This work
pAK67.7	pAK67 containing oligonucleotide giving stop codons in all three forward reading frames	This work
pAK80	Promoter selection vector containing promoterless *lacL* and *lacM* genes	This work
pGEM-7Zf(+)	*E. coli* cloning vector	Promega, Madison, Wis.
pAK80::143	pAK80 derivative containing the 1.7-kb chromosomal DNA fragment from p143	This work
pAK80::162d	pAK80 derivative containing 0.7 kb of the 1.8-kb chromosomal DNA fragment from p162	This work
pAK80::162u	pAK80 derivative containing 1.1 kb of the 1.8-kb chromosomal DNA fragment from p162	This work
pAK80::163	pAK80 derivative containing the 1.0-kb chromosomal DNA fragment from p163	This work
pAK80::170	pAK80 derivative containing the 9.7-kb chromosomal DNA fragment from p170	This work
pAK80::243	pAK80 derivative containing the 1.8-kb chromosomal DNA fragment from p243	This work

DNA was introduced into *Lactococcus lactis* by electroporation of glycine-grown competent cells (17) and into *E. coli* as described by Hanahan (16).

Plasmids containing the chromosomal *Lactococcus lactis* DNA adjacent to the *lacZ* proximal end of Tn*917* were produced from Tn*917*-LTV1 integrants as described by Youngman (40) with the modifications described below. Chromosomal DNA (100 ng) from *Lactococcus lactis* Tn*917*-LTV1 integrants was digested with *Eco*RI, phenol extracted, ethanol precipitated, and ligated in a final volume of 10 μl. The ligation mixture was introduced into *E. coli*, and AMP-resistant transformants were selected.

Construction of pGKV210/C. A *Cla*I site was introduced into the polylinker preceding the promoterless *cat86* gene in pGKV210 as follows. A synthetic oligonucleotide, 5′ GATCGCCATCGATGGC 3′, was self hybridized, producing a double-stranded oligonucleotide with *Bam*HI sticky ends and an internal *Cla*I site. This was inserted into the unique *Bam*HI site in the polylinker of pGKV210, resulting in plasmid pGKV210/C.

Construction of the promoter probe vector pAK80. A new promoter probe vector for *Lactococcus lactis*, pAK80, containing the citrate plasmid minimal replicon (30), a promoterless β-galactosidase operon derived from *Leuconostoc mesenteroides* subsp. *cremoris*, and a gene for erythromycin resistance, was constructed as follows.

A 1.7-kb *Eco*RI fragment containing the minimal replicon of the *Lactococcus lactis* subsp. *lactis* biovar diacetylactis citrate plasmid (*ori*, *repB*, and ~300 bp of flanking DNA [30]) was cloned from pKR41 (13) into the unique *Eco*RI site of pVA891 (24) to produce pAK66, a *Lactococcus*-*E. coli* shuttle vector.

The *Leuconostoc mesenteroides* subsp. *cremoris* β-galactosidase genes were obtained from a clone designated pSB1. This clone was constructed during the course of cloning and sequencing IS*1165* from strain DB*1165* (20). Plasmid pSB1 contains a 5.8-kb *Bgl*II-*Sal*I fragment, containing most of IS*1165* and flanking DNA, inserted in the polylinker of pIC19H (25). DNA sequence analysis revealed that the insert in pSB1 contains the β-galactosidase genes (*lacL lacM*) of *Leuconostoc mesenteroides* subsp. *cremoris* and that they are nearly identical to those of *Leuconostoc lactis* (10). Only three differences were detected in 830 bp sequenced. Since DH5α/pSB1 was blue, in spite of having an insert in the pIC19H polylinker, the *Leuconostoc lacL lacM* genes are expressed in *E. coli.*

Replacement of the *lacL lacM* promoter with a polylinker was done by PCR. The two following primers were used: lac-1, i.e., 5′ ATAGATCTGCAGGATC CCGGGTAACTTTGAAAGGATATTCCTC 3′, and lac-2, i.e., 5′ ATTGAGG GTATACGGTGGGCG 3′. The underlined part of lac-1 is identical to the beginning of the *lacL* gene and contains the ribosome binding site but not the regulatory region. The remaining sequence contains a variety of restriction sites, including *Bgl*II. The lac-2 primer is complementary to the β-galactosidase gene, annealing 20 bp downstream of the unique *Nco*I site. PCR amplification with these primers amplifies from the ribosome binding site to just beyond the *Nco*I site, producing a 360-bp fragment. This 360-bp fragment was purified, digested with *Bgl*II and *Nco*I, and cloned into *Bgl*II-*Nco*I-digested pSB1, replacing the promoter and all upstream *Leuconostoc* sequences with a polylinker. The resulting plasmid was designated pAK67. Sequence analysis revealed no unexpected PCR-induced alterations.

Stop codons were introduced into the polylinker of pAK67 with the following two complementary oligonucleotides: Stop-1, i.e., 5′ GGGTCTAGATTA 3′, and Stop-2, i.e., 5′ TAATCTAGACCC 3′. Annealing gives a blunt-ended DNA molecule containing an internal *Xba*I restriction site. This small fragment was cloned into the *Sma*I site of pAK67. These oligonucleotides were designed in such a way that the *Sma*I site would be retained, a new *Xba*I site would be present in plasmids with this tiny insert, and stop codons would be present in all three reading frames. Cloning was done by digesting pAK67 with *Sma*I, treating with phosphatase, and ligating with a mixture of the two oligonucleotides that had been treated with kinase and annealed. Transformants were purified, and those in which the plasmid had gained an *Xba*I site were analyzed further. DNA sequence analysis revealed one clone, pAK67.7, with the desired structure. The sequence of the polylinker region of pAK67.7 is illustrated in Fig. 2A.

The final step in the production of the promoter probe vector was the combining of the manipulated *lacL lacM* genes with a replicon and selectable marker for *Lactococcus lactis*. A 4.0-kb *Hin*dIII-*Sal*I fragment from pAK67.7 was cloned into pAK66 digested with *Hin*dIII and *Sal*I. The resulting plasmid, pAK80, is a *Lactococcus* promoter probe vector and is illustrated in Fig. 2B.

pAK80 was shown to work as a promoter probe vector by inserting a *Lactococcus lactis* tRNA promoter, resulting in plasmid pAK90 (28). Strain MG1363 harboring pAK80 or pAK90 was streaked on M17 plus X-Gal plates. Only MG1363/pAK90 was blue. The copy number of pAK80 was estimated to be 5 to 10 copies per cell by comparison with a standard of known concentration on agarose gels.

Measurement of β-galactosidase activity in liquid cultures. β-Galactosidase activity was measured as described by Miller (27) with the following modifications. Cultures were grown in M17 or M17arg containing 1.5 times the usual amount of M17 and harvested after 20 h at 30°C or 165 h at 15°C. Cells were harvested by centrifugation and concentrated up to 10-fold in Z buffer. One-half milliliter of bacterial suspension was mixed with 12.5 μl of 0.1% sodium dodecyl sulfate and 25 μl of chloroform on a vortex mixer for 10 s. After 5 min of incubation in a 30°C water bath, 100 μl of ONPG (4 mg/ml of A-medium [27]) was added, and the suspension was vortexed for 2 s and incubated further at 30°C. Reactions were stopped by the addition of 250 μl of 1 M sodium carbonate. After centrifugation, A_{420} and A_{550} values were measured on the supernatant. If A_{550} values exceeded 0.050, the sample was centrifuged again and remeasured. β-Galactosidase activity in Miller units was calculated as $(522 \cdot A_{420})/(t \cdot v \cdot OD_{600})$, where t is time in minutes, v is the volume of culture used in the assay in milliliters, and OD_{600} is the optical density of the culture at 600 nm.

Fermentations. Four fermentors, each containing 1 liter of M17 made with 1.5 times the usual amount of M17 and supplemented with ERY, were set to operate at 30°C. Stirring was kept at 150 rpm without an active supply of air. pH in the growth medium was automatically controlled by the addition of 5 M HCl or 5 M NaOH. Fermentors were set to operate in parallel at pH 5.2 and 7.0. The duplicate fermentors were inoculated with 10 ml of a fresh overnight culture grown in the same medium. Fermentations were run for 22 h, and growth was monitored by measuring the OD_{600}. Samples for measurement of β-galactosidase activity were taken at selected OD_{600} values and time intervals.

RESULTS

Production of promoter fusions and screening for regulated expression of β-galactosidase. A collection of about 2,500 Tn*917*-LTV1 insertions in *Lactococcus lactis* MG1363 was produced and screened for β-galactosidase production. The β-Gal$^+$ integrants displayed varying blue intensities, ranging from pale to strong blue.

Southern hybridizations of *Eco*RI-digested chromosomal DNA from 12 β-Gal$^+$ integrants probed with the transposition vector pLTV1 and a replication region-specific probe showed that 11 integrants have a single copy of Tn*917*-LTV1 and that one integrant, PA179, also contains vector DNA (data not shown). The region of Tn*917*-LTV1 preceding the ribosome binding site and the *lacZ* gene contains stop codons in all three reading frames. Thus, β-galactosidase expression results from transcriptional fusion of chromosomal promoters to the promoterless *lacZ* gene in Tn*917*-LTV1.

All 222 β-Gal$^+$ integrants were screened on plates for regulated expression of β-galactosidase. Twenty integrants showed altered expression of β-galactosidase when the plates contained arginine. The pH of the plates containing arginine is higher than that of the plates without arginine as a consequence of arginine catabolism and the concomitant release of ammonia. Thus, these promoters are regulated by pH and/or arginine. Thirteen integrants showed altered expression at 15°C relative to that at 30°C. In four of these integrants, the expression was also regulated by pH and/or arginine.

Fifteen integrants showing regulated expression of β-galactosidase on plates were selected for further analysis. Since Tn*917*-LTV1 contains two *Sma*I sites (Fig. 1), the location of Tn*917*-LTV1 on chromosomal *Sma*I fragments could be established by pulsed-field gel electrophoresis. Transposon insertion into chromosomal *Sma*I fragments of 50 kb or larger was detectable. PA193 has two copies of Tn*917*-LTV1 in the chromosome. The location of Tn*917*-LTV1 could not be determined in PA222, most likely because the transposon has inserted near the end of a *Sma*I fragment or into a fragment smaller than 50 kb. In the remaining 13 integrants analyzed, Tn*917*-LTV1 is inserted into at least 12 different locations (Table 2).

Regulation of β-galactosidase expression was analyzed in liquid cultures. The integrants showing regulation by pH and/or arginine had the same regulation in liquid media as they did on plates (Table 3), while only two of six integrants regulated by growth temperature on plates were regulated by temperature in liquid media (Table 4).

Characterization of pH-regulated β-galactosidase expression in PA170. The potential pH regulation of β-galactosidase expression in integrant PA170 was analyzed by fermentation at

A

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H
i
n                B        B
d            X   g       Pa     S
I            h   l       sm     m
I            o   I       tH     a
I            I   I       II     I                       R.B.S.
AAGCTTTCGCGAGCTCGAGATCTGCAGGATCCCGGGTCTAGATTAGGGTAACTTTGAAAGGATATTCCTCATG
---------+---------+---------+---------+---------+---------+---------+--
TTCGAAAGCGCTCGAGCTCTAGACGTCCTAGGGCCCAGATCTAATCCCATTGAAACTTTCCTATAAGGAGTAC

K  L  S  R  A  R  D  L  Q  D  P  G  S  R  L  G  *
 S  F  R  E  L  E  I  C  R  I  P  G  L  D  *        ß-galactosidase  M -->
  A  F  A  S  S  R  S  A  G  S  R  V  *
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B

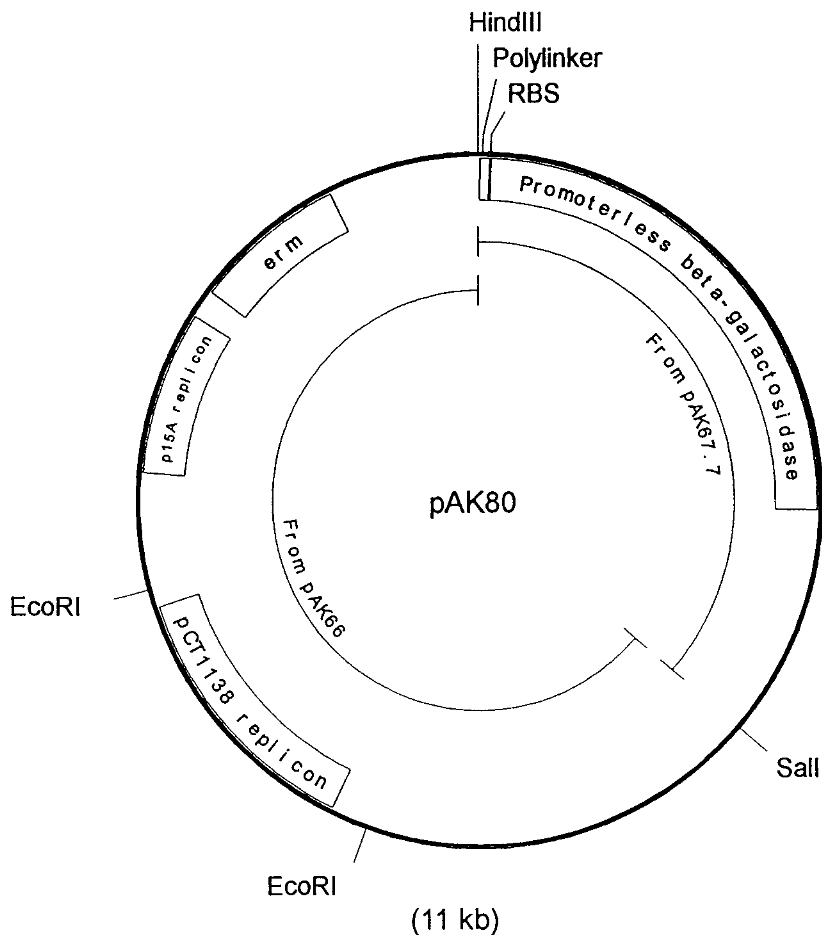

FIG. 2. (A) Polylinker and sequences upstream of the β-galactosidase gene in pAK67.7 and pAK80. Restriction sites which are unique in pAK80 are indicated. R.B.S. indicates the putative ribosome binding site. The last 3 bp constitute the first codon in *lacL*. Stop codons are designated with an asterisk. (B) Physical map of the promoter probe vector pAK80. DNA fragments from pAK66 and pAK67.7 and the restriction sites for construction of pAK80 are indicated. The polylinker region is illustrated in Fig. 2A. RBS indicates the putative ribosome binding site. *erm* is the erythromycin resistance gene.

a constant pH maintained without the use of arginine (Fig. 3A). β-Galactosidase synthesis was clearly dependent on pH. The increase in β-galactosidase activity at pH 5.2 was not proportional to the increase in OD_{600} (Fig. 3B). The slope of the curve is the ratio of change in β-galactosidase relative to change in cell mass. This ratio, $\Delta\beta\text{-Gal}/\Delta OD_{600}$, was less than 1 in the exponential growth phase but increased to about 20 in the late growth phase, i.e., an OD_{600} greater than 1.9. Thus,

TABLE 2. Physical determination of Tn*917*-LTV1 insertion in chromosomal *Sma*I fragments of 15 selected *Lactococcus lactis* Tn*917*-LTV1 integrants

Integrant	Tn*917*-LTV1 target: chromosomal *Sma*I fragment (kb)	Fragment lengths (kb) of *Sma*I-digested target fragments with inserted Tn*917*-LTV1[a]
PA86	65	60, *x*
PA143	280	140, *x*
PA162	310	285, *x*
PA163	310	230, 85
PA170	280	175, 120
PA179	610	500, 125
PA187	610	530, *x*
PA192	175	100, 85
PA193	610	350, 270
	50	30, *x*
PA201	610	470, 160
PA222	ND	
PA229	140	85, *x*
PA237	610	350, 270
PA241	610	350, 270
PA243	610	430, 190

[a] *x* indicates a fragment that could not be detected by pulsed-field gel electrophoresis because it was too small or the relevant band was a doublet.
[b] ND, not determined.

expression of β-galactosidase in PA170 is regulated by pH and growth phase as well as temperature (Table 4).

Cloning of *Lactococcus lactis* DNA fragments potentially harboring regulated promoters and insertion into pGKV210. In addition to the *lacZ* gene, Tn*917*-LTV1 harbors a ColE1 replication region, a β-lactamase gene, and a polylinker (Fig. 1) allowing host DNA adjacent to *lacZ* to be cloned in *E. coli* (40). This is the main advantage in using Tn*917*-LTV1 rather than Tn*917*-TV32. Cloning of adjacent *Lactococcus* DNA was done for integrants PA143, PA162, PA163, PA170, and PA243, resulting in plasmids designated p143, p162, p163, p170, and p243 (Table 1).

The promoter probe vector pGKV210 contains a promoterless *cat* gene preceded by a polylinker and a gene for ERY resistance (37). When a fragment harboring a promoter is inserted into the polylinker in the correct orientation, the plasmid confers CAM resistance. The level of CAM resistance is dependent on the strength of the inserted promoter (38). A *Cla*I site is located in Tn*917*-LTV1 30 bp from the *lacZ* proximal end (Fig. 1). The *Eco*RI-*Cla*I fragments containing lactococcal DNA from plasmids p143, p163, p170, and p243 were inserted into pGKV210/C, a derivative of pGKV210 which carries *Eco*RI and *Cla*I sites in the polylinker. The resulting pGKV210/C derivatives were designated pGKV210/C::143, pGKV210/C::163, pGKV210/C::170, and pGKV210/C::243, respectively (Table 1). The lactococcal DNA of p162 contains a *Cla*I site located 0.7 kb from the *lacZ* end of the transposon. The lactococcal *Eco*RI-*Cla*I fragment of p162 was inserted into pGKV210/C to give pGKV210/C::162u. The lactococcal *Cla*I fragment of p162 was inserted in the proper orientation into pGKV210/C, giving pGKV210/C::162d.

TABLE 3. Effect of final culture pH on β-galactosidase activity

Integrant or strain	β-Galactosidase activity[a] with final pH of:		Ratio (pH 6.7/pH 5.6)
	5.6	6.7	
PA86	1	18	18
PA162	20	50	2.5
MG1363/pAK80::162u	70	100	1.4
PA163	9	0.3	0.03
PA170	2	<0.1	<0.05
MG1363/pAK80::170	20	1	0.05
PA179	4	1	0.25
PA193	7	18	2.6
PA222	9	5	0.6
PA229	2	<0.1	<0.05
PA237	7	15	2.1
PA241	2	5	2.5
PA243	6	20	3.3
MG1363/pAK80::243	700	600	0.9
MG1363/pAK80	<0.1	<0.1	

[a] In Miller units.

TABLE 4. Effect of growth temperature on β-galactosidase activity

Integrant or strain	β-Galactosidase activity[a] when grown at:		Ratio (15°C/30°C)
	30°C	15°C	
PA143	1	2	2
MG1363/pAK80::143	200	35	0.2
PA170	2	15	7.5
MG1363/pAK80::170	20	80	4.0
PA187	1	1	1
PA192	<0.1	2	>20
PA201	1	1	1
PA243	4	6	1.5
MG1363/pAK80::243	700	100	0.1
MG1363/pAK80	<0.1	<0.1	

[a] In Miller units.

The pGKV210/C derivatives were introduced into *Lactococcus lactis* MG1363. Screening for promoter activity was by plating overnight cultures on M17 plates supplemented with ERY and various concentrations of CAM to determine the maximum concentration of CAM at which growth occurs. In MG1363, plasmid pGKV210/C::243 conferred resistance to 12 μg of CAM per ml, pGKV210/C::143 and pGKV210/C::162u conferred resistance to 4 μg of CAM per ml, and pGKV210/C::162d, pGKV210/C::163, pGKV210/C::170, and pGKV210 did not allow growth even on 4 μg of CAM per ml.

Insertion of lactococcal DNA fragments into pAK80 and analysis of regulated β-galactosidase expression in *Lactococcus lactis*. *Eco*RI-*Cla*I fragments containing lactococcal DNA from plasmids p143, p163, p170, and p243 were inserted into pAK80. Because the β-galactosidase genes of pAK80 contain *Cla*I sites, the *Eco*RI-*Cla*I fragments were first subcloned into pGEM-7Zf(+). Similarly, the lactococcal *Eco*RI-*Cla*I and *Cla*I fragments of p162 were subcloned into pGEM-7Zf(+). The pGEM-7Zf(+) derivatives were digested with *Xho*I and *Bam*HI, and the lactococcal DNA was inserted into the polylinker of pAK80. The resulting pAK80 derivatives were named pAK80::143, for example (Table 1). The pAK80 derivatives were introduced into *Lactococcus lactis* MG1363 and screened for promoter activity on plates containing ERY and X-Gal. Of the lactococcal DNA fragments analyzed, all but the p162d and p163 fragments contained a detectable promoter. The PA162 promoter is contained on the p162u fragment, and the p163 fragment was subsequently shown to contain an open reading frame throughout its length (data not shown); therefore, a promoter would not be expected on these fragments.

The regulation of the lactococcal promoters on the pAK80

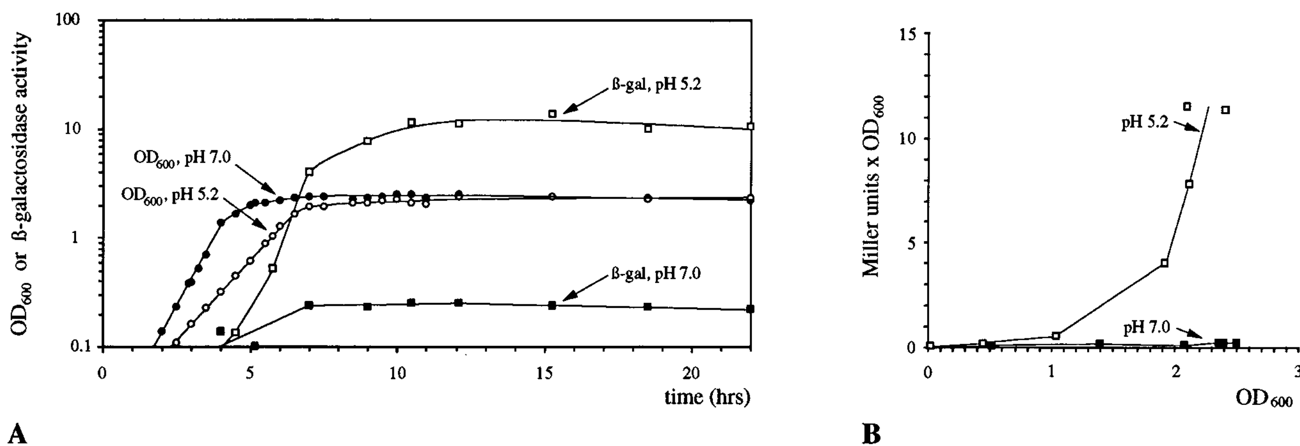

FIG. 3. (A) Semilogarithmic plot of OD_{600} and β-galactosidase (β-gal) activity versus fermentation time for PA 170 at pH 7.0 and 5.2. (B) β-Galactosidase activity versus OD_{600} for PA170 at pH 7.0 and 5.2. β-Galactosidase activity is shown in Miller units · OD_{600}, i.e., activity per volume of culture.

derivatives was compared with the regulation observed in the original Tn*917*-LTV1 integrants (Tables 3 and 4). The regulation of β-galactosidase expression in MG1363/pAK80::170 is analogous to the regulation observed in PA170. The promoters on the remaining fragments either lose the regulation (MG1363/pAK80::243; pH and/or arginine), reverse the regulation (MG1363/pAK80::143; temperature), or gain an unexpected regulation (MG1363/pAK80::243; temperature). Increasing the copy number of the P143 promoter produced a 200-fold increase in β-galactosidase activity at 30°C, whereas increasing the copy number of the P162 promoter produced a fourfold increase and increasing the copy number of the P170 promoter produced a 10-fold increase.

Regulation of the P170 promoter in pAK80::170 by pH was tested in constant-pH fermentations. Synthesis of β-galactosidase is clearly pH dependent (Fig. 4A). The increase in β-galactosidase activity at pH 5.2 was not proportionate to the increase in OD_{600} (Fig. 4B). Thus, expression of β-galactosidase in MG1363/pAK80::170 is regulated in the same way as it is in PA170, i.e., by pH and growth phase as well as temperature.

DISCUSSION

Regulation of gene expression in *Lactococcus lactis* has important scientific and commercial applications. Studies of gene regulation in this bacterium and other gram-positive bacteria led to the identification of new regulation mechanisms not yet observed in gram-negative bacteria (2, 23). The ability to regulate gene expression will be important in the construction of genetically improved strains for industrial applications, including their use as starter cultures and production organisms. In this study, we analyzed gene expression in *Lactococcus lactis* regulated by temperature or pH, factors important in dairy production processes.

Tn*917*-LTV1 was used as a promoter probe in *Lactococcus lactis* and several integrants showing regulated expression of the β-galactosidase isolated. In 15 integrants chosen for fur-

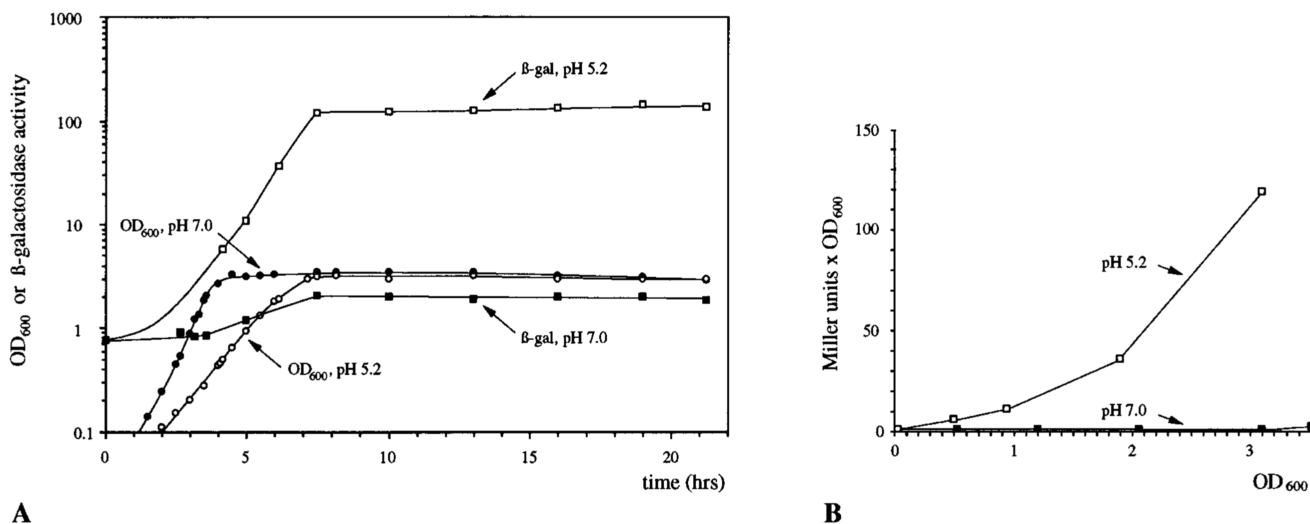

FIG. 4. (A) Semilogarithmic plot of OD_{600} and β-galactosidase (β-gal) activity versus fermentation time for MG1363/pAK80::170 grown at pH 7.0 or 5.2. (B) β-Galactosidase activity versus OD_{600} for MG1363/pAK80::170 at pH 7.0 or 5.2. β-Galactosidase activity is shown in Miller units · OD_{600}, i.e., activity per volume of culture.

ther characterization, Tn*917*-LTV1 was shown to be located in at least 13 different loci on the *Lactococcus lactis* chromosome (Table 2). PA193 has two Tn*917*-LTV1 copies inserted in the chromosome and therefore is different from PA237 and PA241. The basal level of β-galactosidase is lower in PA241 than in PA237 (Table 3), indicating that the transposon resides in different locations in these integrants.

β-Galactosidase activity was measured in stationary-phase cultures because activities were generally too low in the exponential growth phase. Reinforcing the liquid media with extra M17 produced a higher cell yield and higher levels of β-galactosidase. These findings indicate that the *lacZ* gene in Tn*917*-LTV1 is relatively poorly expressed in *Lactococcus lactis* and that the PA integrants have Tn*917*-LTV1 inserted into genes which are expressed in the stationary phase. The latter is supported by the fact that blue color generally emerges in the patches late in the incubation period.

The pH and/or arginine regulation deduced from plate screenings was reproducible in liquid media (Table 3). However, significant consensus on temperature regulation was seen only in two of the six integrants analyzed (Table 4). Reasons for lack of consensus could be the differences in the composition of agar and liquid media and that the blue color observed on plates represents an accumulated effect of β-galactosidase activity, while the activity measured in liquid media reflects the activity at the time of harvest.

The media used for screening of pH- and/or arginine-regulated expression of β-galactosidase differ not only in the final culture pH but also in the concentrations of arginine and glucose. The effect on *lacZ* expression observed could therefore be caused by any of these differences, either separately or in combination. To test for regulation by pH, integrant PA170 was cultivated in fermentors at pH 5.2 or 7.0. Expression of β-galactosidase was regulated by pH and growth phase (Fig. 3). These results do not exclude an additional regulation by arginine or glucose.

We constructed a new promoter probe vector, pAK80, with the β-galactosidase genes from *Leuconostoc mesenteroides* subsp. *cremoris* as reporter genes and a theta replicating replicon from the citrate plasmid of *Lactococcus lactis* subsp. *lactis* biovar diacetylactis. The expression of the β-galactosidase genes is not subject to posttranscriptional regulation, and the presence of a theta replicating replicon provides a vector which is structurally and segregationally stable (7, 21). The range of β-galactosidase activities measurable with pAK80 is very broad, ranging from <0.1 Miller unit for the vector without insert to >5,000 Miller units with a tRNA promoter inserted (28). The promoters measured in the current study produced between 1 and 700 Miller units of β-galactosidase activity. The cloned DNA fragments potentially carrying promoters from selected *Lactococcus lactis* Tn*917*-LTV1 integrants were analyzed in both pGKV210 (37) and pAK80 by plate screenings. When analyzed in pGKV210, no promoter activity could be detected on the DNA fragment from PA170, and a low promoter activity, close to the limit of detection, was detected on the fragments from PA143 and PA162. In contrast, promoter activity was detected on the fragment from PA170 by pAK80, and the promoters harbored on PA143 and PA162 were discriminated by pAK80 (Tables 3 and 4). Thus, pAK80 can detect and discriminate among weak promoters. Defined *Lactococcus* genes have already been analyzed by use of pAK80; the promoters of the *upp* gene and the *trnA* operon were located by use of pAK80 (26, 28).

A comparison of the regulation of β-galactosidase expression in *Lactococcus lactis* harboring the pAK80 derivatives and the corresponding *Lactococcus lactis* Tn*917*-LTV1 integrants (Tables 3 and 4) revealed that the regulation of β-galactosidase expression in MG1363/pAK80::170 is analogous to the regulation observed in PA170. The remaining lactococcal fragments in pAK80 caused either a nonregulated or an unexpected regulation of β-galactosidase expression. Thus, contrasting results may be obtained when promoter characterizations are carried out with a transposon or a plasmid vector.

Promoter P170 is active at pH 5.2 but not at pH 7.0, is more active at 15°C than at 30°C, and has increased activity in the stationary phase. This promoter will be useful for several purposes. The low activity at pH 7.0 should allow genes encoding products toxic to the cell to be cloned. Expression will occur only when the pH is reduced. Low-temperature culture conditions improve the intracellular solubility or secretion efficiency of heterologous proteins expressed in *E. coli* (32). These observations may also apply to *Lactococcus lactis*, making P170 ideal for the expression of heterologous proteins. P170 is also an ideal promoter for genetically improved dairy starter cultures. P170 will cause expression of a desired gene during cheese ripening, as a result of the high promoter activity at low pH, at low temperature, and in the stationary phase. Constructions could include food-grade plasmid vectors (for examples, see reference 13) containing P170 or insertion of desired genes into the chromosome at the locus identified in PA170 as showing the desired regulation of gene expression.

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