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Isolation and Characterization of *Lactococcus lactis* subsp. *lactis* Promoters

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DNA fragments with promoter activity were isolated from the chromosome of *Lactococcus lactis* subsp. *lactis*. For the isolation, a promoter probe vector based on the *cat* gene was constructed, which allowed direct selection with chloramphenicol in *Bacillus subtilis* and *L. lactis*. Four of the putative promoters (P1, P2, P10, and P21) were analyzed further by sequencing, mapping of the 5′ end of the mRNA, Northern (RNA blot) hybridization, and chloramphenicol acetyltransferase activity measurements. From these fragments, −10 and −35 regions resembling the consensus *Escherichia coli* σ^{70} and *B. subtilis* σ^{43} promoters were identified. Another set of promoters, together with a signal sequence, were also isolated from the same organism. These fragments promoted secretion of TEM β-lactamase from *L. lactis*. When the two sets of promoters were compared, it was found that the ones isolated with the *cat* vector were more efficient (produced more mRNA). By changing the promoter part of the promoter-signal sequence fragment giving the best TEM β-lactamase secretion into a more efficient one (P2), a 10-fold increase in enzyme production was obtained.

Interest in molecular biology and genetic engineering of lactic acid bacteria has increased greatly during the past few years. New vectors (27, 29, 30) and integration systems (2, 18, 25) and better transformation methods (11, 16) have become available. An increasing number of genes have also been cloned from these bacteria, including genes involved in protein degradation (13, 15) and lactose utilization (12, 20, 21). These advances have raised the possibility of testing whether lactococci can also be used as hosts in the production of heterologous proteins. Furthermore, current lactococcal starter strains might be modified to include improved fermentation process characteristics.

These approaches require the availability of well-characterized promoters and/or promoter-signal sequence units functional in lactococci. Until now there have been only two reports describing the isolation and characterization of lactococcal promoters, the isolation of promoters from *Lactococcus lactis* subsp. *cremoris* (31) and its bacteriophage BK5-T (17). In addition, a few other promoters have been isolated along with a specific gene (3, 14). So far, few attempts have been made to use these promoters to produce heterologous proteins in lactococci (27a, 29).

In this article, we report the isolation and characterization of transcription-promoting fragments from the *Lactococcus lactis* subsp. *lactis* chromosome. We also describe the construction of a plasmid bearing a new combination of a promoter and signal sequence, both isolated separately from the *L. lactis* chromosome. The use of this plasmid resulted in highly efficient expression and secretion of TEM β-lactamase of *Escherichia coli* into the culture supernatant of *L. lactis*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are listed in Table 1.

Culture media and bacterial transformations. *E. coli* and *Bacillus subtilis* strains were grown in Luria broth (19). For *E. coli*, antibiotic concentrations were 11 μg/ml for chloramphenicol and 12.5 μg/ml for tetracycline; for *B. subtilis*, 5 μg of chloramphenicol per ml was used. Lactococcal strains were grown in M17-glucose (M17G) (28) or M17-glucose-sucrose (M17GS) medium supplemented with chloramphenicol (4 or 5 μg/ml) if necessary.

E. coli was transformed by the method of Hanahan and Meselson (8). *B. subtilis* cells were transformed by the method of Gryczan et al. (7). *L. lactis* protoplast transformation was carried out by the method of von Wright et al. (32), and electroporation was done essentially as described by Holo and Nes (11).

Enzyme assays. For the chloramphenicol acetyltransferase (CAT) assay, cells were grown in the appropriate medium. Cells were harvested from 1-ml culture samples by centrifugation, washed with 50 mM sodium phosphate buffer (pH 7), and suspended in 0.2 ml of the same buffer containing lysozyme (4 mg/ml). Cells were incubated at 37°C for 30 min, followed by sonication. The cell debris was pelleted by centrifugation, and CAT activity in the supernatant was measured by the method of Shaw (26).

For the β-lactamase assay, cell and supernatant fractions were separated by centrifugation after growth in the appropriate medium. Supernatant fractions were assayed for β-lactamase activity by the method of O'Callaghan and Morris (22). For rapid analysis of β-lactamase-positive status, bacterial colonies were suspended in 250 μl of Nitrocefin (Glaxo) in microtiter wells. Positive colonies turned red after 1 to 30 min of incubation at room temperature.

Sequencing of the DNA. DNA was sequenced by the dideoxy chain termination method (24). Denatured plasmid DNA was used as a template (9).

Northern (RNA blot) transfer and hybridization. RNA was isolated from *L. lactis* essentially as described by van der Vossen et al. (31). Cells were cultured in 20 ml of M17G medium with chloramphenicol (5 μg/ml) to 50 Klett units (no. 66 filter). After hot phenol treatment, the RNA was precipitated with ethanol. The pellet was dissolved in distilled water, and the DNA was digested with RNase-free

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Origin or reference
E. coli TG1	K-12Δ (*lac-pro*) *supE thi hsdD5* F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*ΔM15	European Molecular Biology Laboratory
B. subtilis IH6064	*metB5 sacA321*	23
L. lactis subsp. *lactis* MG1614		5
Plasmids		
pBR322		1
pVS2	Plasmid capable of replication in *E. coli*, *B. subtilis*, and *L. lactis*, 5 kb, Em^r Cm^r	33
pSH71	Cryptic plasmid of *L. lactis*, 2 kb	5
pPL603	Promoter cloning vector for *B. subtilis*, 4.8 kb, Km^r	34
pKTH1722	pSH71 replicon containing tetracycline resistance gene	This work
pKTH1734	pKTH1722 containing erythromycin resistance gene	This work
pKTH1736	pKTH1734 containing promoterless *cat-86*	This work
pKTH1750	pKTH1736 containing multiple cloning site in front of *cat*	This work
pKTH1816	Plasmid pKTH1750 bearing *L. lactis* chromosomal DNA, clone P1	This work
pKTH1817	Like pKTH1816, clone P2	This work
pKTH1820	Like pKTH1816, clone P10	This work
pKTH1821	Like pKTH1816, clone P21	This work
pKTH1807	Plasmid pVS2 bearing a *Cla*I-*Pvu*II fragment of ss45	26a
pKTH2001	pVS2 bearing P2-ss45 combination	This work

DNase I (Promega). Denatured RNAs were separated in an agarose gel. Northern transfer to a nitrocellulose membrane (Schleicher & Schuell) was done by the method of Williams and Mason (35). The filter was hybridized in 4× SSC (0.6 M NaCl, 0.06 M sodium citrate)–50 mM NaH_2PO_4 (pH 6.5)–0.1% polyvinylpyrrolidone–0.1% Ficoll–0.1% bovine serum albumin–0.2% sodium dodecyl sulfate (SDS)–200 μg of denatured herring sperm DNA per ml–10^6 cpm of nick-translated pPL603 per ml at 65°C for 18 h. The filter was washed in 2× SSC–0.2% SDS at room temperature for 30 min, at 37°C for 30 min, and at 55°C for 30 min. The hybridized fragments were detected by autoradiography. For quantitation, the films were scanned with a densitometer (Ultroscan XL Enhanced Laser Densitometer; LKB, Bromma, Sweden).

Primer extension. The mapping of the 5′ end of the mRNA was done as follows. A 0.2-pmol amount of a 20-mer primer (5′-ATTCAAAGTCTACCCTGAGC-3′) was added to 5 to 10 μg of RNA. An equal volume of 2× hybridization buffer (100 mM Tris-HCl [pH 8.3], 2 mM EDTA, 0.8 M NaCl) was added. The mixture was heated at 95°C for 2 min and allowed to cool gradually to room temperature.

The RNA-primer hybrid was precipitated with ethanol, and the pellet was dissolved in reaction buffer (50 mM Tris-HCl [pH 8.3]; 10 mM dithiothreitol; 6 mM $MgCl_2$; 50 mM KCl; 0.25 mM each dATP, dTTP, and dGTP; 25 μg of actinomycin C1 [Boehringer] per ml). To this mixture, 1.5 mCi of [α-^{32}P]dCTP (Amersham), 4,000 U of RNasin (Promega), and 700 U of avian myeloblastosis virus reverse transcriptase (Promega) per ml were added. The reaction mixture was incubated at 42°C for 15 min, after which cold dCTP was added to a final concentration of 0.25 mM. Incubation was continued at 42°C for 105 min.

The reaction mixture was then extracted with phenol and phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated with ethanol. The reverse transcriptase reactions were analyzed by electrophoresis on a 6% sequencing gel, with sequencing reactions of one of the promoters used as size markers.

Construction of the promoter probe vector. A promoter probe vector able to replicate in *E. coli*, *B. subtilis*, and *L. lactis* was constructed for the screening of chromosomal DNA fragments containing promoterlike activity (Fig. 1). The construction was done by using *E. coli* as the host. The origin of replication was isolated from plasmid pSH71 by *Cla*I digestion. The larger fragment was isolated and treated with Klenow polymerase. The gene responsible for tetracycline resistance was obtained from pBR322 by *Eco*RI and *Pvu*II digestions. The recessed ends were filled in with Klenow enzyme, and the fragment was ligated to the isolated origin of replication of pSH71. This resulted in plasmid pKTH1722. Ligation of the blunt-ended *Cla*I and *Eco*RI sites regenerated the *Eco*RI site.

The erythromycin resistance marker was isolated from plasmid pVS2 by *Hin*dIII and *Cla*I digestions. The protruding ends were filled in, and the fragment was ligated with *Xmn*I-digested pKTH1722, which created plasmid pKTH1734. pKTH1734 was cleaved by *Eco*RI, and the recessed ends were filled in. The promoterless *cat* gene was isolated from pPL603 as an *Eco*RI-*Pvu*II fragment and ligated to the linearized vector, resulting in plasmid pKTH1736.

The vector was further improved by adding a DNA fragment containing multiple cloning sites to the *Pst*I site upstream of the *cat* gene. The promoter probe vector, pKTH1750, thus obtained contained restriction enzyme recognition sites for *Pst*I, *Eco*RI, *Pvu*II, and *Bgl*II upstream of the *cat* gene.

RESULTS

Screening for *L. lactis* promoters. The promoter probe plasmid pKTH1750 (Fig. 1) contains the origin of replication of pSH71 and antibiotic resistance markers for tetracycline and erythromycin. It also contains a promoterless *cat-86* gene preceded by *Pst*I, *Eco*RI, *Pvu*II, and *Bgl*II sites. Details of its construction are described in Materials and Methods.

To isolate DNA fragments which expressed promoterlike activity, *L. lactis* chromosomal DNA was digested totally with *Sau*3A1, which yielded fairly large fragments (mean size, 2 kb). The mixture of the fragments was ligated with *Bgl*II-digested pKTH1750 vector. The vector pKTH1750 can replicate in *B. subtilis* and *L. lactis*, which allows the use of

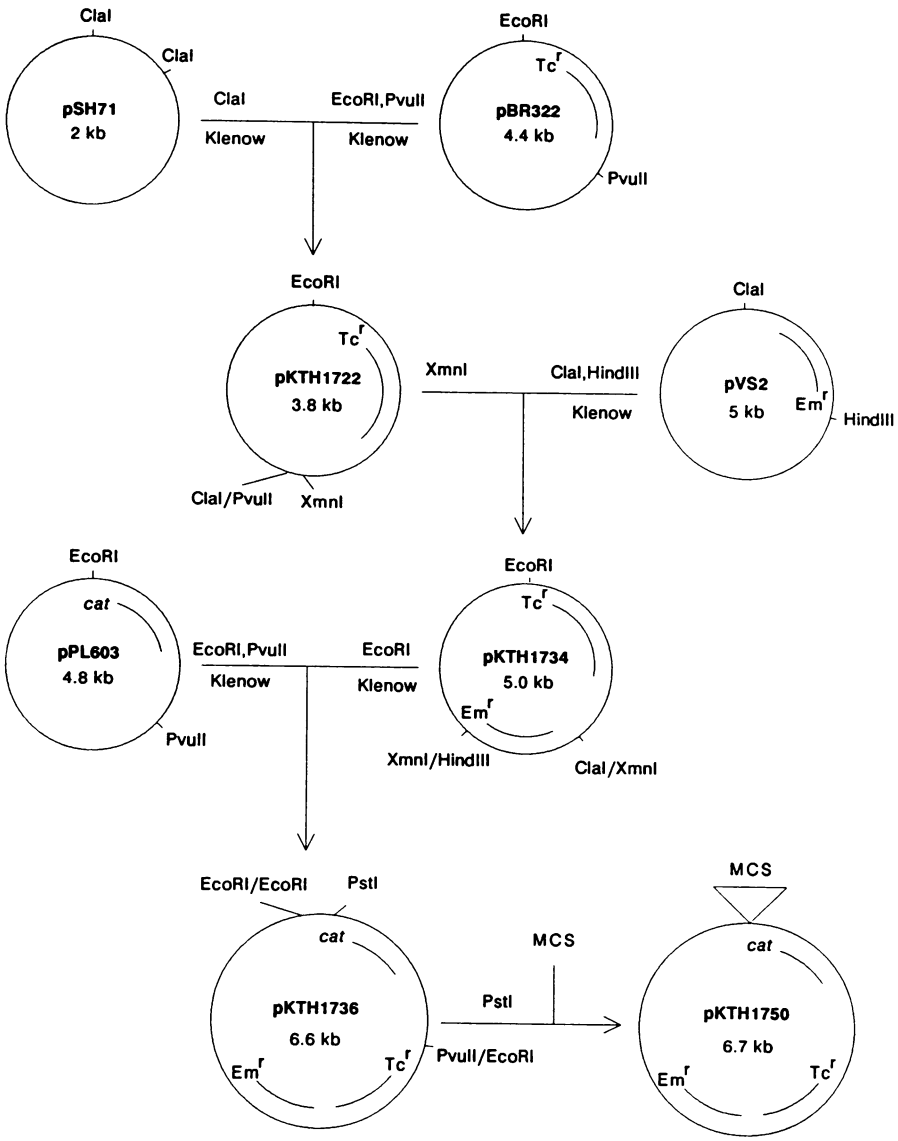

FIG. 1. Schematic representation of construction of the promoter probe vector pKTH1750. The vector has the origin of replication of pSH71 and genes coding for erythromycin (Em^r) and tetracycline (Tc^r) resistance. It also contains a promoterless *cat* gene. Upstream of the *cat* gene there is a multiple cloning site (MCS) containing recognition sites for restriction enzymes *Pst*I, *Eco*RI, *Pvu*II, and *Bgl*II. Klenow refers to Klenow enzyme treatment of the DNA.

both of these strains as transformation hosts. Transformation of *L. lactis* protoplasts with the ligation mixture produced colonies at a very low frequency, ca. 1 transformant per μg of DNA, when the transformation mixture was plated on M17GS plates containing chloramphenicol (4 μg/ml). Transformation to *B. subtilis* produced about 200 transformants per μg of DNA when the transformation mixture was plated onto Luria agar plates containing chloramphenicol (5 μg/ml).

To select the most efficient promoter fragments, the *B. subtilis* transformants were streaked on Luria plates containing chloramphenicol at 5, 15, 45, and 100 μg/ml. Plasmids were isolated from 11 transformants growing at the highest drug concentration and used to transform *L. lactis*. Of these 11 plasmids, 7 transformed *L. lactis* cells to Cm^r.

Four of the promoter clones were characterized in more detail. Two of these, P1 and P2, were from a direct *L. lactis* transformation, and two, P10 and P21, were from a *B. subtilis* transformation. For initial characterization, the cellular CAT activities were measured (Table 2). Although the differences in CAT activities between promoter constructions were more than 20-fold among the lactococci, CAT activities were considerably lower in *L. lactis* than in *B. subtilis*. However, the order of promoter strength remained the same in both hosts.

Promoter characterization by sequencing and mapping of the 5′ end of the mRNA. The sizes of the fragments containing promoter activity were 2,800, 550, 1,200, and 2,900 bp for P1, P2, P10, and P21, respectively. Approximately 500 bp were sequenced upstream from the *cat* gene directly from the plasmid. Relevant parts of the sequences are shown in Fig. 2. The 5′ end of the *cat*-specific mRNA produced by these clones in *L. lactis* was mapped by the primer extension method (Fig. 2 and 3).

TABLE 2. CAT activities of clones screened by the promoter probe plasmid pKTH1750[a]

Plasmid	Promoter fragment	CAT activity	
		L. lactis (mU/ml)	*B. subtilis* (U/ml)
pKTH1816	P1	1.5	0.9
pKTH1817	P2	20.0	4.5
pKTH1820	P10	2.9	1.2
pKTH1821	P21	1.2	0.8
None (control)		0.4	0.1

[a] Activity was measured in samples collected 2 h after the culture had reached the turbidity of 100 Klett units. The control was host strains *L. lactis* and *B. subtilis* without a plasmid.

The putative −35 and −10 sequences closely resembling the consensus *E. coli* σ^{70} and *B. subtilis* σ^{43} (4) recognition sequences TTGACA (−35) and TATAAT (−10) could be identified in clones P1, P2, P10, and P21. The −35 regions were TTGCAT, TTGACA, TTGACA, and TTGAAA and the −10 regions were TAAAAT, TATTAT, TATACT, and TATCAT for P1, P2, P10, and P21, respectively. The spacing between the −35 and −10 regions varied from 17 to 18 bp and was thus similar to the spacing in *B. subtilis* (17 to 19 bp) and *E. coli* (16 to 18 bp) promoters. In front of the −10 regions, separated by 1 nucleotide, a TG nucleotide pair was found. This pair is weakly conserved in *E. coli* (10) but more conserved in gram-positive bacteria (6). Four of the five *L. lactis* subsp. *cremoris* promoters isolated by van der Vossen et al. (31) also contained this pair of nucleotides. The spacing between the 5′ end of the mRNA and the −10 hexanucleotide was 6 bp for P1, 6 bp for P2, 96 bp for P10, and 8 or 11 bp for P21. The reason for the long distance observed in clone P10 is not known. It may be the result of experimental conditions or of in vivo degradation of mRNA.

Transcriptional efficiency of the promoter fragments. There are translational stop codons in all three reading frames between the cloning site and the *cat* gene in the promoter probe plasmid pKTH1750. The *cat* gene is also preceded by a Shine-Dalgarno sequence. Therefore, translation of the gene should initiate at the same position and elongate at the same rate in all promoter clones. However, the secondary structure of mRNA can affect both the rate of initiation of translation and the half-life of the mRNA molecules. Thus, direct measurement of product activity may not reflect actual promoter strength.

To determine whether the mRNA levels are consistent with the CAT activities, the steady-state levels of the *cat* gene-specific mRNAs of the clones were determined. Total RNA was isolated, and the mRNA was detected by Northern blotting and hybridization (Fig. 4). The autoradiogram was scanned with a densitometer. The relative amounts of mRNA were 1 for P21, 5 for P1, 9 for P2, and 6 for P10. The mRNA levels showed a correlation with the CAT activities. For example, P2, with the highest enzyme acivity, also produced the highest amount of mRNA, and the low CAT activity of P21 corresponded to a low mRNA level. However, the mRNA level of P1 was higher than would have been predicted from the CAT activity. This discrepancy could be due to the long distance between the promoter and the *cat* gene (ca. 600 bp).

Comparison of expression units isolated by two different methods. In addition to the promoters isolated during this work, promoter-signal sequence fragments from the *L. lactis* subsp. *lactis* chromosome have been isolated in our laboratory (26a). These fragments promote both expression and secretion of *E. coli* TEM β-lactamase from *L. lactis*. These two sets of promoters were compared at the DNA and RNA levels to test for differences. There was only a low level of protein in the culture medium of lactococci, and therefore it is possible that, in general, the promoters for the secreted proteins are weaker than those for cytoplasmic proteins.

The relevant parts of the nucleotide sequences of the promoter-signal sequence fragments ss30, ss38, ss45, and ss80 are shown in Fig. 5. Putative −35 and −10 regions were also identified in these fragments, but they were not very close to the consensus sequences. The spacing between the −35 and −10 regions for ss30 (13 bp), ss38 (13, 23, or 18 bp), ss45 (20 bp), and ss80 (11 bp) was either shorter or longer than the spacing in P1, P2, P10, and P21 (17 to 18 bp).

The promoter efficiencies of these two sets were compared

```
P1

5' AAAATAAAAATGGACTCAGGCTAGAAAAAT
                            -35
   AAAGGCTTTTATGAAAGAAAGACTTGCATT
                        -10
                                *
   TGTTGTTGAAAAATGCTAAAATACATAAGT
   CCGACTTTTT

P2

5' TAATCCGTAATTTATTGACTTTTATATCAGT
                        -35
   GATTTATGAGTTTTTTCTTGACAGAAGATGG
              -10
                          *
   CGAAAAATGGTATTATATCTAGGTACTGTTT
   TGCGGTGGT

P10

5' GACAAAAACCTAATTTATTTTAAATTTTTGCA
                        -35
   TGTAATGAGTTTATTCTTGACAACTTTTGGGA
            -10
   AACTTGGTATACTAATATAGTCGTTTAAGAGAA
   ACGCAGGCGTGGCTCAACTGGATAGAGTACCTG
   ACTACGAATCAGGCGGTTGTAGGTTCGAATCCT
                *
   ACCGCTTGCATAAATAAAATATGA

P21

5' ACGAGTGCAGCTGAAAAAGCTTATCTTTTTGAT
                            -35
   ATCCTATTAATCAAGTTGACCTTGAAAAAAAAC
                -10
                            *   *
   TGAAAATCTGTTATCATAAATAATGGACATTTT
   ATAATGATGATGATGA
```

FIG. 2. Nucleotide sequences of putative promoters P1, P2, P10, and P21 of *L. lactis*. The sequencing was accomplished directly from the plasmids pKTH1816 (P1), pKTH1817 (P2), pKTH1820 (P10), and pKTH1821 (P21). Only part of each cloned fragment is shown. The putative −35 and −10 regions are underlined, and the 5′ end of the mRNA is marked with an asterisk above the corresponding nucleotide.

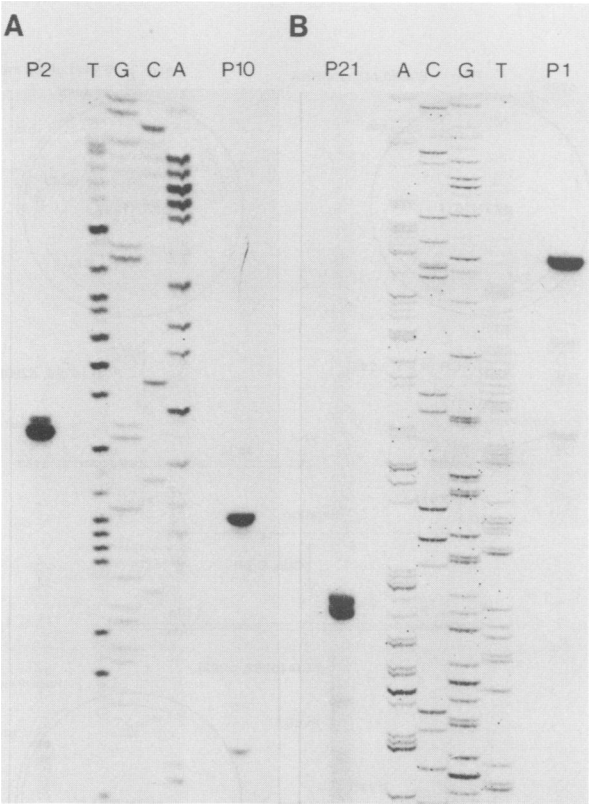

FIG. 3. Identification of the 5′ ends of mRNAs specified by the *L. lactis* promoters P1, P2, P10, and P21. Mapping was done by the primer extension method with [α-^{32}P]dCTP as the label. The primer extension reactions of P1, P2, P10, and P21 were run in a 6% sequencing gel, an autoradiogram of which is shown. The nucleotide sequences of P2 (A) and P1 (B) were used as standards.

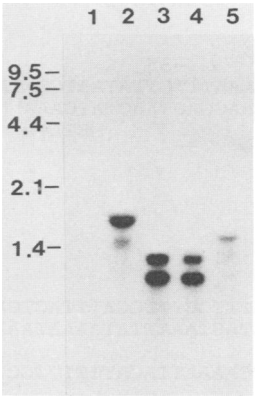

FIG. 4. Northern hybridization analysis of *L. lactis* promoter constructions P1, P2, P10, and P21. Denatured total RNAs were run in an agarose gel, blotted to a nitrocellulose membrane, and hybridized with [α-^{32}P]dCTP-labeled pPL603. The RNAs were isolated from *L. lactis* containing no plasmid (lane 1), pKTH1816 (P1) (lane 2), pKTH1817 (P2) (lane 3), pKTH1820 (P10) (lane 4), or pKTH1821 (P21) (lane 5). As a molecular size marker, a commercial RNA ladder (Bethesda Research Laboratories Life Technologies, Inc.) was used. The fragment sizes are shown in kilobases.

at the mRNA level. The RNA of the promoter-signal sequence constructions was isolated, and β-lactamase-specific mRNA was analyzed by Northern blotting (data not shown) and compared with the mRNA levels produced by the promoter constructions P1, P2, P10, and P21. The promoters isolated with *cat* were found to produce more mRNA than the promoters isolated together with a signal sequence. The differences in the mRNA levels were roughly 10-fold in favor of the former set.

Construction of the hybrid expression and secretion unit. A recombinant plasmid was made to test the possibility of constructing a new functional expression-secretion unit from parts of different origins (Fig. 6). The plasmid contained a hybrid of the most efficient promoter (P2) and the region promoting efficient secretion of TEM β-lactamase (ss45) devoid of its own promoter. The putative promoter region of P2 and the signal sequence and *bla* region of ss45 were isolated from the plasmids pKTH1817 and pKTH1807, respectively, by polymerase chain reaction (PCR). The DNA fragments synthesized contained a recognition site for *Xba*I at the region corresponding to the mRNA start site. The fragments were digested with *Xba*I and ligated. This ligation regenerated the authentic 3′ and 5′ sequences of the mRNA start region for P2 and ss45, respectively. The ligation mixture was digested with *Cla*I and *Bgl*II, and the hybrid fragment was isolated from agarose gel and amplified by PCR. Finally, the fragment was digested with *Pvu*II and ligated to pVS2 vector which had been digested with *Hpa*II and treated with Klenow.

The ligation mixture was transformed into *L. lactis* cells by electroporation and plated on M17GS plates containing chloramphenicol (5 μg/ml). The transformants were screened for β-lactamase production by the Nitrocefin test. One of the positive transformants was selected. Plasmid DNA was isolated, and the promoter-signal sequence joint region was determined by sequencing. This plasmid was designated pKTH2001. The *L. lactis* strains containing plasmids pKTH1807 and pKTH2001 were grown for the analysis of β-lactamase production. The activities were measured in supernatant samples collected 2 h after the culture had reached a turbidity of 100 Klett units (no. 66 filter). The activities were 210 and 2,900 U/ml for pKTH1807 and pKTH2001, respectively. The strain containing plasmid pKTH2001 thus produced 10 times more β-lactamase than the strain containing the original plasmid pKTH1807, directly reflecting the mRNA levels of the two promoters.

DISCUSSION

In this article we describe the isolation and characterization of DNA fragments from the *L. lactis* subsp. *lactis* chromosome containing promoter activity. We also report the use of one of the promoters for the efficient expression of *E. coli* TEM β-lactamase.

The promoters were isolated by using *B. subtilis* and *L. lactis* as transformation hosts, and Cm^r transformants were obtained in both strains. Thus, lactococcal promoters were functional in a *Bacillus* species, which has also been reported by van der Vossen et al. (31). However, not all the promoters that were functional in *B. subtilis* could render the *L. lactis* cells Cm^r. Also, CAT activities were substantially lower in *L. lactis* than in *B. subtilis*. The low CAT activities may not reflect actual promoter function but rather the poor functioning of the *cat* system in lactococci. This was supported by our finding that the steady-state levels of mRNAs from the four promoters were roughly the same in both host strains (data not shown). This suggests that the low CAT

ss30

```
                -35                  -10
5' ATTCCAAAAATTTGTTATAATCGATATGTGAATTTT
   AACACAAAAGGAGTAACTATGAAA
                      METLys
```

ss38

```
             -35                  -10
5' ATTTTCATTTGCTTCCGATTTACTGACTTAAAAGTT
   AAAGATATAGTAAATTATAAAATAAAAATGTATTTT
    -35                        -35  -10
   TTGAAATCAAAATTACATTTTTTGCTTGCTTAAATG
                  -10
   AAACTAAACATGTTATACTCCTAGTATTATGAAA
                                METLys
```

ss45

```
5' TTAAATATTTCATTATGTAAAAACCGCTTACAAAAA
                  -35
   TTTATTACTACTATTGTATCAAAATTCTGTCAAATT
      -10              *
   TGATAAAATAAGGTACGAGTAAGAAGAACTTATACA
   ATGATCATATTGAAAAAATATAATTCAAGAAGAGAG
   ATGTTATAAGAGGTATTTTTGCATTGGGCAAAAAGC
   GATGTGAAATCACATGGCTTTCGAACCTAAAAGAGA
   TGGGTTTGAAACGTATTACTGAAGGGAACCTAGAAT
   AGTGAAA
    METLys
```

ss80

```
5' ATTATTTACAAAACATTTACATAAGAACGCTGTCAT
                -35                -10
   CAAGCGACCTTTGAGGGGGCATTATGTTATAATTAA
   *
   GCTATGAAGAAGAAAATTTTTATTGCTTTGATGGCC
      METLysLysLysIlePheIleAlaLeuMETAla
```

FIG. 5. Nucleotide sequences of putative promoter regions of promoter-signal sequence fragments ss30, ss38, ss45, and ss80. The putative −35 and −10 regions are underlined, and the 5′ end of the mRNA, if mapped, is marked with an asterisk above the corresponding nucleotide. The first amino acids of the signal peptide are also indicated. In ss80, the two possible initiation sites are indicated by boldface (first MET) and lightface (second MET) letters.

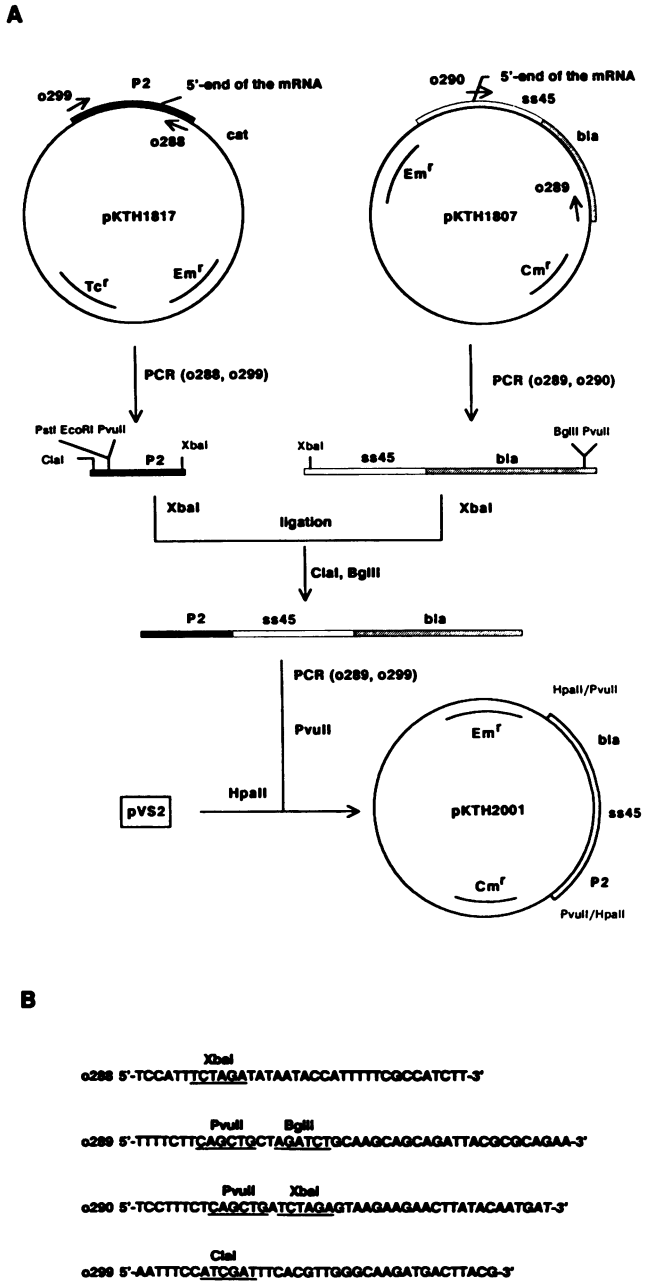

FIG. 6. Construction of a plasmid containing a new promoter signal sequence combination, P2-ss45. (A) Abbreviations and symbols: Cm^r, Em^r, and Tc^r, chloramphenicol, erythromycin, and tetracycline resistance genes, respectively; solid box, promoter P2 region; open box, signal sequence ss45 region; hatched box, β-lactamase coding region; o and number, oligonucleotide primer. (B) Oligonucleotides used in the construction of the hybrid fragment.

activity is due either to decreased translational efficiency or to the shorter half-life of the enzyme. The use, in lactococci, of a *cat*-specific promoter probe vector probably favors the selection of powerful promoters.

From the fragments isolated in this study (P1, P2, P10, and P21), sequences which closely resembled the consensus vegetative promoters of *E. coli* and *B. subtilis* could be identified. The nucleotide sequences of the −35 and −10 regions and the distances between the hexanucleotides matched those of the corresponding regions of *E. coli* σ^{70} and *B. subtilis* σ^{43} promoters (4). This was also the case with the previously isolated promoters of *L. lactis* subsp. *cremoris* (31). The −35 and −10 regions of the promoters isolated from *L. lactis* subsp. *lactis* bacteriophage BK5-T (17), however, showed no obvious similarity to the consensus sequence of *E. coli* promoters. Although the data obtained with four promoters are not sufficient to make general conclusions, it seems that promoter strength correlated with the better match to the consensus promoter sequences, whether the efficiencies were determined as mRNA production or as CAT activity.

Sequence analysis of the clones showed that promoter P2

was nearly identical to the *L. lactis* subsp. *cremoris* promoter 21 cloned by van der Vossen et al. (31). There were 3 nucleotide changes in a 180-bp region. This is a clear indication that these two subspecies are very close relatives, since the sequences lie outside of the structural gene. One of the putative promoters (P1) was accompanied by an open reading frame coding for ribosomal proteins (unpublished data), which suggests that at least this promoter is biologically functional.

We have also isolated putative promoters along with a signal sequence (ss30, ss38, ss45, and ss80). Putative −35 and −10 regions were found for these promoters, but the distance between them was either too short or too long compared with the spacing of typical *E. coli* and *B. subtilis* promoters. These promoters produced less mRNA than the ones isolated by the promoter probe vector, indicating their lower efficiency, although the degradation rate could also have affected the mRNA level. However, these promoters were the most efficient ones that could be isolated by their ability to both express and secrete β-lactamase. This suggests that the lactococcal promoters responsible for the expression of secreted proteins are not very strong.

By changing the promoter of one of the promoter-signal sequence constructions (ss45) to a more efficient one (P2), production of β-lactamase increased 10-fold, up to 2,900 U/ml, in the culture supernatant. It correlated well with the mRNA data, which showed an approximately 10-fold difference in mRNA levels. This suggests that, at least in the case of ss45, the low protein production resulted from the low level of transcription rather than rate-limiting translocation. It appears that better expression and secretion units may be constructed from separate promoters and signal sequences. The results obtained here show that it is possible to construct vehicles for the improvement of starter strains or even the production of heterologous proteins.

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REFERENCES

1. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heynecker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of a new cloning vehicle. II. A multipurpose cloning system. Gene **2:**95–113.
2. **Chopin, M.-C., A. Chopin, A. Rouault, and N. Galleron.** 1989. Insertion and amplification of foreign genes in the *Lactococcus lactis* subsp. *lactis* chromosome. Appl. Environ. Microbiol. **55:**1769–1774.
3. **de Vos, W.** 1987. Gene cloning and expression in lactic streptococci. FEMS Microbiol. Rev. **46:**218–295.
4. **Doi, R. H., and L.-F. Wang.** 1986. Multiple procaryotic ribonucleic acid polymerase sigma factors. Microbiol. Rev. **50:**227–243.
5. **Gasson, M. J.** 1983. Plasmid complements of *Streptococcus lactis* NCDO712 and other lactic streptococci after protoplast-induced curing. J. Bacteriol. **154:**1–9.
6. **Graves, M. C., and J. C. Rabinowitz.** 1986. *In vivo* and *in vitro* transcription of the *Clostridium pasteurianum* ferredoxin gene. Evidence for "extended" promoter elements in gram-positive organisms. J. Biol. Chem. **261:**11409–11415.
7. **Gryczan, T., S. Contente, and D. Dubnau.** 1978. Characterization of *Staphylococcus aureus* plasmids introduced by transformation into *Bacillus subtilis*. J. Bacteriol. **134:**318–329.
8. **Hanahan, D., and M. Meselson.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
9. **Hattori, M., and Y. Sakaki.** 1986. Dideoxy sequencing method using denatured plasmid templates. Anal. Biochem. **152:**232–238.
10. **Hawley, D. K., and W. R. McClure.** 1983. Compilation and analysis of *Escherichia coli* promoter DNA sequences. Nucleic Acids Res. **11:**2237–2255.
11. **Holo, H., and I. F. Nes.** 1989. High-frequency transformation by electroporation of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. Appl. Environ. Microbiol. **55:**3119–3123.
12. **Inamine, J. M., L. N. Lee,and D. J. LeBlanc.** 1986. Molecular and genetic characterization of lactose-metabolic genes of *Streptococcus cremoris*. J. Bacteriol. **167:**855–862.
13. **Kiwaki, M., H. Ikemura, M. Shimizu-Kadota, and A. Hirashima.** 1989. Molecular characterization of a cell wall-associated proteinase gene from *Streptococcus lactis* NCDO763. Mol. Microbiol. **3:**359–369.
14. **Kok, J., K. J. Leenhouts, A. J. Haandrikman, A. M. Ledeboer, and G. Venema.** 1988. Nucleotide sequence of the cell wall proteinase gene of *Streptococcus cremoris* Wg2. Appl. Environ. Microbiol. **54:**231–238.
15. **Kok, J., J. M. van Dijl, J. M. B. M. van der Vossen, and G. Venema.** 1985. Cloning and expression of a *Streptococcus cremoris* proteinase in *Bacillus subtilis* and *Streptococcus lactis*. Appl. Environ. Microbiol. **50:**94–101.
16. **Kondo, J. K., and L. L. McKay.** 1984. Plasmid transformation of *Streptococcus lactis* protoplasts: optimization and use in molecular cloning. Appl. Environ. Microbiol. **48:**252–259.
17. **Lakshmidevi, G., B. E. Davidson, and A. J. Hillier.** 1990. Molecular characterization of promoters of the *Lactococcus lactis* subsp. *cremoris* temperate bacteriophage BK5-T and identification of a phage gene implicated in the regulation of promoter activity. Appl. Environ. Microbiol. **56:**934–942.
18. **Leenhouts, K. J., J. Kok, and G. Venema.** 1989. Campbell-like integration of heterologous plasmid DNA into the chromosome of *Lactococcus lactis* subsp. *lactis*. Appl. Environ. Microbiol. **55:**394–400.
19. **Lennox, E. S.** 1955. Transduction of linked genetic characters of the host by bacteriophage P1. Virology **1:**190–206.
20. **Limsowtin, G. K. Y., V. L. Crow, and L. E. Pearce.** 1986. Molecular cloning and expression of the *Streptococcus lactis* tagatose 1,6-bisphosphate aldolase gene in *Escherichia coli*. FEMS Microbiol. Lett. **33:**79–83.
21. **Maeda, S., and M. J. Gasson.** 1986. Cloning, expression and location of the *Streptococcus lactis* gene for phospho-β-D-galactosidase. J. Gen. Microbiol. **132:**331–340.
22. **O'Callaghan, C. H., and A. Morris.** 1972. Inhibition of β-lactamases by β-lactam antibiotics. Antimicrob. Agents Chemother. **2:**442–448.
23. **Palva, I.** 1982. Molecular cloning of α-amylase gene from *Bacillus amyloliquefaciens* and its expression in *B. subtilis*. Gene **19:**81–87.
24. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
25. **Scheirlinck, T., J. Mahillon, H. Joos, P. Dhaese, and F. Michiels.** 1989. Integration and expression of α-amylase and endoglucanase genes in the *Lactobacillus plantarum* chromosome. Appl. Environ. Microbiol. **55:**2130–2137.
26. **Shaw, W. V.** 1975. Chloramphenicol acetyltransferase from chloramphenicol-resistant bacteria. Methods Enzymol. **43:**737–755.

26a. **Sibakov, M., T. Koivula, A. von Wright, and I. Palva.** 1991. Secretion of TEM β-lactamase with signal sequences isolated from the chromosome of *Lactococcus lactis* subsp. *lactis*. Appl. Environ. Microbiol. **57:**341–348.

27. **Simon, D., and A. Chopin.** 1988. Construction of a vector plasmid family and its use for molecular cloning in *Streptococcus lactis*. Biochimie **70:**559–566.

27a. **Simons, G., and W. M. de Vos.** 1987. Proc. 4th Eur. Congr. Biotechnol., p. 458.

28. **Terzaghi, P. E., and W. E. Sandine.** 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Environ. Microbiol. **29:**807–813.
29. **van de Guchte, M., J. M. B. M. van der Vossen, J. Kok, and G.**

Venema. 1989. Construction of a lactococcal expression vector: expression of hen egg white lysozyme in *Lactococcus lactis* subsp. *lactis*. Appl. Environ. Microbiol. **55:**224–228.

30. **van der Vossen, J. M. B. M., J. Kok, and G. Venema.** 1985. Construction of cloning, promoter-screening, and terminator-screening shuttle vectors for *Bacillus subtilis* and *Streptococcus lactis*. Appl. Environ. Microbiol. **50:**540–542.
31. **van der Vossen, J. M. B. M., D. van der Lelie, and G. Venema.** 1987. Isolation and characterization of *Streptococcus cremoris* Wg2-specific promoters. Appl. Environ. Microbiol. **53:**2452–2457.
32. **von Wright, A., A.-M. Taimisto, and S. Sivelä.** 1985. Effect of Ca^{2+} ions on plasmid transformation of *Streptococcus lactis* protoplasts. Appl. Environ. Microbiol. **50:**1100–1102.
33. **von Wright, A., S. Tynkkynen, and M. Suominen.** 1987. Cloning of a *Streptococcus lactis* subsp. *lactis* chromosomal fragment associated with the ability to grow in milk. Appl. Environ. Microbiol. **53:**1584–1588.
34. **Williams, D. M., E. J. Duvall, and P. S. Lovett.** 1981. Cloning restriction fragments that promote expression of a gene in *Bacillus subtilis*. J. Bacteriol. **146:**1162–1165.
35. **Williams, J. G., and P. J. Mason.** 1985. Hybridization in the analysis of RNA, p. 139–160. *In* B. D. Hames and S. J. Higgins (ed.), Nucleic acid hybridization: a practical approach. IRL Press Ltd., Eynsham, Oxford.