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Isolation and Characterization of Chromosomal Promoters of *Streptococcus salivarius* subsp. *thermophilus*

PHILIPPE SLOS, JEAN-CHARLES BOURQUIN,† YVES LEMOINE,‡ AND ANNICK MERCENIER*

Transgène S.A., 11 rue de Molsheim, F-67082 Strasbourg Cédex, France

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A promoter probe vector, pTG244, was constructed with the aim of isolating transcription initiation signals from *Streptococcus thermophilus* (*Streptococcus salivarius* subsp. *thermophilus*). pTG244 is based on the *Escherichia coli*-streptococcus shuttle vector pTG222, into which the promoterless chloramphenicol acetyltransferase gene of *Bacillus pumilus* (*cat-86*) was cloned. Random *Sau*3A fragments from the *S. thermophilus* A054 chromosomal DNA were cloned upstream of the *cat-86* gene by using *E. coli* as the host. The pool of recombinant plasmids were introduced into *S. thermophilus* and *Lactococcus lactis* subsp. *lactis* in order to search for promoter activity in these hosts. For *S. thermophilus*, it was necessary to first select erythromycin-resistant transformants and then to screen for chloramphenicol resistance among these. Direct selection of chloramphenicol-resistant clones was, however, possible in *L. lactis* subsp. *lactis*. Six fragments exhibiting promoter activity were characterized in *S. thermophilus* by measuring the levels of *cat-86* transcription and/or chloramphenicol acetyltransferase specific activity. Three of the promoter-carrying fragments were sequenced. The 5′ ends of their corresponding mRNAs were determined by S1 mapping and shown to correspond to a purine residue in all cases. Upstream from these potential transcription start points, sequences homologous to the *E. coli* σ70 and the *Bacillus subtilis* vegetative σ43 (or σA) consensus promoters were identified.

Understanding of the genetics of lactic acid bacteria, including thermophilic streptococci, has progressed impressively during the last decade due to the development of recombinant DNA technology. Among these, *Streptococcus salivarius* subsp. *thermophilus* (referred to throughout this paper as *Streptococcus thermophilus*) is an important dairy starter used in the manufacture of yogurt and cheese varieties in which bacterial growth at a high temperature is required. Several gene transfer methods—conjugative mobilization (33), transduction (26), spheroplast transformation (25), and electroporation (5, 36)—have been established for this species, opening the way for starter improvement. However, very little information has been available to date, on the subject of *S. thermophilus* transcription-translation initiation signals. Recently, four genes involved in its carbohydrate metabolism were isolated and sequenced (30, 31, 34a). Although the corresponding promoter sequences have been characterized, none of them have been used yet to express homologous or heterologous genes in *S. thermophilus*. In the case of *Lactococcus lactis* subsp. *cremoris*, promoters were isolated by cloning random DNA fragments in front of a promoterless *Bacillus pumilus cat-86* gene (42). They were subsequently used to drive functional expression of reporter genes inserted in recombinant plasmids (39).

In this article, we describe the construction of a promoter-probe vector, pTG244, carrying a promoterless *cat-86* screening marker. This plasmid allowed us to isolate chromosomal promoters from *S. thermophilus* A054. Measurements of the level of *cat-86* transcription and chloramphenicol acetyltransferase (CAT) specific activities in the corresponding recombinant *S. thermophilus* strains have been performed with six derivatives of pTG244, each containing a different promoter-containing fragment. The nucleotide sequence and the 5′ end of the corresponding mRNA (potential transcription initiation start point) have been determined for the three smallest fragments exhibiting promoter activity. S1 mapping experiments revealed that these sequences are used in vivo by the host, confirming that bona fide *S. thermophilus* promoters were cloned. They also appeared to function in *L. lactis* subsp. *lactis* and other gram-positive bacteria.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *Escherichia coli* ED8739 (29) was used as the host strain in the cloning experiments with pTG244, which is derived from plasmid pTG222 (33) (Fig. 1). *S. thermophilus* A054 (24) and *L. lactis* subsp. *lactis* MG1363 (11) served as recipient strains for the screening of promoter activity.

Culture media and growth conditions were similar to those described previously (33) except that erythromycin (Em) was added at a final concentration of 1 μg/ml when necessary.

Transformation of *S. thermophilus*, *L. lactis* subsp. *lactis*, and *E. coli*. *S. thermophilus* A054 was transformed by electroporation with the Bio-Rad Gene Pulser unit (Bio-Rad Laboratories, Richmond, Calif.). A fresh overnight culture was diluted 100-fold in Belliker medium (Elliker medium [10] containing 1% beef extract) supplemented with 20 mM DL-threonine. Cells were harvested at an A_{660} of 0.25 by centrifugation (6,000 × *g* for 10 min) and washed twice with electroporation buffer (EB; 7 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 6.5], 1 mM EDTA, 272 mM sucrose). The cells were concentrated to a final A_{660} of 2.5 and kept on ice for 5 min. All washes were carried out at 4°C. An aliquot of concentrated cells (0.8 ml) was mixed with 0.5 μg of plasmid DNA resuspended in TE buffer (10 mM Tris-hydrochloride, 1 mM EDTA [pH 7.5]) and transferred to an electroporation cuvette (4-mm elec-

* Corresponding author.
† Present address: Laboratoire de Pathologie Végétale, INRA, 68000 Colmar, France.
‡ Present address: Laboratoire de Génétique Moléculaire des Eucaryotes, 67085 Strasbourg Cédex, France.

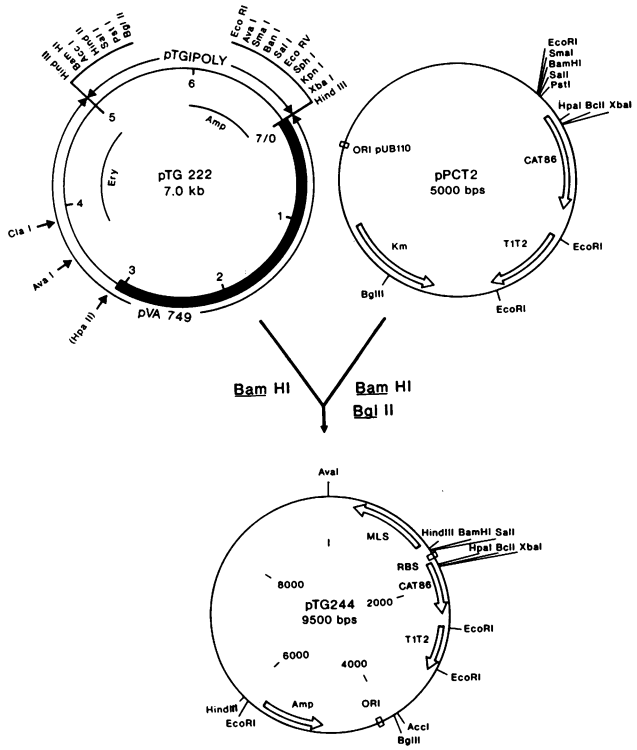

FIG. 1. Construction scheme for the promoter-probe vector pTG244. Only restriction sites relevant to the construction and use of vector pTG244 are given.

trode gap). A single electric pulse was delivered (apparatus setting at 25 μF and 2.5 kV). Immediately following the discharge, the suspension was diluted with an equal volume of Belliker broth and kept at 37°C for 60 min. Aliquots were then overlaid in soft agar on Belliker plates supplemented with the appropriate antibiotics.

L. lactis subsp. *lactis* MG1363 protoplasts were transformed by the protocol of Kondo and McKay (19). Competent *E. coli* cells were prepared and transformed as described by Hanahan (13).

Isolation of plasmid and chromosomal DNA. The plasmid DNA of *S. thermophilus*, *L. lactis* subsp. *lactis*, and *E. coli* was isolated as described previously (33).

For the isolation of *S. thermophilus* chromosomal DNA, an overnight culture was diluted 100-fold in 100 ml of Belliker broth. At an A_{660} of 1.0, cells were harvested by centrifugation. The pellet was resuspended in 40 ml of a 25% sucrose solution containing 2 mg of lysozyme per ml, and the suspension was incubated at 37°C. After 60 min, the weakened cells were collected and resuspended in 5 ml of TES (50 mM Tris-hydrochloride, 5 mM EDTA, 50 mM NaCl [pH 7.5]) containing 1% sodium dodecyl sulfate and RNase (50 μg/ml). To ensure complete lysis, the suspension was kept at 37°C for 30 min prior to the addition of 50 μg of proteinase K per ml. The incubation temperature was maintained at 37°C for 30 min and then increased to 55°C for another 30 min. The chromosomal DNA was further purified by cesium chloride-ethidium bromide density gradient centrifugation (22).

Restriction enzyme analysis, cloning, and DNA sequence determination. Restriction enzymes, polynucleotide kinase, T4 DNA ligase, Klenow fragment of DNA polymerase I, and alkaline phosphatase were purchased from Boehringer GmBH (Mannheim, Federal Republic of Germany) and used as recommended by the manufacturer. DNA was visualized by horizontal agarose gel electrophoresis in TAE buffer (40 mM Tris-acetate, 2 mM EDTA [pH 8]). DNA fragments were isolated by using the Geneclean kit (Bio 101 Inc., La Jolla, Calif.). The nucleotide sequence of the promoter-carrying fragments was determined by the dideoxynucleotidic method of Sanger et al. (34), after cloning in the M13TG130 and M13TG131 vectors (17) with *E. coli* JM103 (27) as the host.

Isolation of *S. thermophilus* RNA and RNA dot blot analysis. Total RNA from strain A054 was extracted by the method of Robbins et al. (32) with a slight modification of the lysis procedure. The cells of a 50-ml culture were grown to an A_{660} of 0.8 before being collected and treated with mutanolysin as described before (25). The resultant spheroplasts were harvested at 4°C by centrifugation (6,000 × *g* for 10 min) in an RNase-free glass tube. The pellet was resuspended in 3 ml of ice-cold lysis buffer containing 4 M guanidinium isothiocyanate, 0.05% Sarkosyl, 0.1 M β-mercaptoethanol, and 25 mM sodium citrate, which led to immediate lysis of the spheroplasts. All subsequent RNA purification steps were done as described by Robbins et al. (32). For RNA dot blot analysis, 4 μl of the RNA suspensions at different concentrations was spotted onto a Hybond-N membrane (Amersham) which was baked for 2 h at 80°C.

Prehybridization (3 h) and overnight hybridization with ^{32}P-labeled oligomer probe TG1769 were performed at 50°C in 5 × SSPE (20 × SSPE is 0.2 M sodium phosphate [pH 7.7], 2 mM EDTA, 3.5 M NaCl)–12 mM $NaPP_i$–2% sodium dodecyl sulfate–5× Denhardt's solution (6). The membrane was then washed three times for 10 min with 2.5× SSPE at 50°C, and hybridization signals were visualized by autoradiography. The corresponding bands were then cut from the membrane, and the radioactivity was measured by scintillation.

Determination of CAT activity. Cell extracts of *S. thermophilus* A054 were obtained following spheroplast formation (25), harvesting, and lysis in 1 ml of 0.25 M Tris-hydrochloride, pH 7.8. RNase (10 μg/ml) and DNase (10 μg/ml) were added to the lysate, which was incubated for 30 min at 37°C. The cell debris was removed by centrifugation (10,000 × *g* for 20 min), and the supernatants were assayed for chloramphenicol acetyltransferase (CAT) activity by the nonchromatographic method of Sleigh (35). The protein concentration was determined by the method of Bradford (3). CAT specific activities are expressed as nanomoles of acetyl groups transferred per hour per milligram of protein.

Plasmid copy number determination. Total DNA was extracted from 25-ml cultures of cells grown to an A_{660} of 0.8. After harvesting, cells were resuspended in 20 ml of a 25% (wt/vol) sucrose solution containing lysozyme (2 mg/ml) and then incubated for 60 min at 37°C.

The treated cells were collected by centrifugation (6,000 × *g* for 10 min) and lysed in 10 ml of acetate buffer (0.15 M sodium acetate [pH 5], 1 mM EDTA, 4% sodium lauryl sarcosine). NaCl was added to a final concentration of 0.1 M, and the lysate was extracted twice with phenol-chloroform. After overnight precipitation with ethanol and a washing step with 70% ethanol, the DNA was resuspended in 2.5 ml of TE. The DNA solution was treated with RNase (50 μg/ml) for 30 min at 37°C, followed by incubation with proteinase K (50 μg/ml) for 45 min at 37°C. After phenol extraction and ethanol precipitation, the DNA was resuspended in 300 μl of

TE. Total DNA (5 μg) was digested with *Hin*dIII. Aliquots of the digestion mixture (2 and 0.2 μg) were loaded on a 0.8% agarose gel. After electrophoresis, the DNA was transferred from the gel to a Hybond-N membrane by the Southern technique (37). The membrane was hybridized under stringent conditions with ^{32}P-labeled probes corresponding to promoter fragments P3, P8, P20, and P25. After washing and autoradiography, the bands corresponding to either plasmid or chromosomal DNA were cut from the membrane and their radioactivity was measured. The copy number of the plasmid was determined by calculating the ratio (cpm in plasmid band/cpm in chromosomal band).

S1 mapping. For single-stranded DNA probe preparation, the following primers, located at the 3′ ends of the promoter fragments (see Fig. 2), were used:

TG8: 5′-TTCCATGGTAATCATGATTTTTAGAG-3′ (1)

TG20: 5′-TTGTAAGGCAGATGCTCTCCCAGC-3′ (2)

TG25: 5′-ATACGATGTTTCCATTATATCATTT-3′ (3)

TG251: 5′-GTCGACGGATCATACGATGTTTCC-3′ (4)

The primers were first 5′ end labeled with T4 polynucleotide kinase and then annealed to single-stranded DNA from M13TG130 containing the corresponding promoter fragments. Subsequently, the DNAs were made double-stranded by using dNTPs and Klenow DNA polymerase I. The DNAs were digested with *Hin*dIII, and the end-labeled probes were isolated from a denaturing polyacrylamide gel. S1 mapping was performed by the method of Hen et al. (15).

RESULTS

Construction of the promoter-probe vector pTG244. The promoter-probe vector pTG244 used in this study was derived from the streptococcal shuttle vector pTG222 (33) (Fig. 1) and pPCT2 (derivative of pPL603 [4a, 42]), which contains a promoterless *cat-86* gene followed by the *E. coli* rRNA T1 and T2 terminators (4). The *Bam*HI-*Bgl*II fragment of pPCT2 (Fig. 1) containing the *cat-86* gene and termination signals was cloned in the unique *Bam*HI site of pTG222, resulting in pTG244. *S. thermophilus* A054 and *L. lactis* subsp. *lactis* MG1363 transformants containing pTG244 were Em^r and Cm^s, indicating that no significant readthrough transcription from the vector sequences occurred. The unique *Bam*HI site of pTG244 was used to insert random chromosomal DNA restriction fragments with compatible cohesive ends. Potential promoter sequences were identified by their ability to drive transcription of the *cat-86* gene, leading to Cm^r. Fragments exhibiting promoter activity were excised from the recombinant plasmids as *Hin*dIII-*Sal*I fragments (Fig. 1) for further analysis.

***S. thermophilus* promoter screening with pTG244.** *Sau*3A fragments from the A054 chromosomal DNA, with an average size of 0.75 kb, were cloned into the unique dephosphorylated *Bam*HI site of pTG244. The corresponding ligation mixture was introduced into *E. coli* ED8739, and ampicillin-resistant colonies were selected. A total of 5,000 recombinant clones were pooled, and their plasmid DNA was extracted after growth under selective pressure (100 μg of ampicillin per ml); 1 μg of this plasmid DNA pool was used to transform *S. thermophilus* A054 and *L. lactis* subsp. *lactis* MG1363.

In the case of *S. thermophilus*, no transformants could be obtained by direct selection on solid medium supplemented with chloramphenicol (Cm) at 5 μg/ml. Therefore, 6,000 Em^r transformants were screened for their Cm^r phenotype. Of the 19 Cm^r Em^r colonies isolated in this way, 8 were studied in more detail. Analysis of their DNA content revealed that they contained recombinant plasmids carrying inserts of 0.15 to 1.7 kb, pTG244-8 to pTG244-25 (Table 1). When transformed into *L. lactis* subsp. *lactis*, all these constructions gave rise to Cm^r colonies (Table 1).

TABLE 1. Characteristics of *S. thermophilus* A054 DNA promoter fragments cloned in pTG244

Plasmid	Promoter insert[a]	Size (kb)	Cm resistance (μg/ml)	
			S. thermophilus A054	*L. lactis* subsp. *lactis* MG1363
pTG244-1	P1	0.2	5	50
pTG244-3	P3	1	5	40
pTG244-4	P4	0.9	5	15
pTG244-6	P6	0.3	<5	5
pTG244-8	P8	0.15	5	10
pTG244-13	P13	0.8	5	5
pTG244-14	P14	0.9	5	5
pTG244-15	P15	1.5	5	5
pTG244-17	P17	1.7	5	10
pTG244-20	P20	0.36	5	10
pTG244-23	P23	0.6	5	10
pTG244-25	P25	0.28	5	15

[a] Promoter fragments P1, P3, P4, and P6 were selected in *L. lactis* subsp. *lactis* MG1363 (see text), while all the others were isolated in *S. thermophilus*.

The same strategy was applied to *L. lactis* subsp. *lactis*. Two recombinant clones could be isolated by direct selection on Cm (5 μg/ml)-containing agar (pTG244-1 and pTG244-3; Table 1), while two additional ones were obtained by toothpicking 250 Em^r transformants onto Cm plates (pTG244-4 and pTG244-6; Table 1). The corresponding recombinant plasmids (insert size varying between 0.2 and 1.0 kb) were introduced into *S. thermophilus* A054, where they all gave rise to Cm^r colonies, with the exception of pTG244-6 (Table 1). In order to evaluate the strength in each host of the 12 promoter fragments described above, the maximum Cm concentration still allowing growth was determined by toothpicking the recombinant clones onto plates containing increasing amounts of the antibiotic. While in *S. thermophilus* none of the constructions tested enabled the host to grow at Cm concentrations higher than 5 μg/ml, in *L. lactis* subsp. *lactis* different promoter fragments led to various maximal levels of Cm resistance (Table 1). Thus, in contrast to the situation existing in other organisms (9, 41, 42), it appeared that the level of Cm resistance could not be used as a first screening to differentiate the putative promoters isolated in *S. thermophilus*.

Analysis of promoter-containing fragments by nucleotide sequence determination and transcript mapping. The three smallest promoter-containing fragments (P8, P20, and P25; Table 1) were analyzed more thoroughly. They were first subcloned as *Hin*dIII-*Sal*I fragments in the phage vectors M13TG130 and M13TG131 in order to sequence both strands. Since it was not possible to clone the fragment carrying the P20 promoter in M13TG131, its sequence was confirmed with three independent recombinant M13TG130 clones. The nucleotide sequence analysis of promoter-carrying fragments P8, P20, and P25 (Fig. 2) revealed that they all contained several potential promoter sequences exhibiting more than 60% homology with the prokaryotic −35 and −10 consensus hexamers, which are spaced by 16 to 18 bp (12, 14). For example, four potential promoter sequences

PROMOTER P8

- 35 - 10

AAGCTTGGGATCTAAACTTGTTCAGAAATTGATATCTCTTTGTTTTTCTAGTATAAT

GAAATAACTACTCGTGCGAGGTTTTTACATATGGAAATGAAACAAATAAACGAG

met glu met lys gln ile asn glu

ACAACTCTAAAAATCATGATTACCATGGAAGATCCGTCGAC

thr thr leu lys ile met ile thr met glu asp

PROMOTER P20

AAGCTTGGGATCAGGTTGATAGGTTAGAAGTGGAAGTGTGGTGACACATGTAGC

GGACTAATACTAATAGCTCGAGGACTTATCCAACGAAAGTAACTGAGATATATT

- 35 (P1) - 10 (P1)

GACAACGATTTGGTTTCTTGATACAGTATAGATATTCAATTTTGAGTTGAGAAT

- 35 (P2) - 10

ACTCAGAGTTAAGTGACGATAGCCTAGGAGATACACCTGTTCCCATGCCGAACA

(P2)

CAGAAGTTAAGCCCTAGTACGCCTGATGTAGTTGGGGTTGCCCCCTGTTAGATA

CGGTAGTCGCTTAGCAAATAGGGAGTTTAGCTCAGCTGGGAGAGCATCTGCCTT

ACAAGCAGAGGGTCAGCGGTTCGATCCGTCGAC

PROMOTER P25

AAGCTTGGGATCTTAAAGGAACAGGGATGACCTTAGTCCCACTTAAGGTTTACCT

AAAAAATGGTTTTGCCAAGGTTCTACTGGGTATCGCCAAAGGGAAGCACGATTAC

GATAAACGTGAGTCAATCAAACGTCGCGAACAAGAGCGTGACATTAAACGTATTA

TCAAGAGTGTTAACCGTTAAAACACCTATCATCTAAGACTGACCTGGTATTAGAT

- 35 - 10

GATTTTTTTGCTTTTTAGCTTTAAGAAATGATATAATGGAAACATCGTATGATCC

GTCGAC

CONSENSUS

TTGACA - 17 bp - TATAAT

FIG. 2. Nucleotide sequence of the fragments carrying promoters P8, P20, and P25 isolated from the *S. thermophilus* A054 chromosome. The overbars indicate the putative promoter −35 and −10 regions, and the dots above the sequences denote the 5′ end of the promoter mRNA (transcriptional start point or precursor mRNA processing site) determined by S1 nuclease mapping experiments. The potential SD sequence of promoter P8 is underlined, and the amino acid sequence of the beginning of the potential ORF is indicated. For comparison, the *E. coli* σ70 (14) and *B. subtilis* σ43 (σA) consensus promoter sequence (28) is shown at the bottom.

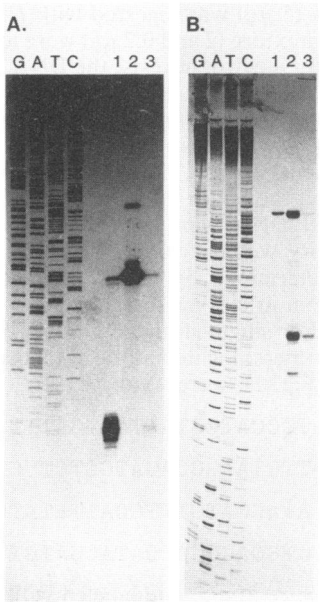

FIG. 3. S1 mapping of the promoter fragments P8 and P20. The experiments were performed as described in Materials and Methods. The sequences of recombinant M13TG130 containing the corresponding promoter fragments served as size markers. For sequencing, the same primers were used as for single-stranded probe preparation (TG8 and TG20 for promoter fragments P8 and P20, respectively). The letters G, A, T, and C indicate dideoxyguanosine-, dideoxyadenine-, dideoxythymine-, and dideoxycytosine-terminated products, respectively. In all S1 mapping experiments, additional bands representing nondigested ^{32}P-labeled probe are present. Control digestions of the probes in the absence of added RNA resulted in their complete disappearance, i.e., no detectable signal after autoradiography (data not shown). (A) Sequence of promoter fragment P8 (lanes G, A, T, C). For the S1 mapping experiments, the probe was hybridized with RNA extracted from A054(pTG244-8) (lane 1) or from plasmid-free strain A054 (lane 3). Lane 2 shows an aliquot of the ^{32}P-labeled probe. (B) Sequence of promoter fragment P20 (lanes G, A, T, C). For the S1 mapping experiments, the probe was hybridized with RNA extracted from A054(pTG244-20) or from plasmid-free strain A054 (lanes 2 and 3, respectively). Lane 1 shows an aliquot of the ^{32}P-labeled probe.

could be identified on fragment P8 (not shown). Computer analysis of the P8, P20, and P25 nucleotide sequences did not reveal the existence of promoter structures recognized by alternative (nonvegetative) sigma factors of gram-positive or gram-negative bacteria.

The presence of multiple potential promoters on the fragments studied prompted us to determine the 5′ ends of the *cat-86* mRNAs by S1 nuclease mapping. Total RNA isolated from *S. thermophilus* A054 and strain A054 containing pTG244-8, pTG244-20, or pTG244-25 was hybridized with the corresponding end-labeled single-stranded probe and further digested with S1 nuclease. The fragments resistant to S1 digestion were run on a sequencing gel and visualized by autoradiography (Fig. 3). The position of the 5′ end of the message, as deduced from the corresponding promoter sequence, is indicated for each fragment in Fig. 2. The corresponding base likely represents the authentic transcription start point, although it may not be ruled out that the observed start results from the processing of a larger precursor.

For the three promoter-carrying inserts (P8 and P20, Fig. 3; data not shown for P25), a protected fragment of identical size was obtained for A054 and A054 containing pTG244-8, pTG244-20, or pTG244-25, which demonstrates that these randomly cloned promoters are also active in vivo. As expected from the copy number (see section below), the signal detected was much stronger in the recombinant strains than in the plasmid-free strains.

The promoter activity of P8 is located on a 150-bp fragment. At 7 bp upstream of the 5′ end of the P8 message, −35 and −10 hexamers completely homologous (with the exception of 1 bp) to the consensus promoter sequences recognized by the major sigma factors of gram-negative and gram-positive bacteria were found. This characteristic structure was followed (at 23 bp) by an open reading frame (ORF) starting with ATG and preceded by a potential Shine-Dalgarno (SD) site (Fig. 2). A free energy of SD–anti-SD binding (ΔG^0) of −9.6 kcal/mol was calculated (38) by postulating that the 3′ end of the *S. thermophilus* 16S rRNA was similar to that from *L. lactis* subsp. *lactis* (21, 23). For the promoter P20, two mRNA 5′ ends were identified which were preceded by two overlapping promoter structures (P20.1 and P20.2). However, these promoter sequences were not followed by SD sequences or ORFs (Fig. 2). Analysis of

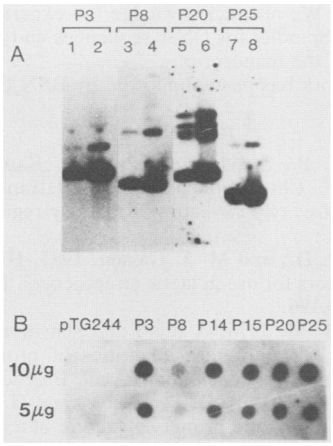

FIG. 4. (A) Copy number determination of pTG244 containing the promoter fragment P3, P8, P20, or P25. For each lane, the lower band represents the smallest *Hin*dIII fragment of the recombinant plasmid (consisting of the cloned promoter fragment and part of the *cat-86* gene). The upper band represents the chromosomal fragment hybridizing with the same probe. Odd- and even-numbered lanes were loaded with 0.2 and 2 μg of total DNA, respectively. (B) Determination of promoter strength by RNA dot blot analysis. Different concentrations of RNA (10 or 5 μg) from *S. thermophilus* A054 containing either pTG244 or recombinant plasmid pTG244-3, pTG244-8, pTG244-14, pTG244-15, pTG244-20, or pTG244-25 were hybridized with the oligonucleotide probe TG1769 (5′-GTGAAAGT GCTCTTTTCGCAG-3′) specific for the *cat-86* transcript (positions +48 to +28 of the *cat-86* sequence [1]).

the nucleotide sequence of the putative promoter P25 revealed the presence of consensus sequences at the 3′ end of the fragment but no flanking ORF. Therefore, the 5′ end of the P25 mRNA was identified by S1 mapping with the TG251 24-mer as a probe. An 18-bp fragment was found to be protected in both A054 and A054 containing pTG244-25 (data not shown).

Relative promoter strength determination. The strength of four putative promoters (P3, P8, P20, and P25) was measured by determining the transcription level of the *cat-86* gene in the corresponding recombinant strains [A054 (pTG244-3), A054(pTG244-8), A054(pTG244-20), and A054 (pTG244-25)]. To rule out any interference by a gene dosage effect, the copy number of the promoter-carrying constructions was determined first. Total DNA of the relevant strains was hybridized with the appropriate ^{32}P-labeled promoter fragment (Fig. 4A). From the result, it appeared that the *S. thermophilus* A054 chromosome contained a single copy of P3, P8, and P25 but, interestingly, four copies of P20. These data were confirmed when the DNA was digested with different restriction enzymes (*Bcl*I and *Pst*I; data not shown). The calculated copy numbers for plasmids pTG244-3, pTG244-8, pTG244-20, and pTG244-25 are listed in Table 2. With the exception of pTG244-25 (copy number of 12), all plasmids were present at seven to eight copies per genome.

The amount of *cat-86* transcript synthesized under the control of different promoter fragments in *S. thermophilus* was determined by RNA dot blot analysis (Fig. 4B). This approach of using an oligonucleotide to detect the *cat-86* mRNA was validated by the fact that only one transcript corresponding to the *cat-86* gene was found in the different recombinant strains by Northern (RNA) blot analysis when the entire gene was used as a probe (data not shown). The radioactivity was normalized to the signal measured for the weakest promoter, P8 (Table 2). When corrected for plasmid copy number variation, promoter P25 appeared to be sevenfold stronger than P8. P3 was the strongest promoter analyzed, giving rise to 10-fold more *cat-86* mRNA than the reference promoter. All of the other potential promoters studied, including the additional promoters P14 and P15 (Table 2), were about fourfold more active than P8. No detectable signal was observed for the control strain A054(pTG244), which confirms that the background of transcription of the promoterless *cat-86* gene was below the detection level (see also first section) in the promoter-probe vector.

TABLE 2. Evaluation of promoter strength activity in *S. thermophilus* A054

Promoter fragment	Relative promoter strength[a]	Plasmid copy no.	Relative CAT sp act[b] (actual CAT sp act)
P3	9.7	6.9	3.2 (25.7)
P8	1	7	1 (8.1)
P14	4	ND[c]	ND
P15	4	ND	ND
P20	5.6	8	2.2 (17.7)
P25	11.8 (7)	12	2.5 (20.5)

[a] Amount of *cat-86*-specific mRNA as deduced from the experiment described in the legend to Fig. 4B and normalized to the value obtained for the putative promoter P8. The value in parentheses for the promoter fragment P25 indicates the relative promoter strength when the increase in copy number of plasmid pTG244-25 was taken into account.

[b] CAT specific activities were normalized to the activity obtained for the putative promoter P8. Values in parentheses represent absolute CAT specific activities, expressed in nanomoles of acetyl groups transferred per hour per milligram of protein.

[c] ND, Not determined.

The hierarchy of promoter strengths established by mRNA studies was compared with that obtained by assaying CAT specific activity in the corresponding recombinant *S. thermophilus* strains (Table 2). The CAT activities measured in cell extracts of strains A054(pTG244-3), A054(pTG244-8), A054(pTG244-20), and A054(pTG244-25) were normalized to the value obtained for P8 (Table 2). It is noticeable that the two methods do not correlate directly: the differences in promoter strength were less pronounced when determined by the CAT activity than by the level of *cat-86* mRNA. From these studies, it also appears that the cloning of promoters of various strengths in pTG244 generally did not affect its copy number.

DISCUSSION

In this article we describe the construction of pTG244, a promoter-probe vector based on the *B. pumilus cat-86* gene, which was used to isolate several *S. thermophilus* genomic DNA fragments exhibiting promoter activity. The relative strength of six random candidates was evaluated by determining the level of *cat-86* mRNA and CAT specific activities in the corresponding recombinant *S. thermophilus* strains. Three of these promoters (P8, P20, and P25) were further characterized by DNA sequencing and S1 mapping experiments. Most of the promoter fragments were isolated after toothpicking Em^r transformants of *S. thermophilus* and *L. lactis* subsp. *lactis* on Cm-containing plates. Surprisingly, a few Cm^r clones could be obtained by direct selection only in the latter host. In addition, the Cm^r *S. thermophilus* transformants resulting from the screening were not able to grow at Cm concentrations higher than 5 μg/ml, which was not the

case for the equivalent *L. lactis* subsp. *lactis* clones. It should be noted that strain A054(pCK17) (2), which carries the pC194 Cm resistance marker (16), was able to grow in the presence of Cm at 100 μg/ml. These results suggest that there may be a specific barrier in *S. thermophilus* curtailing the expression of the *cat-86* gene. Since, for each promoter fragment, similar amounts of *cat-86* mRNA were found in both *L. lactis* subsp. *lactis* (data not shown) and *S. thermophilus*, this limitation most likely lies at the posttranscriptional level. A weak induction and/or translation initiation of the *cat-86* leader peptide or a poor recognition of the authentic *cat-86* ribosome-binding site (20) by *S. thermophilus* would account for the observed data. These hypotheses would also explain the discrepancy between the relative promoter strengths determined by *cat-86* mRNA levels and by CAT assays.

The copy number of the recombinant plasmids carrying promoter fragments of different strength was determined. Only pTG244-25 had a higher copy number than the other plasmids. Computer analysis of the P25 fragment (Fig. 2) showed that a potential promoter can be found on the complementary DNA strand. If this structure is functional in vivo, it could eventually account for the higher copy number of pTG244-25. It appeared that four copies of the fragment P20 (which contains two overlapping promoters) are present in the genome of *S. thermophilus* A054. Possibly, P20 could be part of a tRNA or rRNA gene, though Southern hybridization experiments showed that 16S rRNA, at least, was not involved (data not shown).

For each of the three thoroughly characterized promoter candidates (P8, P20, and P25), the 5′ end of the *cat-86* mRNA was located at a purine residue, as is most frequently observed in *E. coli* (43, 44) and in *L. lactis* subsp. *cremoris* (41). At 6 to 8 bp upstream of the putative transcriptional start sites, hexamers homologous to the −35 (TTGACA) and −10 (TATAAT) regions of the gram-positive consensus promoter sequences (11) were found, which was also the case for *L. lactis* subsp. *cremoris* (41) and *B. subtilis* (28). Interestingly, P8, the promoter candidate which showed the highest degree of homology to the consensus sequence (only one nucleotide difference in the −35 region), appeared to be the weakest potential promoter characterized. This might suggest that the sequences within the −35 and −10 regions do not exclusively determine the promoter strength in *S. thermophilus*, as is found in *E. coli*, for example (7).

Several streptococcal promoters have been reported to have an A+T-rich region upstream of the −35 hexamer (8, 41). The A+T content in the region 50 bp upstream of the putative promoters P8 (only 20 bp), P20.1, P20.2, and P25 were 70, 62, 68, and 68%, respectively. These values are indeed slightly higher than the overall A+T percentage of *S. thermophilus* DNA (60% [18]).

All of the three thoroughly characterized *S. thermophilus* genomic promoter fragments were found to be bona fide transcription initiation signals. Two of them, P8 (weak) and P25 (strong), have been further cloned into a pCK17 derivative in order to obtain expression vectors. These have allowed successful expression of a model gene, the α-amylase gene of *B. licheniformis*, in *S. thermophilus*, *Enterococcus faecalis*, *L. lactis* subsp. *lactis*, and *B. subtilis* (35a).

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