Applied and Environmental Microbiology, Oct. 1987, p. 2452–2457
0099-2240/87/102452-06$02.00/0
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Vol. 53, No. 10

Isolation and Characterization of *Streptococcus cremoris* Wg2-Specific Promoters

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Received 30 March 1987/Accepted 21 July 1987

By cloning *MboI* fragments in the promoter selection vector pGKV210, which replicates in *Streptococcus lactis*, *Bacillus subtilis*, and *Escherichia coli* and carries a promoterless chloramphenicol acetyltransferase gene, we obtained a number of fragments endowed with promoter activity, partly by direct selection for chloramphenicol resistance in *S. lactis* IL1403 and partly by selection in *B. subtilis*. Five fragments were sequenced, and the promoters were mapped with S1 nuclease. The promoters agreed with the *E. coli* promoter consensus and the *B. subtilis* vegetative σ43 promoter consensus. The promoters were preceded by an A+T-rich region (ranging from 64 to 78% A+T). S1 nuclease mapping data showed that the transcriptional start point in three of the fragments was at a TAG sequence 5 to 9 nucleotides downstream from the promoter. Three fragments carried an open reading frame preceded by a ribosome-binding site which can be recognized by *E. coli*, *B. subtilis*, and *S. lactis* ribosomes.

The group N streptococci are important microorganisms in the dairy fermentation industry. To construct dairy starter cultures with improved properties, understanding of gene expression in lactic streptococci is essential. In addition the construction of efficient expression vectors in lactic acid streptococci depends critically on the availability of well characterized expression signals of this group of organisms.

In a previous paper (30) we reported on the construction of the promoter selection vector pGKV210, which replicates in *Streptococcus lactis*, *Bacillus subtilis*, and *Escherichia coli*. This vector, containing a promoterless chloramphenicol acetyltransferase (CAT) gene (*cat-86*), proved to be useful for isolating fragments with promoter activity. However, because of the low transformation frequency of *S. lactis* protoplasts, fragments with promoter activity could be obtained only via precloning in *B. subtilis*. Recently we were able to increase the transformation efficiency of *S. lactis* protoplasts by a factor of approximately 1,600 (manuscript in preparation), which enabled us to select *Streptococcus cremoris*-specific promoters in *S. lactis* in a direct way. In this paper we report on the characterization of a number of *S. cremoris*-specific promoters of various strengths which were isolated partly via precloning in *B. subtilis* and partly by direct cloning in *S. lactis* with pGKV210.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. *S. lactis* IL1403 (5) and *B. subtilis* PSL1 (21) were used as recipients in the promoter-cloning experiments with pGKV210 (30). *S. cremoris* Wg2 (22) was the donor of the promoter-containing DNA fragments. Fusions with the promoter-containing fragments with *lacZ* were made in pMLB1043 (27) in *E. coli* MC1000 (2). pGKV210 and derivatives were routinely isolated from *E. coli* BHB2600, because the copy number of these plasmids is approximately 15 times higher in *E. coli* than in both *S. lactis* and *B. subtilis* (12).

For sequence analysis the promoter-containing fragments were cloned in *E. coli* JM109 (34) with bacteriophages M13mp18 and M13mp19 (34) as vectors.

Media. TY broth (24) was used for culturing *E. coli* and *B. subtilis*. For plating, the TY broth was solidified with 1.5% agar. *S. lactis* and *S. cremoris* were cultured and plated on glucose-M17 broth and agar (28) (Difco Laboratories, East Molesey, United Kingdom). Chloramphenicol was added at final concentrations of 5 to 100 μg/ml for *B. subtilis*, 4 to 30 μg/ml for *S. lactis*, and 10 to 300 μg/ml for *E. coli*. Erythromycin was used at 5 μg/ml for *B. subtilis* and *S. lactis* and at 100 μg/ml for *E. coli*. *E. coli* MC1000 transformants were plated on TY agar with 50 μg of ampicillin per ml and 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-Gal) per ml.

Isolation of plasmid DNA. Plasmid DNA was isolated from *E. coli* by the method of Ish-Horowicz and Burke (10). The same method with minor modifications (12) was used to isolate plasmid DNA from *B. subtilis*. To isolate plasmid DNA from *S. lactis*, the method of Gasson (6) was used.

Isolation of total DNA. High-molecular-weight DNA of *S. cremoris* Wg2 was isolated from a 10-fold-diluted overnight culture grown for 2 h at 30°C in 100 ml of glucose-M17 medium. Cells were harvested by centrifugation for 10 min at 6,000 × *g*, suspended in 10 ml of 1× SSC (0.15 M sodium chloride plus 15 mM trisodium citrate) containing 5 mg of lysozyme per ml, 100 μg of mutanolysin (Sigma Chemical Co.) per ml, and 100 μg of RNase per ml, incubated for 20 min at 37°C, and lysed by addition of sodium dodecyl sulfate (end concentration, 0.5%) and incubation for 10 min at 65°C. After addition of proteinase K (200 μg/ml), incubation was continued for 60 min at 50°C. The lysate was extracted twice with an equal volume of phenol. The aqueous phase was further extracted as described for *B. subtilis* chromosomal DNA extractions by Bron and Venema (1).

Restriction enzyme analysis and molecular cloning. Restriction enzymes and T4 DNA ligase were purchased from Boehringer GmbH, Mannheim, Federal Republic of Germany, and used as recommended by the supplier. DNA digests were separated by agarose gel (0.5 to 2.0%) electrophoresis.

Protoplasts of *B. subtilis* PSL-1 were transformed as described by Chang and Cohen (4). *S. lactis* IL1403 protoplasts were transformed as described by Kondo and McKay (13), except that protoplasts and DNA were incubated in

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22.5% polyethylene glycol for 20 min at room temperature in the presence of liposomes, consisting of cardiolipin and phosphatidylcholine in a molar ratio of 1 to 6. The end concentration of liposomes was 50 μg of lipids per ml. *S. lactis* IL1403 protoplasts were prepared by the method of Okamoto et al. (20), with some modifications (30).

Competent cells of *E. coli* were transformed by the method of Mandel and Higa (15).

DNA sequence determination. Promoter-containing fragments were cloned in M13mp18 and M13mp19 (34) and sequenced by the dideoxynucleotide method of Sanger et al. (25). 17-mer oligonucleotides for internal priming on the promoter fragments were synthesized on the bases of the nucleotide sequence determined by priming DNA synthesis with the universal 17-mer M13 primer.

Isolation of RNA. RNA was isolated from *S. lactis* carrying promoter-containing fragments in pGKV210 and from *S. cremoris* Wg2. The cells were cultured in 200 ml glucose-M17 medium containing 5 μg of erythromycin per ml and 4 μg of chloramphenicol per ml, except for *S. cremoris* cells, which were cultured in the absence of antibiotics. At an A_{660} of approximately 0.4 the cells were harvested by centrifugation (6,000 × *g* for 10 min) and suspended in 100 ml of glucose-M17 medium containing 25% sucrose, 40 mM ammonium acetate, 1 mM magnesium acetate, and 10 mg of lysozyme per ml. After 30 min of incubation at 37°C, the protoplasts were pelleted by centrifugation (6,000 × *g* for 10 min) and suspended in 10 ml of hot (65°C) lysis buffer consisting of 0.2 M sodium acetate (pH 5.0), 1% sodium dodecyl sulfate, and 10 mM EDTA (35). After 10 min, 10 ml of phenol at 65°C was added, and the solution was mixed and cooled to room temperature. Subsequently, chloroform-isoamyl alcohol (24:1, vol/vol) was added and mixed for 10 min (300 rpm). After centrifugation (20,000 × *g* for 10 min), the upper phase was extracted once more with an equal volume of chloroform-isoamyl alcohol (24:1, vol/vol). RNA was precipitated with LiCl (2 M) by the method of Zantinge et al. (36). The RNA was pelleted by centrifugation at 20,000 × *g* for 30 min at 4°C, washed once with 2 M LiCl and twice with cold 80% ethanol, dried under vacuum, and dissolved in 100 μl of distilled water.

End labeling of fragments. Promoter-containing fragments were cut from the promoter selection vector pGKV210 with *Eco*RI and *Sal*I and collected on a DEAE membrane (Schleicher and Schuell, Dassel, Federal Republic of Germany) after gel electrophoresis, as specified by the supplier. The 5' ends of the promoter-containing DNA fragments were labeled after treatment with calf intestinal phosphatase by using [γ-^{32}P]ATP and T4 polynucleotide kinase as described by Maniatis et al. (16).

S1 nuclease mapping assay. RNA (20 μg) was mixed with approximately 150 ng of ^{32}P-5'-end-labeled DNA in a volume of 10 μl containing 80% formamide, 40 mM 4(2-hydroxymethyl)-1-piperazine ethanesulfonic acid (pH 6.4), 0.4 M NaCl, and 1 mM EDTA, as described by Casey and Davidson (3). The mixture was incubated for 5 min at 90°C and subsequently transferred to 48.5°C and incubated for 3 h. The hybridization mixtures were diluted with 200 μl of cold S1 digestion buffer, consisting of 30 mM sodium acetate (pH 4.6), 0.25 M sodium chloride, 1 mM zinc sulfate, 5% glycerol, 200 U of S1 nuclease (Boehringer), and 20 U of RNasin (Boehringer). S1 digestions were done at 37°C for 30 min. S1-resistant hybrids were precipitated with ethanol and analyzed by electrophoresis in 6% polyacrylamide gels containing 7 M urea followed by autoradiography.

β-Galactosidase assays. *E. coli* cells containing *lacZ* fusions were grown in TY broth. Portions of culture (0.1 ml) were diluted in 0.9 ml of Z buffer (18) at an optical density at 600 nm of 0.4 and disrupted with 2 drops of chloroform and 1 drop of 0.1% SDS. Samples were assayed for β-galactosidase activity by the method of Miller (18).

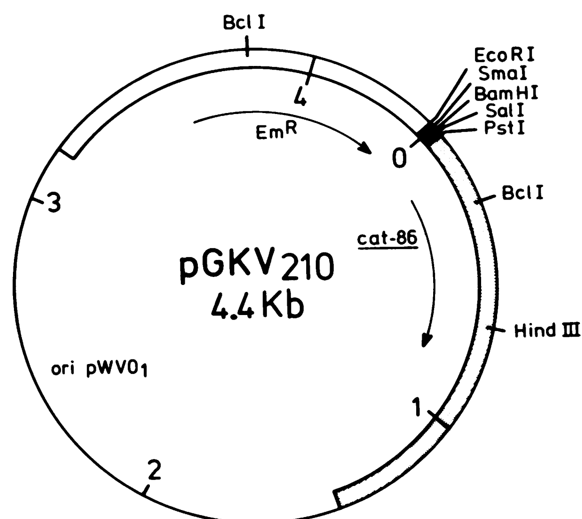

FIG. 1. Physical map of the vector pGKV210 (30). ori indicates the origin of replication of pWVO1 (31). Symbols: ——, pWVO1; ▬, double-stranded M13mp11; ▨, pPL603; ▭, pE194 cop-6.

RESULTS

Promoter screening with pGKV210. Figure 1 shows the promoter selection vector pGKV210, in which the promoterless *cat-86* gene is preceded by a multiple cloning site. *Mbo*I fragments, obtained from *S. cremoris* Wg2 chromosomal DNA, were ligated in the *Bam*HI site of the multiple cloning site, and the ligation mixture was transformed to *S. lactis* IL1403 and *B. subtilis* PSL1 protoplasts. In *S. lactis*, the mixture yielded 34 Cm^r transformants per μg of DNA on glucose-SM17 plates containing 5 μg of erythromycin per ml and 4 μg of chloramphenicol per ml; in *B. subtilis* approximately 300 Cm^r transformants per μg of DNA were obtained on DM3 plates containing 5 μg of erythromycin per ml and 20 μg of chloramphenicol per ml. All fragments which promoted the expression of the *cat-86* gene in *S. lactis* also promoted its expression in *B. subtilis*, but only 70% of the fragments obtained in *B. subtilis* were able to render *S. lactis* Cm^r.

Five randomly selected promoter-containing fragments in pGKV210, active in both *S. lactis* and *B. subtilis*, were further analyzed. Table 1 lists the Cm^r and CAT activities under the control of the different promoters in pGKV221, pGKV223, pGKV232, pGKV244, and pGKV259 in *S. lactis* and *B. subtilis*. In *E. coli* only the level of resistance to chloramphenicol was determined.

Promoter activity giving rise to relatively high levels of Cm^r or CAT activity in one host also gave a relatively high level of resistance and CAT activity in the other host (Table 1). However, resistances and CAT activities observed in *B. subtilis* were considerably higher than those observed in *S. lactis*. The highest Cm^r levels were observed in *E. coli*, but the selection vector pGKV210 also rendered *E. coli* Cm^r.

Nucleotide sequence of promoter-containing fragments. The five different promoter-containing fragments (P21, P23, P32,

TABLE 1. Promoter activity of *S. cremoris* DNA fragments in pGKV210[a]

Plasmid	Insert	Size (bp)	*B. subtilis*		*S. lactis*		*E. coli* Cm^r (μg/ml)
			Cm^r (μg/ml)	CAT activity (U/mg of protein)	Cm^r (μg/ml)	CAT activity (U/mg of protein)	
pGKV210			<5	0.1	<4	0	90
pGKV221	P21	610	40	22	8	1.3	200
PGKV223	P23	680	60	26	20	3.2	300
pGKV232	P32	648	40	17	4	0.6	200
pGKV244	P44	874	40	15	4	0.2	150
pGKV259	P59	551	60	30	24	5.1	300

[a] CAT activity was assayed by the colorimetric method (26) in cell extracts of cells grown in TY broth (*B. subtilis*) or in glucose-M17 medium (*S. lactis*) containing 5 μg of erythromycin per ml. Chloramphenicol was added to a final concentration of 5 μg/ml (*B. subtilis*) or 4 μg/ml (*S. lactis*) 1 h before the cells were harvested.The cells were harvested from an exponentially growing culture in TY broth at an A_{450} of approximately 0.6. The maximal concentration still allowing growth was determined by plating suitably diluted overnight cultures on TY plates (*B. subtilis*) or glucose-M17 medium plates (*S. lactis*) containing 5 μg of erythromycin per ml and increasing amounts of chloramphenicol. The maximal concentrations still allowing growth of *E. coli* were determined on TY plates containing 50 μg of erythromycin per ml and increasing amounts of chloramphenicol.

P44, and P59; Table 1) were cut from the recombinant plasmids with *Eco*RI and *Sal*I (the sites for these enzymes surround the *Bam*HI site in the multiple cloning site of pGKV210 [Fig. 1]) and inserted into the replicative form of M13mp18 and M13mp19 after digestion with *Eco*RI and *Sal*I. To ascertain that no sequence alterations had taken place during the different manipulations which might affect the promoter activity, the fragments were isolated from the replicative form of the M13 clones and reinserted into pGKV210. No change in Cm^r in *B. subtilis* was observed after recloning.

Relevant parts of the nucleotide sequences, determined from the S1 nuclease mapping experiments (see below) and analysis of the sequence data of the five promoter-containing fragments, are shown in Fig. 2.

The sequences shown contain putative promoters, whose −10 and −35 regions (indicated by overbars in Fig. 2) are spaced by 17 to 18 nucleotides and agree with the consensus promoter sequence of *E. coli* (8, 23) and *B. subtilis* vegetative promoters (19), which are presumed to be recognized by σ43 RNA polymerase (formerly denoted σ55 RNA polymerase [7]). Only three of the promoter fragments (P23, P32, and P44) contained an open reading frame (ORF), and all three started with ATG as the translation initiation codon. In all three fragments the translation initiation codon was preceded by a potential Shine-Dalgarno (SD) sequence, capable of base pairing with the 3′ end of the *S. lactis* 16S rRNA (UCUUUCCUCCA) (14). These sequences are underlined in Fig. 2 and have a free energy of binding (ΔG) with the 3′ end of the 16S rRNA of −16.0, −14.4, and −16.2 kcal/mol (−66.9, −60.2, and −67.8 kJ/mol, respectively) for P23, P32, and P44, respectively, according to the rule of Tinoco et al. (29). The spacing between the putative SD sequence and the start codon amounts to 9, 9, and 7 nucleotides for P23, P32, and P44, respectively.

Determination of transcriptional start sites. The transcriptional start sites were determined by S1 nuclease mapping with RNA prepared from *S. lactis* IL1403 containing pGKV221, pGKV223, pGKV232, pGKV244, or pGKV259, as well as from *S. cremoris* Wg2. Before S1 nuclease digestion the ^{32}P-5′-end-labeled promoter-containing *Eco*RI-*Sal*I fragments were hybridized with the specific RNAs at 48.5°C, calculated from the G+C content of the fragment downstream of the putative promoter, which ranged from 35 to 39% G+C, corresponding to a duplex DNA-DNA melting point ranging from 44.4 to 46.4°C in the hybridization buffer used.

The DNA-RNA hybrid-containing mixtures were treated with S1 nuclease, and the protected DNA strands were separated on a sequence gel parallel to a sequence run as a size marker.

The major bands shown in Fig. 3 represent the protected DNA segments. The protected parts of the promoter-containing fragments P21, P23, P32, P44, and P59 were 61, 203, 183, 323, and 178 nucleotides, respectively. The resulting positions of the transcriptional start points are indicated in Fig. 2 as dots. The fact that the sites of transcription initiation were 5 to 9 nucleotides downstream of the putative promoters, as identified by DNA sequencing, indicates that these promoters function as transcriptional-initiating signals in *S. lactis*. In P23, P32, and P59 transcription started at a TAG sequence; in P44 transcription started at GAG; and in P21 it started at TAC. Since initiation of transcription with ATP is preferred over initiation with GTP, and few RNAs are initiated with pyrimidine triphosphates (32, 33), it is likely that the A in the TAG or GAG sequence is the initiating purine. However, the resolution of the signal at the position of the protected DNA fragment in the various promoter-containing fragments does not exclude the possibility that the initiating purine is G instead of A. In P21 transcription starts at an A.

RNA from *S. cremoris* Wg2 also protected the labeled promoter fragments (results not shown). However, as expected, in this case the protected fragments were 12 nucleotides shorter because the RNA synthesized by *S. cremoris* was unable to protect the DNA between the *Mbo*I and the *Sal*I sites, which is a part of the multiple cloning site (results not shown).

Expression of β-galactosidase in fusions with the *lacZ* gene. To determine whether the putative ribosome-binding sites existed in vivo, in frame fusions between the ORFs of the *Mbo*I fragments P32 and P44 were made in pMLB1034 (27), with the *E. coli lacZ* gene deprived of its first 8 codons, SD sequence, and promoter. For this, pMLB1034 was cleaved with *Sma*I and blunt-end ligated with pGKV232 digested with *Mbo*I after the sticky ends had been filled in with the Klenow fragment of DNA polymerase I. Plasmid pGKV244 was digested with *Mbo*I, and the fragments were ligated into the *Bam*HI site of pMLB1034. Then *E. coli* competent cells were transformed with these ligation mixtures. Transformants were selected for blue colonies on ampicillin- and X-Gal–containing TY plates. Blue colonies containing the plasmids pZP32 and pZP44 were isolated. pZP32 and pZP44 had the expected structure, as verified by restriction enzyme analyses and sequence determination around the fusion points (results not shown).

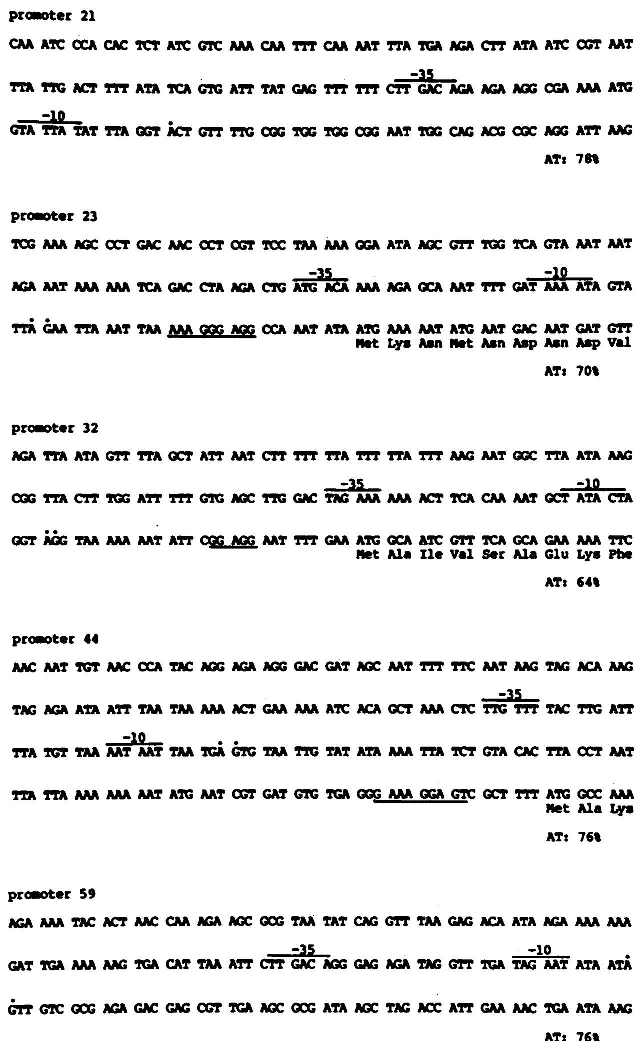

FIG. 2. *S. cremoris* promoter nucleotide sequences determined by the dideoxynucleotide method of Sanger et al. (25). The overbars indicate the promoter −35 and −10 regions, and the dots above the sequences indicate the transcriptional start point determined by S1 nuclease protection experiments. The SD sequences are underlined. The nucleotide sequences were screened for the presence of ORFs with the aid of the Microgenie computer program (Beckman Instruments, Inc.). Starts of the ORFs are given as amino acid sequences. The percent A+T content for a stretch of 50 nucleotides upstream of the −35 hexanucleotide sequence is indicated.

We measured the β-galactosidase activities of the gene fusions in *E. coli* containing pZP32, *E. coli* containing pZP44, and *E. coli* containing pMLB1034, in cultures having an optical density at 600 nm of approximately 0.4. *E. coli* containing pMLB1034 showed negligible activity, whereas the β-galactosidase activities in *E. coli* containing pZP32 and *E. coli* containing pZP44 were 10,000 and 1,200 U, respectively. These results indicate that the ribosome-binding sites deduced from the DNA sequence functioned in vivo.

DISCUSSION

The observations that the *S. cremoris* promoters isolated in *S. lactis* also functioned in *B. subtilis* and that promoters which were more active in *S. lactis* were also more active in *B. subtilis* suggest that the RNA polymerases of both species recognize identical promoters. This was confirmed by the sequence data (Fig. 2), which are summarized in Fig. 4. The promoters which were localized 5 to 9 nucleotides upstream of the start site of transcription by means of S1 nuclease mapping experiments corresponded closely to the consensus −35 (TTGACA) and −10 (TATAAT) regions of *E. coli* promoters (8, 23) and *B. subtilis* vegetative σ43 promoters (19). The spacings between the −10 and −35 sequences of the *S. cremoris* promoters were also similar to those of *E. coli* and *B. subtilis*. Recently we were able to clone and express *B. subtilis* promoters in *S. lactis*, suggesting that at least a certain class of *B. subtilis* and *S. cremoris* promoters are similar (unpublished results).

We observed that 30% of the *S. cremoris* promoters isolated in *B. subtilis* did not function in *S. lactis*. It is conceivable that these promoters are negatively controlled in *S. cremoris*. If this view is correct, one might expect that they are also repressed in *S. lactis*, because *S. cremoris* and *S. lactis* are closely related (between 70 and 100% base sequence homology [11]). Alternatively, since the level of resistance to chloramphenicol and the CAT activities observed in *S. lactis* were always less than in *B. subtilis*, it might be envisaged that from a given promoter *S. lactis*

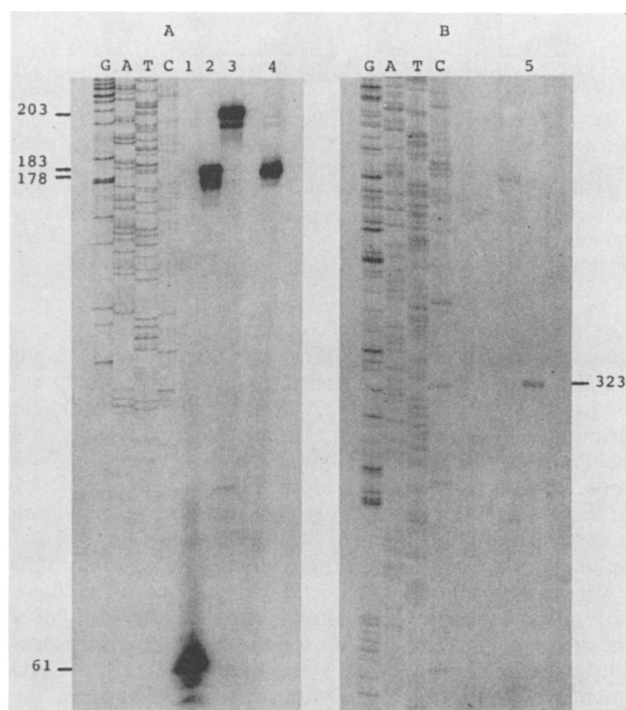

FIG. 3. RNA-protected DNA fragments in *S. cremoris* promoters. Promoter-containing *Eco*RI-*Sal*I fragments P21, P32, P23, P59, and P44 ^{32}P-labeled at their 5′ ends were hybridized to RNA isolated from *S. lactis* containing pGKV221 (panel A, lane 1), pGKV232 (panel A, lane 2), pGKV223 (panel A, lane 3), pGKV259 (panel A, lane 4), and pGKV244 (panel B, lane 5), respectively. The hybrids were treated with S1 nuclease and fractionated on a 6% polyacrylamide–7 M urea gel parallel with a sequence run of a recombinant M13mp18 phage DNA carrying the *Eco*RI-*Sal*I promoter-containing fragment P21, as size marker. The letters G, A, T, and C above the lanes indicate dideoxyguanosine-, dideoxyadenosine-, dideoxythymine-, and dideoxycytosine-terminated products, respectively. The numbers in the margins represent the lengths of the protected fragment in nucleotides.

PROMOTER 44

−35 −10
TCTTGTTTTACTTGATTTTATGTTAAAATAATTAATGAGT

PROMOTER 32

−35 −10
ACTAGAAAAAAACTTCACAAAATGCTATACTAGGTAGGTA

PROMOTER 21

−35 −10
TCTTGACAGAAGAAGGCGAAAAATGGTATTATATTTAGGTAC

PROMOTER 23

−35 −10
TGATGACAAAAAGAGCAAATTTTGATAAAATAGTATTAGAA

PROMOTER 59

−35 −10
TCTTGACAGGGAGAGATAGGTTTGATAGAATATAATAGTT

CONSENSUS E. coli PROMOTER
and VEGETATIVE B. subtilis PROMOTER

−35 −10
tcTTGACA.....17/18..t.tg.TATAAT.. .cat..

FIG. 4. Nucleotide sequences of five *S. cremoris* promoters. The order corresponds to increasing CAT activity. The −35 and −10 regions are indicated above the sequence. The TG sequences upstream of and separated by one nucleotide from the −10 hexanucleotides are boxed. For comparison, the *E. coli* consensus promoter (8, 25) and *B. subtilis* consensus σ43 promoter (21) are indicated.

produces less CAT than *B. subtilis* does. This would account for the observation (unpublished results) that promoters which are weakly active in *B. subtilis* direct insufficient synthesis of CAT in *S. lactis* to render the cells Cm^r. It is conceivable that the reduced synthesis of CAT in *S. lactis* as compared with *B. subtilis* is due to a higher rate of mRNA degradation in *S. lactis*. Interestingly, a similar observation has been made by Hudson and Stewart (9), who showed that CAT activities directed by *Staphylococcus aureus* promoters were always higher in *B. subtilis* then in the original host.

S1 nuclease mapping data showed that the transcriptional start site was A or G in a TAG sequence in three promoter-containing fragments (P23, P32, and P59) or A or G in a GAG sequence of fragment P44. Owing to the resolution of the signal of the protected fragments, it was not possible to determine whether the RNA start was A or G at position +1. Alternatively, it is conceivable that the RNA starts at either of these nucleotides, which would be similar to certain cases in *E. coli*, in which initiation of transcription occurs at two adjacent residues (8, 23). In the promoter-containing fragment P21, transcription starts at the A of a TAC sequence.

The spacing between the −10 region and the start site of transcription ranged from 4 to 8 nucleotides, which has also been reported for *E. coli*-specific promoters (8, 23). Irrespective of the origin of the RNA, namely, RNA obtained from *S. cremoris* or *S. lactis* carrying the promoter-containing fragments, the start points of transcription were found to be the same, indicating that transcription in *S. cremoris* and *S. lactis* is initiated at the same nucleotide.

The minor bands in the autoradiograph of the S1 nuclease mapping experiment (Fig. 3) may be due to RNase activity. Alternatively, these bands may represent secondary start points of transcription; the sequence data of P21, P23, and P59 indicated the presence of a second −10 region 5 nucleotides downstream of the first Pribnow box. Conceivably, this region may be used as a secondary site for the initiation of transcription. The absence of a secondary transcriptional start point on fragment P21 may be explained by the circumstance that the S1-protected fragment will migrate in the buffer front of the sequence gel.

In all five promoter-containing fragments, A+T-rich regions were present upstream of the −35 promoter hexanucleotide. The percentage of A+T in the region 50 bases upstream of the *S. cremoris*-specific promoters ranged from 64 to 78% (Fig. 2). Whether these A+T-rich regions are important for promoter activity in vivo in *S. cremoris* remains to be established, especially since the overall A+T content in the lactic acid streptococci is already relatively high (the mean value of A+T for four different lactic streptococcal strains is 63% [11]).

An interesting question is whether the various promoter sequences can be related to the different levels of *cat-86* gene expression. Although no definite conclusions can be drawn from the present data, it is interesting that the higher Cm^r and CAT activities (Table 1) were observed with promoters which either have complete consensus in their −35 hexanucleotide with the canonical *E. coli* sequences (P21 and P59) or differ from it in just one nucleotide (P23). Since the weaker promoters, P32 and P44, differ in two and three nucleotides, respectively, from the consensus −35 sequence, it seems as if complete consensus in the −35 hexanucleotides yields the stronger promoters, which would be in accord with the observation that in *E. coli* most nucleotide substitutions toward the consensus promoter sequence result in an increase of promoter activity (8).

Four of the five promoters listed in Fig. 4 contain a TG sequence upstream of and separated by one nucleotide from the −10 hexanucleotide. These two nucleotides at this position are weakly conserved in *E. coli* promoters (8), but are much stronger in *B. subtilis* promoters (19) and, as the present work shows, apparently also in *S. cremoris* promoters. Promoter P44 is missing the TG sequence at this site, which might additionally account for the low activity of this promoter.

The promoter fragments P21 and P59 did not contain an ORF. The promoter in fragment P21 is close to the *Mbo*I site of the fragment, which may account for the absence of an ORF. In promoter-containing fragment P59, however, an ORF might be expected, because the length of the transcript from the start to the *Mbo*I site is 166 nucleotides. Possibly this promoter-containing fragment is part of an rRNA gene. In the three promoter-containing fragments P23, P32, and P44, the ORFs started with ATG coding for methionine. Upstream of the start codon an SD sequence is present, which can form stable base pairs with the 3′ end of the 16S rRNA of *S. lactis*, UCUUUCCUCCA (14). The free energy (ΔG) of base pairing was −16.0, −14.4 and −16.2 kcal/mol for the SD sequences in P23, P32, and P44, respectively, and corresponded well to those reported for different gram-positive organisms (17).

Because in-frame fusions of the ORFs of P32 and P44 with the *lacZ* gene had β-galactosidase activity in *E. coli* and *S. cremoris*-specific expression signals functioned in vivo. We

did not succeed in constructing an in-frame fusion with the ORF of fragment P23. It is conceivable that this failure is due to the strong promoter activity of the fragment, which might render the host inviable because of overproduction of the β-galactosidase fusion product.

So far the construction of efficient expression vectors for lactic acid streptococci has not been reported. The identification and characterization of a number of strong *S. cremoris* promoters described in this paper may be useful for the construction of such vectors.

ACKNOWLEDGMENTS

This research was supported by grants GBI-2-054-NL and BAP-0012-NL from the Biotechnology Engineering Program and the Biotechnology Action Program of the Commission of the European Communities, respectively.

We thank Unilever Research Laboratories for synthesising the 17-mer oligonucleotides and for their support of and interest in this work. We are grateful to H. Mulder for preparing the figures.

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