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Characterization of Transcription Initiation and Termination Signals of the Proteinase Genes of *Lactococcus lactis* Wg2 and Enhancement of Proteolysis in *L. lactis*

JOS M. B. M. VAN DER VOSSEN, JAN KODDE, ALFRED J. HAANDRIKMAN,
GERARD VENEMA,* AND JAN KOK

*Department of Genetics, Centre for Biological Sciences, University of
Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands*

The transcription initiation signals of the *prtP* and *prtM* genes specifying the proteolytic activity of *Lactococcus lactis* subsp. *cremoris* Wg2 were mapped by primer extension. The strength of these promoters was analyzed with promoter-screening vector pGKV410, and they appeared to be weaker than previously isolated promoters of strain Wg2. In addition, a putative transcription terminator downstream of the *prtP* gene was characterized by using the terminator-screening vector pGKV259. The putative terminator decreased the transcription activity of lactococcal promoter P59 by approximately 70% in both *Bacillus subtilis* and *L. lactis*. Deletion of a part of the stem-loop structure of the terminator decreased the negative effect on transcription, indicating that the structure could indeed function as a terminator of transcription. The proteolytic activity of the lactococcal host was enhanced by placing the originally oppositely oriented *prt* genes in tandem and replacing the relatively weak promoters upstream of the *prt* genes with the stronger promoter, P32, from the chromosome of *L. lactis* Wg2.

Casein degradation by *Lactococcus lactis* plays a central role in dairy fermentation. Breakdown of caseins by lactococcal proteinase and peptidase activity enables the organism to grow in milk, with concomitant acid production from lactose. The hydrolyzed caseins add to the flavor of the fermented milk product. Because of the importance of proteolysis in dairy fermentations, a considerable amount of research has been carried out with the enzymes composing the proteolytic system of lactococci (for a review, see reference 24). In particular, the genetics of the cell envelope-associated proteinase of *L. lactis* subsp. *cremoris* Wg2 and SK11 and *L. lactis* subsp. *lactis* NCDO 763 have been studied in considerable detail (6, 9, 10, 11–13, 31; for a review, see reference 14).

At least two genes are required for the proteolytic activity of lactococcal strains Wg2 and SK11: the *prtP* gene, which encodes a serine-type proteinase, and the *prtM* gene, which is essential for the maturation of the proteinase, which is initially synthesized as a pre-pro enzyme (9, 31). The two genes of strain Wg2 are divergently transcribed from putative promoters identified on a 345-bp *Cla*I fragment, 87 to 122 bp upstream of the start codon of *prtP*, and 241 to 270 bp upstream of the start codon of *prtM* (12). A second promoter was tentatively identified 46 to 74 bp upstream of the start codon of the *prtM* gene (12). The sequence data of the proteinase genes also suggested the presence of a transcription terminator downstream of *prtP*: an inverted repeat followed by a stretch of T's situated 130 nucleotides downstream of the TAG stop codon of *prtP* has the potential to form a hairpin structure with a calculated ΔG of −24.6 kcal/mol (1 cal = 4.184 J) (12).

Previously, we have reported on the construction of the promoter-screening vectors pGKV110 and pGKV210, in which the promoterless chloramphenicol acetyltransferase

gene (*cat*-86) of *Bacillus pumilus* was used as the reporter gene (29). These vectors have been used to isolate promoters from the chromosome of *L. lactis* Wg2 (29, 30). The relative strengths of these promoters were determined by assaying the levels of chloramphenicol resistance they conferred to the host cell as well as by determining the chloramphenicol acetyltransferase (CAT) activities.

We have also constructed a derivative of pGKV110 containing the *Bacillus subtilis* phage SPO2 promoter, pGKV11, which was suitable for isolation of terminator-containing DNA fragments (29). However, this terminator-screening vector appeared to be unsuitable for discriminating between strong and weak transcription-terminating signals in *L. lactis* (unpublished data), because the SPO2 promoter is too weak in this host. Therefore, we decided to use an improved transcription terminator-screening vector carrying the strong *Lactococcus* chromosomal promoter P59 (30), which discriminates between strong and weak terminator signals in *L. lactis*.

The detailed understanding of the action of the *prtP* and *prtM* genes depends on knowledge concerning the precise starts and stops of transcription of these genes. In addition, to assess the feasibility of increasing the proteinase activity by increased transcription, it was essential to determine the strengths of the *prtP* and *prtM* promoters in relation to other promoters active in lactococci. These considerations prompted us to (i) determine the transcriptional starts of *prtP* and *prtM*, (ii) assay the strengths of these promoters, and (iii) examine the efficiency of the putative transcription terminator of the *prtP* gene. Because the strengths of the promoters of the *prtP* and *prtM* genes were inferior to that of promoter P32, which we had characterized previously (30), we decided to attempt to increase the proteolytic activity of *L. lactis* by placing *prtP* and *prtM* under transcriptional control of P32. This was achieved by first reorganizing the two divergently transcribed genes in one transcriptional unit.

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* Corresponding author.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
B. subtilis PSL1	leuA8 arg-15 thrA recE4 r_m^- m_m^-	19
E. coli MH1	araD139 lacX74 gal hsr hsm$^+$ strA	2
L. lactis MG1363	Plasmid-free strain, Lac$^-$ Prt$^-$	7
Plasmids		
pGKV210	Em^r Cm^s, promoter-screening vector	29
pGKV410	Em^r Cm^s, promoter-screening vector	This work
pGKV232	Em^r Cm^r, pGKV210 with promoter P32	30
pGKV259	Em^r Cm^r, pGKV210 with promoter P59	30
pGKV432	Em^r Cm^r, pGKV410 with promoter P32	This work
pGKV500	Em^r, Prt$^+$, carries 3'-truncated prtP and prtM genes	13
pGKV505	Em^r Km^r Prt$^-$, pGKV500 with a Km^r gene in the BamHI site in prtP	Laboratory collection
pGKV532	Em^r Prt$^-$	This work
pGKV632	Em^r Prt$^-$	This work
pGKV732	Em^r Km^r Prt$^-$	This work
pGKV832	Em^r Prt$^+$	This work
pGKV705	Em^r Km^r Prt$^-$	This work
pGKV805	Em^r Prt$^+$	This work
pGKV905	Em^r Prt$^+$	This work
pGKV424	Em^r Cm^r	This work
pGKV425	Em^r Cm^r	This work
pPT8	Em^r Cm^r	This work
pPT88	Em^r Cm^r	This work

MATERIALS AND METHODS

Strains and plasmids. The strains and plasmids used are listed in Table 1. Culturing of *B. subtilis* and *L. lactis* was as described previously (30). Erythromycin and chloramphenicol were added to *B. subtilis* and *L. lactis* cultures to final concentrations of 5 μg/ml. *Escherichia coli* was grown in TY medium (21). Kanamycin-resistant *E. coli* cells were selected on plates containing 50 μg of kanamycin per ml.

Molecular cloning. Plasmid DNA was isolated as described previously (28). Restriction endonucleases, T4 DNA ligase, Klenow enzyme, and T4 DNA polymerase were purchased from Boehringer GmBH, Mannheim, Germany, and used according to the manufacturer's instructions. Manipulations with DNA were as described by Maniatis et al. (17).

Protoplasts of *B. subtilis* PSL1 were transformed by the method of Chang and Cohen (3). *E. coli* MH1 cells were transformed as described by Mandel and Higa (16). *L. lactis* MG1363 was transformed by electroporation as described by van der Lelie et al. (27) or by the protoplast transformation protocol described previously (28).

DNA sequence determination. Plasmid DNA was sequenced after denaturing in 0.2 N NaOH by using the Sequenase system (United States Biochemical). Single-stranded M13mp18 clones were sequenced according to the dideoxynucleotide sequencing method of Sanger et al. (22). Synthetic oligonucleotides were kindly provided by Unilever Research Laboratories, Vlaardingen, The Netherlands.

Isolation of RNA. RNA from *L. lactis* was isolated by using the method described previously (30), with some modifications. The amount of lysozyme was decreased to 2 mg/ml, and mutanolysin (Sigma, St. Louis, Mo.) was added to the protoplast buffer at a concentration of 10 U/ml. RNA from *B. subtilis* was isolated from 200-ml cultures in TY medium by using the same method, except that mutanolysin was omitted.

Primer extension assay. Two oligonucleotides, KL18 (TG GTCGGTCTGATTTGA) and KL17c (TGGCAAGACAGC CAGCG), served as primers for the synthesis of cDNA on prtM and prtP transcripts, respectively. Approximately 15 μg of total cell RNA was used in the primer extension assay. The RNA was incubated for 5 min at 65°C with 200 to 300 fmol of the synthetic oligonucleotide in hybridization buffer containing 70 mM Tris-HCl (pH 8.3), 14 mM MgCl$_2$, and 14 mM dithiothreitol in a total volume of 14 μl. The mixture was cooled to room temperature and adjusted to a final volume of 20 μl by the addition of dATP, dGTP, and dTTP to final concentrations of 100 μM each. Then dCTP (final concentration, 10 μM) and 15 μCi of [α-^{32}P]dCTP with a specific activity of 3,000 Ci/mmol (Amersham International, Amersham, United Kingdom) were added. cDNA was synthesized by adding 10 U of avian myeloblastosis reverse transcriptase (Boehringer) and incubating the mixture for 30 min at 42°C. The reaction was terminated by ethanol precipitation. The pellet was washed with 80% ethanol and dissolved in 12 μl of distilled water and 8 μl of sequencing loading buffer. Subsequently, the samples were separated on a 6% polyacrylamide-7 M urea gel next to a sequence run representing the size markers for determination of the sizes of the cDNAs synthesized.

Proteinase activity assay. Proteinase activities in cultures grown overnight in GM17 medium were determined with the synthetic substrate methoxy-succinyl-arginyl-prolyl-tyrosyl-p-nitroanilide (MeOsuc-Arg-Pro-Tyr-pNa; Kabi Diagnostica, Stockholm, Sweden). To 200 μl of *L. lactis* culture, diluted 10-fold in fresh GM17 medium, were added 25 μl of 10 mM MeOsuc-Arg-Pro-Tyr-pNa and 25 μl of 100 mM NaHPO$_4$ (pH 6.5). Color development at 405 nm was monitored with a Titertek Multiskan MCC/340 P (Flow Laboratories, Rickmansworth, United Kingdom).

Plasmid constructions. Plasmid pGKV432 was digested with ClaI and HindIII. The largest fragment was ligated to the prtP gene containing the ClaI-HindIII fragment of pGKV500 (13). This fragment lacks all expression signals for the prtP gene. *B. subtilis* PSL1 was transformed with the ligation mixture. This yielded pGKV532. The insertion of the complete prtM gene in pGKV532 was carried out in two steps. First, the terminal part of the prtP gene of pGKV532 was deleted by digestion with PstI followed by incubation

with T4 DNA polymerase to blunt the *Pst*I sticky ends and by digestion with *Hin*dIII. Subsequently, this fragment was replaced with the *Hin*dII-*Xmn*I fragment of pGKV500 containing the largest part of the *prtM* gene. Second, the resulting construction, isolated from a *B. subtilis* transformant, was digested with *Xba*I, the sticky ends were filled in with Klenow enzyme, and the linearized plasmid was subsequently digested with *Hin*dIII. The smaller fragment containing the terminal part of the *cat-86* gene was replaced with the *Hin*dIII-*Dra*I fragment of the double-stranded M13 clone containing the terminal part of the *prtM* gene on the 1.7-kb *Cla*I-*Cla*I fragment of pWV05 (9). The resulting construction was pGKV632, in which the ribosome binding site and the first eight codons of the *prtP* gene were still lacking.

To provide the *prtP* gene with the translational start and the first eight codons, the *Sma*I-*Kpn*I fragment in pGKV632 was replaced by the *Ssp*I (1124)-*Kpn*I (4398) fragment of pGKV505, which contains the *prtP* gene including the ribosome binding site and the first eight codons. In pGKV505, the *prtP* gene had been interrupted by the insertion of the Km^r gene (25) in the *Bam*HI site. This was required for the selection of pGKV732 in *E. coli*.

Plasmid pGKV732 was isolated from the kanamycin-resistant *E. coli* transformants, and the integrity of the *prtP* gene was restored by deleting the Km^r gene through *Bam*HI digestion and religation. The resulting plasmid pGKV832, containing a functional *prtP* and a complete *prtM* gene, was introduced into strain MG1363 by electroporation. These transformants degraded milk caseins.

pGKV410 was linearized with *Pst*I and ligated to the largest *Pst*I fragment of pGKV505, containing the *prtM* gene and the *prtP* gene inactivated by the inserted Km^r gene. The ligation mixture was transformed to *E. coli* MH1. The Km^r transformants contained pGKV705.

Plasmid pGKV705 was digested with *Sma*I and *Mlu*I, and the smallest fragment carrying the truncated 3' end of *prtM* was replaced with a fragment from pSKH1 (9) containing the complete 3' end of the *prtM* gene. To that purpose, plasmid pSKH1 was digested with *Sac*I and the recessed ends were removed with T4 DNA polymerase. The small fragment carrying the 3' end of *prtM* was obtained by cutting the linearized plasmid with *Mlu*I. The pGKV705 derivative containing the complete *prtM* gene was subsequently digested with *Bam*HI to delete the Km^r gene and to restore the integrity of the *prtP* gene. This resulted in pGKV905. *L. lactis* MG1363 transformed by pGKV905 was proteolytically active.

RESULTS

Mapping of the *prtP* and *prtM* promoters. To locate the transcriptional start points of the divergently transcribed *prtP* and *prtM* genes, the primer extension method was used. Oligonucleotides KL18 and KL17c served as primers for the synthesis of cDNA on *prtM* and *prtP* mRNA templates, respectively. The cDNAs were separated on a sequence gel, and a sequence run of fragment P44 in M13mp10 (30) was used as a size marker. The major bands in Fig. 1 represent the cDNA fragments synthesized by reverse transcriptase. The length of elongated primer KL17c was 159 bp, and that of elongated primer KL18 was 324 to 326 bp. Since the sequences of the *prtP* and *prtM* genes are known (12), the transcriptional starts of both genes could be determined. The start positions are indicated by asterisks in Fig. 2, together with the promoters tentatively identified in this region (12),
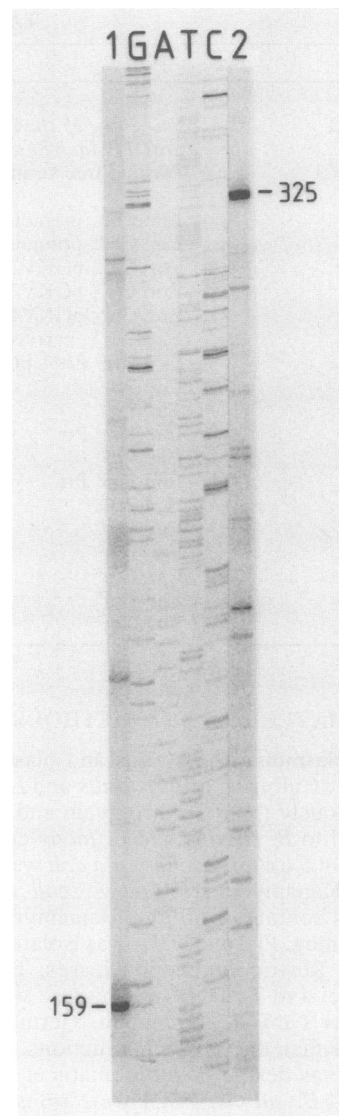
FIG. 1. cDNA fragments synthesized from mRNA of the *prtP* and *prtM* genes. The cDNAs were separated on a 6% polyacrylamide-7 M urea gel next to a sequence run of a recombinant M13mp10 phage DNA carrying the *Eco*RI-*Sal*I promoter-containing fragment P44 (30), which was used as a size marker. Lane 1, extended primer synthesized on the *prtP* mRNA; lane 2, extended primer of the *prtM* mRNA. G, A, T, and C indicate dideoxyguanosine-, dideoxyadenosine-, dideoxythymidine-, and dideoxycytidine-terminated sequencing products of fragment P44, respectively. The numbers in the margins represent the lengths of the synthesized cDNAs corresponding to mRNA initiated at the promoters of the *prtP* and *prtM* genes. For details of the procedure, see Materials and Methods.

and show that transcription of the *prtP* gene started at an A 6 bp downstream of the −10 sequence of the putative promoter and that the transcriptional start point of *prtM* was at the A or C 8 to 9 bp downstream of the putative *prtM* promoter. No cDNA was observed that would correspond to transcription started from a second possible *prtM* promoter located immediately upstream of the *prtM* gene (12).

The *prtM* and *prtP* promoters are relatively weak. To compare the strengths of the *prtP* and *prtM* promoters with

```
         -35      -35                        -10           *
      TTGAATTTGT TCTTCAATAG TATATAAATAT AATAGTATAT AATATTATAT AATATAATCT TAACTACATC AA
      AACTTAAACA AGAAGTTATC ATATATTATA TTATCATATA TTATAATATA TTATATTAGA ATTGATGTAG TT
                            **           -10                         -35
```

FIG. 2. Part of the sequence of the 345-bp *Cla*I fragment of pGKV500 (12) containing the promoters of *prtP* and *prtM* mapped in this study. The −35 and −10 regions of the *prtP* gene are indicated by arrows above the sequence, and those of the *prtM* gene are indicated by arrows under the sequence. The corresponding transcriptional start sites are indicated by asterisks.

the strengths of those randomly isolated from the chromosome of *L. lactis* Wg2 (30), the 345-bp *Cla*I fragment of pGKV500 (13), containing the two oppositely directed promoters, was inserted in two orientations in pGKV410. This plasmid is a pGKV210 derivative (29) in which the *Bam*HI site was converted into a *Cla*I site by digesting pGKV210 with *Bam*HI, filling in the sticky ends with Klenow enzyme, and religating. The insertion of the *Cla*I fragment in two orientations resulted in two plasmids, pGKV424 and pGKV425, in which the promoterless *cat-86* gene was placed under transcriptional control of the *prtP* and *prtM* promoters, respectively. Table 2 lists the maximal resistances of *B. subtilis* and *L. lactis* MG1363 containing pGKV410, pGKV424, or pGKV425 to chloramphenicol and the CAT activities in these strains. The table shows that expression of the *cat-86* gene under control of the *prtM* promoter (in pGKV425) in *B. subtilis* was less than that in pGKV424, in which expression is controlled by the promoter of *prtP*. This situation is reversed in lactococcal strain MG1363, in which the *prtM* promoter was stronger than the promoter of the *prtP* gene. The data listed in Table 2 also show that the levels of Cm^r and CAT activities dictated by the *prtP* and *prtM* promoters in *L. lactis* are less than those of lactococcal promoters P32 and P59, which had been randomly isolated from the chromosome of strain Wg2.

Proteolysis is enhanced by placing *prtP* and *prtM* in an operon structure. The fact that the original *prt* promoters are relatively weak compared with the chromosomal lactococcal promoters present in our collection (30) prompted us to attempt to increase the expression of proteolytic activity in *L. lactis* MG1363 by using promoter P32. As the *prtP* and *prtM* genes are both necessary for proteolytic activity (9) and are transcribed from divergent promoters, placing the two genes under the control of just one promoter required reorganization of these genes. To accomplish this, both *prt* genes were organized in an operonlike structure downstream of promoter P32 (Fig. 3A). This resulted in plasmid pGKV832, in which the two proteinase genes were under the

joint transcriptional control of promoter P32. The proteolytic activity produced by *L. lactis* MG1363(pGKV832) was compared with that produced by MG1363 containing plasmid pGKV905, in which the *prt* genes are expressed by their own expression signals. As with pGKV832, the *prtP* gene present on pGKV905 carries a 3'-terminal deletion of 245 codons from the end of the gene. The truncated proteinase is still active (11). In addition, pGKV905 lacks the SPO2 promoter present in pGKV500 (13). Thus, with respect to the nature of the *prtP* gene product and the absence of additional transcription starts, pGKV905 and pGKV832 are strictly comparable. The construction of pGKV905 is shown in Fig. 3B. The strains were cultured in GM17 medium, and their proteolytic capacities were measured with the synthetic substrate MeOsuc-Arg-Pro-Tyr-*p*Na. The results are presented in Table 3 and show that MG1363(pGKV832) was proteolytically active and that this activity was increased fivefold compared with that of MG1363(pGKV905), in which the two genes had their natural expression signals and organization.

The *prtP* gene is followed by a functional transcription terminator. A putative transcription terminator of the *prtP* gene is present on an *Hpa*II-*Xmn*I fragment of 89 bp (12). This fragment was inserted in the *Sal*I site of pGKV259 downstream of the strong lactococcal promoter P59 (30), after the recessed ends had been filled in with the Klenow enzyme. This placed the putative terminator between the promoter and the *cat-86* gene. *B. subtilis* protoplasts were transformed with the ligation mixture, and a colony that contained plasmid pPT8 was selected. In pPT8, the putative terminator had been inserted in the proper orientation relative to the *cat-86* gene, as was verified by sequence analysis (results not shown). *B. subtilis*(pGKV259) was resistant to 60 μg of chloramphenicol per ml, whereas *B. subtilis*(pPT8) failed to grow in the presence of more than 20 μg of chloramphenicol per ml (Table 2), indicating that the potential stem-loop structure on the *Hpa*II-*Xmn*I fragment decreased transcription and, therefore, may be classified as a

TABLE 2. Promoter activities of *L. lactis* Wg2 DNA fragments in pGKV210 and activity of the terminator of the *prtP* gene of strain Wg2 and its deletion derivative in pGKV259[a]

Plasmid	Insert	Maximum concn (μg/ml) of chloramphenicol for *B. subtilis* growth	CAT activity (U/mg of protein)	Maximum concn (μg/ml) of chloramphenicol for *L. lactis* growth	CAT activity (U/mg of protein)
pGKV424	*prtP* promoter	40	28.1	2	0.04
pGKV425	*prtM* promoter	10	12.5	3	0.08
pGKV232	P32	30	17.7	4	0.43
pGKV259	P59	60	37.6	24	6.1
pPT8	*prtP* terminator	20	8.1	8	1.0
pPT88	*Pvu*II-deleted *prtP* terminator	50	36.2	16	4.7

[a] CAT activity was assayed by the colorimetric method (23) in extracts of cells grown in TY broth (*B. subtilis*) or in glucose-M17 broth (*L. lactis*) containing 5 μg of erythromycin per ml. Chloramphenicol was added to a final concentration of 5 μg/ml (*B. subtilis*) or 2 μg/ml (*L. lactis*) 1 h before cells were harvested. *B. subtilis* cells were harvested from an exponentially growing culture (optical density at 450 nm, approximately 1). Lactococcal cells were harvested from an exponentially growing culture (optical density at 660 nm, approximately 0.5). Protein was measured by the method of Bradford (1). The maximum concentration of chloramphenicol still allowing growth was determined by plating suitably diluted overnight cultures on TY plates (*B. subtilis*) or glucose-M17 plates (*L. lactis*) containing 5 μg of erythromicin per ml and an increasing amount of chloramphenicol.
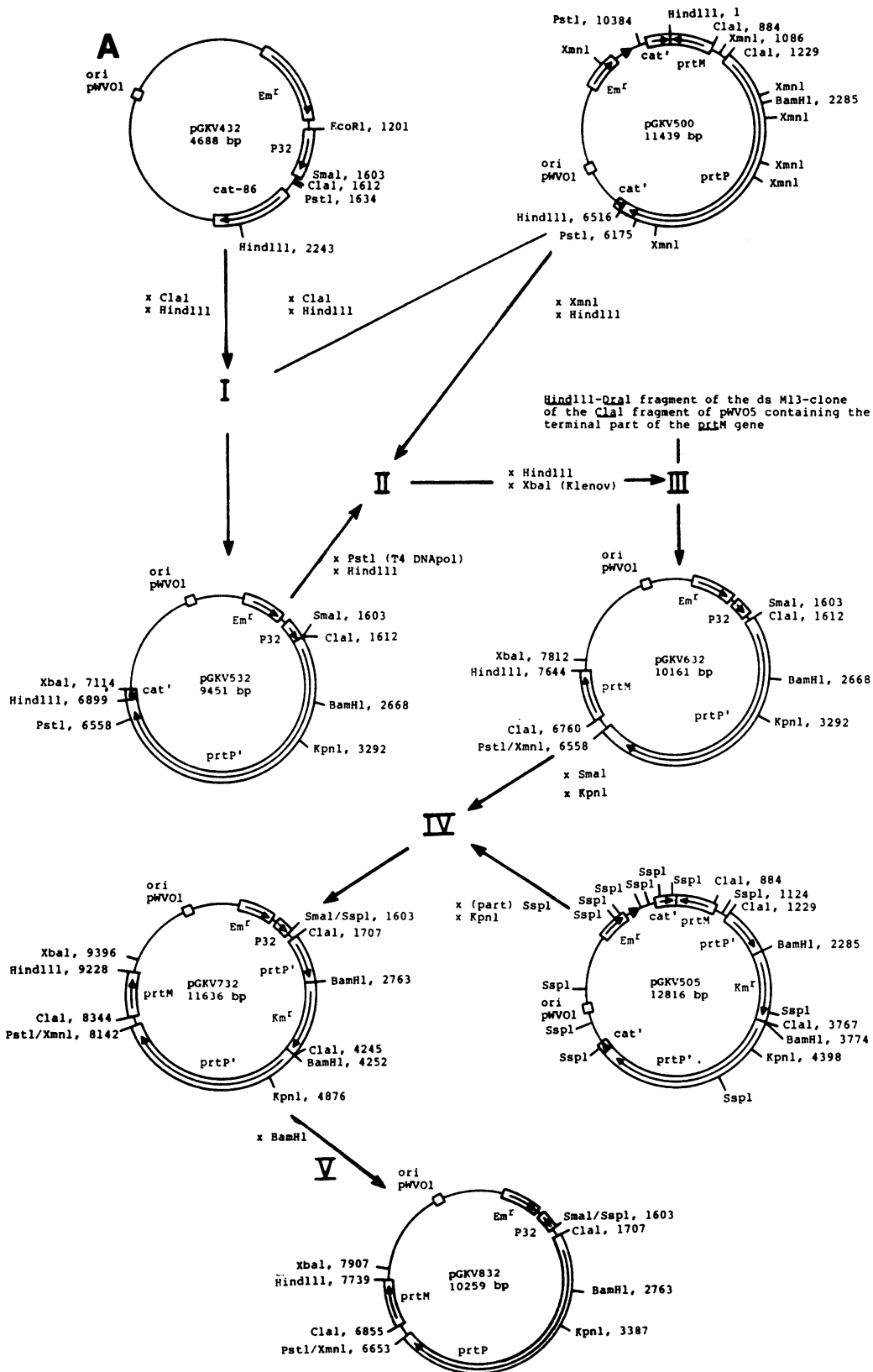
FIG. 3. Plasmid constructions. (A) Construction of pGKV832 in which the *prtP* and *prtM* genes are jointly transcribed from promoter P32. The promoter carrying the *Hpa*I-*Sau*3A fragment of pGKV232 (30) was inserted in the *Eco*RI site of pGKV410 after the recessed ends had been filled in with Klenow enzyme. The ligation mixture was introduced in *B. subtilis* PSL1. One of the resulting Cm^r Em^r colonies contained pGKV432, the starting plasmid for enhanced *prtP* gene expression. (B) Construction of pGKV905 in which the *prtP* and *prtM* genes are expressed by their own promoters and in which the *prtP* gene is truncated at the 3'-terminal *Pst*I site.

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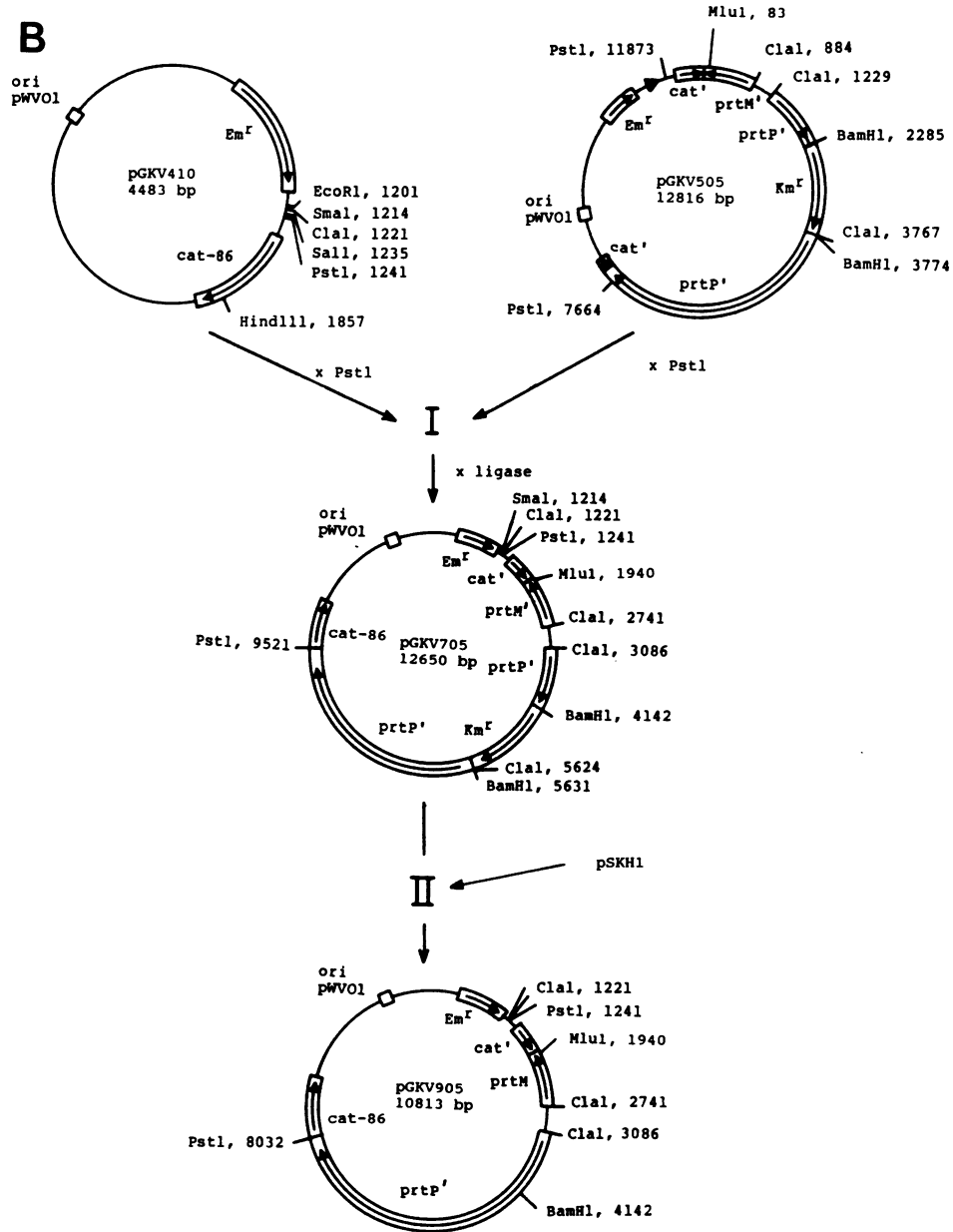
FIG. 3—*Continued.*

functional transcription terminator. In order to investigate the effect of the terminator on *cat-86* gene expression in *L. lactis*, pPT8 was introduced in strain MG1363. Table 2 shows that strain MG1363(pPT8) was resistant to a maximum of 8 μg of chloramphenicol per ml, whereas pGKV259 enabled strain MG1363 to grow at a maximum of 24 μg of chloramphenicol per ml, indicating that the terminator also functioned in *L. lactis*.

The presence of two *Pvu*II sites in the inverted repeat (Fig. 4) allowed us to confirm this conclusion. Digestion with *Pvu*II and religation resulted in a deletion of part of the inverted repeat (in pPT88). Plasmid pPT88 restored the ability of *B. subtilis* to grow at a maximum chloramphenicol concentration of 50 μg/ml and allowed strain MG1363 to grow at a maximum chloramphenicol concentration of 16

TABLE 3. Proteinase activities in overnight cultures of *L. lactis* carrying the indicated plasmids

Plasmid	OD$_{660}$[a]	Activity[b]
pGKV210	0.271	0
pGKV832	0.285	134
pGKV905	0.280	27

[a] Optical density (at 660 nm) of the 10-fold-diluted overnight culture.
[b] Proteinase activity was measured by assaying the release of para-nitroanilide from the synthetic substrate MeOsuc-Arg-Pro-Tyr-*p*Na. Activity is expressed as ΔA_{405}/min/ml of overnight culture with an optical density at 600 nm of 1.

```
              ————————————>        <————————————
                                  -35 ↘              -10↘
          TTTGAACCTT GCTTACGGAG CTGACTTCAC ATTGTTGAGA TCAGCTGCCT TTTGCTTATA GTTC
                          PvuII                       PvuII
```

FIG. 4. DNA sequence of the transcription terminator of the *prtP* gene (12). The arrows above the sequence indicate the inverted repeat, which has the potential to form a hairpin structure in the mRNA with a calculated $\Delta G = -24.6$ kcal/mol (11). The *Pvu*II restriction enzyme sites are indicated below the sequences. The -35 and -10 regions indicated above the sequence represent a promoterlike structure.

μg/ml. Since the deletion in the putative stem-loop structure also increased the transcription of the *cat-86* gene (as probed with pPL608; results not shown), we may conclude that the stem-loop structure downstream of the *prtP* gene is a functional transcription termination signal. Apparently the terminator is relatively weak, as it still allowed 30% expression of the *cat-86* gene on plasmid pPT8.

DISCUSSION

The oppositely directed promoters present on the *Cla*I fragment of the proteinase plasmid pWV05, which drive transcription of the important *prtP* and *prtM* genes of the proteolytic system of lactococci, have been mapped by the primer extension method. In the region -38 to -27 upstream of the transcription start of *prtP*, two possible -35 hexanucleotides are present (Fig. 2). Whether both or only one of these is used in promoter recognition remains to be established. The *prtP* promoter has 16 or 21 bp between the -10 and -35 regions, depending on which of the -35 hexanucleotides is actually used (Fig. 2). This spacing deviates from that in standard gram-positive promoters, which is 17 to 18 bp (8, 18). In addition to the prominent bands in Fig. 2, a number of weaker bands are present. In all probability, these resulted from some degree of degradation of the mRNA template during the extraction and primer extension procedures. The previously characterized lactococcal promoters also conform to this consensus and contained 17 to 18 bp between the -10 and -35 hexanucleotides. The deviation of the *prtP* promoter from this consensus, and the absence in both *prt* promoters of a TG pair upstream of the -10 region, which is characteristic for strong lactococcal promoters (30), may well account for their relative weakness: the promoters of the *prtP* and the *prtM* genes were weaker than any of the five lactococcal chromosomal promoters analyzed earlier (30). Although the major RNA polymerases of *B. subtilis* and *L. lactis* recognize very similar promoter sequences, the observation that in *L. lactis* the promoter of the *prtM* gene was stronger than that of *prtP*, while the situation was reversed in *B. subtilis*, suggests that the two polymerases have undefined differences in promoter recognition.

The putative transcription terminator downstream of the *prtP* gene of strain Wg2 appeared to be functional in *B. subtilis* and *L. lactis* MG1363. However, this structure did not completely prevent transcription of the *cat-86* gene from promoter P59. In both hosts, the level of choramphenicol resistance was reduced to approximately 30%. Two possibilities for the residual transcription can be envisaged. First, the strength of the stem (with a calculated $\Delta G = -24.6$ kcal/mol [12]) correlates with the degree of termination (20) and might be insufficient to fully block transcription from the strong promoter P59. Second, if the promoterlike structure present in the terminator (Fig. 4) was used, this would cause reinitiation of transcription of the *cat-86* gene. Deleting part of the stem-loop structure of the putative terminator by digestion with *Pvu*II resulted in removal of most of the stem and restored resistance to chloramphenicol of both hosts to

almost the level conferred by pGKV259. This result shows that the stem-loop structure is involved in transcription termination. In addition, these experiments show that plasmid pGKV259 is superior to pGKV11 as a terminator-screening vector (29). Because promoter P59 is very efficient in expressing the *cat-86* gene in *L. lactis* (30), vector pGKV259 allowed us to discriminate between efficient and less efficient transcription termination signals in this host.

It is generally believed that the activity of the proteolytic enzymes of dairy starter cultures is largely responsible for the extent of cheese ripening. Enhancement of proteolysis during milk fermentation by increasing the amount of proteolytic enzymes can be realized by carefully adjusting the starter strain composition or by adding (partially purified) proteinases and/or peptidases during the fermentation process (15). The development of genetic engineering techniques for lactococci offers interesting new possibilities in this respect. By using one of the lactococcal promoters from our collection, van de Guchte et al. (26) were able to produce the *B. subtilis* neutral proteinase in *L. lactis*. The *B. subtilis* pre-proproteinase is most probably processed at the proper position and is secreted in an active form into the culture medium. Another way to increase the proteolytic activity of *L. lactis* is to provide the two genes essential for the first step in proteolysis, *prtP* and *prtM*, with strong promoters. Since the promoters of the two genes are apparently rather weak, and our lactococcal promoter collection contained much stronger ones, we attempted to express the two genes under the control of the strong promoter, P32. To accomplish this, the two divergently transcribed genes were arranged in a tandem fashion. This configuration was realized in plasmid pGKV832. By using the synthetic substrate MeOsuc-Arg-Pro-Tyr-*p*Na, it was shown that the proteolytic activity of the cells was enhanced approximately fivefold. Apparently, it is possible to increase the expression of the proteinase genes by applying recombinant DNA technology to lactococci, thus introducing new ways to improve the properties of lactococcal strains for enhanced proteolysis.

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