Cloning, sequencing and expression of the gene encoding the cell-envelope-associated proteinase from *Lactobacillus paracasei* subsp. *paracasei* NCDO 151

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The gene encoding the cell-envelope-associated proteinase of *Lactobacillus paracasei* subsp. *paracasei* NCDO 151 (formerly *Lactobacillus casei* NCDO 151) was cloned and sequenced. The gene was located on the chromosome and encoded a polypeptide of 1902 amino acids. The proteinase is N-terminally cleaved upon maturation. It shows extensive homology to the *Lactococcus lactis* subsp. *cremoris* Wg2 proteinase. Similar to the situation in *Lactococcus*, a maturation gene was found upstream of the proteinase gene. The cloned proteinase gene was expressed in *Lactobacillus plantarum*. However, no expression was observed when the gene was cloned in *Lactococcus lactis*.

Introduction

The lactic acid bacteria are of great economic importance in the dairy industry. They contribute to the flavour and texture of fermented milk products (Fox, 1989; Law & Kolstad, 1983; Thomas & Mills, 1981). Much is known about the proteolytic systems of the lactococci. Several of the proteinases have been purified and their genes sequenced (for a review see Kok, 1990). The genes characterized so far appear to be located on plasmids.

Strains of *Lactobacillus casei* very often occur in hard and semihard cheeses as adventitious bacteria and in many cases contribute to flavour development during the ripening process. Consequently the proteolytic activity of *Lb. casei* is of interest to the dairy industry. *Lb. casei* has been tested for use in mixed starter cultures and shown to contribute to the production of superior-quality cheese (Girgis *et al.*, 1983; Bianchi-Salvadori & Sacco, 1981; Ramos *et al.*, 1981). There has also been interest in employing heat-shocked cells or cell lysates of *Lb. casei* to accelerate cheese ripening (El Abboudi *et al.*, 1991; Trepanier *et al.*, 1991).

Knowledge of the proteolytic systems of the lacto-

bacilli is limited. Some reports on characterization and partial purification of *Lactobacillus* proteinases have been published (Argyle *et al.*, 1976; Ezzat *et al.*, 1985, 1988; Khalid & Marth, 1990; El Soda *et al.*, 1986; Zevaco & Gripon, 1988). We have purified to homogeneity and characterized a cell-envelope-associated proteinase of *Lactobacillus paracasei* subsp. *paracasei* NCDO 151 (formerly named *Lactobacillus casei* NCDO 151) (Næs *et al.*, 1991; Næs & Nissen-Meyer, 1992). This serine proteinase, designated Lp151, shared some properties with known lactococcal proteinases. Very little is known about the *Lactobacillus* proteinase genes. By Southern blotting a gene with homology to the *Lactococcus lactis* subsp. *cremoris* SK11 proteinase gene has been observed in *Lactobacillus lactis* (Kok & Venema, 1988). Kojic *et al.* (1991) recently reported the physical map of a proteinase gene from *Lb. casei* HN14 with similarity to the lactococcal genes.

Here we describe the cloning, sequencing and expression of the *Lb. paracasei* NCDO 151 proteinase gene. Knowledge of the genetics of the proteolytic system in lactobacilli should make it possible to construct strains with genetic characteristics important to the food fermentation industry.

Methods

Bacterial strains and plasmids. *Lactobacillus paracasei* subsp. *paracasei* NCDO 151 (formerly *Lactobacillus casei* NCDO 151) was obtained from the INRA Centre, Jouy-en-Josas, France. λ EMBL3, λ gt10, λ -

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packaging extract plasmids pGEM-7zf(+), M13mp18, pUC19 and T7 DNA polymerase DNA sequencing kit were purchased from Promega, Biotec. λ -ladder DNA size standard (λ concatemers) was from Bio-Rad. RNA molecular mass markers were obtained from Boehringer Mannheim. *E. coli* strains JM109, LE392, TG1, C600 Hfl, DH5 α and *Lactobacillus plantarum* NC8 were from our laboratory stock. Plasmid pVS2, conferring chloramphenicol and erythromycin resistance upon transformed cells, was a gift from Soile Tynkkynen (von Wright *et al.*, 1987). The following gifts were obtained from Dr Jan Kok, University of Groningen, The Netherlands: *Lactococcus lactis* subsp. *cremoris* Wg2; *Lc. lactis* MG1363 P_{rt}⁻ (Gasson, 1983); plasmid pGKV500, harbouring the *Lc. lactis* Wg2 proteinase and maturation gene (Kok *et al.*, 1985); and plasmid pRL12, which is pUC18 containing the ISS1W insertion element downstream of the *Lc. lactis* subsp. *cremoris* Wg2 proteinase maturation gene (*prtM*) on a *Bgl*II–*Acl*I fragment (Haandrikman *et al.*, 1990). M13 phage DNA containing the *Bam*HI–*Eco*RI fragment of the *Lc. lactis* subsp. *cremoris* SK11 proteinase gene was a gift from Willem de Vos, Netherlands Institute for Dairy Research, Ede, The Netherlands (Vos *et al.*, 1989a).

Growth of bacteria. *Lb. plantarum* NC8 and *Lb. paracasei* NCDO 151 were grown in MRS broth (Difco) and MRS broth supplemented with 20 mM-CaCl₂ and 50 mM β -glycerophosphate, respectively, at 30 °C. *Lc. lactis* Wg2 and *Lc. lactis* MG1363 were grown in M17 medium (Oxoid) supplemented with 0.5% glucose. In milk-clotting experiments *Lc. lactis* Wg2 and *Lb. plantarum* NC8 were grown at 30 °C in 5 or 10 ml skimmed milk supplemented with 50 mM- β -glycerophosphate and 0.5% glucose. Transformants were grown in medium containing 5 μ g chloramphenicol ml⁻¹.

Recombinant DNA techniques. Standard procedures were employed for general cloning techniques, largely following the procedures of Maniatis *et al.* (1982). Plasmid DNA from *Lactobacillus* was isolated by the alkaline lysis method using 20 mg lysozyme ml⁻¹ and 250 U mutanolysin ml⁻¹ in the lysis buffer. Chromosomal DNA was purified according to Marmur (1961). DNA fragments used for ligation to phage vectors and plasmids were purified from agarose gels by the freeze-squeeze method. For genomic screening of the proteinase gene (*prtP*), 13–20 kbp chromosomal *Lb. paracasei* NCDO 151 DNA fragments obtained by partial *Sau*3A digestion were ligated into λ EMBL3 vector DNA. Recombinant phages (5×10^5 μ g⁻¹) were plated on *E. coli* LE392. To identify clones containing the *prtM* gene, 4–6 kbp chromosomal *Eco*RI fragments were ligated into λ gt10. In this case recombinant phages were plated on *E. coli* C600 Hfl. To obtain DNA suitable for sequencing, recombinant DNA from positive λ clones was digested with appropriate restriction enzymes and the resulting fragments were subcloned into M13mp18, pUC19 or pGEM-7zf(+) vector DNA. To obtain clones containing both the *prtP* and *prtM* genes, 6–8 kbp *Hind*III fragments of chromosomal *Lb. paracasei* NCDO 151 DNA were ligated with plasmid pVS2 and cloned into *Lb. plantarum* NC8.

In Southern blotting experiments, DNA was transferred to nylon membranes by vacuum blotting according to the manufacturer's instructions (Pharmacia) and hybridization was performed according to Southern (1975). DNA probes were labelled with [α -³²P]dCTP by the random priming reaction (Amersham).

Transformation of lactic acid bacteria. *Lc. lactis* MG1363 was transformed by electroporation according to Holo & Nes (1989). Efficiently transformable *Lb. plantarum* NC8 cells were obtained by growth in MRS broth containing 1% (w/v) glycine to an OD₆₀₀ of 0.6. The cells were first washed with one culture volume 0.01 M-MgCl₂ and then with 30% (w/v) polyethylene glycol (PEG) 1500. The cell pellet was then resuspended in 30% PEG 1500 (1/100 of the initial volume). Electroporation was subsequently performed essentially as for the lactococci (400 Ω , 1.5 kV).

Nucleotide sequencing. Nucleotide sequence analysis was performed by the dideoxy chain-terminating method on single-stranded M13 DNA or directly on denatured plasmid DNA (Kraft *et al.*, 1988). Both strands were sequenced by primer walking. Oligonucleotide sequencing primers were synthesized on an Applied Biosystems synthesizer, model 381A. The computer analyses were performed on an IBM personal computer employing the DNASIS sequence analysis program (Pharmacia) and on a microVAX 3400 computer employing the GCG program package (Devereux *et al.*, 1984).

Pulsed-field gel electrophoresis. The bacterial cells were cast and lysed within blocks of agarose (inserts) according to Lillehaug *et al.* (1991). Lysis was performed with 4 mg lysozyme ml⁻¹ and 400 U mutanolysin ml⁻¹. Restriction enzyme digestion was executed overnight. Pulsed field gel electrophoresis was performed on a CHEF-DR II (Bio-Rad). Electrophoreses were run under two different conditions in a 1.1% (w/v) agarose gel (in 22 mM-Tris-base, 22 mM-boric acid, 0.5 mM-EDTA) at 14 °C: either 200 V, 18 h, pulsetime increasing from 15 s to 70 s, or 200 V, 24 h, pulsetime 1 s to 200 s, according to the manufacturer's instructions.

Results

Identification and chromosomal location of the proteinase gene

To establish whether there was any similarity between the *Lb. paracasei* NCDO 151 proteinase gene and the known lactococcal proteinase genes, total DNA from *Lb. paracasei* NCDO 151 was subjected to restriction enzyme analysis and hybridized to a probe containing the central *Bam*HI–*Eco*RI fragment of the *Lc. lactis* subsp. *cremoris* SK11 proteinase gene. The probe gave one unique signal, indicating the presence of a single-copy *Lb. paracasei* gene with homology to the lactococcal SK11 gene (Fig. 1). This was confirmed by the restriction pattern obtained from several Southern blots (not shown). Similar experiments were performed with a *prtM*-specific probe from *Lc. lactis* Wg2 (the *Hind*III–*Cl*aI fragment), and a *Lb. paracasei* *prtM* gene was detected close to the proteinase gene.

Lb. paracasei NCDO 151 harbours two plasmids. Neither of the plasmids hybridized to the lactococcal probes (not shown). To determine whether the proteinase gene was chromosomally located or resided on a large plasmid which co-purified with the chromosomal DNA rather than with the plasmid DNA fraction, the DNA was subjected to pulsed-field gel electrophoresis under various conditions (Fig. 2). The proteinase apparently resided on a 120 kb *Nor*I fragment and a *Sfi*I fragment of 100 kb. Moreover, the proteinase gene remained with the chromosomal DNA fraction when the cells were lysed and their DNA not digested by restriction enzymes (Fig. 2, lane 1). These experiments strongly indicated that the proteinase gene was chromosomally located.

Lc. lactis Wg2 and SK11 each contain the insertion sequence ISS1 downstream of both *prtM* and *prtP*

Fig. 1. Southern blot analysis of total *Lb. paracasei* NCDO 151 DNA using the central *Bam*HI–*Eco*RI fragment of the SK11 proteinase gene as a probe. (a) Ethidium-bromide-stained gel; (b) autoradiography after blotting and hybridization. λ -*Hind*III was used as a size standard, E, *Eco*RI; B, *Bam*HI; S, *Sal*I; P, *Pst*I.

(Haandrikman *et al.*, 1990). When *Lb. paracasei* DNA was hybridized to the *Acc*I–*Bgl*II fragment of pRL12 containing the ISS/W IS element, no hybridization signals were observed, at either high or low stringency of hybridization (not shown).

Cloning and sequencing of the *prtP* and *prtM* genes

A *Lb. paracasei* NCDO 151 λ EMBL3 library was constructed as described in Methods and 3×10^4 plaques were screened with the central *Bam*HI–*Eco*RI fragment of the *Lc. lactis* SK11 proteinase gene as a probe. DNA from four positive clones was isolated and characterized. Various fragments containing the 5', central and the 3' regions of the proteinase gene were subcloned into M13 phage vector, or into pUC19 or pGEM-7zf(+) plasmid vectors. None of the clones, however, contained the maturation gene *prtM*. A *Lb. paracasei* λ gt10 DNA library was screened with a *Cla*I–*Hind*III fragment

containing the *prtM* gene of *Lc. lactis* Wg2. A 4.0 kbp *Eco*RI fragment from one of the positive clones was subcloned into pGEM-7zf(+). Appropriate subclones containing the *prtP* and the *prtM* genes were sequenced. The sequence obtained was very similar to the sequence of the *Lc. lactis* Wg2 proteolytic system, but apparently carried a deletion in the promoter region common to *prtM* and *prtP*. A 6.7 kbp chromosomal *Hind*III fragment of *Lb. paracasei* NCDO 151 containing both the *prtP* and *prtM* genes in pVS2 was obtained after screening a partial chromosomal library of *Hind*III fragments cloned in *Lb. plantarum* NC8. This construction was named pHN1. Upon sequencing of the *Hind*III fragment, the deletion in the λ gt10 clone was shown to comprise 14 base pairs encompassing the proposed –10 region of the *prtP* gene. The complete sequence of both the *prtP* and the *prtM* genes is presented in Fig. 3. Two divergent open reading frames, ORF1 and ORF2, were detected, encoding polypeptides of 299 and 1902 amino acids, respectively. The N-terminal sequence of the mature proteinase has been determined by Edman degradation to be X-A-K-A-N-S-M-A-N (Næs & Nissen-Meyer, 1992); this indicates the cleavage of the precursor proteinase at position +187 in the nascent polypeptide chain encoded by ORF2. This cleavage would yield an active proteinase, unless otherwise modified, of 1715 amino acid residues and an M_r of 180659. The smaller ORF1 encodes the proposed maturation protein. A typical signal sequence of lipoproteins (von Heijne, 1989) was found at the N-terminus of the primary translation product. A presumed cleavage between amino acid 23 and 24 would give a polypeptide of 276 amino acid residues and an M_r of 30675.

The restriction pattern for the *prtP* and the *prtM* genes obtained after sequencing indicated that the *Lb. paracasei* NCDO 151 proteinase was very similar to the recently identified *Lb. casei* HN14 proteinase (Fig. 4).

When comparing the amino acid sequences of the polypeptides, the Wg2 and SK11 mature proteinases were found to differ from the *Lb. paracasei* proteinase in 58 (96.6% homology) and 77 (95.5% homology) amino acids, respectively (Fig. 5). When comparing the mature *prtM* polypeptides, the homology to the lactococcal maturation peptides was found to be 96.0%.

The DNA sequence homology between the *Lb. paracasei* and the lactococcal proteinase genes ended abruptly just downstream of both the *prtP* and *prtM* genes. Thus the palindromic sequences downstream of the translation stop signal, which may be involved in transcription termination, showed no similarities with the corresponding sequences of the lactococcal counterparts.

Putative Shine–Dalgarno ribosome-binding sites and –10 and –35 proposed promoter sites were located

Fig. 2. Southern blot analysis of total *Lb. paracasei* NCDO 151 DNA employing pulsed-field gel electrophoresis. (a) Ethidium-bromide-stained agarose gel, 200 V, 18 h, pulsetime 15–70 s; (b) autoradiography after blotting and hybridization with the *prtP*-specific probe. U, undigested DNA; N, *NotI*-digested DNA; S, *SfiI*-digested DNA. λ , lambda ladder; one λ unit represents 48.5 kbp.

upstream of both the *prtP* and *prtM* genes. This region also contained several palindromic sequences, two of which encompassed the two suggested –35 regions. These sequences, being able to form hairpin loops, may be involved in transcription regulation of the genes of the proteolytic system.

Expression of the cloned proteinase

No cross-hybridization was observed when *Lb. plantarum* NC8 DNA was hybridized to a *prtP* specific probe. The weakly proteolytic *Lb. plantarum* NC8 used for expression of the cloned proteinase thus contained a proteinase very different from that of *Lb. paracasei* (not

Table 1. Clotting of milk by lactic acid bacteria

Strain	Clotting time (d)*
<i>Lb. plantarum</i> NC8(pHN1) transformant 6	4 ± 1
<i>Lb. plantarum</i> NC8(pHN1) transformant 47	4 ± 1
<i>Lb. plantarum</i> NC8(pVS2)	12 ± 2
<i>Lb. plantarum</i> NC8	12 ± 2
<i>Lc. lactis</i> MG1363(pHN1)†	–
<i>Lc. lactis</i> MG1363	–
<i>Lc. lactis</i> Wg2	1 ± 0

* Experiments were performed twice, with two replicates each time. The results are means ± range. –, No clotting observed.

† Eight transformants containing the proteinase gene were tested.

1	GAATTCGGTGTAAAGCAGCTGCCCTGTGCTGCTGGCGCGCTGCTGCTGAAGATACCTAGCAGCTTGAATTACCGAAGCTGGCCAAAGAAAGTTAAG	120
121	GTTATCAAGCGCTTTCGTGAAATCACTGGTCTTGGCTTGAAGATGCTAAGGTATGGTTGACCGGCACCTAAGGTATATCAAGGAAGCCCTTCCGAAGACGACGCTAACAGCTTAAAG	240
241	GAACAACTTGAAGCGGTGGCGCAACGGTTACCCCTCAATAAACCTAGCTCCATCTTCAATAAAAGCATCTCGCTCAATCGTGTGATACCGGAAAGGCGAGATSCCTTTTATATACAT	360
361	AAAAGCCCCGAAGATCATGTGTGATGACTTTTCGGGGCGTTGATGTCCTCAATTTCTAAAAGAACTGGCTGTTATTTTCATCATACTCTCGGTTAAGCCCTTAATTTCGTTGTCGACGGCTTCTTA	480
299		293
481	TAACTATCTAGCGCATCCGCAAGATCTTTTGTCTTTAATACTGTCACATGCTGTTCTTCAATACCTGACATGATAACGCGTTTCATGATGCTTGAATCGGAGACCACTTTAGCGTAAACGCTG	600
292	Y S D L A D A L D K I T V H Q N K L V Q S I V R Q M I S S D R S W K A Y V S	253
601	GCAGTTAGCGCCTTTTACTGCTAGTAAAGTGCCTTTTGGCGGATGGTTAATCATTTTAACTTCATACCCGTTTTCACATTTGACTGGTGTCTGCGTGTACTCACCATTTTTAAAT	720
252	A T L A K K S S T F T G K A P H N I M K I V E Y G N T V K V P T Q T Y D G N K L	213
721	TTGTAGGCAGCATCCTTAAATGTGGCGTCGAGCGTTTATTGTTTGAATTCAAAATAAATCTTCCCGCGTTATCTTTAGTCGCGAGTATCAATCGGAATCAGTTTTCGCAAGCGTGGCAAAA	840
212	K Y A A D K F T A D L T K N N S E F S I K G G N D K T A T D I S D T K A L T A F	173
841	TCCTTGCCAGCTGCTAAATCACTGATTAACCTTGTAGCAGTCTCCTCGCTAGTCTAGTAAATGTTGGACAGTCACTTTGGGCTGATAGGCTTCCAAACGGCGCTTGGAGCTGGCTTCA	960
172	D K G A A L D S I V Q K A T D E D S T L I H Q V T V K P Q Y T K W V A K L Q S E	133
961	GAAACCTTTTAACTTTTTCAGTCAACTTCACTTAAAGTTGGTTCGTAGGCTTCCCTTGAAGCTACTCGGACTGAACCGCTTTTACACTTAAGAAAGCATCGAAATTTTCGCCCGTAT	1080
132	S V K K L K L A V E S L F N T R L S E K F S S R S F G N Q S L F A D F N E G Y	93
1081	TGTTGTTGTAGCTATCAGGCGTCATTAACTGTTTGTAGCTCAACCGATTTTCCATAGGCAATGATCAATGCAGATGATGAGCAATGATGAGCAATGATGAGCAATGATGAGCAATGATGAGCAAT	1200
92	Q Q K Y S D Y A D N V T K T S V S K G Y A H N L A R Y I L M N A L M T K T T P S	53
1201	TGTTGAGTTCCTTGTAGAAGTTACTTTTCAGTCACTTCCCGCTGAATAGGTGCGCACTTTTGTGCGGCTGATTTGACTGACACCGCTTAGCAGCAGTAAAGCAGTTCGGGTACTT	1320
52	Q K L E K Y F N S E T V K G S Y T A V K Q D A Q N S Q C G S L L L L A T A T S	13
1321	GCCAAATAACTTTTAAAGCGCATTTTTCCTTCATCGACTCGGTCTCTCTGAAATGCTTACAGTAAACGTTGTGTATTTTATTTAGCGTTGGCAATTTGAAATTTTCAATGCAAAATCTC	1440
12	A L L V K L R M K K K M	1
1441	ATTTTCTAATAATGCTAAACACTTTAAACACATCTATAGTCTGTAAATGGCTAAATAATAACGCTAAAGGCTAATTTACAGATAGGAAATTAATAAGATTAAATTTTCGTTGAAATCT	1560
1561	GTTCCTCGAGAGTATATAATCTTAACTACATCAAGCGTACGTTTTCATTTGTTGTTCTGAAACCTTTTGGGAACCTGGAGGCTATTGATGATGCAAGGAAAGAAAGGCTATCGATCTTCTTA	1680
-214		-175
1681	GCCGGTACAGTCCGCTTAGGGGCGCTGCTGCTTGCAGTCCGCGGAAATCCAAAGCAAGGCGCTATCTCGCAGCAAACTAAGTATCATCTCGCAATACGTTAAGGCGCGGACT	1800
-174	A G T V A L G A L A V L P V G E I Q A K A A I S Q Q T K V S S L A N T V K A A T	-135
1801	GCTAAGCAAGCGGCCACTGACACAAACCGCAGCAGCAACGAATCAAGCGATTGCCAGAGTTGCCAGAGTTGATGATTAACAATAGCTGAATAAAGTTACAGCAGCAAGATACCTAT	1920
-134	A K Q A A T D T T A A T T N Q A I A T Q L A A K G I D Y N K L N K V Q Q D T Y	-95
1921	GTTGAGCTCATTTGTTCAAAATGAGCGCGCTGCTCTGAAACCGCACTTTTAAAGTATGATTAATCTCCAGCAGCGGAGATTACAGCAGGAGACCAATAAAGTATGATCGCGGCTCAGGCA	2040
-94	V D V I V Q M S A A P A S E N G T L R T D Y S S T A E I Q Q E T N K V I A A Q A	-55
2041	AGCGTTAAGGCAGCTGTTGAACAGTCAACCAAACTGCCGGTGAAGTATGGCTATGCTTAAACGCTTTTCACTAAAGTTAGGTTGTGATATCCCTAAACTGAAACAAAT	2160
-54	S V K A A V E Q V T Q Q T A G E S Y G Y V V N G F S T K V R V V D I P K L K Q I	-15

EcoRI

PstI

BglII

-10 (P)

SD (P)

ClaI

SD (M)

-10 (M)

-35 (M)

M

SD (P)

-10 (M)

-35 (M)

-10 (P)

SD (P)

ClaI

SD (M)

-10 (M)

-35 (M)

M

SD (P)

-10 (M)

-35 (M)

-10 (P)

SD (P)

ClaI

SD (M)

-10 (M)

-35 (M)

M

SD (P)

-10 (M)

-35 (M)

2161	GCCGGAGTTAAACAGTCACATGGCGAAAGTTTACTATCCGACTGATGCTAAGCCTAACTCGATGGCGAATGTGACGGCGTATGGTCCAAATTACAAAATATAAAGTGAAGCCACAGTT	2280	A G V K T V T L A K A K A N S M A N V Q A V W S N Y K Y K G E G T V
-14		25	
2281	GTCTCGGTTATTGACACTGGCATTCACACATAAAGACATCGCGCTAAGCGATGATAAAGACGTCAAACTAACCAATATGATGTTGAAAAATTCATGATACCGCCCAAGCATGGC	2400	V S V I D T G I D P T H K D M R L S D D K D V K L T K Y D V E K F T D T A K H G
26		65	
2401	CGCTATTTTACTTCAAAAGTGCCATATGGGTTTAAATTACGCTGATAATAACGACACGATTACAGATGATACGGTTGACGAACACACCGGCATGCAATGTTGCTGGGATCATCGGTGCTTAAC	2520	R Y F T S K V P Y G F N Y A D N N D T I T D D T V D E Q H G M H V A G I I G A N
66		105	
2521	GGACAGGTGACGATCCAAACCAAGTCTGTTGCGAGTTGCGCAGAGCACAGCTACTGGCAATGAAAGTTTTCACCACTCTGACACTTCTGCAACACCGGGTCACTACCTTGGTT	2640	G T G D D P T K S V V G V A P E A Q L L A M K V F T N S D T S A T T G S A T L V
106		145	
2641	TCTGCCATTGAAGACTCGGCACAAAATCGGTGCGGATGTCTCAACATGTCTTAGATCTGATTCAGGCACCAACCTTGGAGGATCCAGAAATTCGCGGTGCAAAATGCTAACGAA	2760	S A I E D S A K I G A D V L N M S L G S D S G N Q T L E D P E I A A V Q N A N E
146		185	
2761	TCAGGAACAGCGCGCTCATTTCTGCTGGGAACTCAGGAACATCCGGTTACGAACTCAAGCGCTCAACAAAGATTATTACGGTTTGAAGACAATGAAATGGTGGGAACGCCAGGACA	2880	S G T A A V I S A G N S G T S G S A T Q G V N K D Y Y G L Q D N E M V G T P G T
186		225	
2881	TCAGGAGGACGCCACAGTTGCTCGCTGAGAACACCGATGTCATCAGTCAGGAGTCACCATTAACAGATGGTAAAGACTTACAGCTTGGACCGGAAACCAATTCAGCTTTCGAAGCAAC	3000	S R G A T T V A S A E N T D V I S Q A V T I T D G K D L Q L G P E T I Q L S N
226		265	
3001	GATTTCACTGGTAGCTTTACCAAAAGAGTTTATGTTCTCAAAGATGCTAGTGGGACCTCAGCAAGGGCAGCGGAGTACTGCTCAGCTGAAGGCAAAATTCGCATCGTT	3120	D F T G S F D Q K K F Y V V K D A S G D L S K G A A A D Y T A D A K G K I A I V
266		305	
3121	AAACGTGTGAACCTTAATTTGCTGACAAACAAAATACGCCCAAGCGCTGGTCTGCTGGCTTGATCTTCAACAAGCATGGCACAGCAACACGGTTGACTTCTATTAGTTAACCC	3240	K R G E L N F A D K Q K Y A Q A A G A A G L I I V N N D G T A T P L T S I R L T
306		345	
3241	ACCACCTTCCCAACATTTGGGCTCTCCAGTAAACCGGTCAAAAGCTGGTTCACTGGTCACAGCACACCGGATGATAGTCTCGGTGTCAAGATTGCCCTGACGCTGTTACCAAAATCAG	3360	T T F P T F G L S K T G Q K L V D W V T A H P D D S L G V K I A L T L L P N Q
346		385	
3361	AAATATCTGAAGACAGATGCTGACTTCACATCCGTATGGGCGAGTTTCCATCTTCTCCTTCAACACAGATATTACCGCACCGGCAACATCTGTCACGCAAAACACAAATGGC	3480	K Y T E D K M S D F T S Y G P V S N L S F K P D I T A P G G N I W S T Q N N G
386		425	
3481	TACACAAATATGCTGTACGTCATGGCCCTCGCCATTTATTGCGCGTTTCAACAGCATTTGTTGAACACAGCGTTGAATAACAAAACACCCATTTTATGCTGACTACAAACAACTTAAA	3600	Y T N M S G T S M A S P F I A G S Q A L L K Q A L N N K N N P F Y A D Y K Q L K
426		465	
3601	GGGACAGCGCTCACCGATTTCTTAACACAGTTGAGATGAATCTGCCAGCCATCAACGATATTAACTACAACATGTTATCGTATCGCCCGGCGGCAAGTCCCGTCTGGTTGAT	3720	G T A L T D F L K T V E M N T A Q P I N D I N Y N N V I V S P R R Q G A G L V D
466		505	
3721	GTGAAGGCAGCCATTGATGCATTAGAAAAGAAATCCGTCAACGGTTGTGCGCGGAAACGGCTACCGGGCAGTTGAATTGAAGACTTCACGAGTACGGACACACCTTTTAAACTGACCTTC	3840	V K A A I D A L E K N P S T V V A E N G Y P A V E L K D F T S T D K T F K L T F
506		545	
3841	ACGAACCGACGACCCATGAACCTATCAAAATGGACAGTAAATACGATACTAATGCGGTTTATACATACGCACTGACCCCTAATTCCTGGGGTTTGTGATGACAAGAAGATTGATGGG	3960	T N R T T H E L T Y Q M D S N T D T N A V Y T S A T D P N S G V L Y D K K I D G
546		585	
3961	GCAGCATTAAGCGGCGAGTGACATAACTGTGCCCTGCTGGGAAACGGCGAGATTGAATTCACATCTCTTGGCGAAGTCTTTTGCCACACAGCAATTTGTTGAAGTTTTCTTGAC	4080	A A I K A G S D I T V P A G K T A Q I E F T L S L P K S F D Q Q Q F V E G F L N
586		625	

4081 TTTAAGGTAGCGTGGTTCGCGCTTGAACCTTGCCATACATGGGCTTTTGGTGAAGTGGATGTCGAGTAGTCTCAATGGGATCACTTAGTCTGCTGGTGAAT 4200
 626 F K G S D G S R L N L P Y M G F F G D W N D G K I V D S L N G I T Y S P A G G N 665

 4201 TATGGCACCGTGCCACTATTGACGAACAAAATACAGGCCATCAATATTATGCGCGCATGCTCAGAGCGTGATGGCAACAGACAGTTGACGATCAGCGGATTCGTTTCGAGTGAC 4320
 666 F Y G T V P L L T N K N T G H Q Y Y G G M V T D A D G K Q T V D D Q A I A F S S D 705

 4321 AAGAAATGCTTATATAATGACATCAGCATGCGAGTATTATCTATTCGCGCAATATCAGCAACGCTCCAGTTGATTTCTTGATGGTCAAGGCAATAAAGTTACGACTCTCAGCAGTTCCACC 4440
 706 K N A L Y N D I S M Q Y Y L L R N I S N V Q V D I L D G Q G N K V T T L S S S T 745

 4441 AATCAGACGAACCTTATGATGCTCATTCGCCAAGATACATCTACTACAAATGCTCCAGCGTGGATGGCACTTATGATCAACGTGATGGCAACATCAAGCGGCTGATGTCG 4560
 746 N Q T K T Y Y D A H S Q K Y I Y N A P A W D G T Y Y D Q R D G N I K T A D D G 785

 4561 AGTTACTATTATTCGCGGTACCGGAAGGCGGCGACAAACGTCGAAGTTGATGTCGCTTTCAAGCTCGACTTAAGGCGCCGACAGTTTCGTCATGTCGCTTTGTCACGCCAAA 4680
 786 S Y T Y R I S G V P E G G D K R Q V F D V P F K L D S K A P T V R H V A L S A K 825

 4681 ACGAAATGGGAACCCAGTATTATTGACAGCTGAAGCCAAAGGATTTGATGGTCTTGATGCCACCAAGAGCGTTAAAACTGCAATTAATGAAGTGACGAATCTTGATGCTACC 4800
 826 T E N G K T Q Y Y L T A E A K D D L S G L D A T K S V K T A I N E V T N L D A T 865

 4801 TTTACCGATGCTGGACACGCGTGATGGGTACACCAAATTTGAACGCCATTTATCTGATGAACAGGCCCAAGCACTTGGCAATTCGCGCAATTCGGGTGAGCTGTACTTGACTGATAAT 4920
 866 F T D A G T T A D G Y T K I E T P L S D E Q A Q A L G N G D N S A E L Y L T D N 905

 4921 GCATCCAAATGCCACTAATCAAGATGCCAGCGTTCAAGAAGCGGGCTCTACATCGTTGATTAATTTGTAACGCGCGGTATCCAGACAGAGATCTCAAGTACCACAACTGGCTACGAA 5040
 906 A S N A T N Q D A S V Q K P G S T S F D L I V N G G G I P D K I S S T T T G Y E 945

 5041 GCCAATCTCAAGTGGCGGACGTATACGTTTAGTGAAGTATCCAGACGCGTTACCGTACTTACACTGATGCACAAAGAAACATGATTTGAACACAAACCTATGATGCTGCG 5160
 946 A N T Q G G G T Y T F S G T Y P A A V D G T Y T D A Q G K K H D L N T T Y D A A 985

 5161 ACTAACAGTTTCACATGCCCTCAATGGCGGTACGAAATGCTGATTAGCGCGCGAAGTGAATATATCGCGATAGGGCGCACCCAGTTGCTTAAACATTTTGACACCAAAATTCGGCTG 5280
 986 T N S F T A S M A V T N A D Y A A Q V D L Y A D K A H T Q L L K H F D T K V R L 1025

 5281 ACGCGGCAACCTTTACTGATTTGAAATTCAAATGGCTCGGATCAGACCTCCGAAGCGACCATCAAGGTTACAGGACGGTTAGTTCTGACACCAAGACAGTTAATGTTGGCGACACC 5400
 1026 T A P T F T D L K F N N G S D Q T S E A T I K V T V S S D T K T V N V G D T 1065

 5401 GTAGCAGCACTTGATGCACAACATCACTTTAGTGTGATGTFACCGGTTAATATGTTGACAAATACCATCAAGTGACCGCCACCGACGAGACGCAACACACGCGGACGCAAGACG 5520
 1066 V A A L D A Q H H F S V D V P V N Y G D N T I K V T A T D E D G N T T T E Q K T 1105

 5521 ATTACCTCGTCTTATGATCCTGATGTTGAAGATGCTGTGACGTTCCATCAAGGTGTAATTTGGTCCCAATGATTTCAATGCCACCTCGCTAAGTTCTATGACCTTAAGACCGGG 5640
 1106 I T S S Y D P D V L K N A V T F D Q G V K F G A N E F N A T S A K F Y D P K T G 1145

 5641 ATTGCGACGATTACTGGTAAGTCAAGCACCCACACACACGTTGTCAGGTTGATGTTGAAGCAATTTCAATCAAGATGATCTGACTTTCACTTTACATTTAGTACTCTTGGA 5760
 1146 I A T I T G K V K H P T T T L Q V D G K Q I S I K N D L T F S F T L D L G T L G 1185

 5761 CAAAAACCGTTTGGGTGTTGTTGGGTGACACCACTCAAAACAGACCTTCCAAGAGCGTTGACCTTCATTTTGGATGACGTGGCGCCAAACATTTGTCAATTGACACCTCGACACATGCA 5880
 1186 Q K P F G V V V G D T T Q N K T F Q E A L T F I L D A V A P T L S L D S S T D A 1225

 5881 CCGGTTTATACCAACGATCCGAATCTCCAGATTACCGGAAGCGGCACCTGACAAATGACAATATCTGAGTCTGCAATTAACGGCACTCATGTCGCCACCAATACGACAGCATCAACATC 6000
 1226 P V Y T N D P N F Q I T G T A T D N A Q Y L S L A I N G S H V A S Q Y A D I N I 1265

6001	AATAGCGGCAACCGGGTCAATATGGCTTAATGATCAGCCCGTTAAATGCTCGAAGGCAAAACGCTGCTGACTGTGTTACAGATAGCGAAACCAACACCAACGACCAAGAAATCACA	6120
1266	N S G K P G H M A I D Q P V K L L E G K N V L T V A V T D S E N N T T T K K I T	1305
6121	GTTTACTACCAACCAAGAAACACTGGCAGCACCAACTGTGACGCCCAAGCACTACTGAACCAAGCAAGCGTCTGACGGCAAAACGCTGCCGCAACGGTGAACACTGTTCACTAT	6240
1306	V Y Y E P K K T L A A P T V T P S T T E P A K T V T L T A N A A A T G E T V Q Y	1345
6241	AETGCTGATGGTGGCAAGACATATCAGGATGTTCCGGCAGCCGGTGTCTACGGTCAACGGCAATGGCAACCTCAAGTTAAAGTGCACATGATTAACGTTATACGTTAATGAATCACC	6360
1346	S A D G G K K T Y Q D V P A A G V T V T A N G T F K F K S T D L Y G N E S P A V D	1385
6361	TATGTTGTGACCAATATCAGGCTGATGATCCTGCAAAATTCAGACACACTAGCAGCACTAGCTAATCTGCTCCGCCAAACCGTAAAGTCCAGCGGTAAAGTATGATGATGCC	6480
1386	Y V V T N I K A D D P A Q L Q T A K Q A L T N L I A S A K T L S A S G K Y D D A	1425
6481	ACAACGACTGCTTTAGCAGCGGCAACGAGAGCGCAAGCGGCTTTCATCAGACCGCAAGCGCTCACTTCACTTACTGTGTCCTCAATCAGATCTGCAGAACTGCATCAATCAATTA	6600
1426	T T T A L A A A A T Q K A Q T A L D Q T D A S V D S L T G A N R D L Q T A I N Q L	1465
6601	GCTGCCAAATTAACCTGACGATAGAAGACTTCGCTGCTTAACCAAGTTGCAATCTGTGAAGGCTCGCTGGGAACGAGCTTGGCAATCAAACTGATCCAAAGCACTGGCAAAACATTTACG	6720
1466	A A K L P A D K K T S L L N Q L Q S V K A A L G T D L G N Q T D P S T G K T F T	1505
6721	GCAGCGTTAGACGACCTAGTGGCAAGCTCAAGCAGGTACGCAACGCGCAGCTTCAAGCAGTCTTGCAAGGACTTGTGACGATATTAGCAAAACTTGCAGAGGATATTAA	6840
1506	A A L D D L V A Q A Q A G T Q T A D Q L Q A S L A K V L D A V L A K L A E G I K	1545
6841	GGCGCAACCGCTGAGGTGGCAATGCTAAAGATGCTGCAACTGGCAAACTTGTGATGCGGACATTCGTACACATTCGCTGCTGATCGCTGACAAAGCTT	6960
1546	A A T P A E V G N A K D A A T G K T W Y A D I A D T L T S G Q A S A D A S D K L	1585
6961	GCACATTTACAGCTTTGCAAAAGTCTGAAAACGAAGTGGCAGCTGCCGTTGAAGCGCCCAAGACAGCTGGTAAAGGCGACGATACAAACCGTACTAGCACAAAGCGCGGTCAAGGT	7080
1586	A H L Q A L Q S L K T K V A A A V E A A K T A G K G D D T T G T S D K G G G Q G	1625
7081	ACCCCGCGCCGCTCCAGGCGCACAGTAAGGACAAAGCGGATCAGGCGCAGCAGCTAGTCTTGGCGGTAATATCCCAACAAAGCCAGCACCAACGACGCTCAACAGCAGGATGAT	7200
1626	T P A P A P G D T G K D K G D E G S Q P S S G G N I P T K P A T T T S T S T D D	1665
7201	ACGACTGATCGTAATGGTCAACATACATCCGGTAAGGGGCAATTACCCAGACAGCAGACAACTGACGCGCAGCGCTTGGCTTCTTGGTGTCTTGTGTCAGTCTGATGGGGA	7320
1666	T T D R N G Q H T S G K G A L P K T A E T T E R P A F G F L G V I V S L M G V	1705
7321	TTAGGATTGAACGGAAACACGTCAGGAATAGCCTGTCCATGCGTTACGTTTAGGGCAGCAGCGTATCGGCACAAAGTAACCAACCTATCATGGCTTTGAAGCCATGGCAGTTG	7440
1706	L G L K K Q R E E *	1745
7441	CTGCTTTTATTGTCATATCTTACCGTAAATCAAAATCGGATTGAATCACCGTCTGAGCAATGTCAATCGCGCAAGCCAGCTTTTAACGGCTGCTGCACCGATGGCTGAACATGATGAT	7560
7561	CGATCGTACTCATTTGCAGTAATCGACTGGAGAGCTGATCTCTTGACCCATTAAGAGGATGGCAGGTGATGGTCCAGTACTGCGAAGACCGCGCGTGATATCGTCCCAT	7680
7681	TGCCAGGATAAATTCGGATGCTCATTCGACTAACCGCGCACCGCTTGATAGCCATCTTCGAAGGTTGCGATGTGATAGACATCATTTTCGTTAGCAGGACGTTTACGAAACACATCCAC	7800
7801	ATAGCTGTTAATCGTAA	7817

Fig. 3. Nucleotide sequence of the *priP* and the *priM* genes. The antisense strand (mRNA-like) is shown for the *priP* gene, whereas the sense strand is shown for the *priM* gene. Restriction sites are indicated by horizontal lines above the sequence. Proposed promoter sites and ribosome-binding sites are underlined. Dyad symmetries are indicated by convergent arrows, with the centres of symmetry shown by dots. The upward-pointing arrow indicates the cleavage site to yield the mature proteinase. The downward pointing arrow indicates the cleavage site of the proposed signal sequence of the maturation polypeptide. Amino acids comprising the active site of the serine proteinase are boxed. The deduced amino acid sequences were compared to those of *L. lactis* subsp. *cremoris* Wg2 *priP* and *priM*. Different amino acids of the Wg2 polypeptides are shown below the amino acid sequences of the *Lb. paracasei* polypeptides. Downward pointing arrowheads mark the beginning and the end of DNA sequence homology. The broken line indicates amino acids determined by N-terminal sequencing.

Fig. 4. Restriction enzyme maps of regions containing the proteinase and maturation genes of (a) *Lc. lactis* subsp. *cremoris* Wg2, (b) *Lb. casei* HN14 and (c) *Lb. paracasei* NCDO 151. The divergent arrows mark the positions of the *prtP* and *prtM* genes. The restriction sites for (a) and (c) were determined by sequence analysis; those for (b) are from Kojic *et al.* (1991). C, *Cla*I; B, *Bgl*II; H, *Hind*III; BA, *Bam*HI; E, *Eco*RI.

Fig. 5. Comparison of the amino acid sequences of the primary transcripts of the proteinase genes from (a) *Lb. paracasei* NCDO 151, (b) *Lc. lactis* Wg2, (c) *Lc. lactis* NCDO 763 and (d) *Lc. lactis* SK11. Differences between the *Lactobacillus* and the lactococcal proteinases are shown by vertical lines. The large horizontal box indicates the Lp151 proteinase with the signal sequence (SS, stippled) and the pro-sequence (PRO, hatched). Black boxes mark regions with homology to subtilisins. Active-site amino acids Asp, His and Ser are indicated. Horizontal lines at the C-terminus mark the transmembrane 'anchoring' site. The horizontal arrow indicates the C-terminal duplication of the SK11 proteinase. The bar (top left) represents 100 amino acids.

shown). Plasmid pHN1, containing the 6.7 kbp *Hind*III fragment with the entire *prtM* gene and a slightly truncated *prtP* gene, was transformed into the plasmid-free *Lb. plantarum* NC8 and the proteolytically negative *Lc. lactis* MG1363. Untransformed cells, and cells transformed with pVS2 vector or pHN1 were tested for activity in milk-clotting experiments (Table 1). The results showed a marked increase in proteolytic activity of *Lb. plantarum* NC8 containing pHN1, whereas no expression of *prtP* was observed in *Lc. lactis* MG1363.

Discussion

When proteinase was extracted from the cell wall of *Lb. paracasei* NCDO 151 and purified, two distinct fractions of proteolytic activity were obtained (Næs & Nissen-Meyer, 1992). They represented two proteins, of M_r 135000 and 110000, with an identical N-terminal amino acid sequence. This N-terminal sequence showed homology to the known lactococcal proteinases (Kiwaki *et*

al., 1989; Kok *et al.*, 1988; Nissen-Meyer & Sletten, 1991; Vos *et al.*, 1989a). The hybridization of total *Lb. paracasei* NCDO 151 DNA clearly indicated the presence of only one chromosomal gene with homology to the lactococcal proteinases. This supports the previous conclusion that the smaller protein is a cleavage product of the larger (M_r 135000) proteinase. Catalytically active cleavage products of homologous lactococcal proteinases have been described by Nissen-Meyer & Sletten (1991). Ezzat *et al.* (1988) have reported a further cell-wall-bound proteolytic activity of *Lb. paracasei* NCDO 151. It is still not known whether this is another cleavage product of the Lp151 proteinase or represents a totally different proteinase.

The proteinase genes of the lactococci described so far appear to reside on plasmids. When *Not*I-digested total DNA from *Lb. paracasei* NCDO 151 was subjected to pulsed-field gel electrophoresis, the proteinase gene appeared on a fragment of approximately 120 kbp. Such large plasmids were not observed in *Lb. paracasei* NCDO 151. Plasmids are known to show varying apparent M_r

values when subjected to pulsed-field gel electrophoresis, depending on the conditions used (S.-E. Birkeland, personal communication). For *Lb. paracasei* NCDO 151 the apparent fragment size remained unchanged, regardless of the electrophoretic conditions employed. Moreover, the *prtP*-specific probe hybridized to the undigested chromosomal DNA. These experiments strongly indicated that the *prtP* and *prtM* genes were chromosomally located. A recent report by Kojic *et al.* (1991) indicates a chromosomally located proteinase gene in *Lb. casei* HN14. Judged by the restriction map, the proteinase genes of *Lb. casei* HN14 and *Lb. paracasei* NCDO 151 appear to be very similar, but not identical.

Homologous insertion sequences or parts of insertion sequences are present downstream of both the lactococcal *prtP* and *prtM* genes (Haandrikman *et al.*, 1990). The lactococcal genes thus appear to have been part of the same transposon. This is reflected in the sequence homology outside the coding regions of these genes. The *prtP* and *prtM* genes of *Lb. paracasei* NCDO 151 do not belong to this transposon-like structure. This was verified both by the lack of hybridization to the ISS/W element of the Wg2 proteolytic system and by the abrupt ending of the DNA sequence homology with lactococcal *prtM* and *prtP* downstream of the coding regions. It cannot be excluded, however, that the genes may be flanked by other IS elements with little homology to ISS1.

Sequence comparisons of homologous proteins are important in determining the amino acids responsible for the biochemical and biophysical properties of the proteins. Very few amino acid changes can lead to relatively large changes in protein properties. By construction of Wg2-SK11 hybrid proteinases it has been shown that perhaps as few as seven amino acids were responsible for the conversion of the PI proteinase to a PIII proteinase (Vos *et al.*, 1991). For the distantly related alkaline serine proteinases, the subtilisins, it has been shown that only three amino acid changes may lead to an increase of 7 °C in the denaturation temperature (Narhi *et al.*, 1991). The deduced amino acid sequence of the *prtP* gene of *Lb. paracasei* NCDO 151 showed a high degree of similarity to the known sequences of the lactococcal proteinases, and much of what is known about the lactococcal proteinases is obviously valid for the *Lactobacillus* proteinase. It is a serine proteinase (as was indicated by its inhibition by diisopropyl fluorophosphate and phenylmethylsulphonyl fluoride (Næs *et al.*, 1991). It is first synthesized as a pre-pro-proteinase which is cleaved upon maturation, as was indicated by the N-terminal sequence determination (Næs & Nissen-Meyer, 1992) and it contains the C-terminal hydrophobic anchor sequence that was reported for the SK11 proteinase (Vos *et al.*, 1989a). Despite these similarities

there are distinct differences between the properties of the *Lactobacillus* proteinase and the lactococcal proteinases. For example, when comparing the Lp151 and the 763 proteinases, the Lp151 proteinase showed a lower pH optimum for degrading casein (pH 5.6 vs pH 6.0–6.3) and a lower sensitivity to Cu²⁺ (28% vs 100% inhibition at 1 mM-Cu²⁺) (Næs *et al.*, 1991; Monnet *et al.*, 1987). The *Lactobacillus* proteinase showed a higher degree of sequence similarity to the Wg2 and the 763 proteinases than to the SK11 proteinase (Kiwaki *et al.*, 1989). According to the sequence data, the Lp151 proteinase appears to be more closely related to the PI proteinases (e.g. Wg2), hydrolysing primarily β -casein, than the PIII proteinases (e.g. SK11) hydrolysing α -, β - and κ -caseins. The *Lb. paracasei* NCDO 151 proteinase differs from the Wg2 proteinase in two positions regarded as important for substrate specificity and binding, namely at position 142, where Ala was found as in the SK11 proteinase, and at position 747, where Gln was found. Also the Lp151 proteinase has retained Tyr31 and Tyr112 as in the subtilisins Carlsberg and DY (see Kok, 1990). The influence of these findings on the substrate binding and specificity is at present not known. One would perhaps expect to find differences in the digestion pattern of β -caseins between the Lp151 and Wg2 proteinases on the one hand and the 763 proteinase on the other. Even though the Wg2 and the 763 proteinases are 99% similar, the 763 proteinase carries the Arg747 and Lys748 of the SK11 proteinase which are important for substrate binding and specificity (Kiwaki *et al.*, 1989; Vos *et al.*, 1991). The amino acid differences between the *Lactobacillus* and the lactococcal proteinases appear not to be randomly scattered along the polypeptide chain (Fig. 5). Seemingly, there are long sequences where little change has occurred. The few changes found in these regions are predominantly conservative amino acid changes. The importance of these regions remains obscure. The signal sequences and the prosequences also seem to be highly conserved.

The nascent polypeptide of the *Lactobacillus* proteinase is cleaved at position 187. This would give a polypeptide of M_r 180659.

Less is known about the PrtM protein. It is a membrane-bound lipoprotein (Haandrikman *et al.*, 1991a) with a typical leader sequence of bacterial lipoproteins (von Heijne, 1989). It is somehow involved in the maturation of the proteinase (Haandrikman *et al.*, 1991b; Vos *et al.*, 1989b). No obvious homology to other proteins was found when searching the Swiss protein database (release 19.0) with the PrtM amino acid sequence. The known lactococcal *prtM* genes are virtually identical, and the amino acid changes between the *Lactobacillus* and the lactococcal *prtM* proteins appear to be randomly spread along the polypeptide

chain. Downstream of the *prtP* gene, outside the region of homology to the lactococcal genes, a palindromic sequence followed by a stretch of U residues was found. This may act as a transcription-terminating signal (Rosenberg & Court, 1979). This would give a *prtP* mRNA of about 5860 nucleotides, which corresponds well with that observed by Northern blotting (not shown). The region upstream of the *prtP* gene contained putative non-overlapping promoter sequences for both the *prtP* and the *prtM* genes typical of *E. coli* vegetative promoters (Rosenberg & Court, 1979). For both the *prtP* and *prtM* genes the putative -35 sequences are located within palindromic sequences, which may thus be involved in regulation of transcription. The promoter region of the *prtP* and *prtM* genes carried a deletion of 30 and 34 bp relative to the Wg2 and the SK11 promoter region, respectively. The lactococcal *prtM* gene possesses two proposed promoter sites. The sequence corresponding to the more upstream -10 sequence of the lactococcal *prtM* gene (Kok *et al.*, 1988) is absent in the *Lactobacillus* promoter. The observed lack of proteolytic activity in *Lc. lactis* MG1363 may be due to the loss of the upstream *prtM* promoter.

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