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ORIGINAL PAPER

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Casitone-mediated expression of the *prtP* and *prtM* genes in *Lactococcus lactis* subsp. *lactis* BGIS29

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Abstract Casitone added to chemically defined medium (CDM) specifically influenced the regulation of the proteinase activity in the natural isolate *Lactococcus lactis* subsp. *lactis* BGIS29. Comparative analysis of the influence of casitone present in CDM on the proteolytic activity of strain BGIS29 and of *L. lactis* subsp. *cremoris* strains SK11 and Wg2 indicated that the proteolytic activity of strains BGIS29 and SK11 is casitone-dependent, whereas that of strain Wg2 is not. The regulatory region of the *prt* genes of strain BGIS29 was cloned and sequenced. The nucleotide sequence of the *prt* regulatory region of strain BGIS29 was distinctly different from that of *L. lactis* subsp. *cremoris* strains SK11 and Wg2. Transcriptional gene fusions with the *Escherichia coli* β-glucuronidase gene (*gusA*) were used to study medium-dependent expression of two divergent *prtP* and *prtM* promoters of strain BGIS29 (designated P_{prtP} and P_{prtM}, respectively). β-Glucuronidase assays and Northern blot analysis showed that the activities of P_{prtP} and P_{prtM} are controlled by casitone at the transcriptional level.

Keywords *Lactococcus lactis* · Proteinase · *prtP* gene · *prtM* gene · Regulation

Abbreviations *CDM* Chemically defined medium

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Introduction

The biochemistry and genetics of cell-wall-associated proteinases have been intensively investigated in *Lactococcus lactis* subsp. *lactis* (Gasson 1983), *L. lactis* subsp. *cremoris* (de Vos et al. 1989), and *L. lactis* subsp. *lactis* biovar diacetylactis strains (Kempler and McKay 1979). The proteinase-encoding DNA region of *L. lactis* subsp. *cremoris* Wg2 has been cloned and sequenced (Kok et al. 1985). Interestingly, in close proximity to the proteinase gene (*prtP*), another divergently orientated gene, *prtM*, is located. The *prtM* gene codes for a membrane-located lipoprotein that is essential for activation of the proteinase (Haandrikman 1990). Removal of the *prtM* gene results in elimination of the proteolytic activity, but synthesis and secretion of the proteinase are not affected (Haandrikman et al. 1989). An identical genetic organization of the proteinase genes region of *L. lactis* subsp. *cremoris* SK11 has been found (Vos et al. 1989b).

In contrast to studies of the genetic and biochemical properties of lactococcal proteinases, studies of the regulation of their production are limited. Few studies have been performed in which expression of the enzymes has been analyzed either in different media or in chemically defined media (CDM) with amino acids, peptides or caseins. Published data cannot always be compared directly due to differences in strains, variations in media, etc. In general, the expression of components of the proteolytic pathway of *L. lactis* is highest in media containing amino acids only, while peptides generally down-regulate expression. In *L. lactis* AM1, synthesis of the proteinase is repressed when the growth medium contains casitone, an enzymatic digest of casein that mainly consist of peptides (Exterkate 1985).

All *L. lactis prt* genes analyzed so far are transcribed from a regulatory region within a 340-bp *Cla*I DNA fragment (Vos et al. 1989a). This AT-rich DNA fragment contains two divergently orientated and partially overlapping *prtP* and *prtM* promoters. Around the transcription initiation sites of the *prtP* and *prtM* promoters a region of ex-

Table 1 Bacterial strains and plasmids

Strains or plasmids	Relevant characteristics	Source or references
Lactococcus lactis subsp. *lactis*		
BGIS29	Natural isolate from soft homemade cheese	Laboratory collection
NZ9000	Plasmid-free, Lac^-, Prt^-, *nisR, nisK*, derivative of *L. lactis* MG1363	de Ruyter et al. (1996)
L. lactis subsp. *cremoris*		
SK11	Prt^+, wild-type	Vos et al. (1989a)
Wg2	Prt^+, wild-type	Haandrikman (1990)
Plasmids		
pNZ273	Cm^r, promoter screening vector with the promoterless *gusA* reporter gene	Platteeuw et al. (1994)
pMPE701	Derivative of pNZ273 containing the PCR PRTM700-PRTP700 fragment (720 bp) with the promoter of the *prtM* gene in front of the *gusA* gene	This study
pMPE705	Derivative of pNZ273 containing the PCR PRTM700-PRTP700 fragment (720 bp) with the promoter of the *prtP* gene in front of the *gusA* gene	This study
pMPE701ΔM348	Derivative of pNZ273 containing PCR M300-PRTP300 fragment (348 bp) with promoter of the *prtM* gene in front of the *gusA* gene	This study

tensive dyad symmetry is positioned, though minor differences in the extent of symmetry exist in the different strains (Kiwaki et al. 1989; Van der Vosen et al. 1992; Vos et al. 1989a). Specific sequences within this repeat structure may be involved in the regulation of expression of both the *prtP* and *prtM* genes (Marugg et al. 1996).

By fusing the promoter regions of *prtP* and *prtM* in *L. lactis* subsp. *cremoris* SK11 to the reporter gene *gusA*, a medium-dependent regulation of *prt* genes expression has been observed (Marugg et al. 1995). Expression is repressed at increased nitrogen concentrations or by the addition of specific dipeptides to the growth medium. The data indicate that the internal concentration of the dipeptides, in particular prolylleucine and leucylproline, downregulates the expression of the proteinase genes (Guedon et al. 2001; Marugg et al. 1995). The control mechanism is host-independent, since regulation of the proteinase promoters is observed in both *L. lactis* strains MG1363 and SK11. Since strain MG1363 is plasmid-free, it appears that a chromosomally located gene encodes a regulatory protein (Marugg et al. 1996).

The experiments described here were designed to investigate the role of casitone (pancreatic digest of casein) in the expression of the *prt* genes in the natural isolate *L. lactis* subsp. *lactis* BGIS29. Strain BGIS29 was originally isolated from traditionally homemade soft cheese. This strain produces an extracellular, cell-wall-associated proteinase that, according to the substrate specificity, belongs to the PI-type of lactococcal proteinases, since it hydrolyses primarily β-casein. Preliminary results showed that the *prt* genes are located on a plasmid of at least 130 kb. The obtained results indicate that *prt* gene expression in this strain is influenced by casitone in a different manner than that in *L. lactis* subsp. *cremoris* strains SK11 and Wg2.

Materials and methods

Bacterial strains, media and growth conditions

The bacterial strains used in this study are listed in Table 1. *Lactococcus* strains were grown in M17 broth (Terzaghi and Sandine 1975) containing 0.5% glucose (GM17). In addition, milk citrate agar (MCA) (Gasson 1983) and CDM (Dickely et al. 1995) modified by replacement of casamino acids with casitone (80% of small peptides, 20% of free amino acids) were used for testing proteinase production of the lactococcal strains. CDM contained 2 g $(NH_4)_2SO_4$, 6 g Na_2HPO_4, 3 g KH_2PO_4, 0.011 g Na_2SO_4, and 1 g NaCl dissolved in 200 ml of water; this CDM was called solution A. Solution B contained 0.2 g $MgCl_2$, 0.01 g $CaCl_2$, and 0.0006 g $FeCl_3{\cdot}7H_2O$ dissolved in 800 ml water. Solutions A and B were mixed after autoclaving by pouring A into B, and glucose (5 g l^{-1}), vitamins, sodium acetate (2 g l^{-1}), and asparagine (80 mg l^{-1}) were added. The final concentration of the added vitamins was 0.1 mg l^{-1} for biotin, 1 mg l^{-1} for folic acid, riboflavin, nicotinic acid, thiamine, and pantothenic acid, and 2 mg l^{-1} for pyridoxal (Dickely et al. 1995). Agar plates were prepared by adding agar (1.5%, w/v) (Difco) to each broth when used as a solid medium. The incubation temperature for *L. lactis* strains was 30 °C.

DNA methods, reagents, and enzymes

Plasmid DNA from lactococci was isolated by the method of Bojovic et al. (1991). Large plasmids from lactococci were isolated by the method of O'Sullivan and Klaenhammer (1993). Restriction enzymes and T4 DNA ligase were used according to the manufacturer's instructions (Pharmacia Biotech, Vienna, Austria). All other recombinant DNA techniques were performed as described by Sambrook et al. (1989).

Bacterial transformation

Lactococcus strains were transformed by electroporation and transformants were recovered as described by Holo and Nes (1989). Transformants were selected on GM17 agar plates containing chloramphenicol (5 μg ml^{-1}) (Sigma) and X-glucuronide (0.3 mM) (Clontech).

Table 2 List of primers used in this study. The restriction sites in primer's sequences are underlined

Primer	Sequence
PRTM700	(5'-GCATGAATTCAATGCACGATAAATGAG-3')
PRTP700	(5'-GCTTGAATTCGTTGTCGCTGCGGTTGT-3')
18745	(5'-GGGTTGGGGTTTCTTACAGGACGTA-3')
M300	(5'-TAAAGAATTCCGCTAAAAGTTAATTTACAG-3')
PRTP300	(5'-AGCAGTCGGATCCGTCGACGTATTTGCGAG-3')
P10	(5'-GTCTGTAAACGGCTAAA-3')
P10C	(5'-TTTAGCCGTTTACAGAC-3')
930512	(5'-CCGCCGGAATTCTTTTCGTTGAATTTGTTCTTCAATAGTATATAATATAAT-3')
930513	(5'-AAATTGGTCGACATAGCCCTTTCTTTTTCCTTTGCATCC-3')

Plasmid construction

To make transcriptional fusions of the *prtM* and *prtP* promoters with the promoterless β-glucuronidase gene (constructs pMPE701 and pMPE705, respectively) the regulatory region of strain *L. lactis* subsp. *lactis* BGIS29 was amplified by PCR with primers specific for the 5′-end of the *prtM* gene (PRTM700) and the *prtP* gene (PRTP700) with *Eco*RI restriction sites (Table 2). The ligation mixture was used to transform *L. lactis* NZ9000. Eight blue transformants were obtained. The orientation of the cloned fragment was checked in a PCR by using primer 18745 based on the sequence of the 5'-end of the *gusA* gene and primers based on either the *prtP* or the *prtM* gene sequence (Table 2). It was confirmed that pMPE701 carried the DNA fragment with P_{prtM} in front of the *gusA* gene, while pMPE705 carried the same fragment but in the opposite orientation, i.e. P_{prtP} was located in front of the *gusA* gene. To localize a smaller region that is potentially involved in the regulation of the *prtM* promoter, the region downstream of the P_{prtM} promoter in plasmid pMPE701 was removed (Fig. 1C). A deletion downstream of the *prtM* promoter (construct pMPE701ΔM348) was achieved by PCR using primer M300 with an *Eco*RI restriction site and the PRTP300 primer specific for the 5′-end of the *prtP* gene with a *Bam*HI restriction site (Table 2). All PCR amplifications were performed in 30 successive cycles of DNA melting at 94 °C for 2 min, annealing at 55 °C for 2 min, and elongation at 72 °C for 2 min. Resulting DNA fragments were purified by the Gene Clean procedure (GlassMAX DNA Isolation Matrix System, Gibco BRL), digested with *Eco*RI or with the combination of *Eco*RI and *Bam*HI restriction enzymes and ligated with plasmid pNZ273 digested with the same enzymes.

DNA sequencing

The dideoxynucleotide chain termination procedure for double-stranded plasmid DNA (Sanger et al. 1977) was applied for DNA sequencing and Sequenase (version 2.0 kit; US Biochemicals) was used according to the manufacturer's instructions. The fragments containing the *prt* regulatory region in the plasmids pMPE705, pMPE701 and pMPE701ΔM348 were directly sequenced with primers 18745, PRTM700, PRTP700, P10, 930512, P10C and 930513 (Table 2).

Primer extension and Northern blot analysis

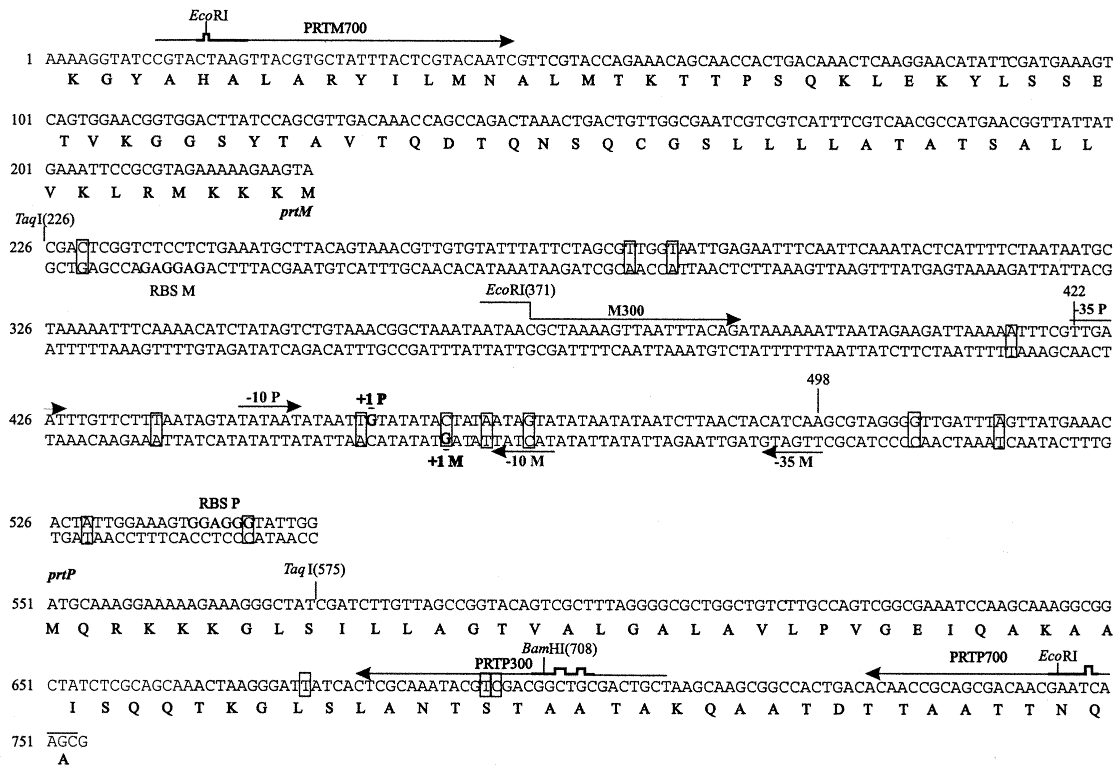
RNA was isolated from 30 ml of exponentially growing cultures of *L. lactis* BGIS29, *L. lactis* NZ9000/pMPE701 and *L. lactis* NZ9000/pMPE705 grown to an OD_{600} of 1.0 in CDM supplemented with different concentrations of casitone (0.1%, 0.5%, 1% and 2%) or in GM17 broth. Microbial biomass was measured in cuvettes of 0.5-cm path length using an Ultrospec II spectrophotometer (LKB). RNA was isolated by the Macaloid method described by Kuipers et al. (1993) with the following adaptation: prior to bead-beating, the resuspended cells were incubated with lysozyme (2 mg ml^{-1}) for 2 min on ice to increase RNA yields. RNA samples were quantified spectrophotometrically (Ultrospec II). Primer-extension reactions were performed by annealing 20 ng of oligonucleotides specific for the 5′-ends of the *prtM* (P10) or the *prtP* (930513) genes to 40 μg of RNA as described previously (Kuipers et al. 1993). The products of the reactions were run next to the DNA sequencing ladder (Sanger et al. 1977) of promoter DNA fragments generated by the same oligonucleotide primers. RNA for Northern blot analysis was fractionated on a 1.0% formaldehyde gel (Sambrook et al. 1989). RNA size markers were obtained from BRL. RNA was transferred to Gene Screen Plus (Dupont) membranes according to the protocols outlined by the manufacturer. Hybridization reactions were performed at 65 °C in a 0.5 M Na-phosphate buffer (pH 7.2) containing 1.0% BSA (fraction V), 1.0 mM EDTA and 7.0% SDS. The blots were washed at 55–65 °C in 0.1×SSC (15 mM NaCl, 1.5 mM Na-citrate). A GlassMatrix (GlassMAX DNA Isolation Matrix System, Gibco BRL) purified *Eco*RI-*Hin*dIII fragment from pNZ273 (*gusA* gene) was used as a probe for P_{prtM}-gusA (pMPE701) and P_{prtP}-*gusA* (pMPE705) fusions. For quantification of the *prtP*- and *prtM*-specific mRNAs, probes were obtained by PCR using primers 930512 and PRTP700 (*prtP*) or P10C and PRTM700 (*prtM*). Probes were labeled by nick-translation with [α-^{32}P]ATP (Amersham) (Sambrook et al. 1989). Computer-assisted quantification of the scanned bands was performed by MD ImageQuant Software, version 3.3.

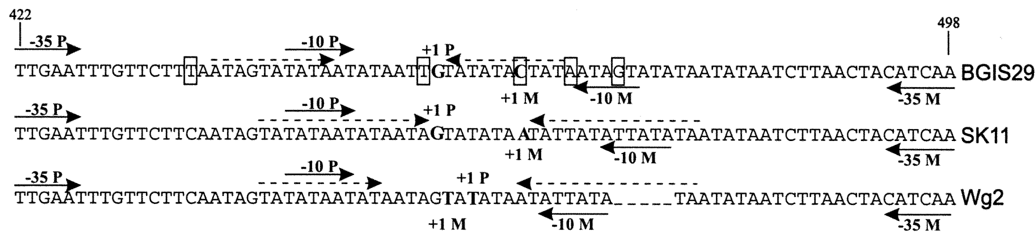
Assay of proteinase activity

Proteolytic activities of lactococcal strains were assayed as described previously (Kojic et al. 1991). For this purpose, *L. lactis* subsp. *lactis* BGIS29, *L. lactis* subsp. *cremoris* strains SK11 and Wg2 were grown either on GM17 plates, or on CDM plates supplemented with 0.1%, 0.5%, 1.0% or 2.0% casitone (Difco) or on milk citrate agar (MCA) plates for 48 h at 30 °C prior to cell collection. Collected cells of strains BGIS29, SK11 and Wg2 (15 mg; approx. density 10^{10} cells per ml) were resuspended in 100 mM NH_4-acetate buffer (pH 6.2). The cell suspension was mixed with substrate (5 mg β-casein ml^{-1}, Sigma) dissolved in the same buffer at a 1:1 volume ratio. After incubation (3 h at 30 °C) cells were pelleted by centrifugation (5 min at 12,000×*g*), the clear supernatant was taken and samples for SDS-PAGE were prepared. Casein hydrolysis was analyzed by SDS-PAGE by loading a 15% (w/v) acrylamide gel with the prepared samples. Gels were run on vertical slab electrophoresis cells (BRL) for 16 h at 10 mA constant current, stained with Coomassie brilliant blue G250 (SERVA) and destained in a mix of methanol (20%) and acetic acid (7%).

Enzyme assays

Activity of the β-glucuronidase (GusA) enzyme was measured as described previously (Kuipers et al. 1995). Protein concentrations were determined with bovine serum albumin as a standard by the method of Bradford (1976).





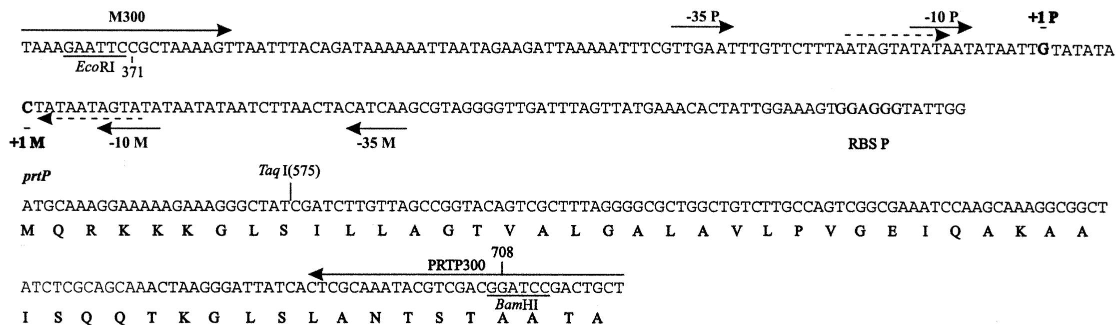


Fig. 1 **A** Sequence of the regulatory region of the *prt* genes in *L. lactis* subsp. *lactis* BGIS29. *Long arrows* above the sequence show the positions of the primers used for PCR; *short arrows* above and below the sequence show the positions of potential –10 and –35 hexamers of the *prtP* (–35 P and –10 P) and *prtM* (–35 M and –10 M) promoters. *+1 P, +1 M* Transcription start points of the *prtP* and *prtM* genes, respectively. RBS sequences (RBS P for the *prtP* gene and RBS M for the *prtM* gene) are given in *bold letters*; only relevant restriction sites are shown. **B** The 77 bp region containing P_{prtP} and P_{prtM} of strains BGIS29, Wg2 and SK11. Differences in sequences between strains BGIS29 and SK11 are *boxed*; the deletion in the sequence of Wg2 is given as a *dashed line*; the inverted repeats in the promoter regions in all three strains are indicated by the *broken arrows*. **C** Sequence of the *prt* promoter region in the deletion derivative pMPE701ΔM348

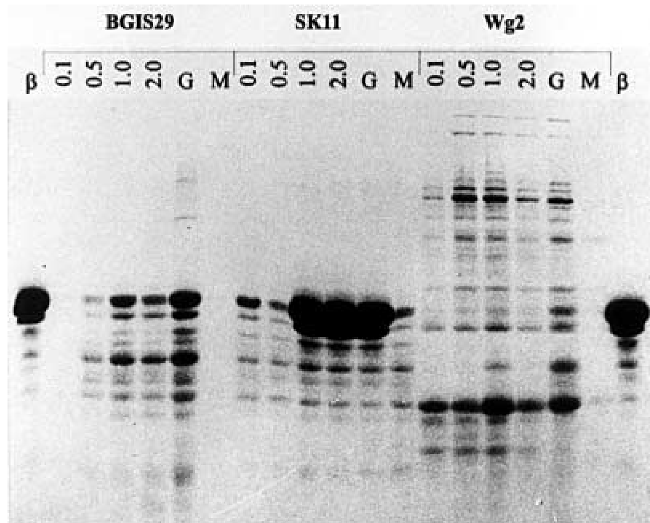


Fig. 2 Influence of growth medium on the proteolytic activity of *Lactococcus lactis* subsp. *lactis* BGIS29, *L. lactis* subsp. *cremoris* SK11 and *L. lactis* subsp. *cremoris* Wg2. *β* β-casein substrate, *0.1, 0.5, 1.0, and 2.0* whole cells collected from CDM plates containing 0.1%, 0.5%, 1% and 2% casitone, respectively, *G* GM17 plates, *M* MCA plates

Results

Influence of the growth medium on proteinase activity

The proteinase activities of *L. lactis* subsp. *lactis* BGIS29 and *L. lactis* subsp. *cremoris* strains SK11 and Wg2 were compared. For this purpose, cells were grown on CDM containing casitone, a protein-rich growth medium (GM17) and MCA plates. The proteolytic activities were determined by following β-casein hydrolysis in 0.1 M NH_4-acetate buffer (pH 6.2). The highest β-casein hydrolysis by whole cells of strain BGIS29 was obtained when the cells were grown on MCA plates or CDM plates supplemented with 0.1% casitone (Fig. 2). Lower proteolytic activities of the BGIS29 proteinase were detected when the cells were grown on CDM plates supplemented with higher concentrations of casitone (0.5%, 1% and 2%). The lowest proteolytic activity of the BGIS29 proteinase was found when the cells were grown on GM17 plates. The proteolytic activity of *L. lactis* subsp. *cremoris* SK11 cells depended on the concentration of casitone in CDM plates as well. Results showed that the proteinase of strain SK11 had a high capacity for β-casein hydrolysis when the cells were grown on MCA plates or CDM plates containing 0.1% or 0.5% casitone. However, hydrolysis of β-casein was negligible when the cells were collected from the CDM plates containing higher casitone concentrations (1% and 2%). Thus, it appears that β-casein hydrolysis by whole cells of strains BGIS29 and SK11 depended on the concentration of casitone in CDM. In contrast, β-casein hydrolysis by the proteinase of strain Wg2 was independent of the concentration of casitone in CDM plates.

Cloning and sequencing of the *prt* regulatory region from *L. lactis* subsp. *lactis* BGIS29

The regulatory region of the *prt* genes was amplified by PCR using primers PRTM700 and PRTP700 (Table 2) and large plasmids of *L. lactis* subsp. *lactis* BGIS29 as a template. Plasmids pMPE701 and pMPE705, containing the cloned PCR product, were sequenced. Nucleotide sequence analysis revealed that cloned fragments contained the regulatory region of the *prt* genes of strain BGIS29 (Fig. 1A). The sequence of the regulatory region of the *prt* genes in *L. lactis* subsp. *lactis* BGIS29 is the same length as that in *L. lactis* subsp. *cremoris* SK11 (Fig. 1B).

Determination of the transcription start points and activities of the *L. lactis* subsp. *lactis* BGIS29 *prtP* and *prtM* promoters

The transcription start points of the *prtP* and the *prtM* genes were determined. Total RNA from strain *L. lactis* subsp. *lactis* BGIS29 was annealed with primer specific for the 5'-ends of the *prtP* or the *prtM* mRNA. The data showed that the transcription start point of the *prtP* gene was a G nucleotide 95 bp upstream of the proposed ATG of the *prtP* gene, and the transcription start point of the

Table 3 β-Glucuronidase (GusA) activities in cell extracts of transformants. *L. lactis* cells (1 ml) were harvested and resuspended in Na-phosphate buffer (50 mM NaPi, pH 7.0) to a final OD_{600} of 2.0. Cells were permeabilized by adding 50 µl of acetone/toluene (9:1) per ml of cells followed by 10 min of incubation at 37 °C; extracts were used immediately in the assay. For the determination of β-glucuronidase activities, 40 µl of extracts were added to 950 µl of buffer (50 mM NaPi, pH 7.0, 10 mM β-mercaptoethanol, 1 mM EDTA, 0.1% Triton X-100) and 10 µl of 100 mM *p*-nitro-β-D-glucuronic acid (Clontech). The mixture was incubated, and the increase in OD_{405} was measured at 37 °C in a Cary 1E UV-visible spectrophotometer (Varian) with a thermostatically controlled compartment. Results are the averages of three independent experiments [β-glucuronidase activity±SD (nmol min^{-1} (mg protein)$^{-1}$]

Transformant	Growth media						Induction ratio 0.1%/4%
	CDM supplemented with casitone					GM17	
	0.1%	0.5%	1%	2%	4%		
NZ9000/ pMPE705 (P_{prtP})	31±19	8± 4	6± 3	5±3	4±2	4±3	7.75
NZ9000/ pMPE701 (P_{prtM})	34±22	15± 9	13± 7	14±7	15±6	6±3	2.27
L. lactis NZ9000/ pMPE701ΔM348 (P_{prtM})	104±61	45±28	31±16	20±9	20±9	6±3	5.20

prtM gene was a G nucleotide 238 bp upstream of the proposed ATG of the *prtM* gene (Fig. 1A).

To study the transcriptional regulation of proteinase gene expression in strain BGIS29, we used plasmid pNZ273, which contains the promoterless β-glucuronidase gene (*gusA*) from *E. coli* (Platteeuw et al. 1994). Transcriptional fusions of the *L. lactis* subsp. *lactis* BGIS29 *prtM* and *prtP* promoters in pNZ273 resulted in constructs pMPE701 and pMPE705, respectively. Transformants *L. lactis* NZ9000/pMPE701 and NZ9000/pMPE705, containing P_{prtM}- and P_{prtP}-*gusA* fusions, respectively, were grown in CDM with 0.1%, 0.5%, 1%, 2% and 4% casitone or in GM17 broth. Results of GusA assays showed that both *prt* promoters of strain BGIS29 are regulated by casitone (Table 3). The activity of P_{prtM} decreased when the casitone concentration increased from 0.1% to 0.5%; higher casitone concentrations had no effect on P_{prtM} activity, i.e. even in the presence of 4% casitone the promoter retained significant activity. The activity of P_{prtP} decreased with the increase of casitone in CDM from 0.1% to 0.5% and minimum activity was achieved in the presence of 4% casitone.

Northern blot analysis

To confirm the transcriptional control of P_{prtM} and P_{prtP} in *L. lactis* BGIS29, Northern blot analysis was performed. Total RNA was isolated from strain BGIS29 cells that were grown in CDM with various concentrations of casitone (0.1%, 0.5%, 1% and 2%) or in GM17 broth. The *prtP*- and *prtM*-specific probes were used to detect the synthesis of specific mRNAs. These results indicated that both *prt* promoters are strongly regulated by a component of casitone at the transcriptional level (Fig. 3).

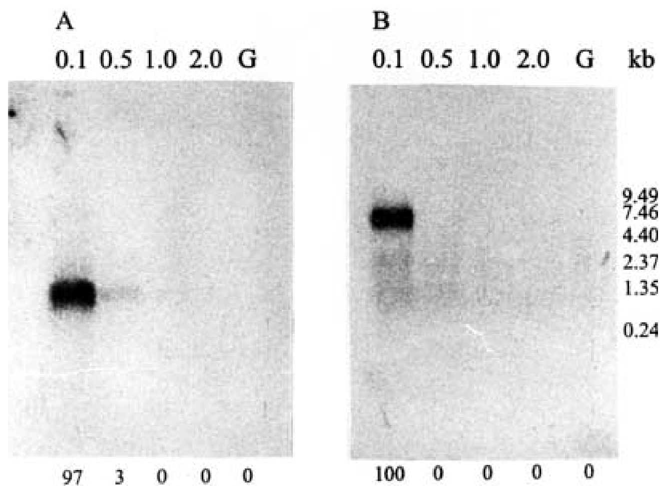


Fig. 3A, B Northern blot analysis of *prt*-specific mRNA in *L. lactis* subsp. *lactis* BGIS29 cells. Total RNA isolated from strain BGIS29 cells grown in GM17 broth (*G*) or CDM containing 0.1%, 0.5%, 1% and 2% casitone (*0.1, 0.5, 1.0,* and *2.0*, respectively) was hybridized to the *prtM*-specific (**A**) and *prtP*-specific (**B**) probes. Single-stranded RNA leader (kb) is given on the *right* side of figure. The *numbers* represent relative quantification of the band intensities

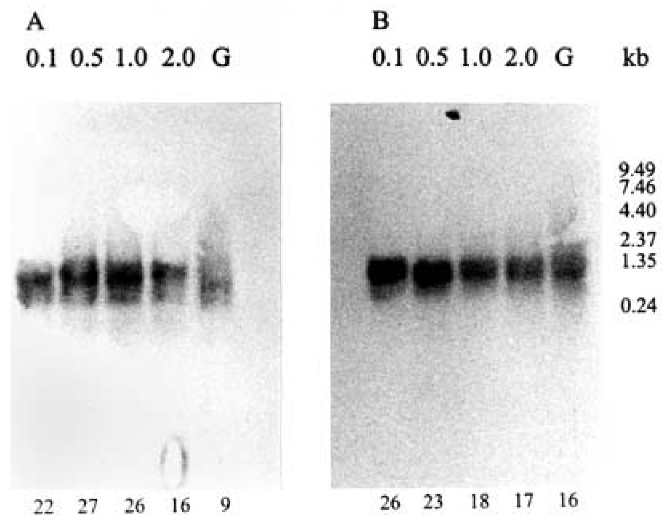


Fig. 4A, B Northern blot analysis of *gusA*-specific mRNA. Total RNA was isolated from *L. lactis* NZ9000/pMPE701 (**A**) and *L. lactis* NZ9000/pMPE705 (**B**) cells grown in GM17 broth (*G*) or CDM containing 0.1%, 0.5%, 1% and 2% casitone (*0.1, 0.5, 1.0,* and *2.0*, respectively). The *gusA*-specific DNA probe was used in the assay to identify localization of *gusA* mRNA. Single-stranded RNA leader (kb) is given on the *right* side of figure. The *numbers* represent relative quantification of the band intensities

Another Northern blot analysis was performed under the same conditions, i.e. by using total RNAs from *L. lactis* NZ9000/pMPE701 and *L. lactis* NZ9000/pMPE705 which had been grown either in CDM with various concentrations of casitone (0.1%, 0.5%, 1% and 2%) or in GM17 broth. Equal amounts of each RNA sample were used for hybridization with a *gusA*-specific probe. The results showed that in the case of the P_{prtM}-*gusA* fusion (pMPE701) there was no significant difference between samples isolated from CDM cultures containing different casitone concentrations, while in samples isolated from GM17 the amount of specific P_{prtM}-*gusA* mRNA appeared to be lower (Fig. 4A). In the case of the P_{prtP}-*gusA* fusion (pMPE705) increased casitone concentrations resulted in steady decreases of mRNA levels (Fig. 4B).

Deletion analysis of the promoter region

To localize a region that could be involved in the regulation of the *prtM* promoter, the region downstream of the P_{prtM} promoter in plasmid pMPE701 was removed. The PCR fragment obtained by using primers M300 and PRTP300 was subcloned into plasmid pNZ273 and construct pMPE701ΔM348 was obtained. The 136 bp of the *prt* regulatory region downstream of the transcription start point of the *prtM* gene was deleted (Fig. 1C). The resulting DNA fragment (from 371 bp to 708 bp) present in pMPE701ΔM348 retained a 180-bp regulatory region and the 5′-end of the *prtP* gene. This fragment also con-

tained P_{prtP} and P_{prtM} as well as their transcriptional start points (Fig. 1). *L. lactis* NZ9000 was transformed with pMPE701ΔM348, and β-glucuronidase activity of the transformants was measured under different conditions. The results indicated that the deleted region is not involved in the regulation of *prtM* gene expression, since the effect of casitone on its expression was retained (Table 3).

Discussion

The influence of casitone in whey permeate on caseinolytic activities of lactococcal proteinases has been reported (Marugg et al. 1995). Testing the proteolytic activity of the natural isolate *L. lactis* subsp. *lactis* BGIS29 grown in CDM containing casitone revealed that casitone also influenced *prt* gene expression in this strain. Comparative analysis of the influence of casitone present in CDM on the proteolytic activity of *L. lactis* subsp. *lactis* BGIS29 and *L. lactis* subsp. *cremoris* strains SK11 and Wg2 strongly indicated that the regulation of *prt* gene expression in *L. lactis* subsp. *lactis* BGIS29 and *L. lactis* subsp. *cremoris* SK11 was casitone-dependent, whereas that in *L. lactis* subsp. *cremoris* Wg2 was not.

The regulatory region of the *prt* genes from *L. lactis* subsp. *lactis* BGIS29 was cloned, sequenced and functionally analyzed. Results of GusA assays and *gusA* Northern blot analysis showed that the *prt* promoters of strain BGIS29 were regulated by a component of casitone at the transcriptional level. The activities of the promoters decreased with increased casitone concentrations in CDM. The difference in expression of the *prt* promoters observed between *gusA* transcriptional fusion constructs (pMPE701 and pMPE705) and the original strain BGIS29 could be a consequence of different concentrations of mRNA, because the number of targets responsible for regulation is much higher in these constructs due to the high copy number of the plasmids.

Nucleotide sequence analysis of the *prt* regulatory regions of strains BGIS29, SK11 and Wg2 showed that the length of the regulatory region containing both *prtP* and *prtM* promoters in *L. lactis* subsp. *lactis* BGIS29 corresponds to that of *L. lactis* subsp. *cremoris* SK11 (Vos et al. 1989a). Five base-pair substitutions were found when the nucleotide sequences of these two regions were compared. The same regulatory region of strain Wg2 is 5 bp shorter than that in either strain BGIS29 or strain SK11 (Kok et al. 1988) (Fig. 1B).

The *prt* regulatory regions of *L. lactis* strains SK11 and Wg2 contain a region of dyad symmetry positioned around the transcription initiation sites of the *prtP* and *prtM* promoters. In strain BGIS29 this inverted repeat is positioned closer to the *prtP* promoter than in *L. lactis* strains SK11 and Wg2. Inverted repeat sequences have been found in or near promoter regions of many prokaryotic genes and are often involved in recognition and binding of transcription factors (Beck and Warren 1988; Harrison and Aggarwal 1990). The present data suggest that any disturbance of the inverted repeats in the *prt* regulatory region in *L. lactis* SK11 influences medium-dependent regulation (Marugg et al. 1996). Deletion analysis of the promoter region starting just downstream of the *prtP* promoter (position +10), which removed half of the dyad repeat and the *prtM* promoter completely, resulted in almost constitutive expression of the *prtP* promoter. Further evidence supporting a regulatory role for sequences within this repeat was obtained by insertion mutagenesis using small DNA linkers that disrupted the palindrome symmetry. The insertions resulted in strong derepression of β-glucuronidase activity and increased mRNA levels at high peptide concentrations, while expression of the *prtP* and *prtM* promoters at low peptide concentrations was hardly affected (Marugg et al. 1996). Comparative analysis of the *prt* promoters in strains SK11, Wg2 and BGIS29 revealed the differences in the nucleotide sequences containing inverted repeats. Therefore, the different proteolytic activities of strains BGIS29 and SK11 in the presence of casitone could be a consequence of a different localization of the inverted repeat sequence in the *prt* promoter region.

To confirm that the different level of regulation of the *prtM* promoter activities of strains BGIS29 and SK11 is correlated to the different localization of the *prtP* and *prtM* promoters within the region of dyad symmetry, deletion analysis of the *prtM* promoter of strain BGIS29 was performed. The results indicate that 180 bp of the regulatory region present in plasmid pMPE701ΔM348 retained regulation of the *prtM* gene expression by casitone (Table 3) indicating that the deleted 136 bp region is not involved in the regulation of *prtM* gene expression. However, the deleted region had an effect on β-glucuronidase gene expression, since the *prtM* promoter in the construct pMPE701ΔM348 had three-fold higher β-glucuronidase activity than the same promoter in pMPE701 (Table 3). The apparent discrepancy between the β-glucuronidase activities in cells harboring pMPE701 or pMPE701ΔM348 could be explained by the presence of two ribosomal binding sites (RBSs) in plasmid pMPE701 and only one RBS in plasmid pMPE701ΔM348. Taken together, these results infer that within this region of dyad symmetry *cis* sequences close to the transcription initiation sites could be responsible for the medium-dependent regulation of *prt* gene expression. The synchronized regulation of expression of the various components of the proteolytic system in lactococci is still largely unexplored. In general, it is known that synthesis of the active proteinase is repressed when the growth medium contains casitone (Marugg et al. 1995). In addition, the expression of the peptide transport systems of *L. lactis* is also affected by the composition of the growth medium (Kunji 1997).

In conclusion, the results presented here confirm the medium-dependent regulation of *prt* expression in *L. lactis* subsp. *lactis* BGIS29. The regulation of P_{prtM} activity, but not of P_{prtP} activity, appeared to be different from that in previously reported *L. lactis* strains. We speculate that the natural isolate BGIS29 is a mutant with respect to the region of dyad symmetry possibly involved in the recognition or binding of a regulator protein. Our further re-

search is aimed at identification of the putative regulator and identification and characterization of its gene.

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References

Beck CF, Warren RAJ (1988) Divergent promoters, a common form of gene organization. Microbiol Rev 52:318–326

Bojovic B, Djordjevic G, Topisirovic L (1991) Improved vector for promoter screening in lactococci. Appl Environ Microbiol 57:385–388

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principle of protein-dye binding. Anal Biochem 72:248–254

De Ruyter PGGA, Kuipers OP, Beerthuyzen MM, van Alen Boerrigter I, de Vos WM (1996) Functional analysis of promoters in the nisin gene cluster of *Lactococcus lactis*. J Bacteriol 178: 3434–3439

De Vos WM, Vos P, de Haard H, Boerrigter I (1989) Cloning and expression of the *Lactococcus lactis* ssp. *cremoris* SK11 gene encoding an extracellular serine proteinase. Gene 85:169–176

Dickely F, Nilsson D, Hansen EB, Johansen E (1995) Isolation of *Lactococcus lactis* nonsense suppressors and construction of a food-grade cloning vector. Molec Microbiol 15:839–847

Exterkate FA (1985) A dual-directed control of cell wall proteinase production in *Streptococcus cremoris* AM1: a possible mechanism of regulation during growth in milk. J Dairy Sci 68: 562–571

Gasson MJ (1983) Plasmid complements of *Streptococcus lactis* protoplast-induced curing. J Bacteriol 154:1–9

Guedon A, Renault P, Ehrlich SD, Delorme C (2001) Transcriptional pattern of genes coding for the proteolytic system of *Lactococcus lactis* and evidence for corrdinated regulation of key enzymes by peptide supply. J Bacteriol 183:3614–3622

Haandrikman AJ (1990) Maturation of the cell envelope-associated proteinase of *Lactococcus lactis*. Ph.D. thesis. University of Groningen, Groningen, The Netherlands

Haandrikman AJ, Kok J, Laan H, Soemitro S, Ledeboer AM, Konings WN, Venema G (1989) Identification of a gene required for maturation of an extracellular lactococcal serine proteinase. J Bacteriol 171:2789–2794

Harrison SC, Aggarwal AK (1990) DNA recognition by proteins with a helix turn helix motif. Annu Rev Biochem 59:933–969

Holo H, Nes IF (1989) High-frequency transformation, by electroporation, of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilised media. Appl Environ Microbiol 55:3119–3123

Kempler GM, Mckay LL (1979) Characterisation of plasmid deoxyribonucleic acid in *Streptococcus lactis* subsp. *diacetylactis*: evidence for plasmid linked citrate utilisation. Appl Environ Microbiol 37:316–323

Kiwaki M, Ikemura H, Shimizu-Kadota M, Hirashima A (1989) Molecular characterisation of a cell wall-associated proteinase gene from *Streptococcus lactis* NCDO763. Mol Microbiol 3:359–369

Kojic M, Fira D, Banina A, Topisirovic L (1991) Characterisation of the cell wall-bound proteinase of *Lactobacillus casei* HN14. Appl Environ Microbiol 57:1753-1757

Kok J, van Dijl JM, van der Vossen JMBM, Venema G (1985) Cloning and expression of a *Streptococcus cremoris* proteinase in *Bacillus subtilis* and *Streptococcus lactis*. Appl Environ Microbiol 50:94–101

Kok J, Leenhouts K, Haandrikman AJ, Ledeboer AM, Venema G (1988) Nucleotide sequence of the cell wall proteinase gene of *Streptococcus cremoris* Wg2. Appl Environ Microbiol 54:231–238

Kuipers OP, Beerthuyzen MM, Siezen RJ, de Vos WM (1993) Characterisation of the nisin gene cluster *nisABTCIPR* of *Lactococcus lactis*: requirement of expression of *nisA* and *nisI* genes for development of immunity. Eur J Biochem 216:281–291

Kuipers OP, Beerthuyzen MM, de Ruyter PGGA, Luesink EJ, de Vos WM (1995) Autoregulation of nisin biosynthesis in *Lactococcus lactis* by signal transduction. J Biol Chem 270:27299–27304

Kunji ERS (1997) The proteolytic system of *Lactococcus lactis*. Ph.D. thesis. University of Groningen. The Netherlands

Marugg JD, Meijer W, van Kranenburg R, Laverman P, Bruinenberg PG, de Vos WM (1995) Medium-dependent regulation of proteinase gene expression in *Lactococcus lactis*: control of transcription initiation by specific dipeptides. J Bacteriol 177: 2982–2989

Marugg JD, van Kranenburg R, Laverman P, Rutten GAM, de Vos WM (1996) Identical transcriptional control of the divergently transcribed *prtP* and *prtM* genes that are required for proteinase production in *Lactococcus lactis* SK11. J Bacteriol 178:1525–1531

O'Sullivan DJ, Klaenhammer TR (1993) Rapid mini-prep isolation of high-quality plasmid DNA from *Lactococcus* and *Lactobacillus* ssp. Appl Environ Microbiol 59:2730–2733

Platteeuw C, Simons G, de Vos WM (1994) Use of the *Escherichia coli* β-glucuronidase (*gusA*) gene as a reporter gene for analysing promoters in lactic acid bacteria. Appl Environ Microbiol 60:587–593

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-termination. Proc Natl Acad Sci USA 74:5463–5467

Terzaghi BE, Sandine WE (1975) Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol 29:807–813

Van der Vosen JMBM, Kodde J, Haandrikman AJ, Venema G, Kok J (1992) Characterisation of transcription initiation and termination signals of the proteinase genes of *Lactococcus lactis* Wg2 and enchancement of proteolysis in *L. lactis*. Appl Environ Microbiol 58:3142–3149

Vos P, Simons G, Siezen R, de Vos WM (1989a) Primary structure and organization of the gene for a prokaryotic, cell envelope-located serine proteinase. J Biol Chem 264:13579–13585

Vos P, van Asseldonk M, van Jeveren F, Siezen R, Simons G, de Vos WM (1989b) A maturation protein is essential for production of *Lactococcus lactis* SK11 serine proteinase located in or secreted from the cell envelope. J Bacteriol 171:2795–2802