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Proteinase Overproduction in *Lactococcus lactis* Strains: Regulation and Effect on Growth and Acidification in Milk

PAUL G. BRUINENBERG, PIETER VOS,† AND WILLEM M. DE VOS*

Molecular Genetics Group, Department of Biophysical Chemistry, Netherlands Institute for Dairy Research (NIZO), P.O. Box 20, 6710 BA Ede, The Netherlands

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Multicopy plasmids that contained the complete or 3′-deleted forms of the proteinase (*prtP*) gene of *Lactococcus lactis* subsp. *cremoris* SK11 under the control of different promoters were constructed and introduced into Prt⁻ lactococcal strains. The production and location of the SK11 proteinase was determined in different hosts grown in industrial and laboratory media. In spite of the 10-fold-higher copy number of the *prt* genes, no overproduction of proteinase was observed in strain SK1128, a Prt⁻ derivative of *L. lactis* subsp. *cremoris* SK112. In contrast, an approximately threefold overproduction of the cell envelope-located or fully secreted proteinase was found in strain MG1820 compared with that of its parental strain *L. lactis* subsp. *lactis* SH4109. In all strains proteinase production appeared to be regulated by the medium composition. Highest proteinase production of the SK11 derivatives was found in milk, in contrast to derivatives of SH4109 that produced most proteinase in whey permeate medium. Analysis of single strains with different levels of proteinase production or mixed cultures containing various ratios of Prt⁺ and Prt⁻ cells indicated that the amount of proteinase produced per cell or culture determines the specific growth rate in milk. Overproduction of cell envelope-located or secreted proteinase in strain MG1820 resulted in a 20%-higher specific growth and acidification rate in milk compared with that in the wild-type strain SH4109. These results indicate that the growth of lactococci in milk is limited by the caseinolytic activity of the proteinase.

Lactococci are gram-positive bacteria used as starters in a variety of dairy fermentation processes. These fastidious bacteria have a complex proteolytic system to break down milk proteins into small peptides and free amino acids that are used as a nitrogen source during growth of the cells in milk (28). This proteolytic system consists of a cell envelope-associated serine proteinase and several intra- and extracellular peptidases (19, 28). The serine proteinase is a key enzyme in this proteolytic system, which is essential for rapid growth of the lactococcal cells in milk, and produces casein peptides that contribute to flavor development in fermented milk products (32).

Genetic studies have shown that two genes, *prtP* and *prtM*, are required for the production of the active serine proteinase (for reviews, see references 6 and 19). The structural *prtP* gene encodes the proteinase precursor that has homology to the subtilisin family of serine proteases, while *prtM* codes for a lipoprotein involved in the maturation of the proteinase precursor (19, 35). Because of the presence of a C-terminal membrane anchor, the proteinase is normally located at the cell envelope of lactococci (3, 34). Proteinases of several strains show a high degree of genetic, biochemical, and immunological heterogeneity (4, 19) and may be divided into type I and type III proteinases on the basis of their caseinolytic specificity (31). Comparisons of the deduced amino acid sequences of those proteinases (19, 34) and analysis of the specificities of hybrid type I-type III proteinases have shown that specific binding and cleavage of caseins is determined by only a few amino acid residues (33).

The production of the lactococcal proteinase has been studied in several strains of *L. lactis* subsp. *cremoris* (9, 10, 16, 17). For strain AM1 it appeared that the synthesis of proteinase was repressed when the growth medium contained Casitone (10), an enzymatic digest of casein containing mainly peptides, whereas the accumulation of proteinase activity increased with the Ca^{2+} concentration of the medium (9). Strains AM1, E8, and Wg2 were found to produce more proteinase in milk than in laboratory media (17). This is particularly relevant since the growth of those and other lactococcal strains in milk was found to be limited by casein (16). The strains used in these studies contained proteinases with distinct caseinolytic specificities (31) encoded by different *prt* genes (3, 19), thus impeding a careful comparison of the control of proteinase production and its effect on growth rate in different strains.

The genes for the type III proteinase of the cheese-making strain *L. lactis* subsp. *cremoris* SK11 have been characterized and expressed in several lactococcal strains (6). This has allowed a further analysis of the location (3, 35), expression (8), and caseinolytic specificity (3, 8, 33) of this lactococcal proteinase. Here we describe the use of genetic methods in analyzing the control of the SK11 proteinase production and its specific contribution to the growth rate of lactococci in milk. The results show that the production of the SK11 proteinase and its regulation is strain and medium dependent. In addition, it appears that overproduction of cell envelope-bound or secreted SK11 proteinase results in a higher growth and acidification rate in milk. The application of lactococci with improved proteolytic capacity may provide an opportunity for accelerating the process of cheese production and ripening.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *L. lactis* strains and plasmids used in this study are listed in Table 1. A schematic representation of proteinase plasmids

* Corresponding author.
† Present address: Keygene, 6700 AE Wageningen, The Netherlands.

TABLE 1. Bacterial strains and plasmids[a]

Strain or plasmid	Relevant properties	Reference
Strains		
L. lactis subsp. *lactis*		
SH4109	Lac$^+$ Prt$^+$ multiplasmid strain	13
MG1363	Lac$^-$ Prt$^-$ plasmid derivative of NCDO 712	13
MG1820	Lac$^+$ derivative of MG1363 harboring the lactose miniplasmid pMG820	22
L. lactis subsp. *cremoris*		
SK112	Lac$^+$ Prt$^+$ multiplasmid strain	7
SK1128	Lac$^+$ Prt$^-$ derivative of SK112	7
Plasmids		
pNZ122	Cm^r Km^r, 2.8 kb, pSH71 replicon	3
pNZ19	Cm^r Km^r, 5.7 kb, pSH71 replicon	29
pPR31	Ap^r, 8.3 kb, ColE1 replicon containing the complete SK11 *prtP* gene	34
pNZ399	Ap^r Tc^r, 2.95 kb, pUC18 containing part of the MG1820 *lac* operon promoter region	30
pNZ521	Cm^r Km^r, pNZ122 carrying the complete *prtP* and the *prtM* gene of pSK111	8
pNZ521ΔH	Cm^r Km^r, pNZ521 lacking a 16-bp *Hin*dIII fragment of the *prtP* gene	This work
pNZ511	Cm^r Km^r, pNZ521 lacking 402 3′ codons and 3′-untranslated region of the *prtP* gene	8
pNZ592	Cm^r Km^r, 11.6 kb, pNZ19 containing the *prtP* gene under control of the *lac* promoter	This work
pNZ582	Em^r, 6.2 kb, pIL253 containing the *prtM* gene (lacking four 3′ codons)	35

[a] The capacity to utilize lactose (Lac$^+$) or produce proteinase (Prt$^+$) is indicated. Cm^r, Km^r, Em^r, Tc^r, and Ap^r indicate resistance to chloramphenicol, kanamycin, erythromycin, tetracycline, and ampicillin, respectively.

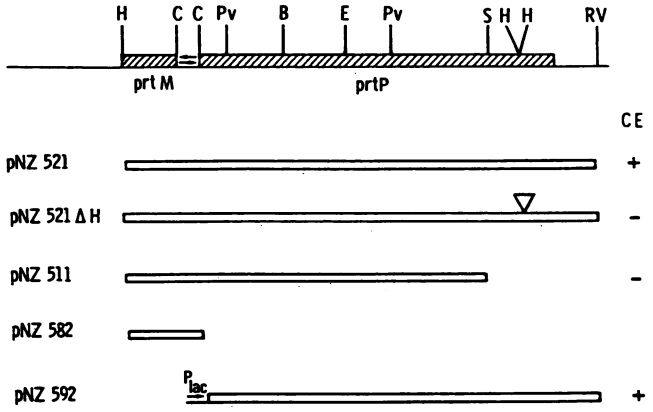

FIG. 1. Schematic representation of proteinase plasmids. At the top is shown the region encoding the *prtP* and the *prtM* genes. Symbols: ▨, coding sequences; ⇆, promoters for both genes (34, 35); □, cloned DNA fragments of the proteinase region in the various plasmids; P_{lac}, part of the promoter region of the *lac* operon of strain MG1820. The location of the proteinase is indicated at the right side (+, a location at the cell envelope; −, proteinase is secreted in the culture medium). Abbreviations for restriction enzymes are as follows: B, *Bam*HI; E, *Eco*RI; C, *Cla*I; H, *Hin*dIII; Pv, *Pvu*II; RV, *Eco*RV; S, *Sal*I.

is shown in Fig. 1. *L. lactis* strains were grown in lactose-M17 broth (E. Merck AG, Darmstadt, Germany) unless otherwise stated. For the assay of proteinase production *L. lactis* cells were grown in 10% (wt/vol) pasteurized, reconstituted skim milk or in whey permeate medium (8) containing 1.9% (wt/vol) β-glycerophosphate and 0.1% (wt/vol) Casitone (Difco Laboratories, Detroit, Mich.) after precultivation in the same media. *Escherichia coli* MC1061 (2) was grown in L broth-based media (25). If appropriate, the media contained chloramphenicol (10 μg/ml), erythromycin (5 μg/ml), ampicillin (50 μg/ml), or 0.5% (wt/vol) glucose.

DNA methodology. Isolation of plasmid DNA from *L. lactis* and transformation of *L. lactis* strains was performed as described previously (8, 35). Isolation of DNA from *E. coli* and standard recombinant DNA techniques were performed according to established procedures (25). The copy number of recombinant proteinase plasmids relative to that of the wild-type proteinase plasmids pLP712 and pSK111 in *L. lactis* subspp. *lactis* and *cremoris* strains (see Table 2) was estimated by hybridization. Plasmid DNA isolated from equal amounts of lactococcal cells (as determined by measuring the optical density at 600 nm) was digested with *Pvu*II, and aliquots were separated by agarose gel electrophoresis and blotted to GeneScreen Plus transfer membrane (Dupont, Boston, Mass.). The DNA was hybridized to a 1.3-kb *Bam*HI-*Eco*RI fragment of pNZ521 labelled with [α-^{32}P]dATP by nick translation. The proteinase plasmid copy number was estimated by quantifying the radioactivity in the 2.7-kb *Pvu*II fragment of the *prtP* gene. Nucleotide sequence analysis of double-stranded plasmid DNA was performed by the dideoxy chain termination method (26). Oligonucleotides were synthesized on a Cyclone DNA synthesizer (Biosearch, San Rafael, Calif.).

Construction of plasmids. Plasmid pNZ521ΔH was constructed by deletion of a 16-bp *Hin*dIII fragment from pNZ521 (Fig. 1), resulting in a frameshift mutation in the C-terminal part of the coding region of the *prtP* gene (34).

A 5.9-kb *Xba*I-*Xho*I fragment from pPR31, containing the structural *prtP* gene, was cloned into the *Xba*I-*Sal*I site of the lactococcal plasmid pNZ19 to yield pLB14. Plasmid pBX5 was constructed by inserting a 18-bp double-stranded *Bss*HII-*Asu*II-*Sca*I-*Xba*I adapter fragment with the sequence 5′-CGCGCTTCGAAAGTACTT-3′ into the *Bss*HII-*Xba*I site of pLB14. Plasmid pNZ399 consists of a 0.35-kb *Ssp*I fragment, containing part of the promoter region of the *lac* operon of *L. lactis* MG1820 (5, 29) inserted in the *Sma*I site of pUC18 in the same orientation as its *lacZ′* gene (30). A 350-bp *Xba*I-*Eco*RI (filled in with Klenow DNA polymerase) fragment from pNZ399 was cloned into the *Xba*I-*Sca*I site of pBX5 to yield pNZ592. All constructs were verified by DNA sequence analysis of relevant regions.

Proteinase isolation and quantification. Lactococcal cells were grown to the mid-log growth phase (optical density at 600 nm = 0.9), and proteinase was released from the cell envelope by incubation in Ca^{2+}-free buffer (cell envelope release fraction) as described previously (12). Secreted proteinase was isolated from the culture medium by freeze-drying of dialyzed samples (8). Proteins were separated on 10% polyacrylamide gels (21) that were stained with Coomassie brilliant blue. Proteinase bands on sodium dodecyl sulfate (SDS)-polyacrylamide gels were quantified with a

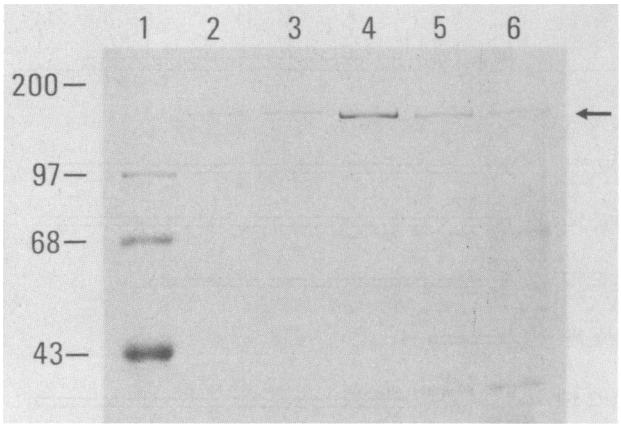

FIG. 2. SDS-polyacrylamide gel electrophoresis of cell envelope-released proteinases. Equal amounts of milk-grown cells of different *L. lactis* strains were harvested, and proteinase was released from the cell envelope and analyzed on 10% acrylamide gels. Molecular mass markers (in kilodaltons) are indicated to the left. The arrow indicates the position of SK11 proteinase. Lane 1, molecular mass markers; lane 2, purified SK11 proteinase; lane 3, strain SH4109; lane 4, strain MG1820 harboring pNZ521; lane 5, strain SK112; lane 6, strain SK1128 harboring pNZ521.

TABLE 2. Proteinase production of *L. lactis* strains harboring different proteinase plasmids

Strain	Proteinase plasmid	Approximate plasmid copy no.[a]	Proteinase production (±SD)[b]	
			Milk	Whey permeate
L. lactis subsp. *lactis*				
SH4109	pLP712	1	4.5 ± 1.3	3.7 ± 1.0
MG1820			—[c]	—
MG1820	pNZ521	10	12.8 ± 2.3	16.5 ± 2.7
MG1820	pNZ521ΔH	10	13.5 ± 2.9	20.9 ± 3.2
MG1820	pNZ511	10	3.1 ± 1.2	2.8 ± 1.5
L. lactis subsp. *cremoris*				
SK112	pSK111	1	6.8 ± 1.3	3.1 ± 0.8
SK1128			—	—
SK1128	pNZ521	10	5.7 ± 1.2	3.4 ± 1.0
SK1128	pNZ511	10	2.8 ± 0.8	1.5 ± 0.5

[a] Relative to pLP712 or pSK111.
[b] Entries are the averages of microdensitometer scans obtained in three independent experiments.
[c] —, no detectable signal.

microdensitometer TLC Scanner CS-900 (Shimadzu Corp., Kyoto, Japan) coupled to a Datamodule Integrator (Waters Associates Inc., Milford, Mass.). Proteinase production was also determined by measuring the proteolytic activity in proteinase fractions towards the chromophoric peptide methoxy-succinyl-arginyl-prolyl-tyrosyl-pNA (Kabi Diagnostica, Stockholm, Sweden) at high-ionic-strength conditions as previously described (11).

Determination of growth and acidification rates. The maximum specific growth rate (μ_{max}) values in pasteurized skim milk were determined by measuring the optical density at 600 nm of cultures clarified by a modified (23) EDTA-borate treatment (18). During cultivation the acidification of milk cultures was monitored with a pH meter.

RESULTS

Overproduction of the SK11 proteinase is host, plasmid, and medium dependent. Plasmid pNZ521 is a high-copy-number plasmid containing the *L. lactis* subsp. *cremoris* SK11 *prtP* and *prtM* genes and, in a manner similar to that of the naturally occurring proteinase plasmids, specifies a functional cell envelope-located proteinase (8) (Fig. 1). To investigate the effect of multiple copies of the SK11 *prt* genes in *L. lactis* subspp. *lactis* and *cremoris* hosts, we introduced pNZ521 into the Prt$^-$ strains MG1820 and SK1128 and compared the proteinase production in milk with that of the wild-type strains SH4109 and SK112 harboring the natural proteinase plasmids pLP712 (13) and pSK111 (7), respectively. The results (Fig. 2) showed an approximately threefold overproduction of proteinase in MG1820 harboring pNZ521 compared with that in strain SH4109 (lanes 4 and 3, respectively). This proteinase overproduction was accompanied by a similar increase in the proteinase activity determined with a chromophoric peptide as a substrate (11; data not shown). Since the proteinase activity values appeared to be less reproducible (especially in the case of secreted proteinase in milk [see below]), SDS-polyacrylamide gel electrophoresis of proteinase fractions was applied for quantifying proteinase production. Milk-grown cells of strain SK1128 harboring pNZ521 showed a level of proteinase production that was comparable to that of wild-type strain SK112 (Fig. 2, lanes 5 and 6). Similar results were obtained upon cultivation of these strains in whey permeate medium (Table 2). In addition, introduction of pNZ521ΔH that encodes a secreted proteinase (Fig. 1) (see below) into *L. lactis* MG1820 also resulted in proteinase overproduction (Table 2). Determination of the amount of *prt*-specific DNA indicated that the recombinant proteinase plasmids had an approximately tenfold-higher copy number than the wild-type proteinase plasmids in both *L. lactis* subspp. *lactis* and *cremoris* strains (Table 2). These results indicate that the production of proteinase is tightly controlled in *L. lactis* subsp. *cremoris* SK112 and its derivatives but not so in *L. lactis* subsp. *lactis* MG1820, in which host overproduction of cell envelope-located or fully secreted proteinase may be realized.

Previously, we have shown that introduction of plasmid pNZ511 that specifies a secreted proteinase (Fig. 1) in strains MG1363 and SK1128 resulted in a reduced level of proteinase production in milk-grown lactococcal cells compared with that in their corresponding wild-type strains (8). Cultivation in whey permeate medium of strain MG1820 and SK1128 harboring pNZ511 yielded similar results, despite the fact that plasmid pNZ511 is also present in an approximately tenfold-higher copy number compared with the wild-type proteinase plasmids (Table 2). Plasmid pNZ511 lacks the putative terminator of the *prtP* gene and encodes a C-terminally truncated proteinase (3, 34, 35). To determine the effect of the location of the proteinase on its production, we constructed plasmid pNZ521ΔH, which contains the 3′-untranslated region of the *prtP* gene but also encodes a truncated proteinase lacking the membrane anchoring domain (Fig. 1). Cells of MG1820 harboring pNZ521ΔH grown on milk and whey permeate medium showed an approximately threefold overproduction of secreted proteinase (Table 2), indicating that complete secretion of the SK11 proteinase does not affect its overproduction.

The production of proteinase by the *L. lactis* subspp.

TABLE 3. Maximum specific growth rates of mixed cultures of the strains MG1820 (proteinase deficient) and MG1820 harboring pNZ521 (proteinase proficient) in milk

Mixed culture (% Prt$^+$)	μ_{max} (h^{-1})[a]	% Prt$^+$ cells[b]
100	0.63	97
80	0.63	77
20	0.48	18
0	<0.06	0

[a] μ_{max} values are the average of two experiments (standard deviation, ±0.03).
[b] Values are derived from the average of counts from triplicate lactose-M17 agar plates with and without chloramphenicol (standard deviation, ±2). The percentage of proteinase-proficient cells at the late-exponential growth phase (optical density at 600 nm = 1.8) was calculated from the fraction of Cm^r cells in the mixed culture.

lactis and *cremoris* strains harboring wild-type or recombinant proteinase plasmids appeared to be dependent on the growth medium (Table 2). No synthesis of cell envelope-located or secreted proteinase could be detected when the lactococcal strains were grown in lactose-M17 broth (data not shown). Furthermore, strain SK112 and its Prt$^-$ derivative SK1128 harboring different proteinase plasmids showed the highest proteinase production in milk (Table 2). In contrast, the proteinase-overproducing MG1820 derivatives appeared to produce more proteinase in whey permeate medium than in milk.

Effects of proteinase production per culture on the growth in milk. To determine the contribution of cell envelope-located SK11 proteinase on the growth rate, mixed cultures of the strains MG1820 (Prt$^-$ Cm^s) and MG1820 carrying pNZ521 (Prt$^+$ Cm^r) were grown in milk, and the maximum specific growth rates were determined (Table 3). A culture inoculated with 80% Prt$^+$ cells showed no apparent difference of the μ_{max} in milk compared with that in a culture inoculated with 100% Prt$^+$ cells. However, a decrease in the μ_{max} of about 25% was observed when the inoculum contained only 20% Prt$^+$ cells. During these growth experiments the fraction of Prt$^+$ cells remained constant in all mixed cultures (Table 3). These results indicate that the amount of proteinase production in a culture determines the maximum specific growth rate of the mixed culture in milk.

Effects of proteinase production and location on growth and acidification rates in milk. We have also determined whether the amount of proteinase production per cell and its location affected the maximum specific growth rate in milk. Previously, we found that the caseinolytic specificity and activity of the cell envelope-bound and secreted SK11 proteinase were similar (6, 8). The results (Table 4) showed that cells of strain MG1820 harboring pNZ521 or pNZ521ΔH, overproducing a cell envelope-located or secreted proteinase, respectively (Fig. 1), had a 20%-higher μ_{max} in milk than that of the wild-type strain SH4109. In contrast, strain SK1128 harboring plasmid pNZ521, which did not overproduce the SK11 proteinase, showed a μ_{max} in milk identical to that of the wild-type strain SK112.

Reduced levels of proteinase production, found in MG1820 and SK1128 harboring plasmid pNZ511, resulted in a decrease of the μ_{max} of these lactococcal strains compared with that of their wild-type counterparts. To further substantiate this correlation between the level of proteinase production and growth rate in milk, we constructed plasmid pNZ592 containing the *prtP* gene under control of part of the *lac* promoter of pMG820 (5, 30). Because pNZ592 lacks the *prtM* gene, this plasmid was introduced into strain MG1363 harboring the compatible plasmid pNZ582 encoding the *prtM* gene (35). *L. lactis* cells containing both plasmids showed a tenfold-reduced level of proteinase production compared with that of wild-type strain SH4109 (not shown), resulting in a strongly reduced μ_{max} in milk (Table 3). Addition of 0.1% Casitone to the milk resulted for all *L. lactis* subspp. *lactis* and *cremoris* strains in μ_{max} values that equalled that of the wild-type strains (Table 3), indicating that the decrease in the maximum specific growth rate is due to limitation in the capacity to produce proteinase.

TABLE 4. Maximum specific growth rates of lactococcal strains harboring different or no proteinase plasmids

Strain[a]	Proteinase plasmid	μ_{max} (h^{-1})[b]	
		Milk	Milk + Casitone
L. lactis subsp. *lactis*			
SH4109	pLP712	0.53	0.69
MG1820		<0.06	0.67
MG1820	pNZ521	0.63	0.68
MG1820	pNZ521ΔH	0.63	0.69
MG1820	pNZ511	0.46	0.70
MG1363	pNZ592 + pNZ582	0.19	0.71
L. lactis subsp. *cremoris*			
SK112	pSK111	0.41	0.43
SK1128		<0.05	0.43
SK1128	pNZ521	0.41	0.45
SK1128	pNZ511	0.35	0.43

[a] Strains were grown in milk or milk with 0.1% (wt/vol) Casitone. The MG1363 derivative was grown in milk containing 0.5% (wt/vol) glucose.
[b] μ_{max} values are the averages of four experiments (standard deviation, ±0.03).

The amount of proteinase production in lactococcal cells appeared to affect also the rate of acidification in milk cultures (Fig. 3) and was found to be highest for strain MG1820 harboring plasmid pNZ521. The proteinase overproduction in this strain resulted in coagulation of milk after 5 3/4 h of cultivation instead of 6 1/4 h for wild-type strain SH4109. In contrast, MG1820 harboring pNZ511 showed a reduced rate of acidification compared with that of the wild-type strain SH4109. However, upon prolonged cultivation of these proteinase-proficient strains, the final pH and cell number of the milk-grown cultures were the same (data not shown). Thus, these results indicate that the amount of proteinase produced per cell, irrespective of its location, determines the growth and acidification rates of lactococci in milk.

DISCUSSION

To analyze the regulation of the SK11 proteinase production and its specific contribution to the growth rate of lactococci in milk, we have constructed multicopy plasmids that upon introduction into lactose-fermenting, Prt$^-$ derivatives of *L. lactis* subspp. *lactis* and *cremoris* strains result in the production of different levels of cell envelope-located or secreted SK11 proteinase.

Regulation of proteinase production in lactococci. Introduction of the multicopy proteinase plasmid pNZ521, containing the complete SK11 *prtP* and *prtM* genes, into *L. lactis* subsp. *lactis* MG1820 or *L. lactis* subsp. *cremoris* SK1128

resulted in different levels of proteinase production and a distinct dependence on the growth medium (Fig. 2; Table 2). A threefold increase in proteinase production was observed in *L. lactis* MG1820 harboring pNZ521. Proteinase overproduction was independent on its location since similar production levels were found in strain MG1820 harboring pNZ521ΔH, which specifies a secreted proteinase. In contrast, no overproduction of the SK11 proteinase was observed when plasmid pNZ521 was introduced in strain SK1128. It also appeared that proteinase production in strain SK1128 harboring different proteinase plasmids, unlike in strain MG1820, was reduced in whey permeate medium that contains Casitone (Table 2). This resembles the end product inhibition of proteinase production described for *L. lactis* subsp. *cremoris* AM1 (10). Since strains AM1 and SK112 are phage-related strains harboring indistinguishable proteinase plasmids (3), it is very well possible that the type III proteinase that is produced by both strains (31) is subject to the same type of regulation.

Plasmids pNZ511 and pNZ521ΔH contain a deletion and frame-shift mutation in the 3′ end of the *prtP* gene, respectively, and both specify a C-terminally truncated, secreted proteinase that lacks the membrane anchor (34). The size, specific activity, and caseinolytic specificity of the secreted proteinase is the same as that of the normally cell envelope-bound wild-type SK11 proteinase (6, 8), and both are subject to C-terminal processing (35). The main difference between pNZ511 and pNZ521ΔH is the presence of the putative *prtP* terminator in the latter one (34). *L. lactis* MG1820 harboring plasmid pNZ511 showed a lower level of proteinase production than MG1820 harboring pNZ521ΔH, in spite of the same high copy number of the plasmids (Table 2). It is possible that the absence of the putative terminator affects the stability of the *prtP* mRNA. Alternatively, the transcriptional elongation of the SK11 *prtP* mRNA with sequences encoded by the vector part of pNZ511 may influence its structure and, subsequently, affect translation initiation. Examples of both types of control have been well documented in other bacteria (14, 24, 27).

It has previously been found that the medium composition determines the proteinase production in wild-type *L. lactis* subsp. *cremoris* strains (17). Here we show that this regulation is also observed in *L. lactis* subsp. *lactis* SH4109 derivatives and is independent of the proteinase plasmid present (Table 2). Unexpectedly, the same proteinase plasmids in *L. lactis* subsp. *cremoris* SK1128 resulted in the production of a differently controlled proteinase production that was highest in milk and not in whey permeate medium (Table 2). This suggests that a specific host function is involved in the tight control in strain SK11. All *prt* genes analyzed so far contain a region of extensive dyad symmetry in the overlapping promoter region of the divergently transcribed *prtP* and *prtM* genes (19). It is tempting to speculate that this structure is involved in *prt* gene expression by functioning as a target for the specific host factor(s) involved in the regulation of proteinase production. Direct and inverted repeats are frequently found in the 5′ flanking sequences of prokaryotic genes and often represent recognition sequences of transcription factors (1, 15). In this respect, it is interesting to note that the promoter region of the SK11 *prt* genes contains an inverted repeat that is 5 bp larger (34) than that of the *prt* genes in *L. lactis* subsp. *cremoris* WG2 (20), a strain that produces a less regulated proteinase (17).

As a consequence of the tightly controlled proteinase production in the SK11 derivatives when grown in the industrial whey permeate medium, strains other than the original host, such as an overproducing MG1820 strain harboring pNZ521 or pNZ521ΔH, should be used for the production of the type III proteinase.

Proteinase production limits growth of lactococci in milk. The contribution of SK11 proteinase production on the growth rate of *L. lactis* MG1820 derivatives in milk was determined in two ways. First, the production levels per culture were varied by mixing different amounts of strain MG1820 (Prt^- Cm^s) and the proteinase-overproducing strain MG1820 harboring pNZ521 (Prt^+ Cm^r) (Table 3). By using the differential resistance to chloramphenicol of these strains, the ratios of Prt^+ and Prt^- cells could be easily monitored during growth in milk and were found to be similar to the initial ratios, indicating the stability of the system. Since use was made of a proteinase-overproducing strain, the growth rate of 20% Prt^+ culture almost equalled that of the wild-type strain SH4109 in milk (cf. Tables 3 and 4). A significant increase of the growth rate was observed when the proteinase production per culture was increased fourfold, as in the culture containing 80% Prt^+ cells. Similar growth rates were found in cultures containing 80% Prt^+ or 100% Prt^+ cells (Table 3), indicating that further increasing the amount of the SK11 proteinase in the culture has no effect on its growth rate in milk. These results demonstrate that the proteinase production per culture is limiting the growth rate of strain MG1820 in milk.

In the second system, the effect of varying the location and amount of proteinase production per cell on the growth rate of pure cultures of MG1820 derivatives in milk was determined (Table 4). Overproduction of the cell envelope-located (pNZ521-encoded) or fully secreted (pNZ521ΔH-encoded) proteinase in MG1820 resulted in an increase of approximately 20% in maximum specific growth rate in milk (Table 4). These results demonstrate that the maximum specific growth rate of the lactococcal cells is not affected by the location of the proteinase. In addition, these data indicate that the growth rate in milk is affected by the amount of proteinase produced per cell. These conclusions are supported by the reduced growth rates in milk that are observed with lactococcal cells harboring the proteinase plasmids pNZ511 or pNZ592, which produce even less (secreted or cell envelope-bound) proteinase per cell than the wild-type strain. Moreover, the correlation between proteinase production level and growth rate in milk is not strain dependent since it was also found in strain SK1128 harboring different proteinase plasmids (Table 4), although proteinase production is subject to tight control in this host. Since all recombinant plasmids specify the same type III proteinase, it is ruled out that the differences in specific growth rate are a consequence of the production of different casein breakdown products that support growth.

An important consequence of the proteinase overproduction is the observation that a faster acidification can be realized during milk fermentations (Fig. 3). Proteinase-overproducing *L. lactis* MG1820 harboring pNZ521 clots milk more than 30 min faster than the other strains, indicating that the amount of proteinase produced determines both the maximum specific growth rate and the rate of acidification in milk.

The results reported here are consistent with those of previous investigations, indicating that casein hydrolysis proceeds too slowly for lactococci to reach their maximum specific growth rates in milk and, consequently, leads to an amino acid limitation during growth (16). Addition of an amino acid mixture or extra casein to the milk resulted in an

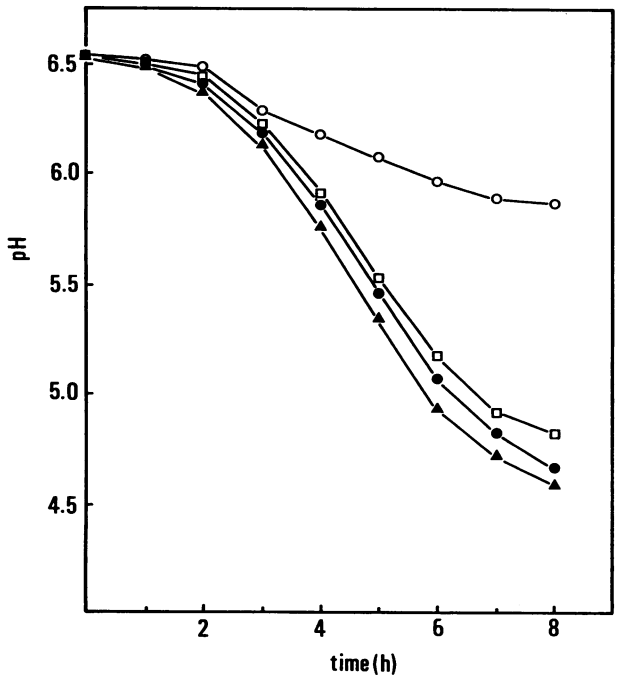

FIG. 3. Rate of acidification during cultivation in milk-based media of *L. lactis* strains harboring no or different proteinase plasmids. All cultures were inoculated at identical cell densities. Symbols: ○, strain MG1820; □, strain MG1820 harboring pNZ511; ●, strain SH4109; ▲, strain MG1820 harboring pNZ521.

increase of the maximum specific growth rates, and it was proposed that the growth rate of lactococci in milk could be increased by proteinase overproduction. The present data provide experimental evidence for this suggestion and demonstrate that the growth and acidification rate in milk may be increased by overproducing a cell envelope-bound or secreted proteinase. The application of lactococci with enhanced proteolytic capacity may provide an opportunity for accelerating both the process of cheese making and the process of cheese ripening.

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REFERENCES

1. **Beck, C. F., and R. A. J. Warren.** 1988. Divergent promoters, a common form of gene organization. Microbiol. Rev. **52**:318–326.
2. **Casadaban, M. J., J. Chou, and S. N. Cohen.** 1980. In vitro fusions that join an enzymatically active β-galactosidase segment to aminoterminal fragments of exogenous proteins in *Escherichia coli*: plasmid vectors for the detection of translation signals. J. Bacteriol. **143**:971–980.
3. **de Vos, W. M.** 1986. Genetic improvement of starter streptococci by the cloning and expression of the gene encoding a non-bitter proteinase, p. 465–471. *In* E. Magnien (ed.), Biomolecular engineering in the European community. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.
4. **de Vos, W. M.** 1987. Gene cloning and expression in lactic streptococci. FEMS Microbiol. Rev. **46**:281–295.
5. **de Vos, W. M., I. Boerrigter, R. J. van Rooyen, B. Reiche, and W. Hengstenberg.** 1990. Characterization of the lactose-specific enzymes of the phosphotransferase system in *Lactococcus lactis*. J. Biol. Chem. **265**:22554–22560.
6. **de Vos, W. M., I. Boerrigter, P. Vos, P. Bruinenberg, and R. J. Siezen.** 1991. Production, processing, and engineering of the *Lactococcus lactis* SK11 proteinase, p. 115–119. *In* G. M. Dunny, P. P. Cleary, and L. L. McKay (ed.), Genetics and molecular biology of streptococci, lactococci and enterococci. American Society for Microbiology, Washington, D.C.
7. **de Vos, W. M., and F. L. Davies.** 1984. Plasmid DNA in lactic streptococci: bacteriophage resistance and proteinase plasmids in *S. cremoris* SK11, p. 201–205. *In* Third European Congress of Biotechnology, vol. III. Verlag Chemie, Weinheim, Germany.
8. **de Vos, W. M., P. Vos, H. de Haard, and I. Boerrigter.** 1989. Cloning and expression of the *Lactococcus lactis* subsp. *cremoris* SK11 gene encoding an extracellular serine proteinase. Gene **85**:169–176.
9. **Exterkate, F. A.** 1979. Accumulation of proteinase in the cell wall of *Streptococcus cremoris* strain AM1 and regulation of its production. Arch. Microbiol. **120**:247–254.
10. **Exterkate, F. A.** 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.
11. **Exterkate, F. A.** 1990. Differences in short peptide-substrate cleavage by two cell-envelope-located serine proteinases of *Lactococcus lactis* subsp. *cremoris* are related to secondary binding specificity. Appl. Microbiol. Biotechnol. **33**:401–406.
12. **Exterkate, F. A., and G. J. C. M. de Veer.** 1985. Partial isolation of and degradation of caseins by cell wall proteinases of *Streptococcus cremoris* HP. Appl. Environ. Microbiol. **49**:328–332.
13. **Gasson, M. J.** 1983. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. J. Bacteriol. **154**:1–9.
14. **Gualerzi, C. O., and C. L. Pon.** 1990. Initiation of mRNA translation in prokaryotes. Biochemistry **25**:5581–5589.
15. **Harrison, S. C., and A. K. Aggarwal.** 1990. DNA recognition by proteins with a helix-turn-helix motif. Annu. Rev. Biochem. **59**:933–969.
16. **Hugenholtz, J., M. Dijkstra, and H. Veldkamp.** 1987. Amino acid limited growth of starter cultures in milk. FEMS Microbiol. Ecol. **45**:191–198.
17. **Hugenholtz, J., H. Veldkamp, and W. N. Konings.** 1985. Detection of specific strains and variants of *Streptococcus cremoris* in mixed cultures by immunofluorescence. Appl. Environ. Microbiol. **53**:149–155.
18. **Kanasaki, M., S. Breheny, A. J. Hillier, and G. R. Jago.** 1975. A rapid method for the estimation of bacterial populations in milk. Aust. J. Dairy Technol. **30**:142–144.
19. **Kok, J.** 1990. Genetics of the proteolytic system of lactic acid bacteria. FEMS Microbiol. Rev. **87**:15–42.
20. **Kok, J., C. J. Leenhouts, A. J. Haandrikman, A. M. Ledeboer, and G. Venema.** 1988. Nucleotide sequence of the gene for the cell wall bound proteinase of *Streptococcus cremoris* WG2. Appl. Environ. Microbiol. **54**:231–238.
21. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **277**:680–685.
22. **Maeda, S., and M. J. Gasson.** 1986. Cloning, expression and location of the *Streptococcus lactis* gene for phospho-β-D-galactosidase. J. Gen. Microbiol. **132**:331–340.
23. **Otto, R.** 1981. An ecophysiological study of starter streptococci. Ph.D. thesis, University of Groningen, The Netherlands.
24. **Platt, T.** 1986. Transcription termination and the regulation of gene expression. Annu. Rev. Biochem. **55**:339–372.
25. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Sanger, F., S. Nicklen, and R. Coulson.** 1977. DNA sequencing

with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
27. **Schmeissner, S., K. McKenney, M. Rosenberg, and D. Court.** 1984. Removal of a terminator structure by RNA processing regulates the gene expression. J. Mol. Biol. **176**:39–53.
28. **Thomas, T. D., and G. G. Pritchard.** 1987. Proteolytic enzymes of dairy starter cultures. FEMS Microbiol. Rev. **46**:245–268.
29. **van Rooijen, R. J., and W. M. de Vos.** 1990. Molecular cloning, transcriptional analysis, and nucleotide sequence of *lacR*, a gene encoding the repressor of the lactose phosphotransferase system of *Lactococcus lactis*. J. Biol. Chem. **265**:18499–18503.
30. **van Rooijen, R. J., M. J. Gasson, and W. M. de Vos.** Submitted for publication.
31. **Visser, S., F. A. Exterkate, C. J. Slangen, and J. C. M. de Veer.** 1986. Comparative study of action of cell wall proteinases from various strains of *Streptococcus cremoris* on bovine α_{S1}-, β-, and κ-casein. Appl. Environ. Microbiol. **52**:1162–1166.
32. **Visser, S., G. Hup, F. A. Exterkate, and J. Stadhouders.** 1983. Bitter flavour in cheese. 2. Model studies on the formation and degradation of bitter peptides by proteolytic enzymes from calf rennet, starter cells and starter cell fractions Neth. Milk Dairy J. **37**:169–180.
33. **Vos, P., I. J. Boerrigter, G. Buist, A. J. Haandrikman, M. Nijhuis, M. B. de Reuver, R. J. Siezen, G. Venema, W. M. de Vos, and J. Kok.** 1991. Engineering of the *Lactococcus lactis* serine proteinase by construction of hybrid enzymes. Protein Eng. **4**:479–484.
34. **Vos, P., G. Simons, R. J. Siezen, and W. M. de Vos.** 1989. Primary structure and organization of the gene for a prokaryotic, cell envelope-located serine proteinase. J. Biol. Chem. **264**:13579–13585.
35. **Vos, P., M. van Asseldonk, F. van Jeveren, R. J. Siezen, G. Simons, and W. M. de Vos.** 1989. A maturation protein is essential for production of active forms of *Lactococcus lactis* SK11 serine proteinase located in or secreted from the cell envelope. J. Bacteriol. **171**:2795–2802.