Expression and release of proteolytic enzymes of *Lactococcus lactis*

- Ripening of UF-cheese -

Promotoren: dr.ir. P. Walstra
emeritus hoogleraar in de zuivelkunde

dr. W.M. de Vos
hoogleraar in de algemene microbiologie

Co-promotor: dr. J. Hugenholtz
projectleider Afdeling Microbiologie, NIZO

WILLEM CORNELIS MEIJER

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PROEFSCHRIFT

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Stellingen

1. Bereiding van kaas uit geültrafiltreerde melk, met alle karakteristieken van Goudse kaas, is onmogelijk.

   - Dit proefschrift

2. Wetenschappelijke conclusies over de groei en het metabolisme van melkzuurbacteriën, gebaseerd op (hogere of lagere) groeisnelheden in melk, moeten met de nodige scepsis bekeken worden.

   - Hoofdstuk 2 van dit proefschrift.

3. De snelheid waarmee de extracellulaire actieve serineproteinase autoproteolyse ondergaat tijdens kweekexperimenten, is een variabele die van wezenlijk belang is voor de interpretatie van de gegevens verkregen uit deze experimenten.

   - **Laan, H., H. Bolhuis, B. Poolman, T. Abee, and W.N. Konings.** 1993. Regulation of proteinase synthesis in *Lactococcus lactis*. Acta Biotechnol. 13:95-101.
   - Hoofdstuk 4 van dit proefschrift.

4. Het kwantificeren van lysis van zuurselbacteriën gedurende de rijping van kaas is onmogelijk zolang er geen methode voorhanden is waarmee lysis *in situ* gevolgd kan worden.

   - Hoofdstuk 6 van dit proefschrift.

5. Exopolysacchariden in roeryoghurt bepalen niet direct de viscositeit van de yoghurt.

   - **Smith, M.R., and F. Kingma.** 1995. The formation of exopolysaccharide by yoghurt bacteria, abstr. JEP 64. *In* Abstracts of the 7th European Congress on Biotechnology 1995. European Federation of Biotechnology, Nice, France.

6. Als bier volgens de classificatie van Nursten kan worden geschaard onder de groep levensmiddelenproducten waarvan de smaak onmogelijk is toe te schrijven aan de gezamenlijke aromacomponenten, hoort Goudse kaas zeker in deze categorie.

   - **Strating, J., and B.W. Drost.** 1987. Limits of beer flavour analysis. *In* G. Charalambous (ed.), Frontiers of flavour. Proceedings of the 5th International Flavour Conference, Porto Karras, Chalkidiki, Greece.
   - **Nursten, H.E.** 1977. The important volatile flavour components of foods. *In* G.G. Birch, J.G. Brennan and K.J. Parker (eds.), Sensory properties of foods. Applied Science Publishers, London.

7. Een goede maatschappelijk werk(st)er zou er naar moeten streven zijn/haar vakgebied overbodig te maken.

8. Voor het behoud van zijn geestelijke gezondheid zou iemand met een lichamelijke handicap zijn handicap nooit voor 100% moeten accepteren.

9. De vrees van de alternatieve konsumentenbond voor allergie veroorzaakt door soortvreemde eiwitten in genetisch gemodificeerde soja is gebaseerd op het principe 'wat de boer niet kent dat eet hij niet'.

   - **Langner, B.** 1996. Hoe veilig is veilig? Kritisch Consumeren **6**:16-17.

10. De kleur oranje in het stoplicht werkt op menige automobilist als een rode lap op een stier.

Stellingen behorende bij het proefschrift "Expression and release of proteolytic enzymes of *Lactococcus lactis* -Ripening of UF-cheese-".

Wilco Meijer
Ede, 19 februari 1997.

Hast thou not poured me out as milk,
and curdled me like cheese?

(Job 10:10)

Aan pa en ma

Voorwoord

Buiten is het nat en guur. De winter breekt weer aan. Binnen is het behaaglijk warm. De atmosfeer is zwanger van een zwoele Zwitsal geur. De tijd is aangebroken voor de waarschijnlijk meest gelezen pagina van dit proefschrift, die tegelijkertijd de minst wetenschappelijke pagina is. Ik had niet verwacht dat het vinden van de juiste bewoordingen voor deze pagina ook zo moeilijk zou kunnen zijn. Waar te beginnen en waar te eindigen?

Allereerst wil ik Jeroen, mijn co-promotor, bedanken voor al zijn hulp. Jeroen, zonder jou was dit proefschrift nooit afgekomen. Je wist altijd weer op de juiste momenten, die wetenschappelijke inzichten op te hoesten die mij voldoende moed gaven om verder te gaan. Naarmate ik je beter leerde kennen heeft onze samenwerking op het lab zich zelfs uitgebreid tot het voetbalzaaltje. Ik hoop dat we met name van het eerste nog lang mogen genieten. Ook mijn twee promotoren, Pieter en Willem, wil ik bedanken voor hun inzet. Pieter, jij was mijn promotor vanaf het eerste uur. Bedankt voor alle wetenschappelijke sturing, met name op die momenten dat ik weer eens te ver van de uiteindelijke doelstelling van het project afdwaalde. Je hebt geprobeerd van mij zowel een microbioloog als een zuiveltechnoloog te maken. Willem, je begeleiding begon helaas een beetje laat. Ik heb me echter gesteund gevoeld door het vertrouwen dat je in me stelde. Joey, de samenwerking met jou is van onschatbare waarde geweest. Dankzij jou werden mijn verhalen pas goed leesbaar. Ad, bedankt voor de wetenschappelijke discussies, maar niet minder bedankt voor alle relativerende opmerkingen. Met name aan onze verschillende buitenlandse congressen heb ik dankzij jou goede herinneringen.

Ook alle leden van de UF-werkgroep wil ik bedanken voor hun suggesties tijdens de vele vergaderingen. Carla, onze projecten zijn enigszins uit elkaar gedreven. Onze samenwerking heeft mijn 'microbiologische' horizon echter aanmerkelijk verbreed.

Het in dit proefschrift beschreven werk had ik zonder de geweldige inzet van verschillende studenten nooit kunnen uitvoeren. Margo, Annika, Annemarie, Fera, Ellen, Connie en Bert ben ik hiervoor bijzonder erkentelijk.

Mijn werk begon in één van de kelders van NIZO. Monique en Marja, nadat we elkaar hadden leren kennen (wat wel een klein jaar heeft gekost) waren jullie de beste collega's die ik me kon voorstellen. Ik heb dagelijks genoten, en doe dat gelukkig nog, van jullie aanwezigheid. Bedankt voor alles! Lucia, Jacqueline en later ook Bert, jullie waren gezellige buren. Marjo, met jouw inspiratie bleek elk opdoemend probleem tijdens de vele fermentaties oplosbaar. Ton, ik heb je initiatieven voor de start van dit project gewaardeerd. Gerrit, het was goed om met jou samen te werken. Ik hoop dat er nog enkele vruchtbare jaren volgen. Ook alle andere microbiologen wil ik hartelijk danken voor hun support, werksfeer, vele belangstellende gesprekken en al wat dies meer zij. De groep is te groot geworden om allen bij naam te noemen, maar iedereen moet zich aangesproken voelen.

Ook wil ik alle overige medewerkers en ex-medewerkers van NIZO bedanken voor hun collegiale werksfeer. De directie, voor de gelegenheid die ze mij bood meer dan vier jaar op NIZO mijn onderzoek te mogen verrichten; de operators van de Technologiehal, voor het

maken van al die 'gekookte kaasjes'; al diegenen die geholpen hebben bij het uitvoeren van de vele verschillende analyses; de medewerkers van de afdeling Documentatie & Services voor hun zeer klantgerichte houding; en de fotografen, voor het eindeloos vergroten en verkleinen van allerlei fotootjes.

Lieve pa en ma, het is ook jullie verdienste dat ik dit boekje heb kunnen schrijven. Al vanaf de kleuterschool hebben jullie mij gestimuleerd. Bedankt voor jullie betrokkenheid en steun. Ook mijn zusjes en zwager wil ik bedanken voor hun constante interesse in mijn werk, alhoewel ze het eigenlijk niet konden volgen. Paulien bedankt voor alle taalkundige correcties.

Bram, bedankt dat je zo lang bij je moeder bent blijven zitten. Dit gaf me net voldoende tijd om het proefschrift af te ronden.

Tot slot, Neelie, je was er altijd als ik weer eens later van het werk thuis kwam. Bedankt voor je constante aanmoediging. Het is een voorrecht om samen met jou te leven.

Wilco

Ede, december 1996

Contents

1

General Introduction

General Introduction

Cheese is the most important fermented milk product from an economical point of view. The Dutch dairy industry has manufactured approximately 690,000 tons of cheese in 1995, for which 55% of the total milk production is used. Two thirds of this is Gouda cheese. The use of ultrafiltration technologies in cheese manufacturing has potential economic advantages. They hold considerable promise as a means of improving cheese yield and efficiency of production, as well as reducing the expenditures for manufacturing equipment and ingredients, as rennet. However, production of semi-hard cheese via ultrafiltration has not been used extensively because of the poor (organoleptic) quality of the cheese. A prerequisite to improve the quality of cheese made from ultrafiltered milk is basic knowledge about all the parameters related to cheese ripening in general. This chapter discusses several microbiological aspects related to cheese maturation in general, and to the specific problems encountered during ripening of cheese made from ultrafiltered milk.

The production of Gouda cheese is schematically demonstrated in Fig. 1. The unripened cheeses are ripened at 13°C for several weeks. During maturation the organoleptic score is expressed by the terms 'young', 'mature' and 'old'. After 6 weeks of maturation the cheese is 'young', after 3 months 'mature', and after half a year the cheese is 'old'. The pH of the cheese is around 5.7-5.9 after 4 h from the start of manufacture, which is approximately during pressing, 5.3-5.5 after 5.5 h and finally around 5.2 in the mature cheese. The starter bacteria are mechanically included in the curd to around 10^8 cfu/g cheese, where they grow to 10^9 cfu/g cheese, which mean that they divide only a few times during manufacturing (see for a detailed review reference 110). For the manufacturing of Gouda cheese a mesophilic, mixed-strain starter culture is used. This starter culture is composed of *Lactococcus lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, *L. lactis* subsp. *lactis* biovar. *diacetylactis* and different *Leuconostoc* species (23, 79). From these strains *L. lactis* subsp. *cremoris* is the most important in quantity.

Essential during cheese ripening is the rapid conversion of lactose into lactic acid by *L. lactis*. However, these bacteria are auxotrophic for several amino acids (50, 105), which limits the growth of *L. lactis* in milk. These bacteria depend on the activity of various proteolytic enzymes that break down the milk protein extracellular into mainly oligopeptides, and subsequently, after transport into the cell by the oligopeptide transport system, into amino acids that are essential for growth (see below for a more detailed overview). During cheese manufacturing primary proteolysis of the casein is for the most part due to the action of the added rennet to the cheese milk. The secondary proteolysis of these casein-derived peptides into small peptides and amino acids is due to the proteolytic enzymes of the starter. These small peptides and amino acids contribute either themselve, or after conversion to volatile flavour compounds, to the organoleptic changes in the fermented milk product (53,

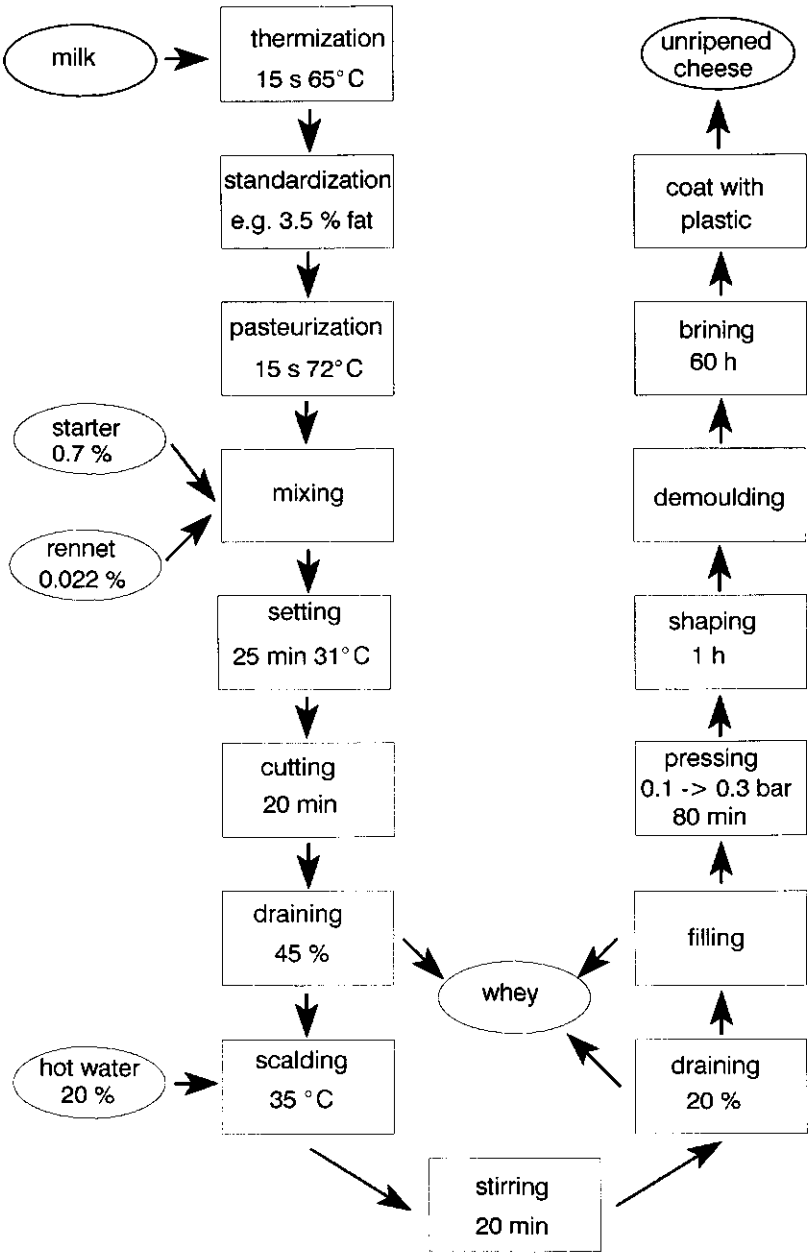

Figure 1. Example of a flow-sheet for the manufacture of 12 kg Gouda cheese by a modern method. Time from start of cutting to start of filling: 60 min. NaCl content of brine: 18%, brine temperature: 14°C. Taken, with some modifications, from Walstra et al. (110).

79, 109). In the young, unripened cheese, the starter cells are deenergized due to depletion of lactose. Active transport of larger peptides into the cell will be drastically reduced. Therefore, it is generally assumed that in the later phase of cheese manufacturing lysis of the starter bacteria results in release of the intracellular peptidases into the curd which guarantee on-going flavour development in the fermented milk product (5, 6, 19) (see discussion below).

The Proteolytic System of *L. lactis*

The extracellular proteinase. The biochemical, genetic and immunological properties of the extracellular serine proteinase (PrtP) in *L. lactis* have been studied extensively (28, 45, 53) and reviewed excellently (54, 84, 87, 104, 106, 109).

The first step in protein degradation is catalysed by the PrtP, which is part of the serine proteases of the subtilisin family. The two divergently transcribed genes, *prtP* and *prtM*, both required for the production of an active proteinase, are generally plasmid located in *L. lactis*. PrtP is synthesized as a pre-pro-protein, in which the structural gene *prtP* encodes a pre-protein with a size of approximately 200 kDa, which directs its own secretion. The *prtM* gene encodes a maturation protein with a size of 33 kDa, which is involved in a proteolytic step that leads to proteinase activation. This results in a mature proteinase of approximately 180 kDa (53, 54).

The proteinase can be partially or completely released from the cell envelope by resuspending the cells in a Ca^{2+}-free buffer (77). The release is most probably the result of an autoproteolytic breakdown as a result of a conformational change induced by the removal of calcium (59, 73).

However, little attention has been paid to the regulation of PrtP synthesis. The first publication dealing with the regulated production of PrtP was in *L. lactis* strain AM1, in which the repression of PrtP by relative low levels of an enzymatic digest of casein was described, while free amino acids did not affect PrtP production (27). Immunological studies showed that strain *L. lactis* AM1 and E8 produces more proteinase in milk than in nitrogen-rich laboratory media (47). For PrtP of *L. lactis* SK110 it was demonstrated, by making use of multicopy plasmids containing the *prtP* gene, that PrtP production is strain and medium dependent. Highest production was found in milk medium, while lowest production was observed in whey-permeate which was enriched with high levels of Casitone, a pancreatic digest of casein containing mainly peptides (8). Currently, it has been found that the production of PrtP and two intracellular peptidases is stringently controlled by the peptide composition of the medium (73, 68, 69) (see Chapters 3 and 4).

Recently, the products resulting from the breakdown of β-casein by PrtP of *L. lactis* Wg2, have been analysed thoroughly by making use of the sensitive detection technique of HPLC in combination with mass spectrometry (51). It was observed that PrtP activity

resulted in the formation of more than 100 different oligopeptides, whereas a significant release of di- and tripeptides could not be observed. The size of the oligopeptides was such that uptake via the oligopeptide transport system of *L. lactis* could be possible (107), which supports the hypothesis that the only proteolytic activity outside the *L. lactis* cell is the PrtP activity.

PrtP activity is necessary for rapid growth of the bacteria in milk and for its subsequent acidification. Moreover hydrolysis of the milk protein into oligopeptides, which are subsequently hydrolysed into amino acids which serve as precursors for flavour, is an essential step in ripening of cheese (81). The importance of the PrtP in flavour development is illustrated by the fact that cheeses manufactured with Prt^- starter cultures appeared to have little or no flavour (29, 100).

The peptide transport systems. Initial casein degradation (by PrtP) occurs extracellularly. Subsequent degradation, however, is catalysed by the intracellularly localized peptidases (102). For efficient utilization of casein by the lactic acid bacteria, peptide transport systems are essential. The peptide transport systems that have been biochemically characterized are: (i) a proton motive force-driven di-tripeptide carrier (DtpT) (40, 57, 96) with a specificity for relatively hydrophilic di-tripeptides, (ii) another energy-driven di-tripeptide transport system (DtpP) (35) with a specificity for more hydrophobic substrates, and (iii) an ATP-driven oligopeptide transport system (Opp) with a specificity for peptides of 4 to at least 8 residues (57, 107).

An interesting aspect is the specific role of the peptide transport system during growth of *L. lactis* in milk. For several reasons activity of the Opp transport system is suggested to be responsible for the internal accumulation of casein-derived peptides: (i) analysis of the extracellular casein-derived peptides hydrolysed by activity of PrtP, indicates that specific peptides are only metabolized when the cell has an active Opp transport system (51); (ii) inactivation of the Opp transport system results in the inability to accumulate amino acids, while a DtpT transport mutant did not show this defect (58); and (iii) Opp transport-deficient strains of *L. lactis* grow poorly in milk, while di-tripeptide transport-deficient mutants grow exactly like the wild-type (52, 58).

Since the DtpT transport system does not play a direct role in growth of *L. lactis* in milk it is postulated that DtpT is involved in the utilization of milk proteins other than β-casein (52). Another possibility is that the DtpT transport system has a signal function for high extracellular dipeptide concentrations by which the lactococcal cell can control its production of the proteolytic enzymes (see Chapters 3 and 4).

The intracellular peptidases. The oligopeptides, generated by PrtP activity and transported by the Opp transport system, are subsequently hydrolysed by the activity of a large variety of peptidases. Approximately 13 different peptidases have been purified and characterized in detail from *L. lactis* (see for detailed description reference 54). Relevant for

this study are the aminopeptidase N (PepN) and the X-prolyl-dipeptidyl aminopeptidase (PepXP). These aminopeptidases need a free N-terminus in their substrate and cleave off either one or two amino acid residues. By cell fractionation studies and immunogold-labelling it was established that PepN and PepXP are both intracellular (30, 102).

The role of the various peptidases in vivo is not yet elucidated. To study the function of the various peptidases, the growth in milk of mutants lacking from one up to five peptidase activities was studied (55, 72, 74, 75, 76, 107). These mutants were still able to grow in amino acid-containing media, indicating that inactivating the peptidases is not lethal for *L. lactis*. It was observed that the growth rate of the cells in milk was gradually decreased with an increase of the amount of peptidase mutations, being up to 10 times slower than for the wild type (74, 76). This indicates that most peptides can be hydrolysed by concerted action of the peptidases, which due to their overlapping substrate specificities, can replace each other to some extent.

The specific role of the various peptidases in cheese ripening has also not been elucidated so far. It was observed that PepN activity had in vitro a debittering effect on a casein hydrolysate (103). In vivo it was observed that cheese manufactured with a PepN deficient starter was very bitter, compared to cheese made with the wild-type starter. Cheeses manufactured with a PepXP deficient variant exhibited a slower flavour development compared to the wild-type (2). On the other hand cheeses manufactured with an overproducing PepN strain did not accelerate flavour development in the cheese (12). This suggests that the peptidases play an important, but very specific role in flavour development of cheese.

Lysis

Autolysis. One of the definitions of autolysis is :"the lytic event which is caused by action of the cell's own intracellular mureinases" (101). The mureinases are enzymes capable of hydrolysing the peptidoglycan of the cell wall. Peptidoglycan consists of a repeating unit of N-acetylglucosamine-β-(1-4)-N-acetylmuramic acid. The glycan strands are cross-linked via tetrapeptide bridges attached to the N-acetylmuramic acid residues (91). The first studied mureinase of *L. lactis* subsp. *cremoris* had the specificity of an endo-N-acetylmuramidase, while no amidase or endopeptidase activity was observed (78). The muramidase hydrolyses the linkage between N-acetylmuramic acid and N-acetylglucosamine. Recently, several lactococcal strains were screened for bacteriolytic activity against *Micrococcus luteus* cells, using electrophoretic profiles on SDS-PAGE gels (82). The authors suggested the presence of at least two different autolytic enzymes involved in autolysis of lactococci, a glycosidase and either a N-acetylmuramyl-L-alanine amidase or an endopeptidase. Maximal autolytic activity was found during the exponential phase of growth at pH values of 6.5-7.5 at 30-45°C (78, 80), which is related to the general tasks of the autolytic enzymes: cell division (11, 86),

including synthesis and remodelling of the cell wall (85, 93), gene transformation (88) and sporulation (for reviews see references 25, 89, 111). Recently, the gene encoding for the major peptidoglycan hydrolase of *L. lactis* was identified. The enzyme was suggested to be an endo-N-acetylmuramidase. Growth of cells of a deletion mutant in long chains, suggested that the hydrolase activity plays an essential role in cell separation (7). Despite the great importance of the autolytic system for the growing cell, nothing is known about regulation of this system in lactococci.

Autolysis of various lactococci has been studied in aqueous systems. Autolysis was observed to be dependent on the growth rate. The use of glucose instead of lactose as a carbon source resulted in an accelerated growth and autolysis rates (108). Comparing lactococcal strains in the stationary phase of growth, strains of *L. lactis* subsp. *lactis* were more autolytic than strains of *L. lactis* subsp. *cremoris* (61). However, comparing lactococcal strains in the late-log phase of growth, strains of *L. lactis* subsp. *lactis* were more resistant to lysozyme treatment than cells of strain *L. lactis* subsp. *cremoris*, which suggests the reverse for their cell wall stability (18).

Autolysis in cheese. The role of autolysis in ripening of cheese has been suggested for the first time by Hansen (41). Lysis was essential for the change in starter composition during ripening of cheese, which seemed to be related to flavour development in cheese. The effect of lysis on the release of intracellular proteolytic enzymes into the cheese matrix and thereby on the ripening of cheese was proposed later (5, 6, 63). Addition of lysozyme-treated starter cells to the cheese milk resulted in a higher level of free amino acids during cheese ripening, due to lysis of the starter culture (62). The main problem with studies on autolysis in cheese is to quantify lysis of the starter in cheese. Therefore, a suitable intracellular marker is needed, of which the extracellular presence should reflect the percentage of lysis of the starter culture. The extraction procedure of such a marker should not induce more lysis of the starter cells, to prevent an overestimation of the lysis in the cheese. The decrease in viable count of the starter culture, the release of cell-bound DNA in cheese (5, 6), and the release fractions of different intracellular marker enzymes have been used to monitor autolysis in cheese (63, 112). Recently, autolysis of two *Lactococcus* strains has been monitored by immunological and enzymatic quantification of the release of two intracellular peptidases during manufacturing of Saint-Paulin type of cheese. Highest release was observed in *L. lactis* AM2, which correlated with a rapid decrease in cell viability, compared to the rather low release by the stable cells of strain *L. lactis* NCDO763. High release of the intracellular peptidolytic enzymes was also reflected in a relatively high amino nitrogen content in the cheese (10). Still, the exact role of autolysis in cheese has not been completely elucidated. A reliable interpretation of the results is difficult, since in this study the effect of autolysis on ripening of cheese is investigated by using two different strains. It has recently been suggested that a balance of autolysed and intact cells of lactic acid bacteria is important for the desired cheese ripening events (19). This highlights the fact that the

mechanism and consequences of autolysis in cheese require further investigation.

Induced lysis. Several methods to increase the fraction of lysed cells in the cheese environment have been proposed: selection of strains with a higher susceptibility to autolysis (10, 61), treating the starter culture with lysozyme (62), using high pressure techniques during ripening of the cheese (114), use of high cooking temperatures (113), addition of phages to the starter culture (67), and induction of prophages in lysogenic strains (31, 32). Lysogenic strains harbour the genome of a virulent phage on their chromosome, which makes the bacterial cell immune for infection with its own phage. The, so called, prophage can be induced spontaneously or actively, resulting in amplification of the virulent phage, and subsequently, in lysis of the host strain (Fig. 2). Feirtag and McKay demonstrated that lysis was induced in cells of *L. lactis* subsp. *cremoris* SK11 by ultraviolet radiation, mitomycin C and by a shift in the growth temperature from 30 to 40°C for 2.5 h (31). This indicates that strain SK11 probably contains a prophage. Since the incidence of lysogeny is widespread in strains of species *Lactococcus* (22, 49, 83), it can be speculated that part of the lysis during cheese ripening is a result of the spontaneous induction of prophages in the starter cultures.

Differentiation of phages of lactic acid bacteria (49), the various phage resistance mechanisms employed by the host strain (44) and the organization and control of the genes involved in lysis induced by the known bacteriophages (115) have been extensively reviewed.

A successful use of phage-assisted lysis in ripening of Cheddar cheese has been shown recently (21). The use of different phage levels in cheese milk resulted in different degrees of lysis of the starter. Increased release of intracellular markers reflected the increased level of phage added. Elevated starter lysis was associated with an increased production of free amino acids and a decreased level of bitter flavour in the cheese.

So far, elevated lysis of the starter culture during cheese manufacturing results in higher amounts of free amino acids and reduced bitterness of the product. No significant effect has been observed on flavour development in general till now.

Lysis in relation to the cell wall structure. Despite the great interest in lysis of lactococci, very little attention has been focused on the characteristics of the barrier which has to be broken down, i.e. the peptidoglycan (Fig. 3). The first studies of the peptidoglycan structure (see before) of *L. lactis* were carried out by Kandler and Schleifer (90, 91, 92). The amino acids composing the tetrapeptide cross bridges between the glycan strains are well conserved in lactococci. The tetrapeptide cross-link usually consists of L-Ala-D-Glu-X-D-Ala. The third amino acid is species-specific, e.g. ornithine such as in *Corynebacterium*, an isomer of diaminopimellic acid such as in *E. coli*, or, as in *L. lactis*, L-lysine. In the interpeptide bridge the L-lysine of one tetrapeptide and the D-alanine of the other tetrapeptide are involved. Other components of the lactococcal cell wall are polysaccharides, lipoteichoic acid and proteins, which are surrounding the peptidoglycan structure. The polysaccharides

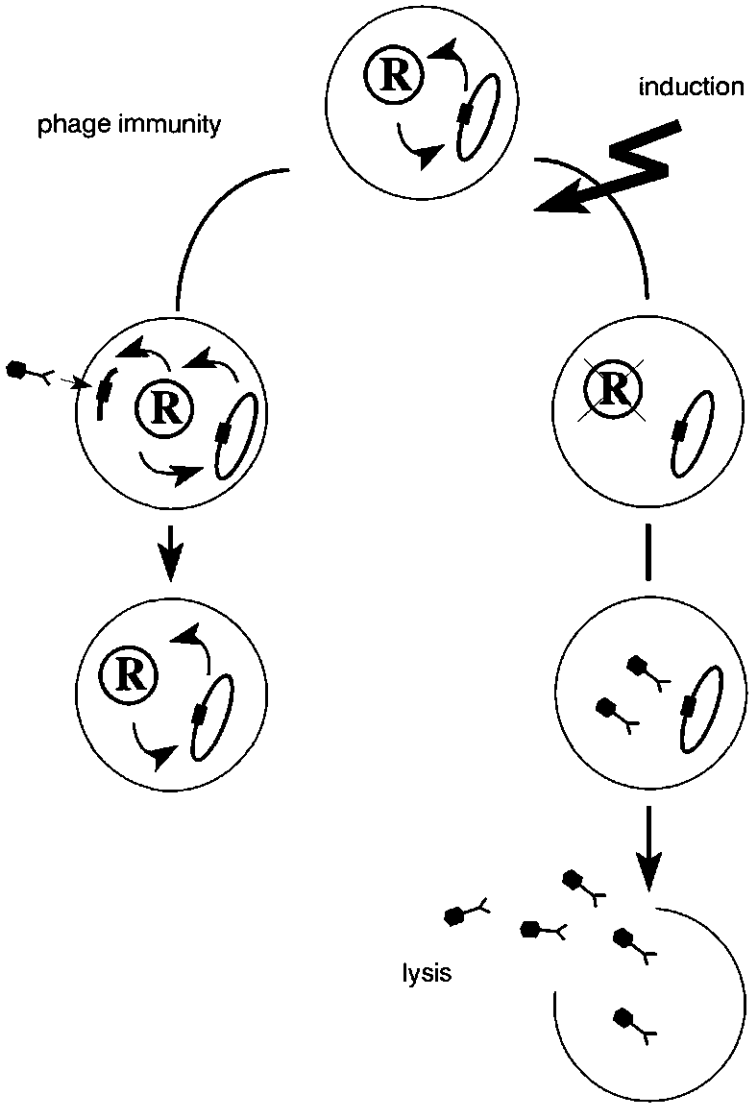

Figure 2. Schematic representation of the process of phage immunity of, and prophage induction in a lysogenic strain. The prophage can be induced spontaneously, or via a temperature, UV-light or chemical mutagenesis treatment. R = repressor molecule.

are composed of rhamnose, glucose, galactose and glucosamine (14, 91). The lipoteichoic acid consists of a linear polymer of 16-40 phosphodiester-linked glycerophosphate residues covalently linked to a lipid moiety in the membrane (34, 95). Substantial amounts of proteins are associated with the cell wall of gram-positive microorganisms (1), such as the

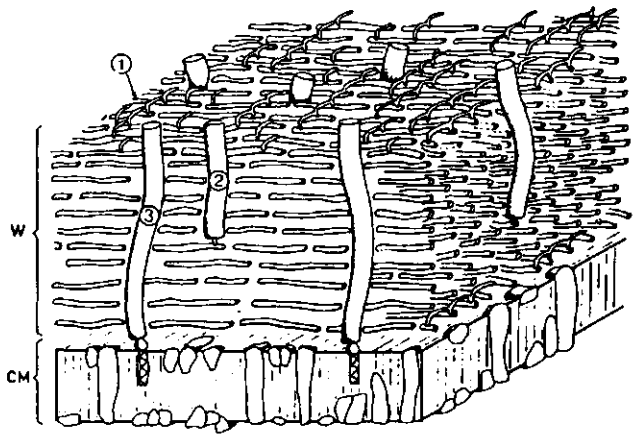

Figure 3. Schematic representation of the cell envelope of a gram-positive bacterium. The wall (W) essentially consists of a thick, multilayered peptidoglycan structure (1) and of anionic polysaccharides (2) and wall teichoic acids (2) which are covalently linked to glycan strands of peptidoglycan. Lipoteichoic acids (3) are anchored in the cytoplasmic membrane. Taken from Sijtsma (94).

extracellular proteinase.

The molecular architecture of the cell wall has been directly related to bacteriophage-resistance and absorption mechanisms (36, 37, 44, 94). Recently, the differences in cell surface among 15 lactococcal strains and their phage-resistant phenotypes have been compared, in order to clarify the cell surface diversity and the relationship between cell surface characteristics and bacteriophage-resistance. Considerable variation was observed in the amount of loosely associated cell surface material, intra and extracellular lipoteichoic acid concentrations and the extracted protein profiles within all the studied lactococcal strains. The variations in cell wall composition found in the 15 phage-resistant variants showed no clear similarity, which indicates the absence of a general phage-resistant, cell wall located, mechanism (20).

Up till now, no direct relation between peptidoglycan composition, which composes 90% of the cell wall of gram-positive microorganisms, and sensitivity to lysis of the lactococcal cell has been reported.

Cheese Manufactured by Ultrafiltration

In the Netherlands the production of cheese is a highly efficient bulk process. However, still approximately 20% of the proteins in milk, the so-called serum proteins, are not

included into the cheese. Incorporation of the whey proteins, normally lost in the whey stream, is for several reasons very attractive: (i) the yield of cheese is increased, and (ii) the financial return is greater when whey proteins are incorporated into cheese rather than converting the whey proteins into functional ingredients for the food industry. Different methods to introduce the whey proteins into the cheese have been described, as the addition of heat-denaturated whey proteins, heat treatment of the cheese milk and ultrafiltration.

Principle of ultrafiltration. Ultrafiltration (UF) is a membrane-separation process, schematically outlined in Fig. 1. Via UF of milk a product can be obtained, called the retentate, which has an increased content of protein, milk fat globules and undissolved salts compared to the initial milk. The permeate is almost completely rid of these milk components. The concentration of lactose, mineral salts and non-protein nitrogen in the water phase in the retentate is comparable to the concentration in the initial milk (26, 39).

UF-cheese manufacturing. The concept of cheese manufacturing from milk concentrated by UF was introduced 25 years ago by Maubois and Mocquot (70). UF technology can be applied in three different ways in the production of semi-hard cheese: (i) limited, at most twofold, concentration followed by the traditional cheese making process for semi-hard cheese, (ii) 4-6 times concentration plus some whey drainage, and (iii) full concentration, including final evaporation of the water, without whey drainage. In the last mentioned way the cheese yield is increased with approximately 17%, which is due to the absence of whey drainage, and subsequently the wash-out of whey proteins (64, 66).

60% of the incorporated whey proteins in UF-cheese is β-lactoglobulin plus α-lactalbumin (64). The rest of the whey protein fraction consists of bovine serum albumin, immunoglobulins, proteose peptone and the caseinomacropeptide split off κ-casein. It is difficult to assess the proportion of denatured whey proteins in UF-cheese. Even the mild temperature during UF of 50°C and an operating time of 2 h, can give rise to some denaturation of whey protein (33). Whey protein denaturation may occur by the incorporation of air during the UF process and, if relevant, by diafiltration, which lowers the protective effect of lactose in the retentates (97) (for a thorough review about the consequences of whey proteins in cheese see references 48, 65).

A logical consequence of the increased protein and mineral content of the UF-retentate is its higher buffering capacity. During UF-cheese manufacturing relative large amounts of lactic acid must be produced to decrease the pH to 5.2, which is the normal pH in standard cheese (16, 56). Selection of highly active starters or higher inoculation sizes of the starter can overcome this problem. However, the growth characteristics and the ability of lactic acid bacteria to develop acid in UF-retentates varies considerably (13, 43, 99).

Ripening of UF-cheese. The first attempts to produce UF-cheese suggested that the ripening and thereby the flavour development of UF-cheese were comparable with

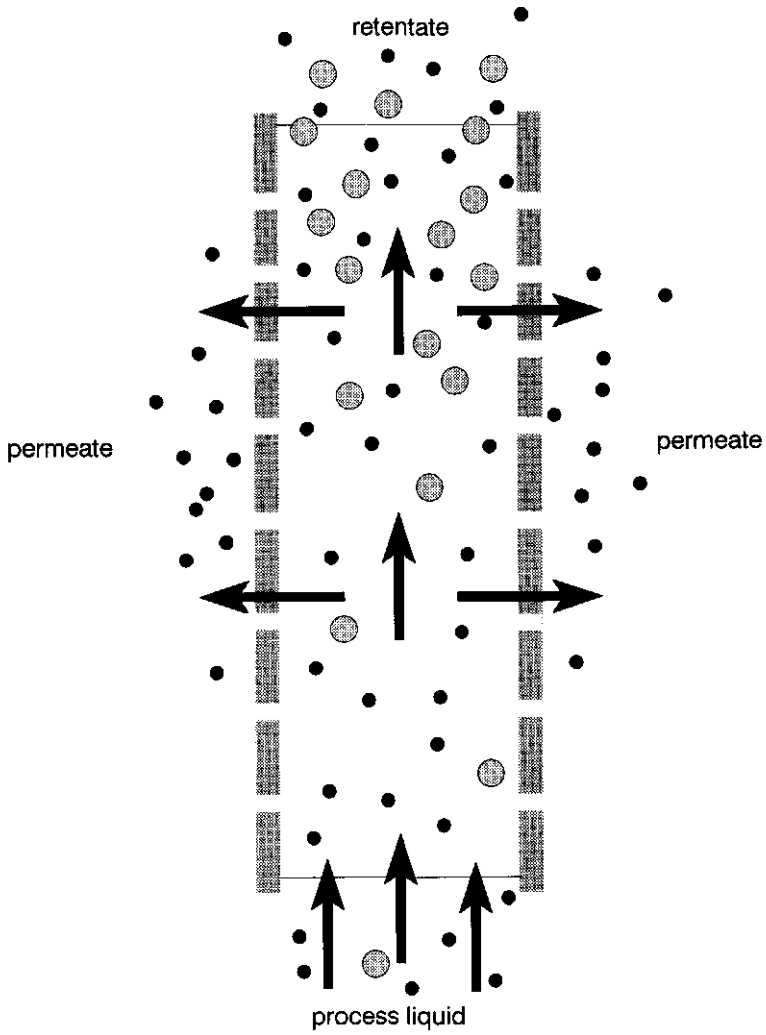

Figure 4. Schematically drawn principle of ultrafiltration.

traditionally manufactured cheese (70, 71). However, at present the procedure is used commercially for only a limited number of cheese types (56, 64). This is mainly caused by the encountered difficulties in texture (15) and flavour development in UF-cheese manufactured from more than fivefold concentrated milk.

It was observed that the increase in soluble N (15, 43, 98) and, thereby, the degradation

of α_{s1}- and β-caseins (24, 38) was much slower during ripening of UF-cheese than in traditionally manufactured cheese. It is known that in the primary hydrolysis of casein, α_{s1}-casein is degraded by chymosin while β-casein is degraded by plasmin, and also chymosin and PrtP activity. The proteolytic system of the starter bacteria is mainly responsible for the secondary hydrolysis of the casein.

By varying the amount of rennet in traditional and UF-cheese it was observed that more residual chymosin is required in UF-cheese than in the traditional cheese to obtain the same casein degradation (9, 17, 24, 38, 98). It has been suggested that the chymosin activity is inhibited by some whey protein component (17, 42). It was clearly demonstrated that hydrolysis of β-casein, catalysed by e.g. plasmin activity, is specifically inhibited by the presence of native β-lactoglobulin (4). This was supported by the observation that the addition to UF-cheese of urokinase, which is an activator for plasmin activity, resulted in an acceleration of β-casein breakdown, suggesting that plasmin activity is the limiting factor in the casein hydrolysis in UF-cheese (3) (for a thorough review of UF-cheese ripening see references 4, 48, 64, 66).

In contrast to the many studies dealing with primary hydrolysis of milk casein in UF-cheese, very little is known about proteolysis of the casein-derived peptides by the proteolytic enzymes of the starter culture in UF-cheese. The influence of the starter amount has been investigated in relation to the ripening of UF-cheese. In one study texture and flavour scores were increased with increasing amount of starter culture used during cheese manufacture (4).

Outline of the Thesis

The technology of cheese manufacturing from milk concentrated by ultrafiltration has potential benefits. One of the benefits, compared to the traditionally produced cheese, is the incorporation of all or part of the whey proteins in the cheese. However, this technology is hardly used for production of semi-hard cheeses, because of the unacceptable organoleptic quality of UF-cheese.

The aim of the work described in this thesis is to unravel different microbiological factors related to cheese ripening in general, and more specifically, factors which are responsible for the poor flavour development of UF-cheese. In *Chapter 2* it is described how the growth of *L. lactis* is affected by cultivation in UF-retentates of various concentration factors. As mentioned before, the proteolytic system of *L. lactis* plays a major role in the degradation of the milk protein into amino acids and thereby in the generation of flavour components. It has been reported that proteinase synthesis in *L. lactis* is dependent on the growth rate of the culture (60) and the medium composition (47). Since growth of *L. lactis* is negatively affected by growth in UF-retentate, it may be speculated that the production level of PrtP, and of the intracellular peptidases is affected by using UF-retentate as the cultivation medium. *Chapter 3* describes the transcriptional regulation of the proteinase gene

expression and *Chapter 4* the control of proteinase and peptidase production during growth of *L. lactis* on various standardized growth media. More specifically, in *Chapter 5* the effect of growth of *L. lactis* cells in UF-retentate on the controlled production of different proteolytic enzymes is described. The studied parameters are the concentration factor of the retentate and the heat treatment of the retentate during the UF procedure.

Flavour development in cheese is not only dependent on the production level of the different proteolytic enzymes, but also on the availability of the extracellular substrates for the intracellular peptidases. Therefore, lysis has been suggested to play a crucial role in cheese ripening. It has been observed that the growth rate of cells of lactococci influences their morphology during growth. Longer cell chains were found at low growth rates, compared to shorter cell chains observed at high growth rates (46), which can be explained by a changed autolytic activity. Therefore, we investigated the relationship between poor growth of lactococci, as observed in UF-retentates, the sensitivity for lysis and the flavour development in cheese. *Chapter 6* describes the effect of the growth rate of *L. lactis* cells on the susceptibility of these cells to lysis. By making use of cells of strain SK110, which demonstrate a typical thermolytic response curve after induction, the relation between lysis and cheese ripening was further corroborated. In *Chapter 7* it is described how the resistance to lysis of the lactococcal cell can affect the debittering capacity of the strain during cheese ripening. Analysis of the lactococcal cell wall established that the sensitivity of the cell to lysis is directly determined by the structure of the peptidoglycan.

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2

Determination of Growth Parameters of Lactococci in Milk and Ultrafiltered Milk

Wilco Meijer, Margo Tacken, Ad Noomen and Jeroen Hugenholtz

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Determination of Growth Parameters of Lactococci in Milk and Ultrafiltered Milk

Fresh skim milk and a highly standardized skim milk powder (Nilac) were ultrafiltrated at 50°C to concentration factors of approximately 2, 2.5, 3.5, and 4.5, based on total protein. Growth characteristics of *Lactococcus lactis* subsp. *cremoris* E8 and the mesophilic mixed-strain Bos starter culture were investigated in the UF-retentates and non-UF skim milk under conditions of regulated pH. The maximum specific growth rate and the maximum specific acidification rate showed extreme variation and, therefore, could not be used to measure the effect of UF on the growth characteristics of *L. lactis* subsp. *cremoris*. The mean total growth of strain E8 and the Bos mixed-strain starter after 22 h decreased by 25 and 40%, respectively, in UF-retentate with a concentration factor of 3.6 compared with growth reached in regular skim milk. Part of this effect could be explained as growth inhibition from the higher concentration of whey proteins in the UF-retentates.

Ultrafiltration is increasingly used in the dairy industry to concentrate the milk proteins (casein and whey proteins) and minerals in dairy products (13). By use of UF, skim milk can be concentrated to a retentate with a composition similar to that of drained soft cheese. An economic advantage of this process, compared with traditionally made soft cheeses, is the incorporation of whey proteins into the cheese, which increases yield by up to 20% (10). Sutherland and Jameson (16) used a similar technique to make hard cheese. They demonstrated that concentrated retentates obtained by UF can be converted into Cheddar cheese using a fairly conventional Cheddar manufacturing process.

Despite the potential economic advantages, the production of semi-hard and hard cheeses from more than twofold concentrated milk is not used extensively in practice because of several biophysical and microbiological difficulties: less acceptable flavour characteristics, problems with consistency (4), and poor growth of the starter bacteria in UF-milk. The reports dealing with the effects of the concentration factor of the milk on the growth and activity of lactic acid bacteria are somewhat contradictory. Some (3) indicate a stimulating effect, but others (12) report an inhibitory effect on growth.

The microbiological problems of making semi-hard cheese from UF-retentates and the conflicting nature of the literature prompted us to undertake this study. Our objectives were to select parameters that were appropriate to define the growth of lactic acid bacteria in milk and to study the growth of *Lactococcus lactis* subsp. *cremoris* E8 and the Bos mixed-strain

starter in UF-retentates compared with growth in milk.

Materials and Methods

Microorganisms. Lactococcus lactis subsp. *cremoris* strain E8, a proteolytic strain, and the Bos mixed-strain starter culture were used. Strain E8 was routinely stored in litmus milk with $CaCO_3$ and 0.5% yeast extract, and the Bos starter was maintained as a starter concentrate (15). Both were kept at -40°C.

Preparation of Milk Retentate. The UF-retentate was prepared from skim milk or Nilac milk powder [a highly standardized low heat spray powder; Netherlands Institute for Dairy Research (NIZO), Ede, The Netherlands]. The milk was ultrafiltered to a concentration factor of approximately 5:1 according to the method reported by Sutherland and Jameson (16). The one-stage UF system contained a spirally wound membrane (Abcor S4, HFK 131 VSV; KOCH membrane Systems Inc., Wilmington, DE) with a total surface of 11 m^2 and a cutoff of 5 kDa. The temperature during the UF process was 50°C, and the inlet and outlet pressures were approximately 500 and 100 kPa, respectively. The maximum volume flow rate over the membrane was 8 m^3/h. The retentate was used to prepare subsequent dilutions, with permeate, for use in the experiments. The non-UF skim milk and reconstituted Nilac milk powder were also kept at 50°C during the UF process.

Preparation of the Whey Protein Concentrate. The whey protein concentrate (WPC) was prepared from mixed cheese whey. The mixed cheese whey was acidified with HCl to a pH of 3.0 and concentrated by a three-stage UF system. Each UF stage contained a spirally wound membrane (Abcor S4, HFK 131 VSV). The total membrane surface of the three-stage UF system was 135 m^2. The UF conditions were the same as during the preparation of the milk retentate. After UF, the retentate was neutralized with 33% NaOH to pH 7.0 and diluted 6-fold with demineralized water. The diluted WPC was concentrated again by the three-stage UF system. The retentate was concentrated to a DM >93% by spray-drying at a temperature of 50°C. The Ca content of the WPC was <0.1%. The functional properties of the WPC have been described by De Wit and De Boer (5).

Media. Lactococcus lactis subsp. *cremoris* strain E8 and the Bos starter were grown in various milk media. In initial experiments, the following media were used: fresh skim milk, and UF-retentates of the skim milk in the concentrations of approximately 1 to 5. In further experiments, UF-retentates were made by dissolving 10% (wt/vol) Nilac low heat milk powder in water (14). Dissolved Nilac milk powder was chosen because its composition is constant, and it contains the same milk protein concentration as fresh skim milk. The compositions of the fresh and Nilac skim milk concentrates are shown in Table 1. In UF-

Table 1. Composition of UF retentates prepared from fresh skim milk (SM) and Nilac SM and of the reconstituted SM (RSM) with a DM content comparable to the UF retentates.

Medium[1] and CF	DM (%)	protein (%)	lactose (%)	ash (%)	Ca (%)
SM	9.14	3.62	ND[2]	0.77	ND
UF SM					
0.9	8.77	3.36	4.60	0.74	0.115
1.8	12.01	6.38	4.52	0.98	0.201
2.6	15.27	9.42	4.50	1.25	0.284
3.5	18.50	12.58	4.39	1.50	0.380
4.3	21.71	15.46	4.36	1.76	0.467
Nilac SM	10.2	3.66	4.88	ND	0.131
UF Nilac					
1.0	10.1	3.81	ND	ND	0.135
1.9	13.9	7.13	4.99	ND	0.231
2.9	17.8	10.56	4.80	ND	0.339
3.6	21.6	13.23	4.69	ND	0.443
4.7	25.7	17.03	4.63	ND	0.534

[1] CF = Concentration factor.
[2] ND = Not determined.

milk, the protein concentration and the amount of Ca^{2+} increased as concentration factor increased. The increase in Ca^{2+} correlated with the higher ash content. The lactose content decreased slightly as the concentration factor increased.

Reconstituted Nilac skim milk, with whey proteins added to a concentration corresponding to the concentration factor of the retentate, was also used. All media were pasteurized at 63°C for 30 min before the growth experiments.

Media used for the viable count determination contained 1% tryptone, 0.3% meat extract, 0.5% yeast extract, 4.0% tomato juice, 2.0% glucose, 0.1% Tween 80, 0.2% K_2HPO_4, and 1.5% bactoagar. After sterilization, pH was adjusted at 6.8 to 7.0. The viable count of the full-grown precultures was determined after 2 to 3 d of incubation at 30°C.

Batch Cultivation. Precultures of the Bos starter were made by 1% inoculation of the starter concentrate in high temperature pasteurized milk (skim milk pasteurized for 30 min at 100°C). The precultures were incubated for 20 h at 20°C. Precultures of strain E8 were obtained by inoculation of 0.2% in high temperature pasteurized milk. The precultures were incubated for 16 h at 30°C. The inoculum used for these precultures was frozen 1-ml fractions, which were identical for all of the described experiments. The full-grown precultures were only used under standardized conditions. The characteristics for the Bos starter and strain E8 full-grown preculture were 1×10^9 cfu/ml and 7×10^8 cfu/ml, the pH

was 4.60 ± 0.05 and 4.50 ± 0.02, and the amount of lactic acid produced was 111 ± 6 and 108 ± 4 mM, respectively. The Bos starter and strain E8 were grown in 1 liter Erlenmeyer flasks containing 0.6 liter of medium. An inoculum of 2% (vol/vol) was used. The pH of the growth medium was maintained at 6.3, in all the experiments, by the addition of 10% (wt/vol) $NaHCO_3$ and 7.5% NH_4OH automatically by an Impulsomat 614 and a Dosimat 655 (both from Metrohm, Applicon, Herisan, Switzerland). Incubation was at 30°C. Anaerobic conditions were achieved by passing N_2 gas over the culture. The contents of the fermenter were mechanically stirred at 250 rpm.

Growth Parameters. The maximum specific growth rate and the total growth of the cultures were determined spectrophotometrically. For the measurement of the optical density, the milk and retentate samples were clarified by mixing 0.5 ml of sample with 4.5 ml of 50 mM EDTA and 0.5 M NaH_3BO_3 (pH 8). After 5 min, the absorbance was measured at 578 nm (Biotron Atom Data Test 366 photometer; Meyvis, Bergen op Zoom, The Netherlands). The measured absorbances at 578 nm were between 0.2 and 1.0. Each measurement was in triplicate. No cell lysis occurred during this treatment, and the absorbance of the diluted cultures remained stable for >30 min. In all cases, the uninoculated medium was used as a blank. The absorbance at 578 nm of the culture was in all cases linearly correlated with the viable count and the amount of cell protein. An absorbance at 578 nm of one corresponded to 225 μg of cell protein/ml and approximately 10^8 cfu/ml of the strain E8.

Maximum specific growth rates (μ_{max}) were calculated during exponential growth according to the equation:

$$\ln X = \ln X_0 + \mu \times t$$

where X_0 = biomass produced at initiation of the experiment, time t_0, and X = biomass produced at time t. The slope of the linear part of the curve of ln X versus t is μ_{max}. The μ_{max} was calculated from slopes with a correlation coefficient higher than 0.99; μ_{max} is expressed per hour.

Total growth is defined as the total amount of biomass that is formed after 22 h of growth at regulated pH. The amount of biomass was determined spectrophotometrically as described.

Maximum acidification rates (μ_{acid}) were calculated in the same manner as the μ_{max} using the following equation:

$$\ln Y = \ln Y_o + \mu_{acid} \times t$$

where Y_0 = quantity of acid produced at initiation of the experiment, time t_o, and Y = acid produced at time t. The acidity of the culture was calculated from the amount of base that was added to the pH-regulated culture and is expressed as millimolar per hour.

Analytical Methods. Protein concentrations and the amounts of nonprotein N of the milk were determined with the micro-N method as described by Koops et al. (9). The lactose concentration was determined according to the method of Schoorl (11). Ash was determined according to International Dairy Federation standard 27 (8). The Ca^{2+} content was measured according to the method of Evenhuis and de Vries (6).

Cell protein was assayed according to Bradford (1), using bovine serum albumin as a standard.

Results and Discussion

Specific Growth Rates. Initial experiments showed clearly the large variation in the specific growth rate of lactococci grown on milk between experiments performed in duplicate on different days. This result, in combination with the conflicting data in the literature (3, 12), prompted us to study this variation in more detail.

The μ_{max}, the specific biomass increase per time interval, of strain E8 was determined in fresh skim milk and in UF-retentate prepared from the same milk and concentrated 4.3-fold. The mean maximum values for specific growth rate on those substrates were 0.60 (SD $= \pm 0.17$; n = 18) and 0.62 (SD $= \pm 0.10$; n = 11), respectively. The μ_{max} on skim milk varied between 0.85 and 0.22/h. These high variations were unexpected because of the standardization of the storage procedure for the bacterial stock, the thawing procedure, the normalized preparation of the inoculum, and the use of completely automated equipment. This variation was not seen with duplicate cultures grown on the same day or when the cultures were inoculated with cultures prepared from different bacterial stocks. Duplicate E8 cultures grown on M17 medium on different days with identical growth conditions showed a mean μ_{max} of 0.82 (SD = 0.02; n = 10). Thus, possible variations in growth, except those induced by the use of different milk preparations, were apparently excluded.

To diminish the variability introduced by the use of fresh skim milk, this medium was replaced by highly standardized Nilac skim milk powder, which is similar in composition to fresh skim milk (14). However, variation in growth rates was the same, using the same number of experiments, as variation on fresh skim milk (data not shown). These findings may explain the conflicting results reported in the literature (3, 12). Srilaorkul *et al.* (12) found that μ_{max} decreased as concentration of the retentate increased. However, an opposite effect was reported by Hickey *et al.* (7) and Christopherson and Zottola (3). The observed variation of the μ_{max} suggests that this parameter is not suitable to describe the growth of lactic acid bacteria in milk and not suitable to determine the effects of UF of milk on growth of *L. lactis* subsp. *cremoris*.

Acidification Rate. To obtain a more reproducible parameter to describe the growth of lactococci in milk and UF-retentates, μ_{acid} was studied in more detail. The μ_{acid} was defined

Table 2. Maximum specific acidification rates (μ_{acid}) per hour of *Lactococcus lactis* subsp. *cremoris* strain E8 (protease positive) and a Bos mixed-strain starter grown in Nilac skim milk (SM) and UF-concentrated Nilac milk [1].

Culture and experiment[2]	Nilac SM	UF			
		1.0	1.9	2.8	3.6
E8					
1	0.62	...[3]	...	...	...
2	0.60	...	...	...	...
3	0.78	...	0.66	0.43	...
4	...	0.55	0.69	...	0.58
5	0.58	...	...	0.95	...
6	...	0.85	0.94	...	...
Bos					
7	...	...	0.44	0.48	0.76
8	0.58	...	0.41	...	0.81
9	0.58	0.55	...	0.51	...
10	0.44	...	...	0.69	...
11	0.69	...	...	0.85	...
12	0.9	...	...	...	...
13	...	...	0.51	...	0.60
14	0.79	0.78	...	0.60	...

[1] Maintained at pH 6.3.

[2] Experiment numbers indicate cultures performed on the same day under identical conditions.

[3] Not done.

as the specific increase in acidity of the culture per time interval. The effect of the concentration factor of the Nilac milk on μ_{acid} was investigated under conditions in which the pH was controlled at pH 6.3 (Table 2). The μ_{acid} increased by about 40 to 25% for a 2.8-fold increase of the concentration factor of the retentate in Experiments 5, 10, and 11, which is consistent with the results of Hickey *et al.* (7). In the other experiments, μ_{acid} did not show an effect or was up to 45% lower in the case of UF-retentate concentrated 2.8- or 3.6-fold. These variations in μ_{acid} were similar to those with the μ_{max} measurements, demonstrating that neither parameter can be used to define growth of *L. lactis* subsp. *cremoris* on UF-milk.

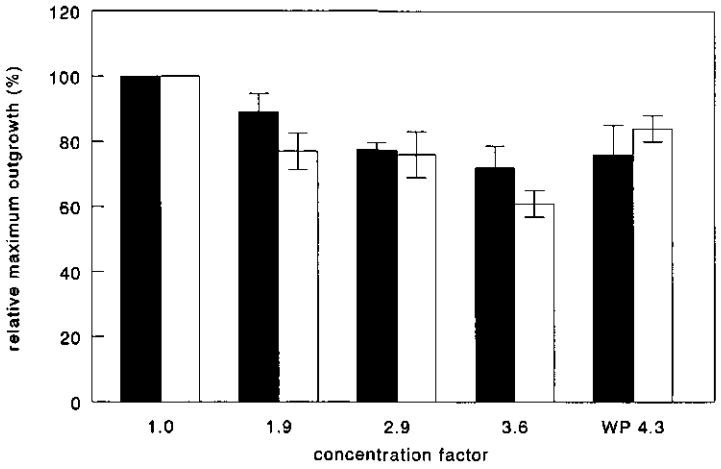

Figure 1. Relative total growth of the Bos mixed-strain starter (black bar) and *Lactococcus lactis* subsp. *cremoris* E8 (protease positive) (white bar) on Nilac skim milk (1.0) its UF retentates and Nilac skim milk (SM) with added whey proteins (WP) in a concentration equal to those in UF retentate concentrated 4.3-fold. Growth was regulated at pH 6.3. The total growth on skim milk was used as control for each experiment and set at 100%. Each bar represents the mean of three experiments.

Total Growth. The effect of concentration of Nilac milk by UF on the total growth of the culture, reached after 22 h of pH-regulated (pH 6.3) growth, was investigated. Total growth was defined as the total amount of biomass, expressed in optical density at 578 nm, which is formed after 22 h of growth under conditions of regulated pH. Strain E8 and the Bos starter were studied. The total growth of strain E8 in UF-retentate of Nilac milk concentrated 3.6-fold decreased by 40% (SD = 4.2) compared with the total growth in Nilac skim milk (Figure 1). For Bos culture, the decrease in growth was 25% (SD = 6.7). In all cases, lactose was the growth-limiting compound. Therefore, the small decrease of lactose in the UF-retentate concentrated 3.6-fold (Table 1), could be responsible for 4% of the decrease in total growth compared with the total growth in milk that had not been concentrated.

Differences in total growth on Nilac skim milk and UF-retentates could not be explained by the inoculum size. The inoculum size for strain E8 and the Bos starter was highly standardized at 2% of 7×10^8 cfu/ml and 2% of 1×10^9 cfu/ml, respectively. In milk that had not been concentrated, the absolute absorption at 578 nm, after correction for dilutions, varied from 9.0 to 13.0. The corresponding colony-forming units per milliliter were 1×10^9 and 6×10^9, respectively. Despite the variation in absolute values, in all cases, the concentration factor of the milk correlated with the decrease in the total growth. The same result was found when the number of colony-forming units or the amount of cellular protein was measured (data not shown). These results show that the total growth can be used as a

parameter to define growth of *L. lactis* in milk.

Effect of Whey Proteins. The total growth of strain E8 and the Bos starter decreased in UF-retentates compared with the total growth reached in regular skim milk. The concentration of whey proteins is increased specifically by the UF process. To study the effect of the increased WPC on the total growth, purified whey proteins were added to Nilac skim milk in concentrations similar to those found in UF-retentates concentrated 4.3-fold. This addition caused a mean decrease of 15% in the total growth of the culture E8 and 20% of the Bos culture in 10 independent experiments compared with the decrease in total growth of 40% and 25% on UF-retentates (Figure 1). This result indicates that the decrease in total growth, observed in cultures grown on UF-retentates, can be partly explained by the increase in concentration of whey proteins in UF-retentates. An opposite effect has been described by Broome et al. (2). They suggested that additions of WPC to milk stimulated the specific growth rate and the acid production of *Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus helveticus*.

Further research should focus on the possible mechanisms that are involved in the inhibiting effect of whey proteins on the growth of lactic acid bacteria and on which whey proteins are involved in this process. In addition, more attention should be directed to the other factors responsible for the growth inhibition of lactic acid bacteria on UF-retentates.

Conclusions

Despite the use of highly standardized conditions, μ_{max} (the specific biomass increase per time interval) and μ_{acid} (the specific increase in acidity of the culture per time interval) could not be used to characterize the growth of lactic acid bacteria on milk and UF-retentates because of the huge variations in the measurements. Thus, growth experiments in milk that have been described in the literature have to be interpreted with much care.

Concentration of milk by UF clearly inhibited the total growth (the total amount of biomass, expressed in optical density at 578 nm, which is formed after 22 h of growth at pH-regulated conditions) of *L. lactis* subsp. *cremoris* E8 and the Bos starter. Part of the inhibition could be explained by the increased concentration of whey proteins in UF-retentates.

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3

Medium-Dependent Regulation of Proteinase Gene Expression in *Lactococcus lactis*: Control of Transcription Initiation by Specific Dipeptides

Joey D. Marugg, Wilco Meijer, Richard van Kranenburg, Peter Laverman, Paul G. Bruinenberg, and Willem de Vos

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Medium-Dependent Regulation of Proteinase Gene Expression in *Lactococcus lactis*: Control of Transcription Initiation by Specific Dipeptides

Transcriptional gene fusions with the *Escherichia coli* β-glucuronidase gene (*gusA*) were used to study the medium- and growth-dependent expression of the divergently transcribed genes involved in proteinase production (*prtP* and *prtM*) of *Lactococcus lactis* SK11. The results show that both the *prtP* and *prtM* genes are controlled at the transcriptional level by the peptide content of the medium and, to a lesser extent, by the growth rate. A more than 10-fold regulation in β-glucuronidase activity was observed for both *prtP* and *prtM* promoters in batch and continuous cultures. The level of expression of the *prtP* and *prtM* promoters was high in whey permeate medium with relatively low concentrations of peptides, whereas at increased concentrations the expression of the promoters was repressed. The lowest level of expression was observed in peptide- and amino acid-rich laboratory media, such as glucose-M17 and MRS. The addition of specific dipeptides, such as leucylproline and prolylleucine, to the growth medium negatively affected the expression of the *prtP-gusA* fusions. The repression by dipeptides was not observed in mutants defective in the uptake of di-tripeptides, indicating that the internal concentration of dipeptides or derivatives is important in the regulation of proteinase production.

Lactococci are gram-positive bacteria used in the production of a great number of fermented milk products. These bacteria have multiple amino acid auxotrophies, and for growth in milk they require efficient proteolytic and transport systems to degrade milk proteins into small peptides and free amino acids that can be taken up and used as a nitrogen source (31). The cell envelope-associated serine proteinase is a key enzyme in the proteolytic system, which is essential for rapid growth of lactococcal cells in milk and generates casein peptides that contribute to flavour development in fermented milk products (33, 34).

Biochemical and genetic studies have shown that the proteinases of several *Lactococcus lactis* strains are highly related but differ in their caseinolytic specificities (17, 18). Complete nucleotide sequences of plasmid-located proteinase genes from *L. lactis* Wg2 (19), SK11 (35), and NCDO 763 have been determined (16). Comparison of the deduced amino acid sequences and further analysis revealed small differences that clarified the variations in their caseinolytic specificities. The proteinase genes of the different strains have a similar organization, and further genetic studies have shown that two divergently transcribed genes,

prtP and *prtM*, are required for the production of the active serine proteinase (5, 17, 18). The structural *prtP* gene encodes a proteinase precursor with a size of approximately 200 kDa that has homology to the subtilisin family of serine proteases (27). The *prtM* gene encodes a *trans*-acting maturation protein with a size of 33 kDa that is required for the activation of the inactive proteinase precursor (12, 36).

Despite the wealth of genetic and biochemical data on the lactococcal proteinases, studies on the regulation of their production are limited. In *L. lactis* AM1, the synthesis of proteinase was repressed after the addition of Casitone, a pancreatic digest of casein containing mainly peptides, to the growth medium (10). Strains AM1, E8, and Wg2 produced more proteinase in milk than in laboratory media (14, 15). It has also been reported that proteinase production in strain Wg2 is dependent on both the nitrogen source and growth phase (21). Recently, the regulation of *L. lactis* SK11 proteinase production in different lactococcal strains has been analysed by using multicopy plasmids containing the *prtP* gene (2). The results showed that production of the SK11 proteinase and its regulation were strain and medium dependent. Moreover, this regulation appeared to be independent of the copy number in the strains investigated. However, in all these cases, regulation of proteinase production was studied at the enzyme level, and no differentiation between gene expression, proteinase secretion or processing, and autoproteolytic breakdown could be made.

To study the transcriptional regulation of proteinase gene expression, we decided to use plasmids containing the promoterless β-glucuronidase gene (*gusA*) from *Escherichia coli* under control of the SK11 *prtP* and *prtM* promoters. *gusA* is a well-studied reporter gene and has been used in the isolation and analysis of promoters from various species of lactic acid bacteria (24). The expression of *prt-gusA* gene fusions has been analysed in *L. lactis* cells that were grown in industrial and laboratory media containing different amino acid sources including Casitone, Casamino Acids, or yeast extract. We observed a medium-dependent regulation of both the *prtP* and *prtM* promoters in batch and continuous cultures. Expression is repressed at increased nitrogen concentrations or by the addition of specific dipeptides to the growth medium. The results indicate that proteinase production in *L. lactis* is regulated at the transcriptional initiation level and is mediated by a specific host factor.

Materials and Methods

Bacterial strains and growth conditions. E. coli MC1061 (4) was grown in L-broth-based medium (25). The *L. lactis* strains used in this study are listed in Table 1. *L. lactis* strains were grown in glucose-M17 broth (GM17) (Merck GmbH Darmstadt, Germany) unless otherwise stated. For the assay of β-glucuronidase production, *L. lactis* cells were grown in whey permeate medium (9) consisting of 0.5% ultrafiltrated whey permeate powder and 1.9% (wt/vol) β-glycerophosphate supplemented with various concentrations of the nitrogen sources Casitone (0.1% to 2% [wt/vol]) or Casamino Acids (0.1% to 4% [wt/vol]) (both

Table 1. *L. lactis* strains and plasmids used in this study.

Strain or plasmid	Relevant characteristic(s)[a]	Reference
Strains		
MG1363	Lac$^-$ Prt$^-$ plasmid-free and prophage-cured derivative of NCDO 712	11
MGDT1	DtpT$^-$ mutant of MG1363	20
MGAD4	AlaT$^-$ mutant of MGDT1	20
MG1363 Opp$^-$	Opp$^-$ Sm^r mutant of MG1363	8
SK112	Lac$^+$ Prt$^+$ strain; harbours proteinase plasmid pSK111	6
SK1128	Lac$^+$ Prt$^-$ derivative of SK112, obtained by plasmid curing	6
Plasmids		
pNZ122	Cm^r, 2.8-kb pSH71 replicon	7
pNZ521	Cm^r, 10.7-kb pNZ122 derivative carrying the complete *prtP* and *prtM* gene of pSK111	9
pNZ124	Cm^r, 2.8-kb derivative of pNZ122 carrying a polylinker sequence	24
pNZ272	Cm^r, 4.7-kb pNZ124 derivative carrying promoterless *gusA* gene from *E. coli*	24
pNZ544	Cm^r, 5.1-kb pNZ521 derivative carrying *gusA* gene fused to *prtP* promoter	This work
pNZ554	Cm^r, 5.1-kb pNZ521 derivative carrying *gusA* gene fused to *prtM* promoter	This work
pNZ555	Cm^r, 4.8-kb derivative from pNZ544 by deletion of the 0.35-kb promoter fragment	This work

[a] Lac$^+$, lactose-fermenting phenotype; Prt$^+$, ability to produce a functional proteinase; DtpT$^-$, di-tripeptide transport deficient; AlaT$^-$, alanine(/glycine) transport deficient; Opp$^-$, oligopeptide transport deficient; Cm^r and Sm^r, resistance to chloramphenicol and streptomycin, respectively.

from Difco Laboratories, Detroit, Mich.) after precultivation in GM17. In some experiments the following di- and tripeptides were added to the whey permeate medium (supplemented with 0.1% Casitone): alanylglutamine, glutamylglutamine, glutamylglycine, glycylproline, leucylleucine, leucylproline, prolylglutamine, prolylleucine, prolylphenylalanine, prolylproline, prolyltyrosine, and prolylvalylglycine (all obtained from Bachem Feinchemikalien AG, Bubendorf, Switzerland). If appropriate, the media contained chloramphenicol (10 μg/ml), erythromycin (5 μg/ml), or 0.5% (wt/vol) glucose. Continuous cultures were grown in whey permeate medium containing 0.19% (wt/vol) β-glycerophosphate and 0.15 to 2% (wt/vol) Casitone as the nitrogen source under glucose-limited conditions. They were grown anaerobically under an N_2 atmosphere in glass fermenters containing a working volume of 500 ml. The pH of the medium was controlled at 6.3 by titration with a mixed buffer solution containing 7.5% (wt/vol) NH_4OH and 10.0% (wt/vol) $NaHCO_3$.

DNA methods, reagents, and enzymes. Isolation of plasmid DNA from *E. coli* and standard recombinant DNA techniques were performed according to the methods of Sambrook et al. (25). Isolation of plasmid DNA from *L. lactis* and transformation of *L. lactis* strains were performed as described previously (9). 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.). All enzymes were purchased from Bethesda Research Laboratories (Gaithersburg, Md.). *para*-Nitrophenyl-β-D-glucuronic acid was purchased from Clontech Lab. Inc. (Palo Alto, Calif.).

Construction of plasmids. A schematic representation of the different plasmids used in this study is shown in Fig. 1. Plasmid pNZ521 (9) contains the complete *prtP* gene and the *prtM* gene from pSK111 cloned into pNZ122 carrying the pSH71 replicon (7). Plasmid pNZ272 contains the promoterless *gusA* gene encoding β-glucuronidase from *E. coli* cloned into pNZ124, a pNZ122 derivative with a polylinker sequence (24). Plasmid pNZ544 carrying the *prtP* promoter fused to the *gusA* was constructed as follows. Both the Klenow-treated 0.35-kb *Cla*I fragment from pNZ521 carrying the *prt* promoter region and the 1.9-kb *Eco*RI-*Hin*dIII fragment from pNZ272 carrying the *gusA* gene were ligated into the 2.8-kb vector part of pNZ521 obtained by digestion with *Sal*I and *Sst*I and subsequent treatment with Klenow and T4-DNA polymerase, respectively. The resulting plasmid, pNZ544, contains intact *Sal*I and *Eco*RI restriction sites (restored after the fusion to the filled-in *Cla*I fragment) flanking the promoter region. Therefore, digestion of pNZ544 with these enzymes, filling in with Klenow DNA polymerase, and ligation with T4 DNA ligase resulted in either inversion of the 0.35-kb promoter fragment (pNZ554) or its deletion (pNZ555). All constructions were carried out in *L. lactis* and verified by restriction enzyme analysis and DNA sequence analysis of relevant regions.

Enzyme assays. Unless otherwise stated, *L. lactis* strains were grown to the mid-log growth phase (A_{600} of 0.7). Cells were harvested, washed twice, and resuspended in GUS buffer (50 mM $NaHPO_4$, 10 mM β-mercaptoethanol, 1 mM EDTA, 0.1% Triton X-100). Cell extracts of lactococcal cells were prepared by using a bead beater as described previously (24). The cell extracts (10 to 50 μl) were added to GUS buffer supplemented with 1 mM *para*-nitrophenyl-β-D-glucuronic acid as a substrate. The initial rates of β-glucuronidase activity were measured at 405 nm in a Uvikon 810 spectrophotometer (Kontron, Zurich, Switzerland) with a thermostatically controlled cell compartment at 37°C. Protein concentrations were determined according to the Bradford method (1) with bovine serum albumin as a standard.

Results

Expression of gusA under control of SK11 prtP and prtM promoters. The 352-bp *Cla*I fragment from pNZ521 contains the partially overlapping, divergently orientated *prtP* and *prtM* promoters (Fig. 1) (35). The promoterless *gusA* gene with its ribosome binding site was

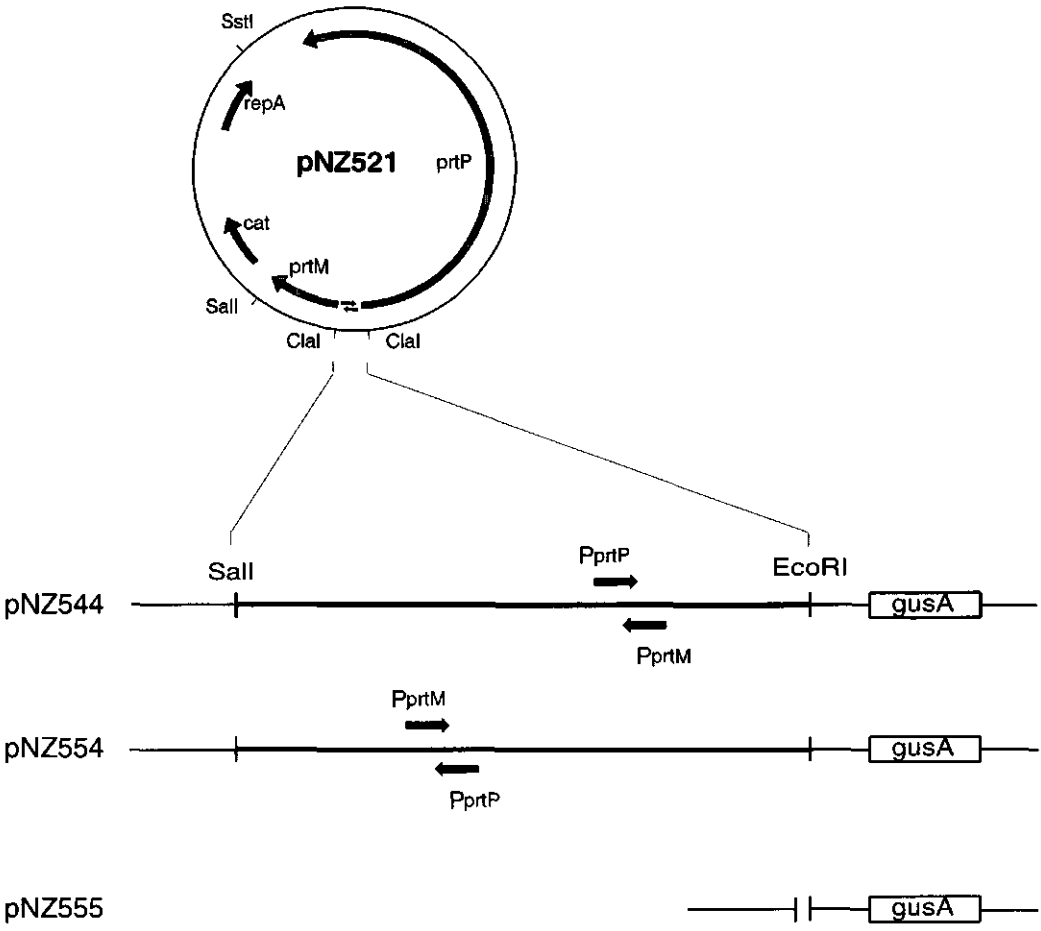

Figure 1. Schematic representation of transcriptional fusions of *prtP* (pNZ544) and *prtM* (pNZ554) to promoterless *gusA* gene encoding β-glucuronidase. The 0.35-kb *Cla*I fragment from pNZ521 containing the partially overlapping divergent promoters of the *prtP* and *prtM* genes (indicated by the bold lines) was cloned into the multiple cloning site upstream of the *gusA* gene to obtain pNZ544. Plasmids pNZ555 and pNZ554 were derived from pNZ544 by deletion or inversion of the 0.35-kbp *Sal*I-*Eco*RI fragment, respectively. The promoters are represented by the solid arrows.

placed under control of each promoter to create transcriptional fusions. In the resulting plasmids, designated pNZ544 and pNZ554, *gusA* expression is dependent on the activities of the *prtP* and *prtM* promoter, respectively (Fig. 1). In each orientation of the *Cla*I fragment, the start codons and preceding ribosome binding sites of either the *prtP* or *prtM* genes are still present. However, translation initiates at the ATG of *gusA*, as in-frame stop codons are present in both plasmids in the region upstream of the *gusA* ribosome binding site. Plasmid pNZ544 and its derivative pNZ555, in which the promoter region was deleted, were introduced into the proteinase-deficient *L. lactis* model strain MG1363, and expression was analysed after growth in media supplemented with various nitrogen sources. Extracts were prepared from cells harbouring pNZ544, and β-glucuronidase activities were determined (Table 2). The highest β-glucuronidase activities directed by the *prtP* promoter were observed in extracts of cells that were grown in whey permeate medium supplemented with a low concentration of Casitone (0.1%). Casitone is a pancreatic digest of casein consisting of mostly small peptides and amino acids (in a proportion of about 80 to 20%, respectively [32]). At Casitone concentrations lower than 0.1%, growth rates decreased dramatically (data not shown). When cells of strain MG1363 harbouring pNZ544 were pregrown in whey permeate containing 2% Casitone and subsequently diluted in fresh medium containing only 0.1% Casitone, β-glucuronidase levels increased rapidly from the beginning of the early log phase. Maximum levels were reached at the end of the exponential phase (Fig. 2A). In extracts of cells grown in whey medium with a high (2%) Casitone concentration, β-glucuronidase levels specified by pNZ544 remained low during the whole growth period and showed an approximately six- to eightfold reduction compared with the maximal levels obtained after growth in whey permeate supplemented with 0.1% Casitone (Fig. 2B). At

Table 2. β-Glucuronidase activity in cell extracts of MG1363 directed by *prtP* promoter (pNZ544) or *prtM* promoter (pNZ554) after growth in industrial and laboratory media.

Medium	Avg β-glucuronidase activity ± SD (nmol[min × mg protein]$^{-1}$)[a]	
	pNZ544	pNZ554
WP[b] + 0.1% casitone	39 ± 5	64 ± 1
WP + 0.5% casitone	21 ± 2	30 ± 1
WP + 1.0% casitone	11 ± 1	20 ± 4
WP + 2.0% casitone	7 ± 2	14 ± 3
GM17	3 ± 1	6 ± 1
MRS	3 ± 0	4 ± 1
Elliker	2 ± 1	4 ± 1

[a] Results are the averages of two independent measurements.
[b] WP, whey permeate.

increasing Casitone concentrations (0.1 to 2%) in the whey permeate medium, the β-glucuronidase activity in the cell extracts decreased gradually, whereas in nitrogen-rich laboratory media, such as GM17, MRS, and Elliker, an approximately 10-fold reduction was observed (Table 2). As expected, cells harbouring pNZ555 did not produce β-glucuronidase activity at all in any of the media (data not shown). These differences in β-glucuronidase activities strongly suggest medium-dependent control of the *prtP* promoter. Transcription of the *prtP* promoter seems to be decreased especially when the concentration of peptides or amino acids in the medium is increased.

The medium-dependent regulation of the *prtP* promoter was also investigated in the original host *L. lactis* SK112, harbouring the proteinase plasmid pSK111, and its proteinase-deficient derivative, strain SK1128, from which plasmid pSK111 has been cured (6). Plasmid pNZ544 was introduced in both strains, and β-glucuronidase activities were determined after growth in media with various nitrogen sources. Both strains exhibited control of β-glucuronidase production similar to that observed in strain MG1363 (data not shown). This indicates that regulation of the *prtP* promoter located on pNZ544 is strain independent and, moreover, is not affected by the presence of additional copies of the *prt* genes carried on the proteinase plasmid pSK111. Strain SK112 harbouring pNZ544 was also grown in milk, and β-glucuronidase activity was determined in cell lysates. The *gusA* expression levels obtained were similar to those obtained after growth on whey permeate containing 0.1% Casitone, indicating that the *prtP* promoter is maximally active during exponential growth in milk (data not shown).

The control of the *prtM* promoter was analysed in the same way as that of the partially overlapping *prtP* promoter (Fig. 1). As with the *prtP* transcriptional fusion, β-glucuronidase activity directed by a transcriptional fusion of the *prtM* promoter and the *gusA* gene located on pNZ554 was repressed approximately 10-fold in peptide- and amino acid-rich media (such as GM17, MRS, and Elliker broth) compared with that obtained in the whey permeate medium containing 0.1% Casitone (Table 2). With an increase in the Casitone concentration in the whey permeate medium, β-glucuronidase activity specified by pNZ554 decreased approximately fivefold, indicating that both the *prtP* and *prtM* promoters are regulated in a similar way.

Expression of prtP-gusA fusion in transport mutants. To determine the role of the nitrogen source (peptides or amino acids) in the control of the proteinase promoters more precisely, we used three isogenic MG1363 derivatives defective in the uptake of oligopeptides or amino acids: an Opp^- strain defective in the uptake of oligopeptides, a $DtpT^-$ strain defective in the uptake of di-tripeptides, and an $AlaT^-$ $DtpT^-$ strain impaired in the uptake of di-tripeptides and of both alanine and glycine (Table 1) (8, 20). Plasmid pNZ544 was introduced into each of these strains, and β-glucuronidase activity was determined after growth in GM17 medium or in whey permeate medium containing 0.1 or 2.0% Casitone (Table 3). The wild-type strain and all the peptide transport mutants exhibited high levels of

Table 3. β-Glucuronidase activity in cell extracts of MG1363 and its isogenic transport mutants harbouring plasmid pNZ544[a].

Medium	Avg β-glucuronidase activity ± SD (nmol[min × mg protein]$^{-1}$)[a]			
	MG1363	MG1363 Opp$^-$	MG1363 AlaT$^-$ DtpT$^-$	MG1363 DtpT$^-$
WP + 0.1% casitone	38 ± 4	30 ± 2	68 ± 5	42 ± 6
WP + 2.0% casitone	5 ± 1	4 ± 2	18 ± 5	9 ± 2
GM17	3 ± 1	2 ± 0	16 ± 2	10 ± 1

[a] Results are the averages of two independent experiments.

β-glucuronidase activities when cells were grown in whey permeate with 0.1% Casitone. The highest levels of β-glucuronidase activities were found in the double mutant AlaT$^-$ DtpT$^-$. When cells were grown in GM17, only cells of strain MG1363 and those of the Opp$^-$ mutant harbouring pNZ544 exhibited an approximately 10-fold reduction in β-glucuronidase activity. In contrast, the β-glucuronidase activity in the AlaT$^-$ DtpT$^-$ mutant harbouring pNZ544 was reduced when cells were grown in GM17, but only to a level that was fourfold lower than that obtained in whey permeate containing 0.1% Casitone; compared with the level in the wild-type strain grown in GM17, the cells of this derivative showed a four- to fivefold increase in β-glucuronidase activity. In whey permeate containing 2% Casitone, the double mutant showed a similar increase in β-glucuronidase activity compared with wild-type levels under the same conditions. Similarly increased levels of β-glucuronidase activity, although less pronounced, were also observed for cells of the DtpT$^-$ derivative of MG1363 harbouring pNZ544 grown in GM17 or whey permeate containing 2% Casitone (Table 3). The increased levels of β-glucuronidase activity observed in the AlaT$^-$ DtpT$^-$ or DtpT$^-$ transport mutant in nitrogen-rich medium indicate that the uptake of small (di-tri)peptides, and possibly of amino acids, from the medium plays an essential role in the control of the *prtP* promoter.

Specific dipeptides repress the expression of the prtP-gusA fusion. To investigate whether control of the *prtP* and *prtM* promoters is related specifically to the presence of free amino acids or peptides in the medium, β-glucuronidase activities were also assayed after growth of the cells in whey permeate medium containing 0.1% Casitone and supplemented with increasing concentrations of Casamino Acids, an acid hydrolysate of casein in which free amino acids and small peptides are present in a ratio of 82 to 18%, respectively (according to manufacturer). In extracts of cells grown in the presence of 0.1% Casitone and 0.1% Casamino Acids, the level of β-glucuronidase activity was almost as high as that obtained in the 0.1% Casitone medium. The results revealed that β-glucuronidase activities encoded by

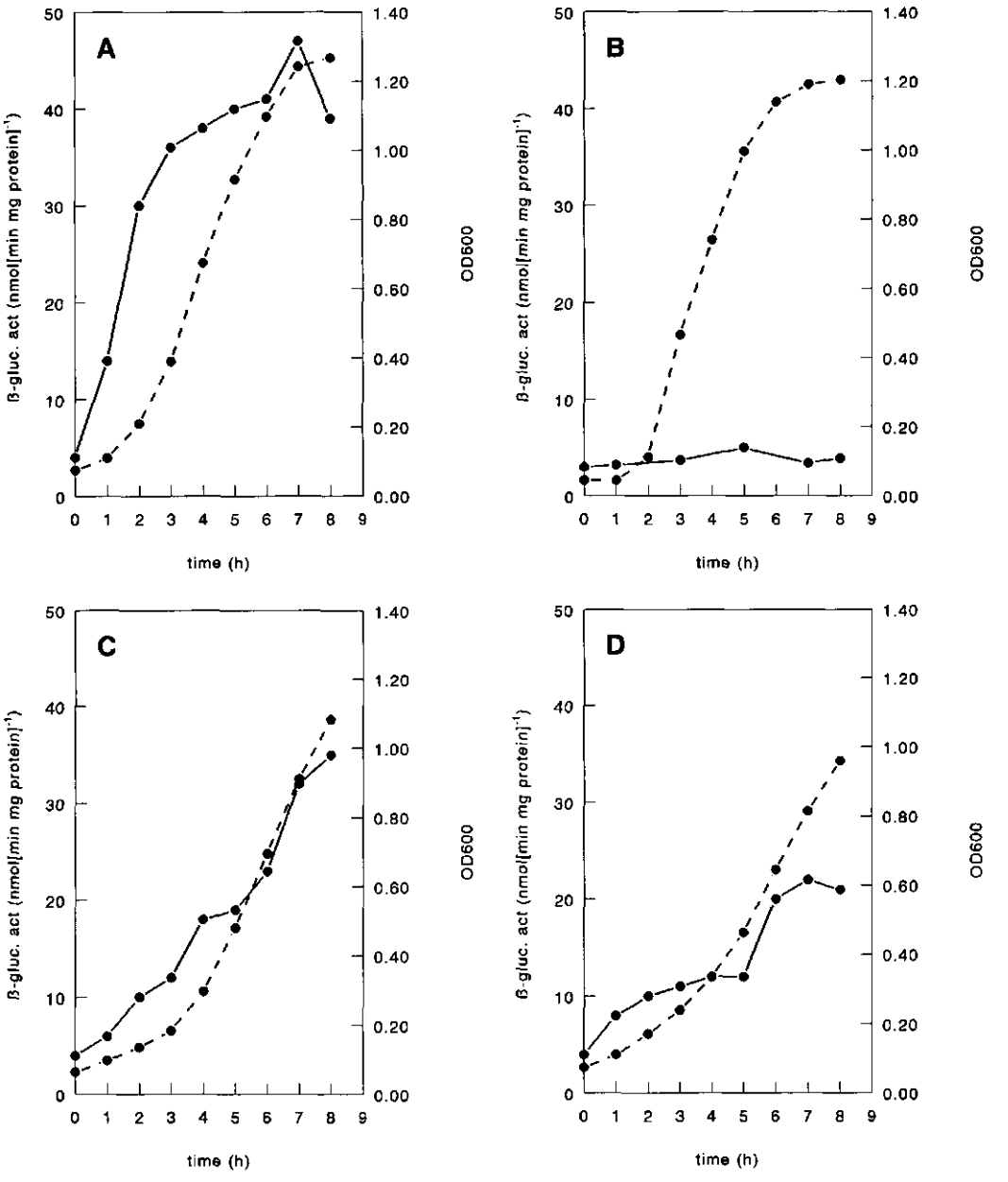

Figure 2. Changes in *prtP*-directed β-glucuronidase activity (solid lines) during growth (optical density at 600 nm [OD600]; broken lines) of *L. lactis* MG1363 harbouring pNZ544 in whey permeate medium with various (concentrations of) peptide sources. The nitrogen source(s) added to basic whey permeate medium was 0.1% Casitone (A), 2% Casitone (B), 0.1% Casitone and 2 mM leucylproline (C), or 0.1% Casitone and 1 mM prolylleucine (D).

pNZ544 or pNZ554 decreased when the cells were grown in media with increasing Casamino Acid concentrations, but that this reduction was less drastic than that with the equivalent concentration of Casitone (data not shown). Apparently, peptides, predominantly present in Casitone, rather than free amino acids may reduce *prtP* and *prtM* expression more effectively. Similarly, the effect by individual amino acids was investigated. The presence in the growth medium (whey permeate medium supplemented with 0.1% Casitone) of relatively high concentrations (10 mM) of each of the 20 amino acids did not significantly affect the β-glucuronidase activity (data not shown).

To further corroborate the role of (di-tri)peptides in the regulation of *prtP* expression, we tested 11 specific dipeptides and 1 tripeptide for their effect on the expression of the *prtP-gusA* fusion after their addition to the growth medium (final concentration, 0.5 to 2 mM). With the exception of leucylproline and prolylleucine, none of the di(tri)peptides tested showed any influence on β-glucuronidase activity. The presence of leucylproline or prolylleucine (final concentration of 2 or 1 mM, respectively) in whey permeate medium containing 0.1% Casitone during growth of MG1363(pNZ544) cells resulted in significantly reduced β-glucuronidase activity compared with that obtained in the same medium without the addition of the dipeptides (Fig. 2C and D). This repression of the P*prtP*-directed β-glucuronidase activity in strain MG1363 harbouring pNZ544 by either of the two dipeptides appeared to be transient, as the β-glucuronidase activity increased again either in the mid-exponential-growth phase (2 mM leucylproline [Fig. 2C]) or at the end of the exponential-growth phase (1 mM prolylleucine [Fig. 2D]). At lower concentrations the repressive effect of the two dipeptides disappeared in the early-exponential-growth phase (data not shown). In the AlaT$^-$ DtpT$^-$ transport mutant harbouring pNZ544, the addition of either of the two dipeptides affected β-glucuronidase activity to a significantly lesser extent than observed in the wild-type strain, again indicating that uptake of (specific) peptides is needed to affect the expression of the proteinase promoter (data not shown).

Expression of prtP-gusA fusion in continuous cultures. The addition of the dipeptide leucylproline or prolylleucine not only affected β-glucuronidase activity but also diminished the (initial) growth rate of the cells, which was evident from a comparison of the growth curves (Fig. 2A through D). Therefore, repression could result from this change in growth rate. To investigate the influence of different growth rates on the expression of the *prtP* promoter, β-glucuronidase activity was determined during growth of MG1363 harbouring pNZ544 in glucose-limited continuous cultures with 1% Casitone as a nitrogen source at various imposed growth rates (Fig. 3). The results show that the β-glucuronidase activity varied about twofold with the dilution rate (D) at values between 0.1 and 0.5 h^{-1}. Only at very low D values (0.05 h^{-1}) did the β-glucuronidase activity increase almost threefold compared with that at a high dilution rate (D = 0.5 h^{-1}). Apparently, under growth conditions that are normally observed in all batch culture experiments (μ values between 0.1 and 0.5 h^{-1}) the expression of the *prtP* promoter is only marginally affected by the growth

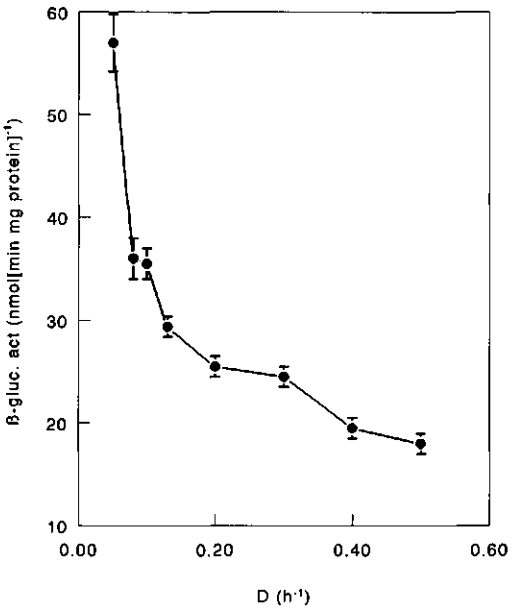

Figure 3. *prtP*-directed β-glucuronidase activity at different imposed dilution rates (D hour^{-1}) of continuous cultures of *L. lactis* MG1363 harbouring pNZ544. The data are mean values for duplicate measurements. Error bars indicate standard deviations.

rate.

Subsequently, β-glucuronidase activities were determined in a glucose-limited continuous culture of MG1363 harbouring pNZ544 at a constant dilution rate (D = 0.19 h^{-1}), with various Casitone concentrations (0.15 to 2%). With increasing Casitone concentrations, β-glucuronidase levels decreased approximately eightfold, similar to the decrease observed in the batch cultures, indicating that the medium-dependent regulation is independent of the growth rate (data not shown).

The repression by dipeptides of the expression of the *prtP* promoter was also studied during growth of MG1363 harbouring pNZ544 in continuous culture. At a growth rate of 0.19 h^{-1}, the addition of prolylleucine to a final concentration of 1 mM resulted in an immediate decline of β-glucuronidase levels, which reached a minimum approximately 3 h after the pulse (Fig. 4). Subsequently, β-glucuronidase activities increased to the levels prior to the addition of the dipeptide. The observed decrease had the same kinetics as the calculated dilution curve, indicating that the synthesis of β-glucuronidase had come to a complete stop. The addition of leucylproline (1 mM) to the culture had a similar effect (data not shown).

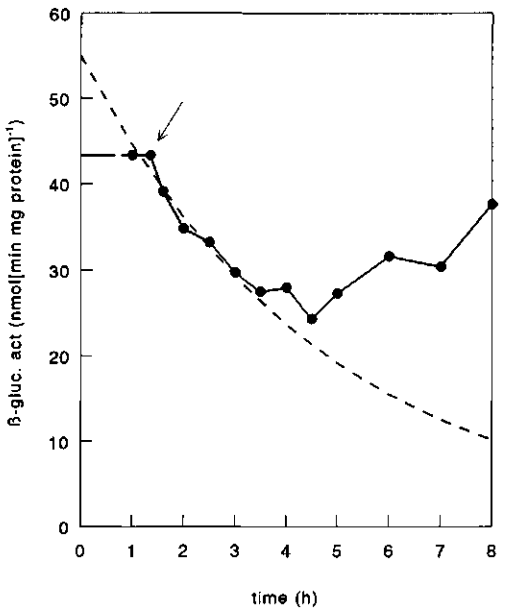

Figure 4. Changes in *prtP*-directed β-glucuronidase activity in a continuous culture of *L. lactis* MG1363 harbouring pNZ544 at a growth rate of 0.19 h^{-1} (in whey permeate medium with 1% Casitone as the nitrogen source) after addition of 1 mM prolylleucine (indicated by the arrow). The calculated dilution curve is indicated by the broken line.

Discussion

We have studied the medium-dependent expression of the *prtP* and *prtM* genes required for proteinase production in *L. lactis*. Transcriptional fusions of both the *prtP* and *prtM* promoters with the promoterless *gusA* gene from *E. coli* were constructed, and expression was analysed in batch and continuous cultures of *L. lactis* by using different growth media. The results show that the level of expression of both promoters is regulated approximately 10-fold by the peptide content of the medium. The level of expression of the *gusA* gene under control of the *prtP* or *prtM* promoters was high under low-peptide conditions, i.e., in whey permeate medium containing 0.1% Casitone, while a decrease in enzyme activity was observed when the peptide concentration in the medium was increased; minimal expression was observed in GM17 broth or in whey permeate medium containing high (>1%) concentrations of Casitone.

The results discussed here are supported by quantitative primer extension studies of *L. lactis* using mRNA preparations that were derived from cultures grown in medium containing increased nitrogen concentrations (23). Comparison of *prtP*- or *prtM*-specific mRNA levels produced in medium with a low- or high-nitrogen content showed a degree of regulation

similar to that found in β-glucuronidase activity assays. Together, the data strongly suggest that the medium-dependent expression of the *prt* promoters is controlled at the level of transcription initiation. The similarity in regulation of the divergently but partially overlapping promoters is remarkable and suggests a delicate, identical control by the transcription machinery in the cell that results in stoichiometric amounts of PrtM and PrtP. The control mechanism was found to be host independent, since regulation of the proteinase promoters was observed in both *L. lactis* MG1363 and SK11. Moreover, since *L. lactis* MG1363 is a plasmid-free strain, regulation encoded by the host chromosome is suggested.

Apart from the tight control by the nitrogen source, the growth rate of the cells has an influence, albeit minor, on the expression of the *prt* genes. In continuous cultures at increasing dilution rates ($0.05 < D < 0.5\ h^{-1}$), a maximally threefold decrease in *prtP*-directed β-glucuronidase levels was observed. A different growth rate dependency has been observed for synthesis of *L. lactis* Wg2 proteinase, the level of which in continuous cultures with amino acids as the sole nitrogen source was found to be maximal at a dilution rate of $0.23\ h^{-1}$ but decreased at higher dilution rates (21).

Specific peptides appear to play an important role in the medium-dependent regulation of the *prt* genes. In particular, of the 11 dipeptides and 1 tripeptide that were tested, the addition of the dipeptide leucylproline or prolylleucine to the growth medium specifically affected the expression of the *prtP-gusA* fusion. Our results further demonstrate that the addition of free amino acids to the medium did not affect *prtP*-directed β-glucuronidase activity significantly. We have obtained several lines of evidence which show that dipeptides rather than amino acids are more effective in this regulation: (i) promoter activity is controlled more effectively during growth of the cells in the presence of the peptide-rich nitrogen source Casitone than in the presence of equivalent amounts of Casamino Acids, a nitrogen source which contains mostly free amino acids; (ii) during growth in peptide-rich media, β-glucuronidase production directed by the *prtP* promoter is increased in mutants which are defective in the uptake of di- and tripeptides (Table 3); and (iii) the di-tripeptide transport deficient mutants showed no significant reduction in *prtP* promoter activity by the addition of dipeptides. The results with the transport mutants show that active uptake of (di)peptides via the specific transport system for di-tripeptides is essential for control of the proteinase promoter. The high degree of effectiveness of the dipeptides in the regulation may reflect their efficient uptake by lactococci. It has been shown that the di-tripeptide transport system has a high affinity for proline-containing dipeptides (29, 30). Remarkably, the oligopeptide transport system does not seem to be involved in the regulation of proteinase production, since an Opp^- mutant showed a regulation pattern similar to that observed for the wild-type. This lack of response may be due to a lower transport efficiency of the oligopeptide transport system than that of the di-tripeptide transport system (20). Alternatively, it could also indicate that peptides containing more than three amino acid residues, which are known to be transported via the Opp system, are not involved in the regulation of proteinase production. Altogether, the data indicate that the internal

concentration of the dipeptides (or derivatives) plays an important role in the regulation of the proteinase genes. After growth in low-nitrogen media, the AlaT$^-$ DtpT$^-$ mutant exhibited higher levels of β-glucuronidase activity than did the wild-type (Table 3), which can be explained by its lower growth rate than that of the wild type (22). A regulatory role of peptides, and in particular of the dipeptide leucylproline, in proteinase production in *L. lactis* Wg2 has previously been suggested (21). However, in these experiments, proteinase production was found to be inhibited long after the addition of (di)peptides to the culture. Therefore, the decline in proteinase levels could also be explained by autoproteolytic degradation, which is known to occur rapidly (18).

The repression by dipeptides appears to be a very rapid and efficient process, as the addition of prolylleucine to a steady-state continuous culture resulted in an immediate and complete stop of β-glucuronidase production. This means that upon the uptake of the dipeptide a signal is transmitted to the *prt* promoters. It may be speculated that this regulation is mediated by the interaction of the dipeptide or its derivatives with a regulator protein capable of regulating the transcription initiation at the *prtP* and *prtM* promoters. At this moment, our data do not provide information about whether this interaction is achieved by dipeptides, their constituting amino acids, or derivatives thereof. However, because of the high levels of intracellular peptidase activity, peptides are almost immediately hydrolysed in the cell (13, 20, 28). Therefore, we favour the possibility that the cell senses a temporary increase in the intracellular concentration of the dipeptides.

Another important aspect to be addressed is the question of whether the *prtP* and *prtM* promoters are induced or repressed. The interaction of the dipeptides (or derivatives) with a putative regulator may affect its affinity for the *prt* operator region, resulting either in induction or in repression of expression. A regulation mechanism for proteinase production in which peptides play a major part is quite feasible. As the proteinase is able to release (small) peptides into the growth medium, which can be taken up by the cells, one could speculate that expression of the *prt* genes is controlled by a transcriptional feedback mechanism. At the moment it is not clear whether the putative regulator protein is specific for the *prt* genes. In *E. coli* the leucine-responsive regulatory protein (Lrp) is an autoregulated pleiotropic factor that controls a large number of genes of metabolic pathways in response to the availability of amino acids and nitrogen bases (for a recent review, see reference 3). Lrp can function as both an activator and a repressor, and its activity is modulated positively or negatively by leucine. In analogy to *E. coli*, it is tempting to speculate about the presence of a similar global regulator in *L. lactis*, which may regulate a number of genes involved in nitrogen metabolism, including genes for proteinase and peptidases and genes involved in nitrogen transport across the membrane. Preliminary results indicate that the *pepN* gene encoding the aminopeptidase N is regulated in a way similar to that of the *prt* genes (22), and further research is aimed at characterizing this global regulation system in *L. lactis*.

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4

Regulation of Proteolytic Enzyme Activity in *Lactococcus lactis*

Wilco Meijer, Joey D. Marugg, and Jeroen Hugenholtz

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Regulation of Proteolytic Enzyme Activity in *Lactococcus lactis*

Two different *Lactococcus lactis* host strains, *L. lactis* subsp. *lactis* MG1363 and *L. lactis* subsp. *cremoris* SK1128, both containing plasmid pNZ521, which encodes the extracellular serine proteinase (PrtP) from strain SK110, were used to study the medium and growth-rate-dependent activity of three different enzymes involved in the proteolytic system of lactococci. The activity levels of PrtP and both the intracellular aminopeptidase PepN and the X-prolyl-dipeptidyl aminopeptidase PepXP were studied during batch and continuous cultivation. In both strains, the PrtP activity level was regulated by the peptide content of the medium. The highest activity level was found during growth in milk, and the lowest level was found during growth in the peptide-rich laboratory medium M17. Regulation of the intracellular peptidase activity appeared to be a strain-dependent phenomenon. In cells of strain MG1363, the activity levels of PepN and PepXP were regulated in a similar way to that observed for PrtP. In cells of strain SK1128, the levels of both peptidases were not significantly influenced by the peptide content of the medium. The presence of specific concentrations of the dipeptide prolylleucine could mimic the low activity levels of the regulated proteolytic enzymes, even to the activity level found on M17 medium. The effect of the presence of the dipeptide prolylleucine in the medium on the activity level of the regulated proteolytic enzymes was confirmed at fixed growth rates in chemostat cultures.

Lactococci, gram-positive, facultatively anaerobic bacteria, depend on the availability of a proteolytic system for growth on milk. The enzymes of the proteolytic system, composed of the extracellular cell wall-bound serine proteinase (PrtP) and various well characterized peptidases, supply essential amino acids by concerted action in degradation of the casein (12, 13). The proteolytic system has an important role in the generation of casein peptides, which contribute to organoleptic changes in fermented milk products (14, 18, 25, 26).

The first step in the breakdown of casein occurs via PrtP activity. The biochemical, immunological, and genetic properties of the membrane-bound proteinase have been well investigated for various lactic acid bacteria (6, 9, 15). The casein peptides produced by PrtP are subject to further degradation by the different peptidases. Nucleotide sequence analysis of the different peptidase genes (15) together with immunological studies (24) strongly suggests that these enzymes are located intracellularly.

There have been relatively few studies published on the regulation of proteinase

production, and to our knowledge there is no information available about the regulation of the various peptidases in lactococci. Proteinase activity of *Lactococcus lactis* subsp. *cremoris* AM1 was effectively repressed with an increased concentration of a pancreatic digest of casein (containing mainly peptides) in the medium, and the highest proteinase activity level was found in the late stationary phase of growth (5). Immunological studies showed that strains E8, AM1, KH, and TR produced more proteinase in milk than in minimal media (9). It has been reported that proteinase production in strain Wg2 is dependent on the growth rate (17). Continuous cultures showed a maximum proteinase production at a growth rate (μ) of 0.23 h^{-1}. Remarkably, inhibition of the rate of proteinase production was observed after the addition of casein (which does not contain free peptides) and peptides (17). These data, showing that production of proteinase is medium dependent, were recently supported by studies on gene expression level. By using transcriptional gene fusions of the *prt* promoters with the *Escherichia coli* β-glucuronidase gene, it was shown that expression of both *prtP* and *prtM* (maturase) genes is controlled, at the transcriptional level, by specific dipeptides in the medium and by growth rate. A greater-than-10-fold decrease in both the *prtP* and *prtM* promoter activity was observed in peptide-rich laboratory media, like M17 medium, as compared with that in whey permeate medium with relatively low concentrations of peptides (19).

Because of the important role of proteinase in combination with the peptidases in flavour development of cheese, the regulated production of these enzymes was studied. Enzyme activity was used as a measure of total enzyme production (2, 11). We have investigated whether the regulation of expression of the *prt* genes, as described on the transcriptional level (19), is also observed at the level of enzyme production. We have studied not only PrtP production but also the production of two intracellular peptidases, the aminopeptidase PepN and the X-prolyl-dipeptidyl aminopeptidase PepXP. In the case of a potential strain dependency of this regulation, enzyme activities were measured in two derivatives of strains, *Lactococcus lactis* subsp. *lactis* MG1363 and *L. lactis* subsp. *cremoris* SK1128, both encoding the complete proteinase gene from strain SK110. The regulation pattern, previously presented for PrtP (19), was confirmed, and furthermore, it was found that the production of the intracellular peptidases was regulated in a strain-dependent manner.

Materials and Methods

Bacterial strains and growth conditions. L. lactis subsp. *lactis* MG1363 (7) and *L. lactis* subsp. *cremoris* SK1128 (3), both harbouring plasmid pNZ521, were used. Plasmid pNZ521 is a high-copy-number plasmid containing the *L. lactis* subsp. *cremoris* SK110 *prtP* and *prtM* genes, which together encode a functional cell wall-bound proteinase (4). *L. lactis* strains were routinely stored as a frozen stock at -80°C in M17 broth (Merck Darmstadt GmbH) with 15% (wt/wt) glycerol added. *L. lactis* cells were grown in glucose or lactose M17 broth

containing 10 mM $CaCl_2$ or in fresh skim milk, pasteurized for 30 min at 100°C, or in sterilized (15 min, 120°C) nitrogen-free, milk-derived, whey permeate (4) containing 1.9% (wt/vol) β-glycerophosphate, 10 mM $CaCl_2$, and different concentrations of the nitrogen source Casitone (0.2 to 2.0% [wt/vol]; Difco Laboratories, Detroit, Mich.), in all cases after precultivation in M17 broth. If relevant, the medium contained chloramphenicol (10 μg/ml), 0.5% (wt/vol) glucose, or 0.5% (wt/vol) lactose. In some experiments, the dipeptide prolylleucine (Bachem, Feinchemikalien AG, Bubendorf, Switzerland) was added to the whey permeate medium. In pH-regulated batch cultures, the pH was maintained at 6.3 by the addition of 10% (wt/vol) $NaHCO_3$ and 7.5% NH_4OH.

Continuous cultures of *L. lactis* MG1363 were grown in a 1-liter vessel (Applicon Dependable Instruments, Schiedam, The Netherlands) containing 0.5 liter of fresh skim milk, sterilized at ultra-high temperatures in a vacutherm instant sterilizer (Alfa-laval, Lund, Sweden) for 5 s at 144°C, and 0.35% (wt/vol) glucose. The culture was adjusted to a dilution rate (D) of 0.06 to 0.5 h^{-1} and a pH of 6.3. Under steady-state conditions, cells in the culture grow at a specific rate equal to the imposed dilution rate (D). Casitone (0.5% [wt/vol]), the dipeptide prolylleucine (final concentration, 0.5 mM), or the amino acid leucine (final concentration, 5 mM) was added to the medium when indicated. Anaerobic conditions were achieved by continuous flushing of the headspace with approximately 50 ml of N_2 gas per min.

The total number of cells in the continuously growing culture and the proteinase stability were determined by plating cells on GMA agar as described previously (10).

Preparation of standardized cell suspensions and cell extracts. Cells grown in batch cultures were harvested at the late log growth phase (A_{650}, 0.9 $\pm$ 0.1). Cultures grown on milk were clarified by an EDTA-borate treatment as described previously (20). The cells were washed twice with imidazole buffer (50 mM, pH 6.5), supplemented with 10 mM $CaCl_2$, and resuspended in the same buffer. The cell suspension was standardized at an A_{650} of 20, where an A_{650} of 1 corresponds to 225 μg of cell protein per ml. The cell extract was prepared as described previously (21).

Enzyme assays. L-Lactate dehydrogenase activity was assayed as described previously (8). PrtP activity was measured by a modification of the assay described previously (6) by use of the chromogenic substrate 3-carbomethoxypropionyl-L-arginyl-L-prolyl-L-tyrosine-*p*-nitroaniline hydrochloride (S2586; Kabi Diagnostica, Stockholm, Sweden). The assay mixture, a combination of 45 μl of imidazole buffer, 150 μl of NaCl (final concentration, 1.5 M), 100 μl of $CaCl_2$ (final concentration, 10 mM), 135 μl of demineralized water, 50 μl of substrate (10 mM; dissolved in demineralized water), and 20 μl of the standardized cell suspension, was incubated at 35°C for 10 min. The reaction was terminated by adding 500 μl of glacial acetic acid, the samples were centrifuged for 3 min at 6,000 $\times$ g, and subsequently, the released nitroaniline was measured at 410 nm in an Ultrospec Plus

spectrophotometer (Pharmacia LKB). For the assay of PepN and PepXP, the substrates L-leucine-4-nitroanilide-*p*-toluenesulfonate (Boehringer GmbH, Mannheim, Germany) and glycyl-prolyl-*p*-nitroanilide-*p*-toluenesulfonate (Sigma), respectively were used. Both peptidases were assayed in a 1-ml volume containing 20 μl of the standardized cell suspension, 780 μl of 50 mM Tris-HCl buffer (pH 7.5), and 200 μl of 4 mM substrate in 50 mM Tris-HCl (pH 7.5). The release of nitroaniline was measured on line at 410 nm in an Ultrospec Plus spectrophotometer with a thermostatically controlled cell compartment at 35°C.

Protein assay. Protein was determined by the method of Bradford (1) with bovine serum albumin as a standard.

Results

Activity level of the SK110 proteinase in host strains MG1363 and SK1128. Enzyme activity levels were measured after growth of *L. lactis* subsp. *lactis* MG1363 and *L. lactis* subsp. *cremoris* SK1128, both harbouring plasmid pNZ521, in media with different peptide sources. The results showed that for strain MG1363, the highest specific proteinase activity was observed at the end of the exponential growth phase in cells which were grown in milk medium (Fig. 1). The proteinase activity level obtained in cells that were grown on whey permeate medium supplemented with 0.5% Casitone and whey permeate medium supplemented with 2.0% Casitone were two- and fivefold lower, respectively, than the maximal proteinase activity on milk medium at the late exponential phase of growth (Fig. 1 and Table 1). Incubation of cells in whey permeate supplemented with Casitone at concentrations lower than 0.5% resulted in a sharp decrease of the specific growth rate together with a dramatic increase of the autoproteolytic breakdown of the proteinase (data not shown). When the MG1363 cells were grown on the nitrogen-rich M17 medium, the level of proteinase activity was approximately sixfold lower than the values obtained with the milk medium.

The effect of the medium composition on the activity level of the SK110 proteinase in strain SK1128 was also studied (Table 1). Cells of this strain showed a medium dependency of proteinase activity similar to that of strain MG1363 but to a lesser extent. The proteinase activity in cells of strain SK1128 grown in M17 medium was 2.5-fold lower than the activity level in milk. In whey permeate medium with increasing Casitone concentrations (0.5 to 4%), the proteinase activities were 3- and 1.3-fold lower in strains MG1363 and SK1128, respectively (data not shown), than the proteinase activities in the same strains grown in milk.

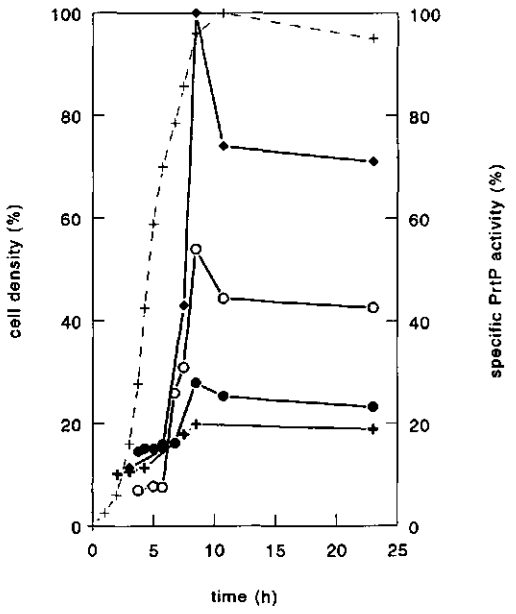

Figure 1. Specific PrtP activity of a batch culture of *L. lactis* MG1363 harbouring pNZ521 grown on either milk (♦), whey permeate with Casitone at 0.5% (○) or 2.0% (●), or M17 medium (+) at controlled pH conditions (pH 6.3) relative to the highest activity level reached on milk medium. The growth curve of strain MG1363 in the M17 medium is indicated by the broken line. In the four different media, the cultures reached the early stationary growth phase after the same time intervals. The data represent mean values of duplicate measurements.

Medium-controlled activity of two intracellular peptidases is strain dependent. Activity levels of PepN and PepXP were determined in cells of strain MG1363 and strain SK1128 grown in media with different peptide sources (Table 1). In cells of strain MG1363, a medium dependency similar to that observed for PrtP activity was found for the activity levels of PepN and PepXP. The highest levels of PepN activity were found in the late exponential growth phase in cells that were grown on milk. Increases in the Casitone concentration by 0.5 and 2.0% resulted in decreases of the PepN activity by factors of 2 and 4, respectively, as compared with the activity levels obtained after growth in milk. The lowest activity was found in cells grown in M17 medium, i.e., five times lower than the activity level in milk. PepXP activity appeared to be similarly controlled as PepN activity but to a lesser extent. The activity level in cells grown on M17 medium was lower only by a factor of two (Table 1) compared with the level found in cells grown in milk. In strain SK1128, neither PepN nor PepXP activity seemed to be affected by growth in the different media, except for the PepXP activity in cells that were grown in milk medium, which was approximately twofold lower than the activity levels obtained in the other media.

Table 1. Specific PrtP, PepN, and PepXP activities in whole cells of strains MG1363(pNZ521) and SK1128(pNZ521) after growth in milk, the nitrogen-rich M17 medium, or whey permeate (WP) with Casitone at different concentrations.

Enzyme	Medium	Specific activity[a]	
		MG1363(pNZ521)	SK1128(pNZ521)
PrtP	milk	65	19
	WP 0.5% Casitone	37	17
	WP 2.0% Casitone	13	14
	M17	10	7
PepN	milk	37	11
	WP 0.5% Casitone	17	9
	WP 2.0% Casitone	9	7
	M17	7	10
PepXP	milk	155	87
	WP 0.5% Casitone	136	139
	WP 2.0% Casitone	93	118
	M17	81	133

[a] Values are the average of at least two independent experiments. Enzyme activities are given in nmol(min × mg protein)$^{-1}$. The standard deviation is in all cases < 10%.

Since Casitone consists of mainly small peptides (19), these results suggest that in cells of strain MG1363, the activity levels of PepN and, to a lesser extent, of PepXP are controlled by the peptide concentration in the medium. The absence of this peptide dependency in cells of strain SK1128 indicates a strain-dependent regulation of these peptidases.

Effect of the dipeptide prolylleucine on the activity levels of PrtP, PepN, and PepXP. Previously, we had demonstrated that the addition of leucylproline or prolylleucine to a growth medium negatively affected the expression level of the *prtP* gene (19). In the present work, the effect of the dipeptide prolylleucine (final concentration, 0.05 to 0.5 mM) on the activity level of the PrtP, PepN, and PepXP in strain MG1363 after its addition to the growth medium containing 0.5% Casitone was studied. In control experiments, it was established that prolylleucine did not have any direct inhibitory effect on the activity of these enzymes (data not shown). At increasing prolylleucine concentrations in the whey permeate medium, the PrtP, PepN, and, to a slightly lesser extent, PepXP activity levels decreased gradually in cells of strain MG1363 (Table 2). The addition of 0.5 mM prolylleucine to the whey permeate medium resulted in PrtP, PepN, and PepXP activity levels that were similar to those found after growth of cells in peptide-rich M17 medium.

Table 2. Specific PrtP, PepN, and PepXP activities in whole cells of MG1363(pNZ521) after growth on whey permeate (WP) with the dipeptide prolylleucine (Pro-Leu) at various concentrations.

Enzyme	Addition to basic WP medium containing 0.5% Casitone	Specific activity[a]
		MG1363(pNZ521)
PrtP	none	37
	0.05 mM Pro-Leu	34
	0.1 mM Pro-Leu	30
	0.2 mM Pro-Leu	21
	0.5 mM Pro-Leu	10
PepN	none	17
	0.05 mM Pro-Leu	14
	0.1 mM Pro-Leu	14
	0.2 mM Pro-Leu	12
	0.5 mM Pro-Leu	10
PepXP	none	123
	0.05 mM Pro-Leu	120
	0.1 mM Pro-Leu	138
	0.2 mM Pro-Leu	104
	0.5 mM Pro-Leu	83

[a] Values are the average of two independent experiments. Enzyme activities are given in nmol(min × mg protein)$^{-1}$. The standard deviation is in all cases $< 10\%$.

Activity levels of PrtP, PepN, and PepXP of strain MG1363 in continuous cultures. The use of the different growth media and the addition of the dipeptide had a direct effect on the growth rate of the cells. Therefore, continuous-culture studies were carried out to investigate the effect of the growth rate on the repression of the different proteolytic enzymes by the dipeptide prolylleucine. Activity levels of PrtP, PepN, and PepXP were determined in cells of strain MG1363 during growth in a glucose-limited continuous culture with milk as the growth medium at various imposed growth rates (μ) (Fig. 2). The results show that the PrtP activity increased by a factor of three at increasing μ values from 0.06 to 0.50 h^{-1}. The PepN activity increased 1.5-fold with an increase of μ from 0.08 to 0.50 h^{-1}. The PepXP activity in cells of strain MG1363 was highest at a μ of 0.22 h^{-1} and decreased at μ values lower and higher than the optimum value. Apparently, the normal variation in the growth rate in batch cultures, ranging from 0.2 to 0.5 h^{-1}, as caused by the addition of prolylleucine, cannot completely explain the repression found for the various proteolytic enzymes.

Previously, we demonstrated that *prtP* gene expression decreased threefold with an increase of D from 0.05 to 0.5 h^{-1} (19), which seems to be in contrast with the results presented for the activity level (Fig. 2). This apparent discrepancy could be explained by the

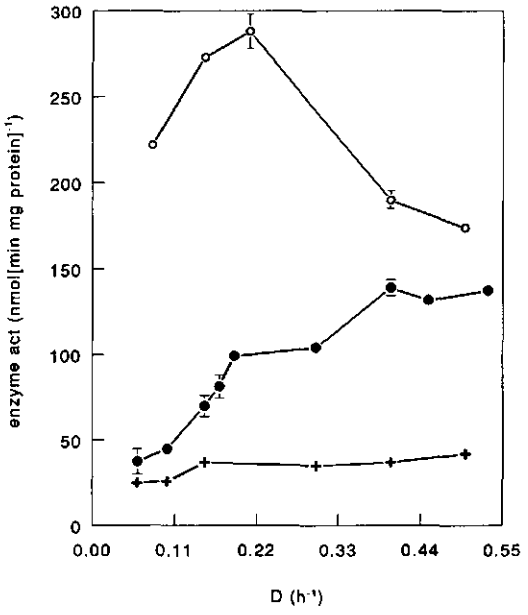

Figure 2. PrtP (●), PepN (+), and PepXP (○) activity (nmol[min × mg protein]$^{-1}$) at different dilution rates (*D*) of a continuous culture of *L. lactis* MG1363 harbouring pNZ521 grown on milk. The data represent mean values of duplicate measurements.

fact that proteinase would be more susceptible to autoproteolysis at a lower μ value, resulting in lower activity levels. To test this assumption, the specific proteinase activity was measured in MG1363 cells starting at the moment that the μ of the continuous culture was lowered from 0.45 to 0.10 h^{-1}. A decrease in the proteinase activity that was much faster than that of the calculated dilution curve was observed (Fig. 3). This rapid decline in the proteinase activity within 6 h to the low level which corresponds to a μ of 0.1 h^{-1} can be explained only by a growth-rate-dependent autoproteolytic breakdown, with the proteinase being more susceptible to autoproteolysis at a lower growth rate.

The repression by Casitone and the dipeptide prolylleucine was also studied during growth of MG1363 in continuous culture. An extra addition of 0.5% Casitone (final concentration) at a fixed μ of 0.2 h^{-1} resulted in an immediate decline of the PrtP and PepN activity levels and, to a much lesser extent, of the PepXP activity level (Fig. 4A). The minimum level was reached approximately 4 h after the addition. Over this period, the PrtP, PepN, and PepXP activity levels decreased by 40, 30, and 13%, respectively. Subsequently, the enzyme activity increased to the levels that existed before the addition. Comparable results were found in an experiment in which 0.5 mM prolylleucine (final concentration) was added (Fig. 4B). The minimum activity levels of PrtP and PepN were reached after approximately 2 h, whereas the activity levels of PepXP seemed to be insensitive to the increased prolylleucine concentrations in the medium. During the pulse experiments, the

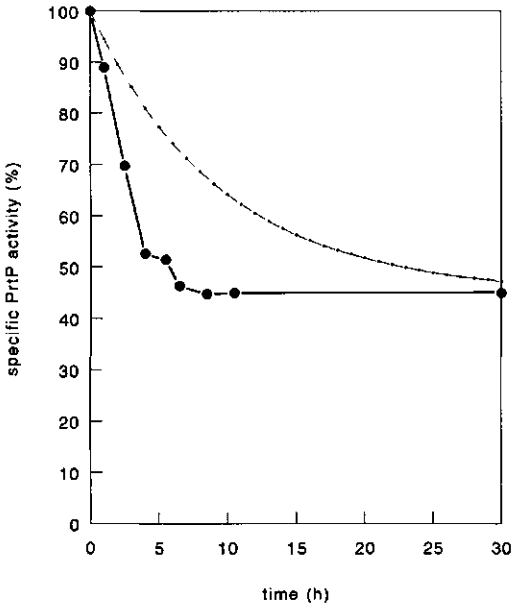

Figure 3. Changes in the specific PrtP activity (●) in a continuous culture of *L. lactis* MG1363 harbouring pNZ521 after changing the dilution rate (D) of 0.45 h^{-1} to 0.10 h^{-1} at time zero. The calculated dilution curve of the PrtP activity is indicated by the broken line. Steady-state activity at a D of 0.45 or 0.10 h^{-1} corresponds to 132 and 50 nmol(min × mg protein)$^{-1}$, respectively.

amount of biomass remained stable in the culture, and in all cases, the cells were growing under glucose limitation. Lactate dehydrogenase, which was measured as a marker enzyme, showed a constant activity during the 10 h after pulsing the continuous culture with Casitone or prolylleucine (data not shown).

To investigate the possible role of the amino acids leucine and proline as effector molecules, we carried out some growth experiments in batch cultures with the addition of leucine or proline (final concentrations, 1, 5, and 10 mM) to the whey permeate medium. Only leucine slightly inhibited the activity level of the PrtP of cells of strain MG1363 (data not shown). To rule out the effect of the growth rate, the extra addition of leucine (final concentration, 5 mM) was repeated in a continuous culture (Fig. 4C). At a μ of 0.20 h^{-1}, the addition resulted in a slight decrease in the activity level of PrtP, parallel to the theoretical washout curve, which reached a minimum 2 h after the pulse. PepN and PepXP activity levels seemed not to be affected by the increased leucine concentration in the medium.

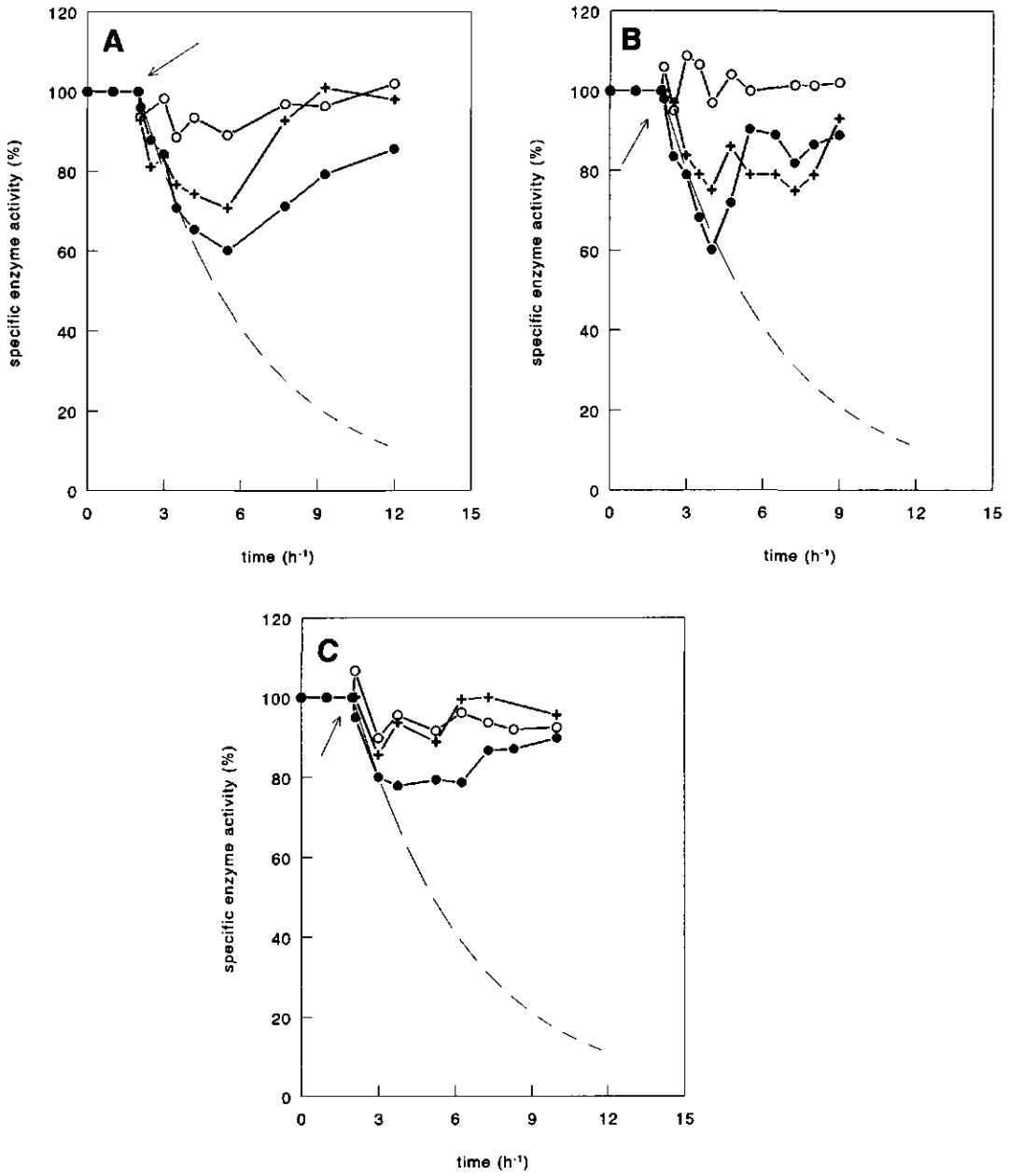

Figure 4. Changes in the specific PrtP (●), PepN (+), and PepXP (○) activity in continuous cultures of *L. lactis* MG1363 harbouring pNZ521 at a dilution rate of 0.20 h^{-1} in milk after the addition of 0.5% Casitone (A), 0.5 mM prolylleucine (B), and 5 mM leucine (C) (arrow indicates time of addition). The calculated dilution curve in the case of complete inhibition is indicated by a broken line.

Discussion

We have studied the regulation of three different enzymes involved in the proteolytic system of lactococci by making use of activity measurements. The activity levels of PrtP, PepN, and PepXP of two different host strains were measured in batch and continuous cultures with different growth media. The results show that the PrtP activity in cells of strain MG1363 is decreased by a factor of six, while in cells of strain SK1128 the PrtP activity is decreased approximately by a factor of three by increasing the peptide content of the medium. In both cases, the activity level was highest under low peptide concentrations, such as in milk, while the lowest activity was found in the peptide-rich media, such as M17 medium. Since a direct relationship between PrtP activity and PrtP production has been demonstrated (2, 11), these data imply a medium dependency of PrtP production for several reasons. First, the enzyme activity itself was not directly affected by the addition of the dipeptide. Second, the medium-dependent regulation of PrtP, which is observed at the activity level, is in agreement with our previous work which showed that expression of both the *prtP* and *prtM* genes is controlled at the transcriptional level by the peptide concentration in the medium (19). Apparently, no other regulation mechanisms are active between gene expression and enzyme activity.

Whereas the PrtP activity level is controlled in both *L. lactis* strains, the medium-dependent regulation of the intracellular peptidases was observed only in cells of strain MG1363 (Table 1). The results show that the activity levels of PepN and PepXP are repressed approximately by factors of five and three, respectively, by the peptides in the medium, whereas in cells of strain SK1128, no repression is observed for either peptidase. This indicates that the intracellular peptidases PepN and PepXP are regulated by the peptide content of the medium but, in contrast to the PrtP activity, in a strain-dependent manner. Although the intracellular peptidases and PrtP may be controlled by the same mechanism, it is more likely that two different control mechanisms are involved. In this respect, the mechanism for the production of the PrtP may be controlled by a more general, strain-independent mechanism, whereas the production of the intracellular peptidases may be controlled by a strain-dependent mechanism.

The growth rate of the cells also has an effect on the production of PrtP. The PrtP activity level in cells of strain MG1363 increased with an increase of the dilution rate from 0.06 to 0.50 h^{-1} (Fig. 2). For synthesis of the proteinase of *L. lactis* subsp. *cremoris* Wg2, a different growth rate dependency has been observed. In minimal medium, with amino acids as the sole nitrogen source, a maximal production of PrtP was found at a dilution rate (D) of 0.23 h^{-1}; production decreased at lower and higher dilution rates (17). However, the potential effect of autoproteolysis on PrtP production level was not discussed. The influence of the growth rate on the activity levels of PepN and PepXP was studied only in cells of strain MG1363 and appeared to be of minor influence.

Dipeptides, in particular prolylleucine, play an important role in the medium-dependent

regulation of the proteolytic enzyme activity in lactococci (19). In cells of strain MG1363, a concentration of 0.5 mM of the dipeptide prolylleucine is needed to repress the activity of PrtP and the two intracellular peptidases to the minimal level found in cells grown on the peptide-rich M17 medium. Our studies with the continuous cultures show that the decrease of proteolytic activity, caused by Casitone and more specifically by prolylleucine, can be observed independent of the growth rate. In pulse experiments, the addition of the peptides to a steady-state culture resulted in an immediate decline of the PrtP and PepN activity in cells of strain MG1363, parallel to the kinetics of the theoretical washout curve. This indicates that the overall synthesis of the proteolytic enzymes had come to a complete stop. In contrast, PepXP activity in cells of strain MG1363, grown at a fixed rate, seems to be insensitive to the addition of prolylleucine. This suggests either that not all of the peptidases are controlled in their activity level by the peptide content of the medium or that other peptides are involved in the regulation of the level of PepXP activity.

Previously, we have presented a model in which dipeptides (or derivatives thereof), after their uptake by the cells, serve as effector molecules in the controlled production of proteolytic enzymes (19). The addition of leucine in a 10-times-higher concentration had less effect than the addition of the dipeptide prolylleucine, although the uptake systems for the amino acid leucine and the peptide prolylleucine have similar efficiencies (22, 23). This emphasizes the specific role of dipeptides in this control mechanism.

Repression of proteolytic activity is normally not encountered during growth of *L. lactis* in milk. PrtP, PepN, and PepXP had the highest activity levels under these conditions as compared with growth on complex amino acid and peptide-containing growth media. Apparently, no regulating peptides are accumulated during growth of *L. lactis* in milk. This is in agreement with earlier reports (13) that PrtP activity does not release detectable amounts of di- and tripeptides during hydrolysis of casein. This and previous work suggest that only during growth on (di)peptide-rich medium will the cell control its proteolytic activity by repression of PrtP, PepN, and PepXP (19). In this regulation, the presence of the di- and tripeptide transport system (DtpT) seems to be essential, as indicated by the increased expression level of the *prtP* gene and PrtP enzyme and the reduced level of regulation observed in a DtpT-negative mutant (16, 19).

Future studies will focus on the transcriptional basis for the medium-dependent regulation of the different peptidases. Furthermore, we aim to identify the intracellular repressor protein which senses the intracellular concentration of the dipeptide. Special attention will be paid to the host strain dependency of this regulation in relation to the selection of strains suitable for different starter cultures used in the dairy industry.

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5

Proteolytic Enzyme Activity in Lactococci Grown in Different Pre-Treated Milk Media

Wilco Meijer, and Jeroen Hugenholtz

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Proteolytic Enzyme Activity in Lactococci Grown in Different Pre-Treated Milk Media

Lactococcus lactis subsp. *lactis* MG1363, harbouring plasmid pNZ521, which encodes the extracellular serine proteinase (PrtP) from strain SK110, was used to study the effect of two different treatments of the growth medium milk on the activity levels of PrtP and the intracellular aminopeptidase PepN and X-prolyl-dipeptidyl aminopeptidase PepXP. All three proteolytic enzymes showed lower activity levels in cells grown in high heat treated milk as compared to cells grown in non-heat treated milk. Highest activity levels of the three studied enzymes were found in cells grown in milk heat treated for 30 min at 63°C. Using cells of strain *L. lactis* subsp. *lactis* MG1363, harbouring plasmid pNZ544, which encodes reporter gene *gusA* under control of the *prtP* promoter, it was demonstrated that the regulation of PrtP takes place at the transcription initiation level. After separation of the pH 4.6 soluble fraction of high heat treated milk with reverse phase HPLC it was found that the hydrophilic small peptide fraction of the milk was responsible for this regulation. Amino acid analysis of this fraction confirmed that this fraction consisted of peptides only. Ultrafiltration of milk, which increases the dry matter of the milk specifically through increase of its protein content, only significantly affected the levels of PrtP and PepN in cells of strain MG1363. Highest activity was found during growth in un-concentrated milk, and the lowest level was found during growth in four times concentrated retentate. Using cells of strain MG1363(pNZ544), it was demonstrated that also in this case regulation of PrtP takes place at the level of transcription initiation. Approximately 40% of the decrease in activity of PrtP and PepN could be explained by the presence of higher amounts of purified whey proteins and higher amounts of dry mass. This suggests the presence of another factor, concentrated by ultrafiltration, which controls the production of different proteolytic enzymes in concentrated retentate.

The proteolytic system plays an essential role in the nitrogen metabolism of lactococci. The extracellular cell wall-bound serine proteinase (PrtP) is a key enzyme in this system, the activity of which is necessary for growth of lactococci in milk by initiating the breakdown of the casein to smaller peptides, mainly oligopeptides (9). After transport to the intracellular environment by the oligopeptide transport system (11), these peptides are further degraded into smaller peptides and amino acids by intracellular peptidases (10). This is essential for

growth of the lactococcal cells in milk, as they have multiple amino acid auxotrophies (21). The amino acids also play a crucial role in the flavour development in different fermented milk products, as they are precursors for many volatile aromatic cheese components (5, 22, 23).

Although the biochemical and genetic properties of the proteolytic system of lactococci have been extensively studied and excellently reviewed (10, 21), regulation of the production of some of the proteolytic enzymes has been addressed just recently. The activity of PrtP, aminopeptidase PepN and X-prolyl-dipeptidyl aminopeptidase PepXP was shown to be controlled by the peptide content of the medium (17). It was shown that the PrtP, PepN and PepXP activities were highest in cells grown in milk, compared to activities in cells which were grown in a peptide-rich medium. A specific increase of the concentration of the dipeptides prolylleucine or leucylproline resulted in a similar controlled repression as observed in the peptide rich M17 medium. By using transcriptional gene fusions of the *prtP* and *prtM* promoters with the promoterless β-glucuronidase gene from *Escherichia coli*, it has been shown that this medium-dependent regulation of PrtP takes place at the level of transcription initiation (13, 14). It is currently assumed that the di-tripeptide transport system (Dtp-T) may function as a sensor for (di)peptide-rich media, by which the cell can control its proteolytic activity by repression of the *prtP* gene expression level. This hypothesis was supported by the facts that (i) PrtP activity levels were highest during growth of *Lactococcus lactis* in milk (17), (ii) PrtP activity hydrolyses casein mainly into oligopeptides and not in detectable amounts of di-, tripeptides (9), and (iii) a DtpT-negative mutant, grown in peptide-rich media, showed increased *prtP* gene expression levels and subsequent PrtP enzyme production (11, 13).

The peptide-composition of milk, which is the basic medium used for the production of all fermented dairy products, can be influenced by a variety of treatments. Pasteurization, to obtain a better conservation, or more severe heat treatments of milk, applied for instance to ensure the incorporation of extra whey proteins in cheese (18), can affect the composition of the milk by e.g. hydrolysis of casein or by increased plasmin activity at the higher pasteurization temperatures. Ultrafiltration of milk, which is also used to increase cheese yield, changes the composition of the milk by increasing the protein content of the milk (15). The altered peptide composition of milk as a result of both treatments negatively affects the flavour development in cheeses made from this milk (1, 12).

Because of the important role of the proteolytic enzymes in flavour development of fermented milk products, we investigated the effect of different heat treatments of the milk and concentrating the milk by ultrafiltration on (i) the activity levels of the proteolytic enzymes, PrtP, PepN and PepXP in cells of strain *Lactococcus lactis* subsp. *lactis* MG1363 grown on these various milk media and, subsequently, (ii) the peptide composition of the milk media. The results indicate that the activity level of the enzymes is negatively affected by peptides produced during the different treatments of the milk. By making use of transcriptional gene fusions it was confirmed that the regulation for PrtP was on the level of

transcription initiation.

Materials and Methods

Bacterial strains and growth conditions. Lactococcus lactis subsp. *lactis* MG1363 (6), harbouring plasmid pNZ521 or pNZ544, was used. Plasmid pNZ521 is a high-copy-number plasmid containing the complete *prtP* and *prtM* genes of *L. lactis* subsp. *cremoris* SK110, which together encode for the functional extracellular cell-wall bound serine proteinase (4). Plasmid pNZ544 contains the transcriptional gene fusion of the promoterless β-glucuronidase gene of *Escherichia coli,* placed under control of the *prtP* promoter (13).

L. lactis strains were routinely stored as a frozen stock at -80°C in M17 broth (Merck Darmstadt GmbH) with 15% (wt/wt) glycerol added. *L. lactis* cells were grown in fresh, not heat treated skim milk, reconstituted skim milk or in ultrafiltrated (UF) retentates all containing 1.9% (wt/vol) ß-glycerophosphate; or in sterilized (15 min, 120°C) M17 broth (Merck GmbH Darmstadt, Germany) containing 10 mM $CaCl_2$; or in sterilized (15 min, 120°C) nitrogen free, milk derived whey permeate (4) containing 1.9% (wt/vol) ß-glycerophosphate, 10 mM $CaCl_2$ and Casitone (Difco Laboratories, Detroit, Mich.) as nitrogen source. For growth of strain MG1363 with plasmid pNZ544 in whey permeate medium 0.1% (wt/vol) Casitone was added, and for growth of strain MG1363 with plasmid pNZ521 0.5% (wt/vol) Casitone. In all cases the strains were precultivated in M17 broth. The fresh skim milk was used after various heat treatments, 30 min at 63°C, 30 min at 100°C, and 10 or 20 min at 120°C. The reconstituted skim milk, heat treated for 30 min at 120°C, was dissolved to a dry matter (DM) content comparable with the DM of the UF-retentates as described previously (16). The UF-retentates, heat treated for 30 min at 100°C, were prepared by an ultrafiltration procedure as described previously (16). All media contained chloramphenicol (10 μg/ml) and 0.5% (wt/vol) glucose. In some experiments separated peptide fractions of the milk heat treated for 20 min at 120°C, were added to the whey permeate medium (0.25 mg/ml). The initial pH of the milk medium was adjusted to 6.8. In pH-regulated batch cultures, the pH was maintained at 6.3 by the addition of 10% (wt/vol) $NaHCO_3$ and 7.5% NH_4OH. Where indicated, a whey protein concentrate was added to the milk medium, which was prepared as described previously (16).

Preparation of standardized cell-suspensions and cell-free extracts. Cells grown in batch-cultures were harvested at the exponential growth phase (A_{650nm} of 0.9 ± 0.1). The cultures grown in milk were clarified by an EDTA-borate treatment as described previously (16). The cells were washed twice with imidazole buffer (50 mM, pH 6.5), supplemented with 10 mM $CaCl_2$ and resuspended in the same buffer. The cell suspension was standardized at an A_{650nm} of 20, where A_{650nm} = 1 corresponds to 225 μg cell protein per ml. The cell-free extract was prepared as described previously (19).

Enzyme assays. L-Lactate dehydrogenase (LDH) (8), PrtP, aminopeptidase N (PepN) and the X-prolyl-dipeptidylaminopeptidase (PepXP) (17), pyruvate kinase (PK) (3), and β-glucuronidase activity (13) were assayed as described previously.

Protein assay. Protein was determined according to Bradford (2) using bovine serum albumin as a standard.

Peptide analysis of the milk media. To prepare milk samples for analysis of the small peptide fractions with HPLC, the milk samples were adjusted with 2 N HCl to a pH 4.6 and subsequently clarified by centrifuging 10 min at 14,000 rpm. The supernatant was 1:1 (vol/vol) diluted with buffer A (see below), centrifuged again and prepared for HPLC analysis. For the semi-preparative HPLC the pH 4.6 soluble fraction was concentrated 5 times. A response curve for the dipeptide prolylleucine (Bachem Feinchemicalien AG, Bubendorf, Switzerland) of a known nitrogen content was made.

Peptide analysis was carried out using HPLC equipment consisting of two M 6000A pumps (Waters Assoc.), an ISS-100 automatic sample injector (Perkin-Elmer), a Kratos Model 783G UV detector and a Waters Type 680 automated gradient controller. The equipment was linked to a data acquisition and processing system (Turbochrom, Perkin-Elmer). Buffer A was acetonitrile-water-trifluoroacetic acid (100:900:1 vol/vol/vol) and buffer B was the same mixture with the proportions 900:100:0.7 (vol/vol/vol).

For analytical RP-HPLC a 250 mm × 4.6 mm I.D. Hipore RP-318 column (Bio-Rad Labs.) was used with a C_{18} cartridge (Bio-Rad Labs.) as a guard column. Starting from 0% of buffer B a gradient was generated immediately after injection by gradually increasing this proportion to 50% after 60 min, 70% after 65 min, no increase for 5 min, before returning to starting conditions in 5 min. The column temperature was 30°C and peak detection was at 220 nm. The flow rate was 0.8 ml min^{-1} and the injection volume 100 μl. The system pressure was 1500 p.s.i..

For semi-preparative RP-HPLC a 250 mm x 10 mm RP-318 column (Bio-Rad Labs.) was used at 30°C. Using a flow rate of 8.8 ml min^{-1} the slope of the above-mentioned solvent gradient was adjusted to obtain optimal separation for the various fractions. Peak detection was at 220 nm and the injection volume was 450 μl. Fractions of the effluent were collected, concentrated by evaporation at reduced pressure, and then freeze dried.

Amino acid analysis. Amino acid analysis was performed on a 4151 Alpha Plus amino acid analyser (Pharmacia/LKB, Uppsala, Sweden) after hydrolysis of the samples with 6 M HCl in evacuated tubes at 110°C for 24 h.

Results

Enzyme activity levels of L. lactis MG1363 grown in various heat treated milk media. Enzyme activity levels were measured after growth of *L. lactis* MG1363, harbouring plasmid pNZ521 or pNZ544, in various heat treated milk media. The results showed that the specific PrtP activity of strain MG1363(pNZ521), was highest in cells grown in the milk heat treated for 30 min at 63°C (Table 1). The PrtP activity decreased in cells grown in milk that received more severe heat treatments. The activity level in the milk heat treated for 20 min at 120°C was twofold lower than the maximal proteinase activity found in the milk medium heat treated for 30 min at 63°C.

Activity levels of PepN and PepXP were also determined in cells of strain MG1363(pNZ521) grown in various heat treated milk media (Table 1). For both enzymes a similar effect of heat treatment on activity was observed, as was found for PrtP activity. The highest activity level was found in cells grown in milk which was heat treated for 30 min at 63°C, i.e. approximately two times higher than the activity level found in milk which was heat treated for 20 min at 120°C. Activities of two glycolytic enzymes, LDH and PK, were also assayed in MG1363(pNZ521) cells grown in various heat treated milk media. In contrary to the proteolytic enzymes, neither LDH nor PK activity seemed to be significantly affected in cells grown on heat treated milk.

The effect of the heat treatment on the proteinase activity is mediated at the expression level. To investigate whether the specific proteinase activity is affected at the level of activity or at the level of gene expression during growth of cells in different heat treated milk media,

Table 1. Specific activities[a] of PrtP, PepN, PepXP, LDH and PK in cells of strain MG1363(pNZ521) and of β-glucuronidase (GUS) in cells of strain MG1363(pNZ544) after growth in fresh milk that received various heat treatments.

heat treatment milk	specific activity					
	PrtP	PepN	PepXP	LDH	PK	GUS
-	92	81	88	97	107	116
30 min 63°C	100	100	100	100	100	100
30 min 100°C	72	63	70	88	100	100
10 min 120°C	73	56	65	98	93	69
20 min 120°C	56	44	64	101	111	53

[a] Entries are the average of at least two independent experiments. Enzyme activities are given in % relative to the activity level reached on the milk medium heat treated for 30 min at 63°C, the generally used heat treatment for cheese production. Relative activity of 100% corresponds to 96, 54, 245, 9600, 500 and 64 μmol(min × mg protein)$^{-1}$ for PrtP, PepN, PepXP, LDH, PK and GUS respectively. SD is in all cases < 10%.

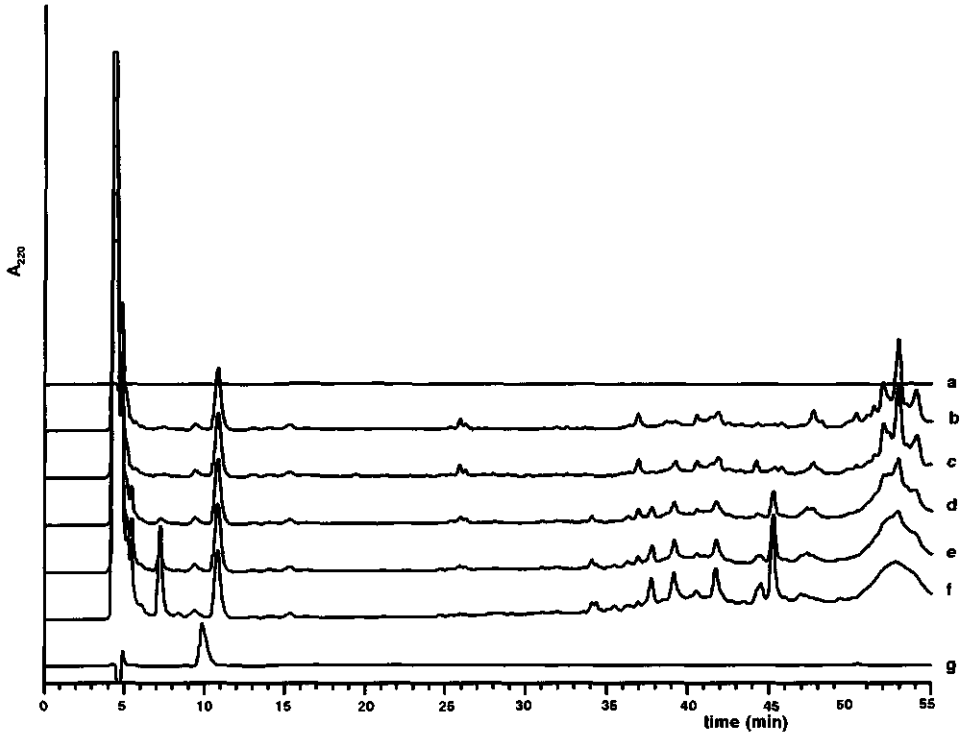

Figure 1. Reversed-phase HPLC chromatograms of buffer A as a control (a), and the pH 4.6 soluble fractions of milk samples subjected to various heat treatments; non-heated milk (b), 30 min 63°C (c), 30 min 100°C (d), 10 min 120°C (e), 20 min 120°C (f), and 1 mM of the dipeptide prolylleucine (g). The chromatographic conditions were those described for separation on the analytical column (see Materials and Methods). A_{220} is the absorbance at 220 nm.

activities of the reporter enzyme β-glucuronidase, which is under control of the *prtP* promoter in cells of strain MG1363(pNZ544), were measured in those milk media. The specific β-glucuronidase activity was highest in cells grown in the non-heat treated fresh skim milk (Table 1). The activity level obtained in cells that were grown in the 20 min 120°C milk was twofold lower than the maximal activity found in the non-treated milk medium. This indicates that the proteinase activity of cells grown in milk subjected to different heat treatments is regulated at the transcription initiation level.

Peptide accumulation in various heat treated milk media. Previously, we have demonstrated that specific peptides repress the production level of the PrtP, PepN and PepXP in lactococci (17). Therefore, we compared the composition of free peptides in the milk after the different heat treatments (Fig. 1). The RP-HPLC pattern show that small, hydrophilic and

Table 2. Specific activities[a] of PrtP, PepN and PepXP in cells of strain MG1363(pNZ521) and of β-glucuronidase (GUS) in cells of strain MG1363(pNZ544) after growth in whey permeate (WP), in the nitrogen-rich M17 medium, or WP enriched with different peptide fractions A to D, isolated using semi-preparative RP-HPLC as shown in Fig. 2.

medium	specific activity			
	PrtP	PepN	PepXP	GUS
WP	100	100	100	100
M17	26	25	52	11
WP + A	15	58	78	37
WP + B	56	63	101	74
WP + C	48	120	98	100
WP + D	59	58	98	74

[a] Entries are the average of at least two independent experiments. Enzyme activities are given in % relative to the activity level reached in whey permeate medium. Relative activity of 100% corresponds to 35, 24, 128 and 20 μmol(min × mg protein)$^{-1}$ for PrtP, PepN, PepXP and GUS respectively. SD is in all cases < 10%.

larger, hydrophobic peptides are accumulated as a result of the heat treatment of the milk medium.

To investigate which accumulated peptide fraction was responsible for the decreased enzyme activity levels of the various proteolytic enzymes in cells grown in high heat treated milk, we separated the peptides present in the milk heat treated for 20 min at 120°C using semi-preparative RP-HPLC (Fig. 2). The HPLC eluent is separated into six peak fractions and four base fractions, from which these peaks are excluded, indicated as 1-6 and A-D, respectively. Activity levels of PrtP, PepN and PepXP were determined in cells of strain MG1363(pNZ521) grown in the peptide-rich M17 medium and in whey permeate with and without addition of the various freeze dried eluent fractions of the RP-HPLC (final concentration 0.25 mg dry weight/ml). The activity levels of all three proteolytic enzymes studied were not affected by the individual addition of fraction 1 to 6 to the growth medium. The addition of fraction A to the growth medium whey permeate resulted in a sixfold lower PrtP activity in cells of strain MG1363(pNZ521), relative to cells grown in whey permeate without additions (Table 2). Addition of fractions B, C or D resulted in a twofold reduction of the PrtP activity; whereas growth of cells of strain MG1363(pNZ521) in M17 caused a fourfold reduction in PrtP activity as compared to the activity level in cells grown in whey permeate, as was shown before (17). Activity of PepN in cells grown in whey permeate was approximately two times reduced by the addition of fraction A, B or D compared to the activity in whey permeate, whereas addition of fraction C caused a slight increase. The PepXP activity level was only slightly reduced by the addition of fraction A to the growth

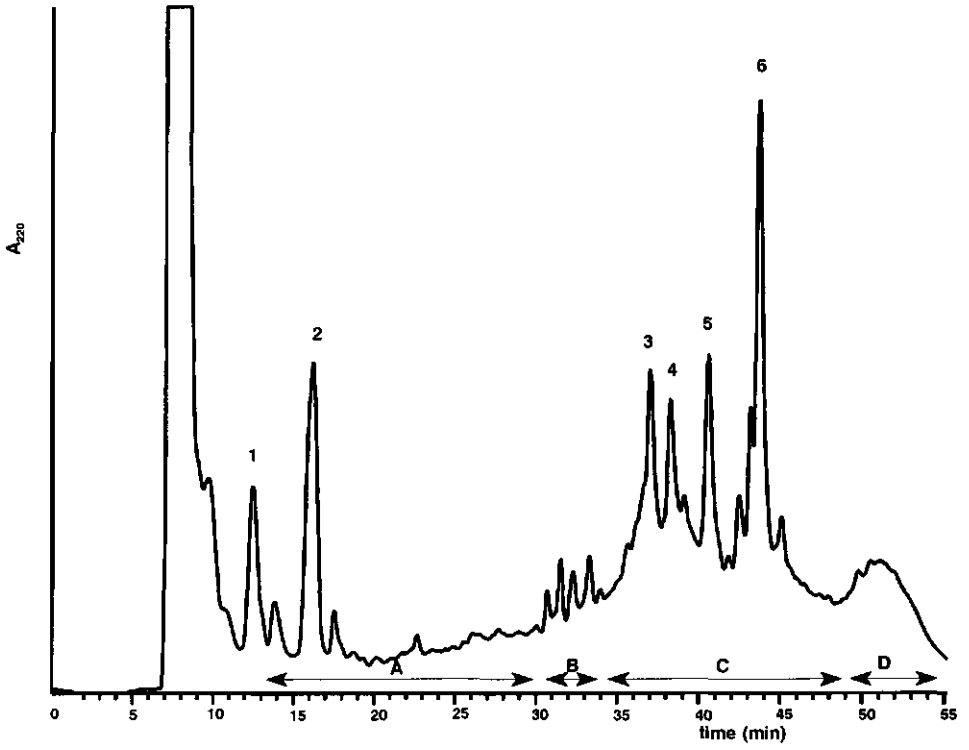

Figure 2. Reversed-phase HPLC chromatogram of the pH 4.6 soluble fraction of milk treated for 20 min at 120°C. Effluent fractions according to numbers 1-6, indicating six peptide fractions and letters A-D, indicating the rest fractions without the peak areas, were separated. The chromatographic conditions were those described for separation on the semi-preparative column (see Materials and Methods). A_{220} is the absorbance at 220 nm.

medium.

Activities of the reporter enzyme β-glucuronidase in cells of strain MG1363(pNZ544) were also measured in whey permeate supplemented with the various fractions. Addition of fraction A resulted in a three times lower β-glucuronidase activity in cells of strain MG1363(pNZ544), relative to cells grown in whey permeate, whereas addition of fractions B or D just slightly reduced the β-glucuronidase activity. Remarkably, addition of fraction C had no effect on the β-glucuronidase activity in cells of strain MG1363(pNZ544), which does not correlate with the effect found on the activity level of PrtP.

To further establish that peptides actually control the production of the investigated proteolytic enzymes in cells grown in extensively heat treated milk, we analysed the composition of fraction A, B and C. After hydrolysis of these fractions it was demonstrated using amino acid analysis that fraction A, B and C exclusively contained amino acid residues.

Table 3. Specific activities[a] of PrtP, PepN, PepXP, LDH and PK in cells of strain MG1363(pNZ521) and of β-glucuronidase in cells of strain MG1363(pNZ544) after growth in milk, UF-retentates with different concentration factors (CF), and reconstituted skim milk (RSM) with a dry matter content comparable to the UF retentates.

medium and CF	specific activity					
	PrtP	PepN	PepXP	LDH	PK	GUS
milk	100	100	100	100	100	100
UF 2.5	65	61	86	88	95	86
UF 4.0	64	57	83	92	102	59
RSM 1.0	100	100	100	ND[b]	ND	ND
RSM 2.5	104	88	90	ND	ND	ND
RSM 4.0	91	75	84	ND	ND	ND

[a] Entries are the average of at least two independent experiments. Enzyme activities are given in % relative to the activity level reached on the non-concentrated milk medium. Relative activity of 100% corresponds to 65, 34, 190, 9600, 497 and 63 μmol(min × mg protein)$^{-1}$ for PrtP, PepN, PepXP, LDH, PK and GUS respectively. SD is in all cases < 10%.
[b] ND, not determined.

The amino acid composition of the fractions reflected the total amino acid composition of the milk proteins.

Enzyme activity levels of L. lactis MG1363 grown in UF-retentates. To investigate the effect of the use of milk concentrated by ultrafiltration on the production level of different proteolytic enzymes, we carried out growth experiments with strain MG1363(pNZ521) on milk and UF-retentates. An approximately 1.5 times reduction in the specific PrtP and PepN activity was observed in cells grown in 4 times concentrated retentate compared to the activity level found in non-treated milk (Table 3), while PepXP activity was not significantly affected by the concentration factor of the retentate. For PrtP and PepN activity almost the same decrease in activity was found in cells which were grown in UF-retentate with concentration factors of 2 and 4. In a control experiment it was demonstrated that the temperature and pressure of the milk during the UF process did not affect the activity level of the three proteolytic enzymes (data not shown). It was also shown in pH-regulated batch cultures that the different buffering capacities of the milk and UF-retentates did not affect the activity level of the proteolytic enzymes (data not shown). The addition of a mixture of non-denaturated, purified whey proteins, prepared from mixed cheese whey, to the milk medium in a concentration similar to those found in UF-retentates concentrated fourfold resulted in a small decrease of the activity level of PrtP and PepN in cells of strain MG1363(pNZ521) compared to the activity on fresh skim milk; PepXP activity was not significantly affected by the addition of the purified whey proteins (data not shown). The addition of the whey

proteins accounted for approximately 40% of the decrease in activity in cells grown in UF-retentates as compared to cells grown in milk, indicating that another fraction which is concentrated by the ultrafiltration procedure is also responsible for the inhibition of enzyme production.

The effect of the growth of lactococci in UF-retentates on the activity level of some glycolytic enzymes was also studied (Table 3). Cells of strain MG1363(pNZ521) showed no significant change in LDH and PK activity when grown in four times concentrated retentate, as compared to the activity level found in milk.

Subsequently, β-glucuronidase activities in cells of strain MG1363(pNZ544) grown in different UF-retentates were determined. The activity level was twofold lower in cells grown in four times concentrated UF-retentate, as compared to the activity on fresh skim milk, which establishes that for the proteinase the inhibition is mediated at the transcription initiation level.

Effect of dry matter on the activity level of the proteolytic enzymes. The increased dry matter (DM) content in the UF-retentates was simulated by using reconstituted skim milk (RSM) with a dry matter content comparable to the dry matter of the UF-retentates (Table 3). Cells of strain MG1363(pNZ521) grown on RSM with a four times increased DM content showed that the specific activity of PepN was slightly reduced compared to the activity level on non-treated milk. In contrast, PrtP and PepXP activities are not significantly affected at all by the increased DM content of the milk.

Discussion

The activity levels of PrtP, PepN and PepXP in *L. lactis* MG1363(pNZ521) cells were determined after growth in milk which was heat treated at various intensities. The results obtained show that the highest proteolytic activity was found in milk that was heat treated for 30 min at 63°C. With more severe heat treatments of the milk medium the activity level of all three proteolytic enzymes decreased, the lowest activity levels were found in milk heat treated for 20 min at 120°C. With the transcriptional gene fusion of the *prtP* promoter and *gusA* genes, it was demonstrated that this regulation of proteolytic enzyme activity takes place at the gene expression level. Addition of fractions containing small and/or hydrophilic peptides, isolated by reverse phase HPLC from milk heat treated for 20 min at 120°C, to the growth medium whey permeate also decreased the activity level of the three proteolytic enzymes. These results suggest that small peptides, accumulating in milk due to intense heat treatment, play a role in the regulation of the production of proteolytic enzymes. This is in good agreement with our earlier work, which showed that specific dipeptides, such as prolylleucine, could function as effector molecules for the controlled production of the proteolytic enzymes (13, 14, 17).

PrtP activity, and to a lesser extent also PepN, are affected by the peptides present in the effluent of fraction A mainly (Fig. 2, Table 2). The PrtP and PepN activity levels in cells of strain MG1363(pNZ521) are repressed approximately 6 and 1.7-fold, respectively, by the addition of peptide fraction A to the growth medium whey permeate (Table 2). As suggested from the position of the reference peak prolylleucine in the reverse phase pattern (Fig. 1) this fraction A consisted mainly of di- and tripeptides, at an estimated concentration of 0.8 to 1.2 mM in the growth experiments. This concentration range is in good correlation with earlier presented work, in which addition of 0.5 mM prolylleucine decreased the activity of PrtP in cells grown in whey permeate to the level obtained in cells grown in M17 (17). Remarkably, the addition of fraction C to the growth medium whey permeate, only decreased the activity level of PrtP, while the *prtP* expression was apparently not affected by the addition of fraction C. This contradiction suggests the presence of peptides in fraction C which inhibit (i) PrtP at the enzyme level, maybe allosterically, which has only been described earlier for peptidases (7), or (ii) the translation, secretion or processing of the PrtP protein. This could also explain the apparent contradiction between the PrtP activity and *prtP* expression level in cells grown in milk that was heat treated for 30 min at 100°C.

The fact that PepXP is almost insensitive to the addition of the small peptide fractions indicates that PepXP is either not controlled by the peptide content of the medium, or, more likely, is regulated by other peptides, as we concluded before (17).

Whereas the PrtP activity level is mainly controlled by small hydrophilic peptides, PepN activity is also controlled by peptides present in fractions B and D, which contain large and or extremely hydrophobic peptide residues, as can be suggested from the retention time on HPLC of these fractions. Previously, we discussed the possibility of two separate control mechanisms for the PrtP and for the intracellular peptidases, respectively (17). The general repression of the PepN activity by a broad spectrum in size and charge of peptides, implies that the control mechanism for the intracellular peptidases, or at least for PepN, is less specific than the control mechanism for PrtP.

The effect of concentrating the milk by ultrafiltration on the activity level of the proteolytic enzymes in cells of strain MG1363(pNZ521) was studied. The results show that the PrtP and PepN levels in cells of strain MG1363(pNZ521) are decreased approximately by a factor 1.6 and 1.8, respectively, by increasing the concentration of the milk medium by a factor 4, while PepXP is not significantly affected by the concentration factor of the growth medium. The decreased PrtP and PepN activity can only partially be explained by an increased dry weight of the UF-retentate (Table 3) and by the amount of whey proteins in the UF-retentate. This suggests the presence of a regulating component in UF-retentates which is concentrated by the ultrafiltration procedure. This component could well be one or more of the peptides functioning in the control mechanism as we described before (13, 17). This is supported by the observation that (i) the *prtP* gene expression level is affected by the concentration factor of the growth medium, in a similar way as the PrtP activity level in cells of strain MG1363(pNZ521), and (ii) other, non-proteolytic, enzymes are not affected by the

concentration factor of the growth medium (Table 3).

Production of fermented milk products from high heat treated milk or UF concentrated retentates looks economically attractive, because of the extra incorporation of whey proteins in the product, especially cheese (15, 18). Also the keeping quality range of fermented milk products is much longer if high heat treated milk is used. However, in cheeses made via these techniques the flavour development is retarded compared to traditionally produced cheese, or off-flavours such as bitter are prominent (1, 12). In this respect the decreased production levels in high heat treated milk or UF concentrated milk of proteolytic enzymes important for flavour development can play a major role. This is emphasized by e.g. the specific debittering role of PepN (20).

Future studies should be directed on the identification and characterization of the peptides that are accumulated in the milk after the various treatments and which play an important role in the controlled production of the proteolytic enzymes. This could be instrumental knowledge in the improvement of the organoleptic quality of cheeses produced from high heat treated milk and ultrafiltered milk.

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6

Thermoinducible Lysis of *Lactococcus lactis* subsp. *cremoris* SK110: Implications for Cheese Ripening

Wilco Meijer, Connie Dobbelaar, and Jeroen Hugenholtz

Submitted for publication

Thermoinducible Lysis of *Lactococcus lactis* subsp. *cremoris* SK110: Implications for Cheese Ripening

The key physiological parameters for the thermoinducible lysis of *Lactococcus lactis* subsp. *cremoris* SK110 cells were characterized. The thermolytic response, observed after subjecting a culture of strain SK110 2.5 h to a raise in temperature from 30°C to 40°C in the exponential phase of the growth, resulted in the induction of a prophage in the cells. This was established with electron microscopy by which isometric phages with an average head size of 55 nm were observed in the induced culture. The sensitivity of the induction of the prophage was shown to be dependent of the growth rate and the pH. The highest thermolytic response was found at neutral pH values and at high growth rates. The effect of the thermolytic response of cells of strain SK110 on the release of the intracellular enzymes, aminopeptidase PepN, X-prolyl-dipeptidyl aminopeptidase PepXP and lactate dehydrogenase was monitored. Upon thermoshock treatment a three- to sevenfold higher release was observed. When strain SK110 was used as a starter culture for the manufacturing of Gouda cheese, a significant increase in the level of free amino acids was measured after six weeks of ripening when a temperature shock was used during the cheese-making process. This indicates that lysis of starter bacteria plays a significant role in cheese ripening, and moreover that temperature-induced lysis of lysogenic strains is a valuable tool to affect the ripening of cheese.

Ripening of cheese is a complex process, in which the balanced breakdown of the caseins into small peptides and amino acids, as precursors for flavour development, is crucial (16, 28, 29). Apart from added renneting enzymes used in cheese-making, the proteolytic enzymes from the lactic acid bacteria, used as starter organisms, play a major role in proteolysis (17, 28).

It is generally assumed that one of the key steps during proteolysis in cheese is the release of intracellular proteolytic enzymes into the cheese matrix as a result of lysis of the starter culture (1, 2, 10). The addition of phages to the starter culture (4, 19), or the induction of prophages in lysogenic starter cultures (11), are promising methods to increase lysis of the starter culture. The latter seems to be the most practical since it has been reported that the incidence of lysogeny in lactococcal strains is very high (5, 15).

Most studies of phages of lactic acid bacteria have been focused on their differentiation on the basis of morphology, serology and DNA characterization (15), and on phage

resistance systems employed by the host strain (12). In contrast, little is known about physiological parameters affecting the induction of prophages in lysogenic lactic acid bacteria. It has been reported that the effectivity of the lysogenic induction was dependent on the inducible agent (15), the temperature of the incubation and the growth phase of the culture (22). No direct relation with the growth rate has been reported. For *Lactococcus lactis* subsp. *cremoris* SK11 it was shown that lysis was induced by ultraviolet radiation, mitomycin C and by a shift in the incubation temperature from 30°C to 40°C for 2-2.5 h (11). This indicates that strain SK11 probably contains a prophage (31).

Because of the important role of lysis in the control of flavour development in cheese, various physiological aspects influencing the heat-inducible lysis were studied. We have investigated the effect of growth conditions, such as the growth rate, on the heat-inducible lysis of *L. lactis* subsp. *cremoris* SK110. Furthermore, practical relevance of the heat-inducible lysis was studied by measuring the release of two intracellular peptidases, aminopeptidase PepN and X-prolyl-dipeptidyl aminopeptidase PepXP in vitro, and by following the ripening of semi-hard cheese in situ. This paper shows that the heat-inducible lysis is strongly dependent on the growth rate and the pH of the growth-medium. Moreover, heat-inducible lysis was found to be a valuable tool to enhance ripening of cheese.

Materials and Methods

Bacterial strains and growth conditions. The proteolytic strain *Lactococcus lactis* subsp. *cremoris* SK110, derived from starter culture SK11 (7), and *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* C17 were routinely stored in litmus milk with $CaCO_3$ and 0.5% yeast extract (Difco Laboratories, Detroit, Mich.) at -42°C. Cells of strain SK110 were grown in pH-regulated batch cultures in M17 broth with 0.5% (wt/vol) lactose, after precultivation in M17. The pH was maintained at 6.3, unless stated otherwise, by the addition of 10% (wt/vol) $NaHCO_3$ and 7.5% NH_4OH. Anaerobic conditions were achieved by continuous flushing of the headspace with N_2 gas. The cells of strain SK110 were grown at 30°C. To induce lysis, the growth temperature was shifted in the mid-exponential phase of growth (5.5 h after inoculation) to 40°C for 2.5 h, unless stated otherwise.

Continuous cultures of strain SK110 were grown in a 1 liter vessel (Applicon Dependable Instruments, Schiedam, The Netherlands) containing 0.5 liter M17 medium with 0.5% (wt/vol) lactose. A dilution rate (D) of 0.1 to 0.5 h^{-1} and a pH of 6.3 were applied to the culture. The culture was grown anaerobically at 30°C. To induce lysis, the temperature of a steady state culture was shifted to 40°C within 5 min.

The degree of lysis of the batch and continuous cultures was determined spectrophotometrically by measuring the absorbance at 600 nm (Biotron Atom Data Test 366 photometer, Meyvis, Bergen op Zoom, The Netherlands) and the degree of cell death from viable counts on GMA agar, as described by Hugenholtz (14).

For cheese making, cells of strain SK110 and C17 were precultivated for 20 h at 20°C in milk and in milk with 0.1% yeast-extract (Difco Laboratories, Detroit, Mich.), respectively.

Electron microscopy. A sample for electron microscopy was taken from a batch culture of cells of strain SK110 in M17 medium, 3 h after the induction of lysis via a temperature shock. The sample (1 liter) was centrifuged for 10 min at 3,000 g to remove whole cells and cell debris. Subsequently, the supernatant was ultra-centrifuged for 2.5 h at 100,000 g. The pellet containing phage particles was dissolved in 1 ml potassium phosphate buffer (50 mM, pH 7.5). Approximately 50 μl of this sample was placed onto a carbon-coated 400-mesh copper grid. Phage preparations were stained with 1% uranyl acetate and examined with a Jeol JEM 1200 EX transmission electron microscope (Jeol, Japan).

Preparation of cell-free extracts. Cells grown in batch and continuous cultures were harvested at different time intervals of growth or at different steady state conditions, respectively, washed twice in potassium phosphate buffer (50 mM, pH 7.5) and resuspended in the same buffer containing 1 mg lysozyme per ml. The cell suspension was incubated for 60 min at 35°C. Subsequently, the cells were disrupted using a Heatsystems Sonicator XL2020 (Farmingdale, N.Y.) for 10 times 15 s. The disrupted cell suspension was clarified by centrifugation for 30 min at 25,000 g. To measure the release of intracellular enzymes in the supernatant during growth, 100 ml cell culture was centrifuged for 20 min at 25,000 g. The supernatant was concentrated 6 to 10 times by ultrafiltration at 4°C (Amicon, Danvers, Mass., USA), using a Filtron Omega filter with a nominal cutoff of 30 kDa.

Enzyme assays. L-Lactate dehydrogenase activity was assayed as described previously (13). Aminopeptidase N (PepN) and X-prolyl-dipeptidyl-aminopeptidase (PepXP) activities were measured by the assay described previously (21), with a slight modification. Both peptidases were assayed in 50 mM potassium phosphate buffer (pH 7.5). The release of nitroanilide was continuously measured at 410 nm in a Uvikon 810 spectrophotometer (Kontron, Switzerland), with a thermostatically controlled cell compartment at 35°C.

Protein determination. Protein was determined according to Bradford (3) using bovine serum albumin as a standard.

Cheese production. Gouda cheese was made from 200 liter portions of pasteurized (10 s; 74°C) milk in the manner characteristic for Gouda cheese (30). Cheese milk was inoculated with 1% SK110 and 1% C17 in the standard cheese manufacture, and with 5% SK110 and 1% C17 in the 'heat treated' cheese manufacture. To induce lysis during cheese making, the curd/whey mixture was heated for 2.5 h at 40°C after the first washing step of the curd, that is 30 min after inoculation of the cheese milk with the starter culture. The

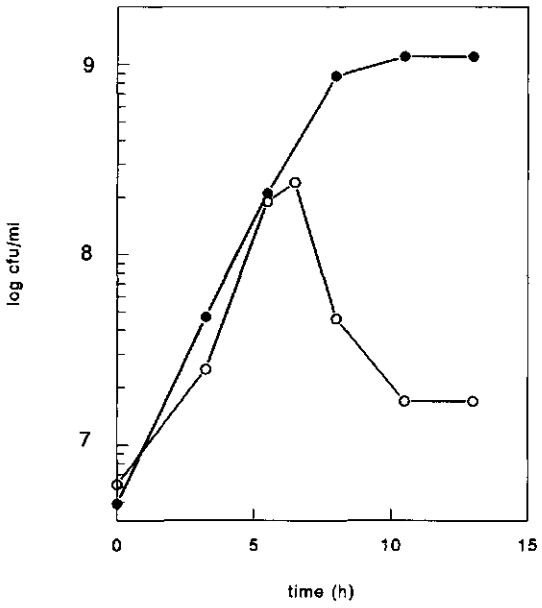

Figure 1. The growth curve (colony forming units vs. time) of cells of strain *Lactococcus lactis* subsp. *cremoris* SK110 in M17 medium at controlled pH conditions (pH 6.3) at 30°C (●) and at 30°C followed by a temporarily increase to 40°C, 5.5 h after inoculation of the culture (○).

normal 'cooking' temperature is 34-35°C; the time from renneting to brining was about 5.5 h and the ripening temperature was 13°C.

Amino acid analysis. Amino acid compositions were determined on a 4151 Alpha Plus amino acid analyser (Pharmacia LKB, Uppsala, SE), directly in the soluble nitrogen fractions of the cheese slurry, prepared as described previously (9).

Results and Discussion

Induction profile of the temperature induced lysis in strain SK110. Lysis was induced in cells of strain *Lactococcus lactis* subsp. *cremoris* SK110 by subjecting the culture temporarily to a temperature raise from 30°C to 40°C. The induction profile of the culture subjected to the heat-shock showed a dramatic 90% decrease in the amount of colony-forming units in the culture to about 10^7 colony-forming units per ml (Fig. 1). Concomitantly, the absorbance of the culture also strongly decreased, indicating disintegration of the cells (data not shown). The observed decrease in count did not occur immediately after the raise in temperature, but started only 0.5-1.0 h after the growth temperature was shifted to 40°C. Subjecting a culture

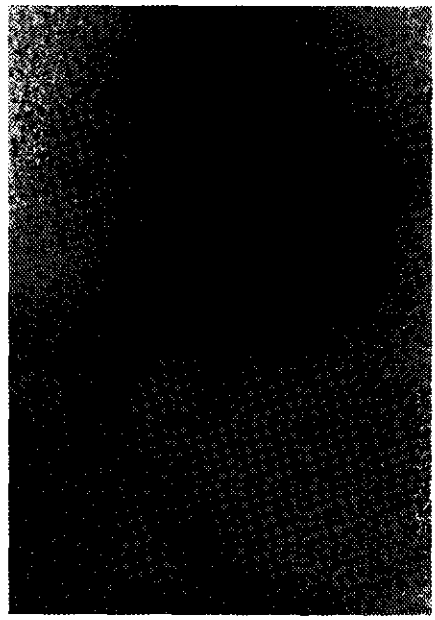

Figure 2. Electron micrograph of bacteriophage head observed in a heat-shocked culture of *Lactococcus lactis* subsp. *cremoris* SK110 (head-size of 55 nm).

of strain SK110 to a shorter heat shock than 2.5 h at 40°C resulted in a lower decrease in viable count and absorbance of the culture than observed in the culture that was heat treated for 2.5 h at 40°C (data not shown). The observed thermolytic response is typical for the process of induction of prophages in lysogenic strains (11, 24, 31).

To correlate the temperature-induced lysis in cells of strain SK110 to the induction of a prophage, cell lysates were analysed for the presence of temperate phage particles. Despite the mild isolating strategy used, no intact phage particles could be observed. Electron micrographs showed the presence of a multitude of isometric phage heads, without tails, in cell lysates of the induced culture (Fig. 2), while no phage particles could be observed in cell lysates of the non-induced culture. The average head size was approximately 55 nm. The presence of incomplete phage particles suggests a disturbed gene expression of the prophage.

Temperature-induced lysis is dependent on the pH and the growth rate. Temperature-induced lysis was analysed in cultures of strain SK110 grown in M17 medium at various controlled pH-values. The temperature response curve showed the highest decrease in absorbance in the culture grown at pH values of 6.0-6.3, the optimal pH for growth of these bacteria. Cultures of strain SK110 grown at pH values lower than 5.8 were less sensitive to the temperature treatment (data not shown).

Previously, it was demonstrated that the induction of bacteriophages was most efficient

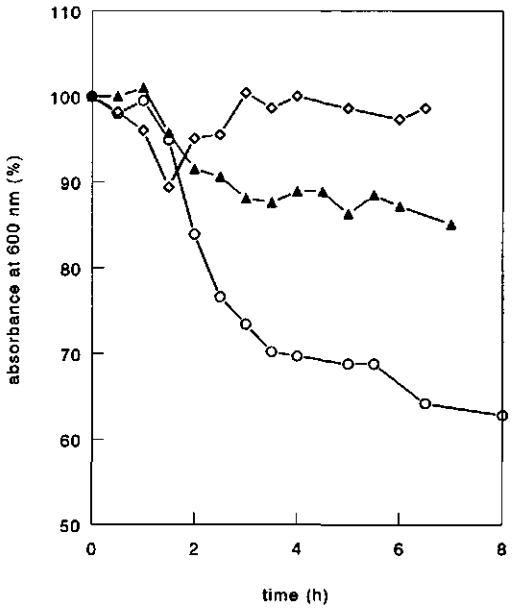

Figure 3. Relative changes in absorbance (%) of a lactose-limited continuous culture of cells of strain SK110 at a dilution rate of 0.30 h^{-1} after a temporary raise in temperature to 40°C for 2.5 h. The starvation condition of the culture was initiated by switching off the medium supply 1.5 h (◇), 2.0 h (▲) or 2.5 h (○) after start of the temperature shock.

in cultures in the mid-exponential phase of growth (22). To investigate whether this effect is due to the growth phase of the culture or to the growth rate, continuous culture studies were carried out. In order to optimize the heat-induced lysis in chemostat-grown cultures a lactose-limited culture of cells of strain SK110, with an imposed growth rate (μ) of 0.3 h^{-1}, was subjected to a temperature shock for 2.5 h at 40°C, after which the temperature was decreased to 30°C. Parallel with the start of the temperature shock, starvation for the growth-limiting substrate was initiated by switching off the medium supply after 1.5 to 3.0 h (Fig. 3). The results show that the strongest decrease in optical density was found in the culture which was actively growing during the complete temperature shock of 2.5 h. The heat treatment did not affect the amount of biomass in the culture, expressed by the absorbance, when the culture reached the starvation phase 1.5 h after initiation of the temperature shock. The culture in which the starvation phase was started 2 h after initiation of the heat shock, showed less decrease in absorbance than the culture which was starved directly after the heat shock, which is probably due to a wash-out of the induced phage particles, and a subsequent recovery of the biomass in the culture. This demonstrated that the thermolytic response was highest when the culture was in an active growth-state during the whole heat treatment.

The effect of the growth rate on the temperature-induced lysis (2.5 h, 40°C) was studied

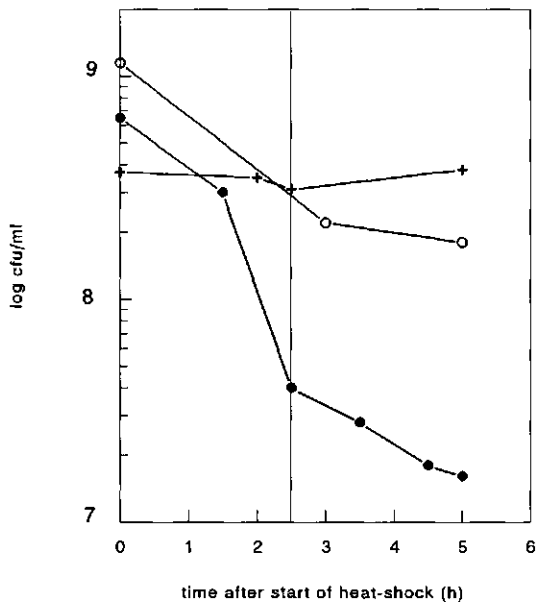

Figure 4. Changes in the viable count in lactose-limited chemostat cultures of strain SK110 with growth rates of 0.1 h^{-1} (+), 0.3 h^{-1} (○) and 0.5 h^{-1} (●), during and after a raise in temperature to 40°C for 2.5 h. After the heat shock the growth temperature was decreased to 30°C and the starvation condition was initiated by switching off the medium supply.

in continuous cultures of cells of strain SK110 grown at various imposed growth rates. Immediately after the heat treatment the temperature was decreased to 30°C and the starvation condition was initiated by switching off the medium supply (Fig. 4). The highest decrease in the number of colony-forming units was observed after the heat shock in the cultures grown at a growth rate of 0.5 h^{-1}, whereas the culture grown at a growth rate of 0.1 h^{-1} did not show lysis. The number of viable cells in the cultures with the growth rate of 0.3 h^{-1} and 0.5 h^{-1} decreased by approximately 85 and 97%, respectively. Remarkably, 15 and 50%, respectively, of this decrease was observed after initiation of the starvation condition. The decrease in absorbance values showed the same trend (data not shown). In control experiments, it was established that only imposing the starvation condition did not affect the amount of biomass in the chemostat culture in the first 10 h after initiation at any of the growth rates tested.

The standard induction time of 2.5 h, used at different imposed growth rates, gives rise to different cell division cycles during the heat shock period. The apparent control by the growth rate of the heat-inducible lysis could therefore also be explained by a different number of cell division cycles. To exclude this possibility, the decrease in biomass was measured in cultures with growth rates of 0.1 and 0.3 h^{-1}, that were heat treated for 7.5

Table 1. Activity of PepN, PepXP and LDH in the supernatant of a culture of strain SK110 without a heat treatment and with a heat treatment, 5.5 h after inoculation of the culture, from 30°C to 40°C for 2.5 h. Cells of strain SK110 were grown in M17 medium under pH-controlled conditions (pH 6.3).

Enzyme	Time (h)	Released enzyme activity (nmol[min × ml cell culture]$^{-1}$)[a]	
		non heat treated culture	heat treated culture
PepN	5.5	ND[b]	ND
	8.0	ND	0.7
	10.5	0.8	2.3
PepXP	5.5	2.0	1.6
	8.0	3.1	14.6
	10.5	4.2	22.7
LDH	5.5	<50	<50
	8.0	251.2	1507.0
	10.5	279.0	2066.0

[a] Released activity is expressed per ml culture with an A_{600nm} of 1.
[b] ND = non-detectable

and 2.5 h, respectively. Despite the same number of cell division cycles during the heat treatment in both cultures, lysis occurred only in the 0.3 h^{-1} culture. It is concluded that the extent of temperature-induced lysis is controlled by the growth rate of the cells only.

This observation is in agreement with earlier reports (22) in which temperature-induced lysis was found to be optimal in the mid-exponential growth phase. Also in various lactococci and in *Lactobacillus helveticus* lysis was observed to be highest in the exponential growth phase (18, 23). A similar optimum has been reported for the production of autolytic enzymes by *Staphylococcus aureus* (20). In *L. lactis* a variation in growth rate has been reported to affect the viability of the cells (26). For many gram-negative bacteria a direct effect of the growth rate on the size and composition of the cell wall has been reported (6, 8, 25). It is plausible that such a change in cell wall composition affects the stability of the cells and their sensitivity to (induced) lysis. Therefore, attention should be paid to the exact relationship between thickness or composition of the cell wall and the lysis efficiency in *L. lactis*.

Release of intracellular peptidases. In order to apply thermolysis in the acceleration of cheese ripening, the release of intracellular peptidases is essential, because of their crucial role in flavour development in dairy products (27). Therefore, the release of three intracellular marker enzymes, lactate dehydrogenase (LDH), aminopeptidase N (PepN) and X-prolyl-dipeptidyl aminopeptidase XP (PepXP) was determined in (non-) heat treated cultures of cells of strain SK110 (Table 1). The results show that the activity of PepN,

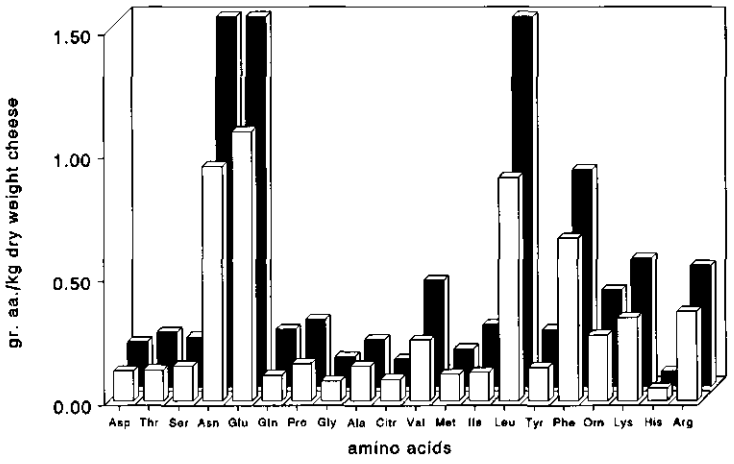

Figure 5. Free amino acid profile of cheeses after six weeks of ripening determined in cheese slurries from cheeses prepared with (black bars) and without (white bars) a raise in temperature from 30°C to 40°C of 2.5 h during the production process. Amino acid levels are expressed in g per kg dry mass of cheese.

PepXP and LDH in the supernatant of the heat treated culture after 10.5 h of growth is 3-, 5.4- and 7.4-fold higher, respectively, than in the non-treated culture. The sharp increase of the activity of the studied cytoplasmic enzymes in the non-cellular fraction (on average 5.3-fold after 10.5 h of growth) coincides with the decrease in the amount of viable cells (more than 90% after 10.5 h of growth) in the culture after the temperature-induced lysis in the culture (Fig. 1, Table 1). However, a quantitative relation between lysis and release is not evident because the extent of lysis is not exactly known and the released enzymes, presumably, do not retain 100% activity.

To investigate whether the temperature-induced lysis can indeed be used to enhance cheese ripening, cheese trials were carried out. Cultures of strain SK110 were used in combination with cells of *L. lactis* subsp. *lactis* biovar. *diacetylactis* C17 as the starter culture for cheese making. Free amino acid levels were determined in cheese after six weeks of ripening in control cheese and in cheese where the curd was heat-shocked during the production process for 2.5 h at 40°C (Fig. 5). The results show that the level of all free amino acids was approximately 1.4-fold higher in the heat treated cheese, compared with the levels found in control cheeses. The total free amino acid level observed in the heat treated cheese was 10.0 g amino acids per kg dry weight of cheese, compared to 7.3 g amino acids per kg dry weight of cheese in the non-heat treated cheese. Also the flavour characteristics of the heat treated cheese and the control cheese were clearly different. The total outgrowth of the starter bacteria was identical in both cheeses. The numbers of viable cells of strain

SK110, were 8 * 10^8, 24 h after inoculation of the cheese milk in the control cheese, and 9 * 10^8, 3 h after the inoculation of the cheese milk in the heat treated cheese. This suggests that the potential pool of intracellular proteolytic enzymes was comparable in both cheeses.

In conclusion, the susceptibility of starter cells for induced lysis is strongly dependent on the physiological state of the starter bacteria before and during cheese manufacturing. This is very relevant for the quality of the cheese since we have shown that temperature-induced lysis of lysogenic strains is a valuable tool to affect the ripening process. It is interesting to speculate on a central role of (induced) lysis by lysogenic strains in the maturation of dairy products. To effectively use this process in improvement of dairy products, further research on the mechanism and control of (induced) lysis is essential.

Acknowledgements

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Lysis of *Lactococcus lactis* subsp. *cremoris* SK110 and its Nisin-Immune Transconjugant in Relation to Flavour Development in Cheese

Wilco Meijer[1], Bert van de Bunt[1], Boudewijn de Jonge[2], Gerrit Smit[1], and Jeroen Hugenholtz[1]

[1] Department of Microbiology, NIZO, Ede, The Netherlands
[2] Laboratory of Microbiology, The Rockefeller University, New York, USA

Lysis of *Lactococcus lactis* subsp. *cremoris* SK110 and its Nisin-Immune Transconjugant in Relation to Flavour Development in Cheese

Strain *Lactococcus lactis* subsp. *cremoris* SK110 and its transconjugant containing a mutated transposon Tn*5276* (Tn*5276*-NI), defective in the production of nisin but still containing the genes encoding for nisin immunity, sucrose utilization and phage resistance, were compared in their sensitivity for (induced) lysis. The results show that the thermolytic response, normally encountered after subjecting the culture for 2.5 h to an increased temperature of 40°C in the exponential phase of the growth, was absent using the transconjugant SK110::Tn*5276*-NI. In continuous cultures it was demonstrated that the observed difference was independent of the growth rate. In an agar diffusion assay with *Micrococcus flavus* it was demonstrated that strain SK110 and its transconjugant had the same autolytic activity. Cell walls of strain SK110 showed a two times higher sensitivity to mutanolysin than cell walls of its transconjugant. The muropeptide compositions of the purified peptidoglycan of strain SK110 and its transconjugant were compared by using high-performance liquid chromatography. The muropeptide composition of transconjugant SK110::Tn*5276*-NI showed a significantly higher amount of tetrapeptides compared to the composition of strain SK110. These tetrapeptide fraction could well be involved in the cross-linking of the glycan strands. The observed difference indicates that either a factor located on the mutated transposon Tn*5276*, or the specific integration site on the genome of the lysogenic host strain, is important in the composition of the peptidoglycan and thereby in the resistance to lysis.

Lactococci are used worldwide as starter bacteria for cheese manufacture. Enzymes of the proteolytic system of lactococci play a major role in the breakdown of large, casein-derived, peptides into amino acids. This breakdown is considered the rate limiting step in the maturation of cheese (10). The peptidases, which are intracellular, are crucial for this degradation (29). It is generally assumed that lysis of the starter bacteria results in the release of the intracellular peptidases into the curd, resulting in the hydrolysis of casein-derived peptides into amino acids and thereby, the enhancement of the flavour in cheese (1, 2, 6, 17).

The lytic activity of lactococcal strains has been studied extensively. The autolysin of *Lactococcus lactis* subsp. *cremoris*, having an endo-N-acetylmuramidase specificity, reaches highest activity in the exponential phase of growth (18). Studies in liquid media showed that

the extent of autolysis may be influenced, depending on the strain, by carbon source, temperature and pH (15, 19, 28). Recently, the gene encoding for the major peptidoglycan hydrolase of *L. lactis*, which is supposed to be involved in cell separation, has been cloned and sequenced (5).

The use of lysogenic strains as starter culture, to enhance lysis of the starter cells during cheese ripening, has been proposed by Feirtag and McKay (11). It was shown for *L. lactis* subsp. *cremoris* SK110 that lysis was induced by ultraviolet radiation, mitomycin C and by an increase in incubation temperature from 30°C to 40°C for 2.5 h. Recently, by electron microscopy, evidence supporting for the lysogenic character of strain SK110 was provided (17). When strain SK110 was used as starter culture for the manufacturing of Gouda cheese, a significant increase in the level of free amino acids was measured in the mature cheese when the prophage was induced during the cheese-making process.

In cheese trials we have used, as starter cultures, strain *L. lactis* SK110 and its transconjugant *L. lactis* SK110::Tn*5276*-NI, which carries the mutated nisin-transposon Tn*5276*-NI, that is defective in nisin production. Organoleptic evaluation showed that the mature cheese manufactured with the transconjugant as the starter culture was much more bitter than cheese manufactured with the parental strain, which suggested a difference in extent of lysis between strain SK110 and its transconjugant. This prompted us to determine the sensitivity for (induced) lysis under different growth conditions, the activity of the lytic enzymes, and to analyse the peptidoglycan structure by RP-HPLC of strain SK110 and SK110::Tn*5276*-NI. The results indicate that the cell wall of the transconjugant has an altered composition, which correlates with the higher stability of the cell wall.

Materials and Methods

Bacterial strains and growth conditions. The proteolytic strain *Lactococcus lactis* subsp. *cremoris* SK110, an isolate from starter culture SK11 (8), strain *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* C17, and strain *L. lactis* subsp. *cremoris* SK110::Tn*5276*-NI, a transconjugant from strain SK110, harbouring a variant of transposon Tn*5276* defective in nisin production, were used in this study. Transposon Tn*5276* is a conjugative nisin-sucrose transposon from host strain *L. lactis* R5 (NIZO, Ede, The Netherlands) (21). Transposon Tn*5276*-NI was derived from host strain *L. lactis* R520 (NIZO, Ede, The Netherlands). Transfer of Tn*5276*-NI to the recipient strain SK110 was done via the intermediate recipient strain *L. lactis* MG1614. The conjugal matings were carried out on milk agar plates (25), and the transconjugants were selected as described previously (22). All strains were routinely stored in litmus milk with $CaCO_3$ and 0.5% yeast extract (Difco Laboratories, Detroit, Mich.) at -42°C. *L. lactis* cells were grown in M17 medium with 0.5% lactose, after precultivation in milk. Transconjugants containing Tn*5276*-NI were routinely grown in

medium containing 50 U/ml nisin. In pH-regulated batch cultures the pH was maintained at 6.3, by the addition of a solution containing 10% (wt/vol) $NaHCO_3$ and 7.5% NH_4OH. The growth temperature was 30°C, and anaerobic conditions were achieved by continuous flushing of the headspace with N_2 gas. The thermolytic response was induced by raising the growth temperature of the culture for 2.5 h to 40°C in the mid-exponential phase of growth.

Continuous cultures were grown in a 1 liter vessel (Applicon Dependable Instruments, Schiedam, The Netherlands) containing 0.5 liter LM17 medium under lactose-limited conditions. They were grown anaerobically under an N_2 atmosphere at a constant pH of 6.3. The thermolytic response was induced by an increase of the growth temperature to 40°C for 2.5 h at steady state conditions. Immediately after the heat treatment the temperature was decreased to 30°C and starvation condition was initiated by switching off the medium supply (17).

The total number of cells in the batch and continuous cultures was determined by measuring the absorbance at 600 nm (Biotron Atom Data Test 366 photometer, Meyvis, Bergen op Zoom, The Netherlands) and by plating cells on GMA agar, as described previously (14).

Cheese manufacturing. Gouda cheese was made from 200 liter portions of pasteurized (10 s, 74°C) milk by standard procedure (30). Cheese milk was inoculated with either 0.5% of the *L. lactis* strains SK110 and C17, or 0.5% SK110::Tn*5276*-NI and 0.5% C17.

The total number of cells in the matured cheese was determined in a cheese sample which was 10 times diluted in a 2% trisodium citrate solution (wt/vol), and subsequently, homogenized for 5 min in a stomacher (Lab-Blender 400, Seward, London). Viable count was determined on GMA agar (14).

Cell extracts. Cell extracts were prepared as described previously (20).

Electron microscopy. Samples for electron microscopy were taken from a batch culture of cells of strain SK110 in M17 medium 3 h after the induction of lysis and from a batch culture of its transconjugant 0.5 h after induction. Phage particles were concentrated from the culture supernatant of strain SK110 as described before (17). Cells of a 100 ml culture of transconjugant SK110::Tn*5276*-NI were harvested and resuspended in 2 ml potassium phosphate (50 mM, pH 6.5). The cells were extracted and, subsequently, the sample was centrifuged for 10 min at 12,000 g to remove whole cells and cell debris. Samples were further prepared as described previously (17) and examined with a Jeol JEM 1200 EX transmission electron microscope (Jeol, Japan).

Protein assay. Protein was determined according to the Bradford method (3) using bovine serum albumin as a standard.

Determination of lytic activity. Lytic activity of cell-free extracts of cells of strain SK110 and transconjugant SK110::Tn*5276*-NI was qualitatively determined by using an agar diffusion bioassay (27) with *Micrococcus flavus* as the indicator strain. Plates were prepared by adding a 0.1% inoculum of an exponentially growing culture of the indicator strain to 25 ml of bio-assay medium solidified with 1.5% agar. After solidification, wells were made with a sterile stainless-steel cork bore and filled with 20 μl cell-free extract of cells from strain SK110 and transconjugant SK110::Tn*5276*-NI, harvested in the mid-exponential growth phase, with a protein content of 4 mg/ml. After incubation at 30°C for 48 h lytic activity was measured as the diameter of the clearing zone including the well.

Lytic activity was quantitatively assayed by exposing 0.4 mg/ml purified cell walls from *M. lysodeicticus* (Sigma), suspended in a sodium phosphate buffer (50 mM, pH 6.5) to cell-free extracts, with a protein concentration of 40 μg/ml (final concentration), of cells of strain SK110 and transconjugant SK110::Tn*5276*-NI in a sodium phosphate buffer (50 mM, pH 6.5) at 25°C. The decrease in absorbance was followed at 600 nm.

Isolation of cell wall material. Cells of strain SK110 or transconjugant SK110::Tn*5276*-NI were harvested in the mid-exponential growth phase, and resuspended in 100 ml distilled water. Subsequently, the cells were disrupted by sonicating 40 times for 1 min, using a Heatsystems Sonicater XL2020 (Farmingdale, N.Y.) at maximum power with a 1.25 cm macrotip; 1 min sonication was alternated with 2 min cooling in ice. The suspension was heated at 100°C for 15 min and, after cooling to 37°C, DNase, RNase and phosphate buffer (final concentrations 5 μg/ml, 30 μg/ml and 0.1 M [pH 6.7], respectively) were added. After 2 h at 37°C, the cell walls were pelleted by centrifugation for 20 min at 12,000 g. The walls were resuspended in 300 ml Tris-HCl buffer (50 mM, pH 7.6) containing trypsin (0.6 mg/ml). Sodium dodecylsulfate (2%, wt/vol) was added after 4 h incubation at 37°C, and the suspension was stirred for 1 h at room temperature. The pure white cell walls were collected by centrifugation (12,000 g, 20 min), washed three times with 0.9% NaCl and three times with distilled water and freeze dried.

Enzymatic digestion of cell walls. Purified cell walls (1.4 mg/ml) were digested by mutanolysin (10 U/ml) (Sigma) at 37°C in a NaH_2PO_4 buffer (50 mM, pH 6.8). The decrease in optical density was followed at 600 nm.

Muropeptide analysis. The peptidoglycan isolation, the preparation of the muropeptides and the separation of the muropeptides by HPLC was performed by the method described by Driehuis and Wouters (9).

Table 1. Overall flavour and bitter scores of 4 month-old cheeses manufactured with either 0.5% SK110 and 0.5% C17, or 0.5% SK110::Tn*5276*-NI and 0.5% C17.

starter culture	Organoleptic evaluation of the cheeses	
	bitterness[1]	overall[2]
0.5% SK110 + 0.5% C17	0.4	6.4
0.5% SK110::Tn*5276*-NI + 0.5% C17	2.2	4.8

[1] Bitter score on a scale of 0 (absent) to 4 (very strong).
[2] Overall score on a scale of 4 (very poor) to 8 (excellent).

Results

Cheese manufacture with strain SK110 and transconjugant SK110::Tn5276-NI. Gouda-type cheese was manufactured with *Lactococcus lactis* subsp. *cremoris* SK110 and its transconjugant SK110::Tn*5276*-NI, as the starter cultures. The acidification rate in both cheeses was identical (data not shown). Also the number of viable cells of strain C17, used as adjunct starter culture was 3.5×10^8 cfu/ml in both cheeses, 24 h after inoculation of the cheese milk. Organoleptic evaluation of the cheese manufactured with cells of strain SK110 showed a good quality cheese, with a low bitter score, whereas cheese manufactured with SK110::Tn*5276*-NI resulted in a bitter product (Table 1). To determine the stability of the starter culture during ripening in cheese environment, the number of viable cells was counted (Fig. 1). The results show that after 40 days the number of cells of starter culture SK110 in cheese was about 10^{-4} times the number for starter SK110::Tn*5276*-NI. This suggests a large difference in stability of strain SK110 and transconjugant SK110::Tn*5276*-NI.

Thermolytic response of strain SK110 and transconjugant SK110::Tn5276-NI in batch and continuous cultures. To compare the sensitivity for (induced) lysis between cells of strain SK110 and its transconjugant, lysis was induced in both types of cells by a raise in growth temperature from 30°C to 40°C for 2.5 h. The culture of cells of strain SK110 subjected to the temperature shock showed a typical prophage induction profile, measured in the absorbance of the culture and the number of colony-forming units of the culture. A lower increase in biomass in the first 0.5-1.0 h of the heat treatment was observed, compared to the biomass increase in the non-heat treated culture, followed by approximately 90% reduction in the number of viable cells (Fig. 2) (17). The absorbance of the heat treated culture resembled the same trend as observed for the amount of viable cells (data not shown). Subjecting a culture of cells of transconjugant SK110::Tn*5276*-NI to a heat shock did not result in any reduction in cell viability. The growth curve observed for the heat treated culture of cells of transconjugant SK110::Tn*5276*-NI was identical to the growth curve

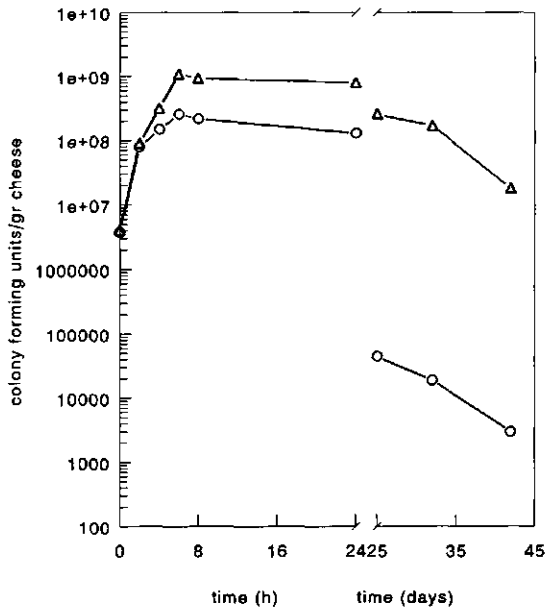

Figure 1. Growth and decline of lactococci in cheese manufactured with either strain *L. lactis* subsp. *cremoris* SK110 (○), or transconjugant SK110::Tn*5276*-NI (△) as starter.

observed for the non-treated cultures of strain SK110 and transconjugant SK110::Tn*5276*-NI (Fig. 2).

Previously, we demonstrated that the growth rate of the cells affected the extent of lysis in lysogenic cells (17). Therefore, the different thermolytic response curves from strain SK110 and transconjugant SK110::Tn*5276*-NI could have resulted from a difference in growth rate. To rule out such an effect, continuous culture studies were carried out to investigate the sensitivity to lysis at one fixed growth rate (Fig. 3). The results show that at the imposed growth rate of 0.5 h^{-1} cells of strain SK110 were more sensitive to the imposed heat shock than cells of transconjugant SK110::Tn*5276*-NI. The number of colony-forming units in the culture of cells of strain SK110 and transconjugant SK110::Tn*5276*-NI decreased by 98 and 67%, respectively, 8 h after imposing the continuous culture to the heat shock. Remarkably, the number of colony-forming units in the culture of strain SK110 was still decreasing after the heat treatment, while the amount of biomass in the culture of its transconjugant was stable after the heat treatment. Apparently, cells of transconjugant SK110::Tn*5276*-NI show, a growth temperature-induced, reduced growth rate, whereas cells of strain SK110 show a combined effect of the heat treatment and the induced prophage.

To investigate whether cells of transconjugant SK110::Tn*5276*-NI were disturbed in the synthesis of phage particles, an attempt was made to visualize the temperate phage particles by transmission electron microscopy. In cell lysates prepared from a culture of cells of

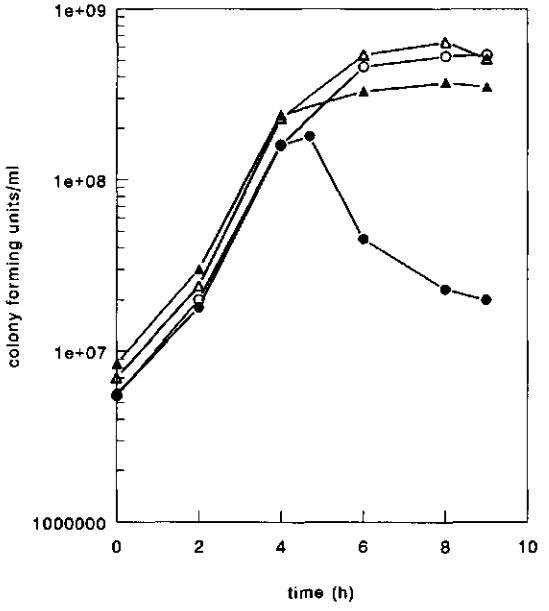

Figure 2. Growth curves of cells of strain *L. lactis* subsp. *cremoris* SK110 (○, ●) and cells of transconjugant SK110::Tn*5276*-NI (△, ▲) in M17 medium at 30°C (open symbols) and at 30°C followed by a raise in temperature of 2.5 h to 40°C, 4.5 h after inoculation of the culture (closed symbols).

transconjugant SK110::Tn*5276*-NI, 0.5 h after the start of the heat treatment, low numbers of incomplete isometric phage particles, with the average head size of approximately 55 nm, were observed. No phage particles were observed in the supernatant of the culture (data not shown). The number and size of the observed phage heads was identical to the phage heads observed in the culture supernatant of strain SK110, 3 h after the start of the heat treatment (17).

Lytic activity of strain SK110 and its transconjugant. The different sensitivity for temperature induced lysis in cells of strain SK110 compared to cells of transconjugant SK110::Tn*5276*-NI could be due to a different expression of the autolytic system. Therefore, lytic activity in cell-free extracts of cells of strain SK110 and cells of transconjugant SK110::Tn*5276*-NI were compared using *Micrococcus flavus* as indicator strain. The results showed that the lytic activity, expressed as the lytic zone on bio-assay agar, is the same for both cell-free extracts after the incubation time of 48 h (data not shown). In order to compare lytic activity of cells of strain SK110 and cells of transconjugant SK110::Tn*5276*-NI more quantitatively, purified cell walls from *M. lysodeicticus* were treated with cell-free extracts from both cells. It was observed that the decrease in absorbance for both cell-free extracts

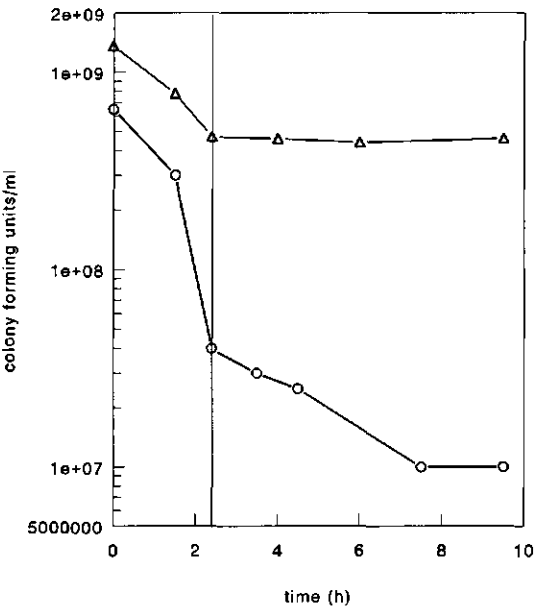

Figure 3. Changes in the amount of colony forming units of a lactose-limited continuous culture of cells of strain *L. lactis* SK110 (○) and its transconjugant SK110::Tn*5276*-NI (△), at a dilution rate of 0.5 h^{-1}, during and after a raise in growth temperature to 40°C for 2.5 h. Immediately after the heat shock the growth temperature was decreased to 30°C and the starvation condition was initiated by switching off the medium supply.

was comparable (data not shown). It was demonstrated via both tests that neither cells of strain SK110 nor cells of transconjugant SK110::Tn*5276*-NI showed higher phage encoded lytic activity plus autolytic activity levels after the induction of the prophage (data not shown).

Cell wall hydrolysis of strain SK110 and its transconjugant SK110::Tn5276-NI. Another possible explanation for the different thermolytic response in cells of strain SK110 compared to cells of the transconjugant SK110::Tn*5276*-NI could be a different sensitivity of the cell wall for lytic activity. Therefore, isolated cell wall material of cells of strain SK110 and transconjugant SK110::Tn*5276*-NI, harvested in the same growth phase at identical growth conditions, was used to study the sensitivity to mutanolysin, an enzyme that hydrolyses the N-acetylmuramyl-1,4-β-N-acetylglucosamine bonds in the peptidoglycan structure (Fig. 4). Treating cell walls of strain SK110 with mutanolysin resulted in a 1.7 times faster decline in absorbance of the cell wall suspension than in the suspension of transconjugant SK110::Tn*5276*-NI. The observed decrease in absorbance after the addition of 10 U/ml mutanolysin to a cell wall suspension of strain SK110 and transconjugant SK110::Tn*5276*-NI

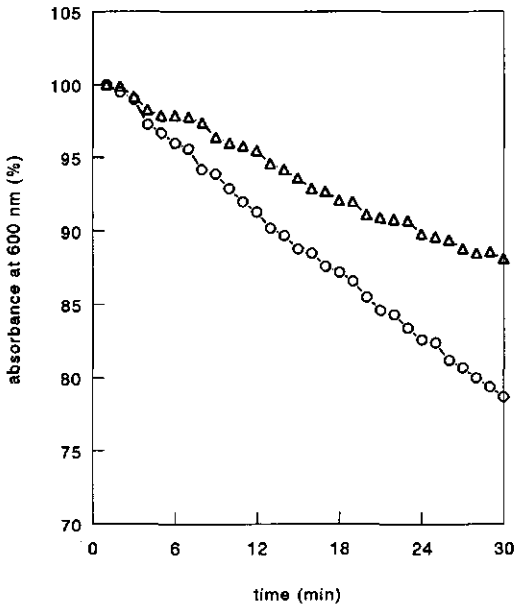

Figure 4. Relative changes in absorbance (600 nm) after treating 1.4 mg/ml isolated cell walls of strain *L. lactis* SK110 (○) and transconjugant SK110::Tn*5276*-NI (△) with mutanolysin (10 U/ml) at 37°C.

was 22 and 13%, respectively, after 30 min incubation.

In order to obtain more detailed information about the cell wall composition and structure, the isolated cell walls of strain SK110 and transconjugant SK110::Tn*5276*-NI were digested with muramidase and, subsequently, the muropeptides were separated by reversed-phase HPLC (Fig. 5). As observed from the elution profile of the RP-HPLC, cells of strain SK110 and transconjugant SK110::Tn*5276*-NI contained the same set of muropeptides. The fraction of the muropeptides, marked with an asterix, in the peptidoglycans of transconjugant SK110::Tn*5276*-NI was higher than the fraction in the peptidoglycans of strain SK110. After comparison with the elution profile of the known muropeptides from *Streptococcus faecium* (7), which elution profile is closely related to the elution profile for *L. lactis*, it was observed that the marked muropeptides consist mainly of tetrapeptides.

Discussion

We have studied strain *Lactococcus lactis* subsp. *cremoris* SK110 and the transconjugant SK110::Tn*5276*-NI as starter cultures for cheese manufacturing. Organoleptic evaluation of the cheese showed a much higher bitter score in the cheese manufactured with the

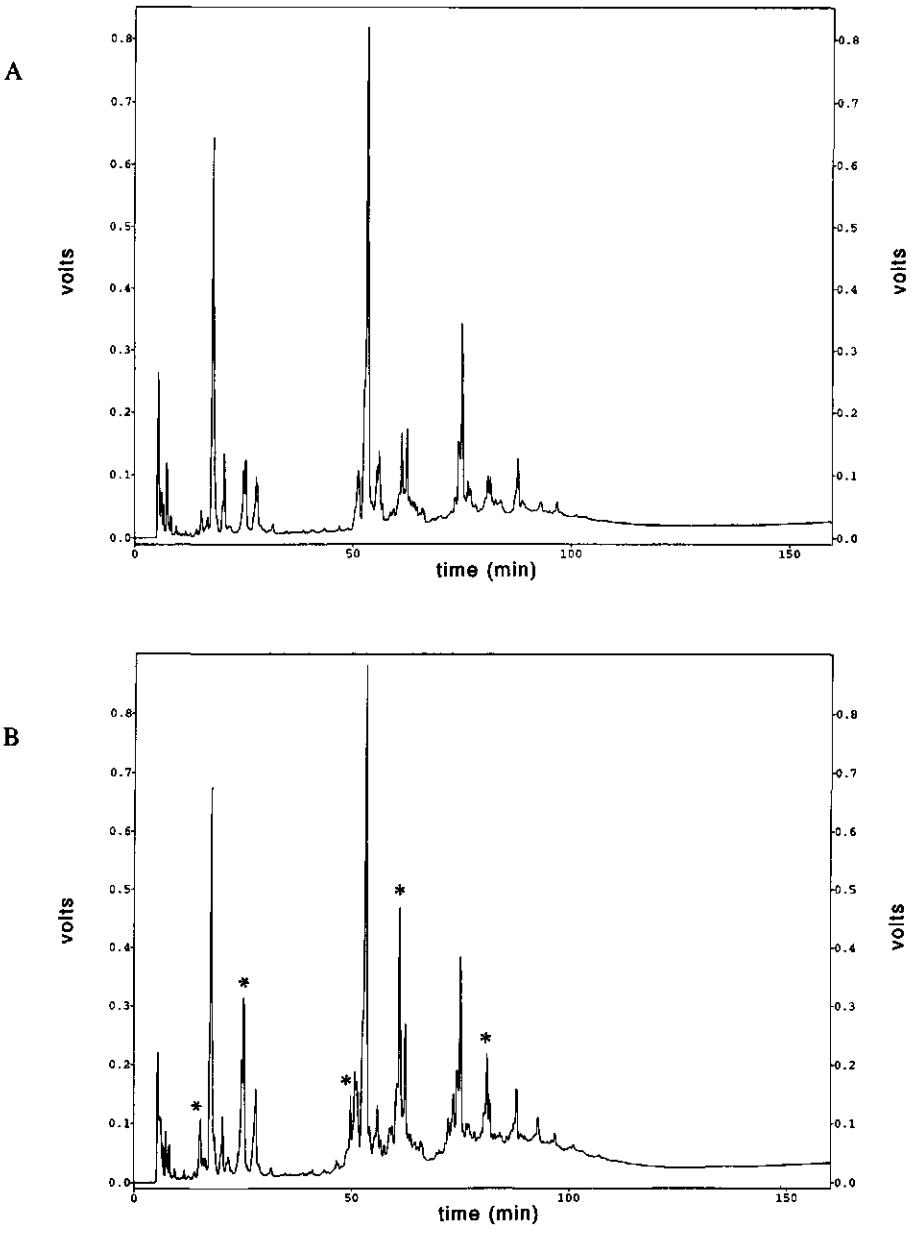

Figure 5. Reversed-phase HPLC chromatograms of separated muropeptides from peptidoglycan which was obtained from cells from strain *L. lactis* SK110 (A) and transconjugant SK110::Tn*5276*-NI (B) grown in batch cultures in M17 medium, harvested at completely identical conditions, and digested with muramidase as described in the Materials and Methods. * indicates differences in reversed-phase pattern A and B.

transconjugant compared to cheese manufactured with the parental strain. To elucidate the mechanism behind this phenomenon we studied the different sensitivity for lysis of strain SK110 and the transconjugant in relation to the cell wall structure. In the maturing cheese it was observed that the decrease in number of viable cells of strain SK110 was much faster than the decrease in viable cells of the transconjugant (Fig. 1). The thermolytic response of cells of strain SK110 and the transconjugant in batch and continuous cultures was also investigated. The results show that cells of the transconjugant SK110::Tn*5276*-NI were insensitive for the temperature treatment, while cells of strain SK110 showed the thermolytic response curve as we published before (17) (Fig. 2). In continuous cultures it was demonstrated that the observed difference was independent of the growth rate (Fig. 3). The higher decrease in number of viable count for strain SK110 compared to the decrease in number of viable cells of the transconjugant, suggest that cells of the transconjugant have a higher resistance to lysis than cells of strain SK110. Further evidence supporting the hypothesis that the different sensitivity for (induced) lysis could be explained by a difference in cell wall structure was: (i) cells of strain SK110 were still lysogenic after being conjugally transferred with transposon Tn*5276*-NI; (ii) cells of strain SK110 and the transconjugant showed the same autolytic activity on cells of strain *M. flavus* and *M. lysodeicticus*, and; (iii) purified cell walls of strain SK110 were more sensitive to mutanolysin compared to cell walls of the transconjugant (Fig. 4).

Analysis of the cell wall composition of strain SK110 and transconjugant SK110::Tn*5276*-NI revealed that the peptidoglycan of cells of transconjugant SK110::Tn*5276*-NI contained significantly higher amounts of tetrapeptides than the peptidoglycan of strain SK110 (Fig. 5). Schleifer and Kandler described the peptidoglycan structure of *L. lactis* as a repeating disaccharide unit, composed of β-(1-4) polymerized N-acetylglucosamine and N-acetylmuramic acid (23). These peptidoglycan strands are cross-linked via linear tetrapeptides attached to the N-acetylmuramic acid, which results in a rigid cell wall structure. Therefore, the higher amount of tetrapeptides observed in the transconjugant could have a positive effect on the rigidity of the peptidoglycan, and thereby, alters the susceptibility of the cell wall for lytic activity. Because lysis of starter cells is a prerequisite for the release of debittering enzymes into the cheese matrix, such as the aminopeptidase N (26), the defect bitter observed in cheese manufactured with the transconjugant could well be explained by the decreased sensitivity of the cells for lysis.

For *E. coli* it has been described that the type and amount of cross-links between the glycan strands are affected by the growth rate of the cells (9). Since, as far as we know, nothing has been reported about a controlled peptidoglycan synthesis, we can only speculate that the higher amount of tetrapeptides in the transconjugant SK110::Tn*5276*-NI is caused by a decreased L,D-carboxypeptidase activity, normally active in synthesis of peptidoglycan (4).

The observed difference in peptidoglycan composition between strain SK110 and the transconjugant indicates that either (a) gene(s) encoded on transposon Tn*5276*-NI, or the

specific integration site of Tn*5276*-NI in the genome of the lysogenic strain SK110, is involved in this phenomenon. Transposon Tn*5276*-NI is defective in the production of nisin and contains the genes encoding for nisin immunity, the sucrose operon and a bacteriophage resistance system (21, 25). The mechanisms for some of the bacteriophage resistance systems and nisin immunity are currently unclear. Studies on bacteriophage resistance in strains E8 and SK110 have revealed that a factor located outside the cell wall is involved in the adsorption of phages (12, 13, 24). Especially, the higher amount of sugar residues in the phage-resistant variants was supposed to function as an steric barrier for phage receptors in the cell wall. Recently, it was reported that two nisin resistant variants of a strain of *Listeria innocua* showed a thicker cell wall, and a higher resistance to phage attack and to three different cell wall attacking antibiotics, as mutanolysin, as compared to the parental strain. However, no difference was observed in the purified cell wall composition between the parental strain and the nisin resistant variant (16). So far, no studies are available in which the peptidoglycan structure itself is shown to play a direct role in the defense mechanisms of the cell. This makes it impossible to identify which gene on the transposon is responsible for the observed difference in peptidoglycan composition.

In further work we aim to elucidate the mechanism by which incorporation of Tn*5276*-NI, in the genome can affect the peptidoglycan composition of the lysogenic host strain SK110. More in general, future studies will focus on physiological parameters affecting the synthesis and maturation of the peptidoglycan composition in relation to the cell wall stability in lactococci.

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8

Summary and Concluding Remarks

Summary and Concluding Remarks

Semi-hard cheese types, such as Gouda, cannot be satisfactorily produced when using ultrafiltration technology. Although the cheese yield increases using this method, the higher financial return is completely lost by the (poor) quality of the cheese. The work described in this thesis is directed at improving, by microbiological methods, the quality of e.g. Gouda cheese made from ultrafiltered milk. In the course of the work, some fundamental questions were raised on growth behaviour of lactic acid bacteria in UF concentrated milk in relation to regular milk, on survival and stability of the bacteria in regular cheese and on the principles of flavour development during cheese ripening.

Growth characteristics of starter bacteria in milk concentrated by ultrafiltration. Chapter 2 describes the growth behaviour of *Lactococcus lactis* in UF concentrated milk in relation to regular milk. The total amount of biomass of *L. lactis* subsp. *cremoris* E8 and the mixed strain starter culture Bos decreased gradually by 25 and 40%, respectively, when growing in UF-retentates with increasing concentration factors up to a factor 3.6 compared with the total growth reached in regular milk. The cause of the decreased outgrowth found in UF-retentates is not precisely known, but it is clearly related to the increased concentration of whey proteins in the UF-retentate.

Regulated production of proteolytic enzymes. The enzymes of the proteolytic system, composed of the extracellular serine proteinase and the intracellular peptidases, hydrolyse in concerted action the milk proteins into amino acids. The proteolytic activity of lactococci is crucial for growth in milk. In cheese milk the degradation of casein is hydrolysed by the combined activity of chymosin and proteolytic enzymes. During maturation of cheese the pool of amino acids contribute, either directly or as precursor for flavour compounds, to the final cheese flavour (8). In contrast to the wealth of knowledge on the biochemical and genetic characterization of the proteolytic enzymes, little is known about the regulation of these enzymes, e.g. their medium and growth dependent activity.

Chapter 3 describes the use of a reporter gene, β-glucuronidase (*gusA*) of *Escherichia coli*, to study expression of the *prtP* and *prtM* genes under different conditions. Both, *prtP* and *prtM* promoters, were stringently controlled by the peptide content of the medium. Specifically, addition of the peptides leucylproline or prolylleucine to the growth medium negatively affected the expression level of the *prtP-gusA* fusions. In mutants defective in the uptake of di-tripeptides the repression by these dipeptides was not observed, which suggests a role of the di-tripeptide transporter as a sensor for the extracellular small peptides.

Chapter 4 describes the regulation of the extracellular PrtP and two intracellular peptidases, aminopeptidase N (PepN) and X-prolyl-dipeptidyl aminopeptidase (PepXP), in

two different host strains, *L. lactis* subsp. *lactis* MG1363 and *L. lactis* subsp. *cremoris* SK1128, both containing plasmid pNZ521, which encodes the PrtP from strain SK110. Production levels of all three enzymes were found to be highest in the late exponential phase of growth. The production was only slightly affected by the growth rate, PrtP and PepN production levels increased with increasing growth rates whereas PepXP showed an optimum at growth rate of 0.22 h^{-1}. The PrtP production level showed a medium-dependent control, which correlated with the controlled expression of the *prt* promoters. Highest production level was observed during growth of *L. lactis* cells in milk, lowest levels during growth in a peptide rich medium. The two peptidases were found to be regulated in a similar way as PrtP in strain MG1363, while in host strain SK1128 no regulation was observed. The regulating effect of the dipeptide prolylleucine appeared to be independent of the growth rate of the cells.

The basic mechanism for the controlled production of the proteolytic enzymes is not yet clarified. Deletion and mutation analyses of the *prt* promoter region revealed that a 90 bp sequence (operator), which contains the *prtP* and *prtM* promoter, is sufficient for their full expression and regulation (5). As already speculated in *Chapter 3*, a putative negative regulator may bind to this *prt* operator region. The affinity of the regulator for the *prt* operator region is increased after a conformational change induced by interaction with an effector, resulting in repression of transcription (Fig. 1). Specific dipeptides, such as prolylleucine, are supposed to act as the effector molecules. Whether the dipeptide plays a direct role in the conformational change of the regulator protein, or that the activity of the di-tripeptide transport system itself facilitates the conformational change of the regulator, is still an intriguing question.

The basic knowledge of the control mechanism of proteolytic enzyme production can be used to influence the proteolytic enzyme activity in starter bacteria using cultivation media with varying peptide concentrations.

Chapter 5 describes the controlled production of proteolytic enzyme activity in *L. lactis* cells grown in different pre-treated milk media, when milk was subjected to increased heat treatments and higher UF concentration factors. Cells of *L. lactis* showed decreased activity of PrtP, PepN and PepXP when grown in milk with increased heat treatments and milk with different concentration factors concentrated by ultrafiltration. This medium-dependent regulation of PrtP was confirmed at the level of transcription initiation. Analysis of the peptide composition of the heat treated milk showed higher concentrations of small, probably hydrophobic, peptides, than in non-treated milk. Therefore, it is suggested that small peptides present in the milk medium, due to the heat treatment of the milk, control the production of the different proteolytic enzymes. It is speculated that the control of proteolytic enzyme production in UF-retentates is directed via the same mechanism. The observation that the increase in soluble N is much slower during ripening of UF-cheese than in traditional manufactured cheese is in agreement with the reduced proteolytic activity of the starter cell grown in UF concentrated milk (4, 7).

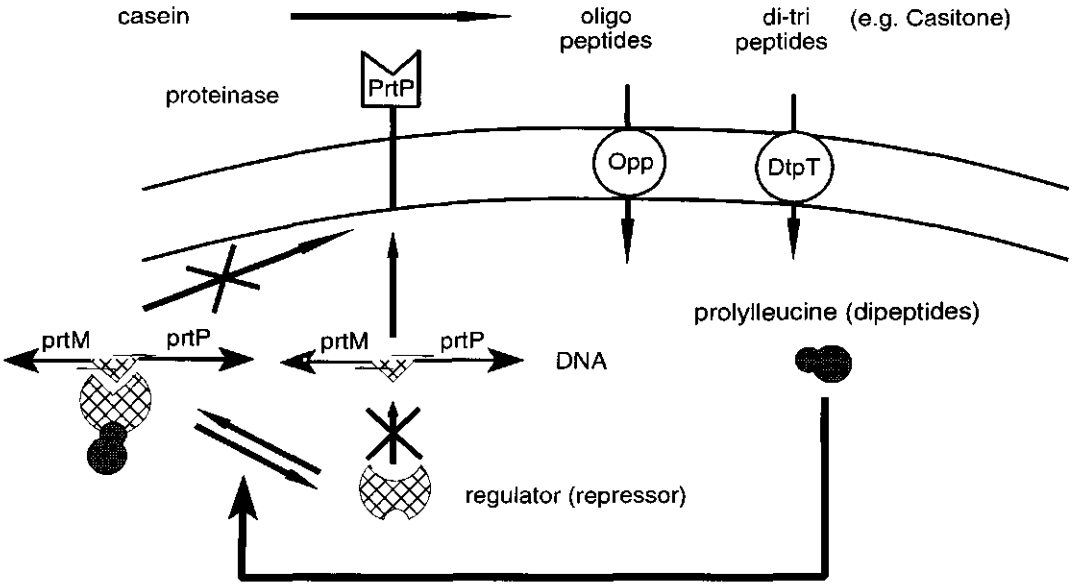

Figure 1. Proposed model for the medium-dependent regulation of PrtP in *L. lactis* during growth in milk. (Opp = oligopeptide transport system, DtpT = di-tripeptide transport system).

Lysis of starter bacteria in relation to flavour development in cheese. During maturation of cheese the starter cells are metabolically inactive. This excludes the energy driven transport of oligopeptides, degraded from the milk protein by the hydrolytic activity of PrtP, into the cell. To assure the production of amino acids from the oligopeptides intracellular peptidases have to be released into the cheese matrix by lysis of the starter bacteria (2).

Chapter 6 describes the use of the lysogenic *L. lactis* subsp. *cremoris* SK110 to study the influence of different growth conditions on lysis. Lysis was induced via a temporary increase in growth temperature from 30°C to 40°C for 2.5 h. Highest sensitivity of the lactococcal cell for induced lysis was observed at neutral pH values and at high growth rates. Using electron microscopy, it was confirmed that lysis was indeed a result of prophage induction. The induced lysis resulted in an increased release of peptidases from the cytoplasm. Lysis induced in starter culture SK110 during cheese manufacturing leads to an enhanced pool of amino acids and a clearly distinguishable cheese flavour in the matured cheese, compared to the control cheese.

Remarkably, the reduction in number of viable cells as a result of induced lysis, is not quantitatively reflected by the increased amount of released intracellular enzymes. This

suggests the existence of non-viable, stable protoplasts after the prophage-induced lysis. Subsequently, the 6 times higher release of intracellular proteolytic enzymes in a prophage-induced culture of strain SK110, just enhances the amount of free amino acids 1.4-fold in cheese. This clearly demonstrates that the release of intracellular peptidolytic enzymes is a rate limiting step in flavour development in cheese. However, elimination of this rate limiting step is not sufficient to ensure satisfactory ripening. It may be speculated that another rate limiting step become apparent, for instance the degradation of the free amino acids into volatile flavour compounds.

Finally, *Chapter 7* deals with the relationship between the cell wall composition and the susceptibility of the cell for lysis. Cheese was manufactured with strain *L. lactis* SK110 and its transconjugant containing the mutated nisin transposon Tn*5276*, which encodes for nisin immunity but not production, bacteriophage resistance and the sucrose operon. The bitter score was rather high in cheese produced with the transconjugant compared to the cheese made with strain SK110. Cells of transconjugant SK110::Tn*5276*-NI showed less susceptibility for (induced) lysis than cells of strain SK110. It was observed that the peptidoglycan of the transconjugant SK110::Tn*5276*-NI was less sensitive to mutanolysin than the parental strain. The peptidoglycan of the transconjugant SK110::Tn*5276*-NI showed a significantly higher amount of tetrapeptides, involved in cross-linking of the glycan strands, than the peptidoglycan of strain SK110. The changed peptidoglycan composition of transconjugant SK110::Tn*5276*-NI could decrease the susceptibility of the cell wall for lytic enzymes. This explains the observed higher bitter score via a reduced release of (debittering) intracellular peptidases during cheese ripening.

The observation that the presence of the nisin-sucrose transposon Tn*5276*-NI affects the lactococcal cell wall composition, suggests either a disturbed gene expression in the host strain due to the specific integration site of Tn*5276*-NI in the genome of the host strain, or the presence of genes on Tn*5276*-NI that play a role in peptidoglycan synthesis. The first option can be revealed by using different, non-isogenic, host strains for Tn*5276*-NI, the second via inactivation studies of the various genes located on Tn*5276*-NI. Until now nothing is known about the control of peptidoglycan synthesis in lactococci. For application in the dairy industry, this knowledge would allow the development of nisin-immune, industrial strains which are still able to develop proper flavour characteristics.

In general, it can be concluded that the release of peptidolytic enzymes, due to lysis of the cell, is an important, rate limiting, factor in cheese ripening. Since lysogenity seems to be wide-spread among lactococci, it is interesting to speculate that the required lysis of the starter culture during maturation of cheese is based on (spontaneous) induction of the prophage in the early stage of ripening. The fact that a lysogenic strain is immune to infection with its own phage, prevents lysis of the whole starter population and leads to the desired balance between lysed and intact cells. Therefore, induction of lysis may well be come a strong tool to accelerate the ripening of cheese and to alter the flavour characteristics of the product. However, induction of lysis via a heat treatment during cheese manufacturing

induces considerably enhanced syneresis, which may prevent attaining the desired moisture content of the cheese. Alternative inducers to be considered are treatments with salt, as already used during brining of the cheese, high pressure or UV-light. Another possibility is the use of the controlled expression of lytic enzymes. Recently, de Ruyter et al. have developed a controlled expression system using the autoregulated promoter of the nisin operon for overexpression of bacteriophage lysins (3).

Prospects of UF-cheese ripening. The studies described in this thesis can be related to various aspects encountered during ripening of UF-cheese. The less favourable growth characteristics of starter cells grown in UF-retentates, compared to normal milk (*Chapter 2*) gives rise to (i) a lower total proteolytic activity expressed per g cheese, (ii) reduced production levels of the different proteolytic enzymes which is due to the changes in growth behaviour and the changes in cultivation medium of the starter bacteria (*Chapter 4, 5*), and (iii) to a reduced sensitivity for lysis of the starter culture, which results in a reduced release of intracellular flavour generating enzymes into the cheese matrix (*Chapter 6*).

Other studies, directed at elucidating the technological problems encountered during UF-cheese manufacturing, showed that the relative activity of chymosin in UF cheese-milk gradually decreased with increasing the concentration factor of the milk (1). To improve the ripening of UF-cheese it is important, therefore: (i) to increase the total addition of chymosin to the UF-cheese milk, (ii) to increase the inoculation size of the starter culture to the UF-cheese milk, (iii) to select starter cultures with high production levels of proteolytic enzymes, and (iv) to select lysogenic starter cultures, which gives the possibility to induce lysis during cheese manufacturing. Preliminary results showed that these measures can significantly enhance the organoleptic quality of UF-cheese.

Another promising possibility for manufacturing UF-cheese is the use of thermophilic strains as an additional starter culture. Thermophilic strains have been successfully used because of their debittering activity (6), which is probably due to their high proteolytic activity and their high susceptibility for release of the intracellular peptidolytic activity. Although, the use of thermophilic strains gives rise to particular organoleptic characteristics, which deviate from the traditional Gouda cheese flavour, these strains are very successfully used for rapid flavour development in semi-hard cheeses.

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Samenvatting

Samenvatting

Kaas is, met een jaarproductie in 1995 van 690.000 ton, het belangrijkste gefermenteerde melkproduct in Nederland. In het traditionele kaasbereidingsproces wordt de kaasmelk enzymatisch gestremd. De gestremde melk wordt vervolgens gescheiden in de wrongel - de toekomstige kaas met daarin alle caseïne-eiwitten - en de wei, met daarin alle wei-eiwitten. Een veelbelovende manier om het kaasproductieproces te optimaliseren, is de toepassing van ultrafiltratietechnologie. Kaas bereid uit geültrafiltreerde melk, is op een fysische manier geconcentreerd, hetgeen leidt tot de insluiting van meer wei-eiwitten in de kaas. Het economisch voordeel, verkregen door de insluiting van eiwitten die normaal verloren gaan in de weistroom, wordt helaas teniet gedaan door de slechte kwaliteit van de kaas. Het doel van dit promotieonderzoek was het verbeteren van de kwaliteit van harde en half-harde kazen bereid uit geültrafiltreerde melk. Hierbij is in het bijzonder aandacht besteed aan het groeigedrag van melkzuurbacteriën in geültrafiltreerde melk, ten opzichte van dat in standaardmelk, de overleving en de stabiliteit van melkzuurbacteriën in kaas en enkele principes van smaakontwikkeling tijdens de rijping van kaas.

Hoofdstuk 1 is een algemene inleiding over de rol van melkzuurbacteriën bij de bereiding en de rijping van Goudse kaas. Hierbij is primair aandacht besteed aan de enzymen van melkzuurbacteriën die een rol spelen in de afbraak van het melkeiwit (de zogenaamde proteolytische enzymen) en aan het belang van het openbreken (zogenaamd lysis) van de melkzuurbacteriën voor de aromavorming in kaas. Verder geeft dit hoofdstuk een uitgebreid overzicht van vooral de microbiologische problemen die optreden bij bereiding en rijping van kaas bereid uit geültrafiltreerde melk.

Hoofdstuk 2 beschrijft het groeigedrag van *Lactococcus lactis* in geültrafiltreerde melk in vergelijking met de groei in standaardmelk. De maximale uitgroei van zowel *L. lactis* subsp. *cremoris* E8 als het samengestelde kaaszuursel Bos, gebruikt bij de bereiding van Goudse kaas, is respectievelijk 25% en 40% lager in 3.6 maal geültrafiltreerde melk dan in standaardmelk. Aangetoond is dat de verhoogde concentratie aan wei-eiwitten in geültrafiltreerde melk verantwoordelijk is voor deze verlaagde uitgroei.

De proteolytische activiteit van lactokokken is essentieel voor de groei in melk. De extracellulaire, celwand-gebonden proteïnase, breekt als eerste de melkeiwitten af tot oligopeptiden. Deze oligopeptiden worden via een oligopeptide-transportsysteem in de bacteriecel gebracht, waarna intracellulaire peptidasen deze peptiden kunnen afbreken tot aminozuren. De aminozuren die enerzijds noodzakelijk zijn voor de groei van melkzuurbacteriën, dragen anderzijds direct of indirect, als precursors, bij aan de aromavorming in kaas. In tegenstelling tot de gedetailleerde genetische en biochemische kennis van het proteolytische systeem, was er bij aanvang van dit onderzoek weinig bekend over de regulering.

Hoofdstuk 3 beschrijft de constructie van een plasmide, waarmee de aflees-frequentie van het proteïnase-gen van *L. lactis* subsp. *cremoris* SK110 (zgn. transcriptie), bepaald kan

worden via de activiteit van een verklikkereiwit. Dit is mogelijk gemaakt door het gen voor het verklikkereiwit achter de aan/uit schakelaar (de zgn. promoter) van het proteïnase-gen te plaatsen, zodat productie van dit eiwit alleen plaats kan vinden wanneer de promoter aanstaat. Aangetoond is dat de promoteractiviteit van de proteïnase sterk afhangt van het peptidegehalte van het kweekmedium. Met name de toevoeging van specifieke dipeptiden, zoals leucylproline, heeft een sterk negatief effect op de promoteractiviteit. In mutanten waarin opname van kleine di/tripeptiden niet meer mogelijk is, wordt deze remming door kleine peptiden niet waargenomen. Aangezien *L. lactis* voor groei in melk alleen het oligopeptide-transportsysteem nodig heeft (zie boven), wordt gesuggereerd dat het di/tripeptide-transportsysteem een rol speelt als sensor voor de extracellulaire concentratie aan kleine peptiden, waardoor de proteïnase-activiteit naar behoefte wordt gereguleerd.

Hoofdstuk 4 beschrijft de regulatie van de extracellulaire proteïnase en twee intracellulaire peptidasen, aminopeptidase N (PepN) en X-prolyl-dipeptide aminopeptidase XP (PepXP) in twee verschillende *L. lactis*-stammen, MG1363 en SK1128, waarbij de activiteit als maat voor het productieniveau is genomen. Het productieniveau van elk proteolytisch enzym in cellen van stam MG1363 is het hoogst in de laat-exponentiële groeifase. In continue cultures is aangetoond dat de productie door verschillen in de groeisnelheid slechts heel weinig wordt beïnvloed. De proteïnaseproductie vertoont, onafhankelijk van de stam, een medium-afhankelijkheid die overeenkomt met de gereguleerde promoteractiviteit. Het hoogste productieniveau is aangetoond in *L. lactis*-cellen gekweekt in melk, de laagste productieniveaus in peptiderijke kweekmedia. De regulatie van de peptidaseproductie blijkt een stamafhankelijk karakter te hebben; peptidasen worden alleen in cellen van stam MG1363 gereguleerd door de peptideconcentratie in het kweekmedium.

Het mechanisme dat ten grondslag ligt aan de gereguleerde productie van de proteolytische enzymen is nog niet opgehelderd. Een mogelijkheid is dat een hypothetisch regulatoreiwit, na een verandering in conformatie, bindt aan de proteïnasepromoter, waardoor verdere aflezing van het proteïnase-gen niet meer mogelijk is. Deze conformatieverandering zou op gang gebracht kunnen worden door activiteit van het di/tripeptide-transportsysteem of door interactie met de intracellulair aanwezige dipeptiden.

Hoofdstuk 5 beschrijft de productie van verschillende proteolytische enzymen in cellen van *L. lactis* gekweekt in verschillend voorbehandelde melkmedia. Zowel bij groei in melk die een intensievere hittebehandeling heeft ondergaan als bij melk die verder is geconcentreerd door ultrafiltratie, neemt de productie van de extracellulaire proteïnase en PepN en PepXP af, vergeleken met het productieniveau in standaardmelk. Deze regulatie in de verschillende soorten melk is ook aangetoond voor de promoteractiviteit van de proteïnase. De toename in concentratie van peptiden in melk als gevolg van een intensievere hittebehandeling suggereert dat ook hier het hierboven beschreven controlemechanisme van toepassing is.

Tijdens de rijping van kaas zijn de cellen, door het opraken van de koolstofbron, metabolisch inactief. Dit sluit actief transport van de extracellulaire oligopeptiden naar de intracellulaire peptidasen uit. Om de vorming van aminozuren te bewerkstelligen, is daarom

uitlek van de peptidasen in de kaasmatrix, door lysis van de melkzuurbacteriën, noodzakelijk.

Hoofdstuk 6 beschrijft de gevoeligheid van *L. lactis* subsp. *cremoris* SK110 voor lysis bij verschillende groeisnelheden en de rol van lysis bij de aromavorming in kaas. Het lysogene karakter van stam SK110 biedt de mogelijkheid om met een temperatuurschok lysis te bewerkstelligen, ten gevolge van de inductie van het in het gastheergenoom geïntegreerde faag DNA. Allereerst wordt aangetoond dat cellen van *L. lactis* SK110 gevoeliger zijn voor (geïnduceerde) lysis bij hoge groeisnelheden dan bij lage groeisnelheden. Het vrijkomen van intracellulaire peptidasen neemt sterk toe als gevolg van de inductie. Inductie van lysis in kaas, bereid met stam SK110 (als zuursel), resulteert in een verhoogde concentratie aminozuren en een duidelijk andere smaak in de gerijpte kaas, in vergelijking met de standaardkaas.

In *hoofdstuk 7* wordt de relatie tussen de celwandsamenstelling en de gevoeligheid voor lysis van cellen van *L. lactis* beschreven. Met het zuursel *L. lactis* SK110 en een transconjugant, een afgeleide van stam SK110 die een stukje overdraagbaar DNA bevat dat onder andere codeert voor nisine-immuniteit, is kaas gemaakt. De kaas gemaakt met de transconjugant wordt duidelijk bitter, vergeleken met de kaas bereid met stam SK110. In analyses van het gezuiverde peptidoglycan, de bouwsteen voor bacteriële celwanden, is aangetoond dat de celwand van de transconjugant minder gevoelig is voor mutanolysine, een celwand-afbrekend enzym, en relatief meer dwarsverbindingen binnen het peptidoglycan bevat dan het peptidoglycan van stam SK110. De stabielere celwandstructuur, in combinatie met de geringere gevoeligheid van de transconjugant voor (geïnduceerde) lysis, zou de verklaring kunnen zijn voor het gebrek "bitter" in kaas geproduceerd met deze transconjugant.

In *hoofdstuk 8* wordt tenslotte een samenvatting gegeven van de onderzoeksresultaten in het licht van de problematiek omtrent rijping van kaas bereid uit geültrafiltreerde melk.

Het in dit proefschrift beschreven onderzoek leidt allereerst tot een beter begrip van verschillende processen die een rol spelen bij de aromavorming in kaas. Tevens biedt het inzicht in de microbiologische factoren die een rol kunnen spelen bij de gebrekkige rijping van kaas bereid uit geültrafiltreerde melk. In ander onderzoek, gericht op de technologische problemen die optreden bij de bereiding van kaas bereid uit geültrafiltreerde melk, is aangetoond dat de relatieve stremsel activiteit afneemt met een verhoging in de concentratiefactor van de melk. Een cumulatie van factoren, zoals de vermindering van de uitgroei, een verlaging van de proteolytische activiteit, een verlaagde gevoeligheid voor lysis van het zuursel gekweekt in geültrafiltreerde melk en een verlaagde stremsel activiteit, zou een verklaring kunnen zijn voor de gebrekkige rijping van kaas bereid uit geültrafiltreerde melk. Deze kennis kan gebruikt worden voor het verbeteren van de kwaliteit van Goudse kaas bereid via ultrafiltratie.

Curriculum vitae

Curriculum vitae

De schrijver van dit proefschrift werd geboren als Willem Cornelis Meijer op 16 juni 1967 te Apeldoorn. Vervolgens heeft iedereen hem Wilco genoemd. In 1983 behaalde hij het MAVO diploma op de Brouwerskamp te Nunspeet. Na een kort verblijf op de HAVO werd in 1986 het VWO diploma behaald op het Lambert Franckens College te Elburg. Aansluitend begon hij de studie Biologie aan de Rijksuniversiteit Groningen. De doctoraal fase omvatte als hoofdvakken Biochemie, Moleculaire Genetica en Microbiële Fysiologie. De bijbehorende stages werden uitgevoerd op de afdeling Bioprocestechnologie van TNO Zeist onder leiding van dr. P.G.M. Hesselink, en op de afdeling Microbiële Fysiologie van de Rijksuniversteit Groningen onder leiding van Prof. dr. L. Dijkhuizen. De beide stages werden afgewisseld met een kort, 3 maanden durend, docentenbestaan op de Hanzehogeschool Groningen als docent Microbiologie en Celbiologie. Het doctoraalexamen werd in september 1991 behaald. In januari 1992 trad hij als assistent in opleiding in dienst bij de sectie Zuivel en Levensmiddelennatuurkunde van de Landbouwuniversiteit Wageningen, die hem 4 jaar lang detacheerde op het Nederlands Instituut voor Zuivelonderzoek (NIZO) te Ede. Binnen de afdeling Microbiologie van NIZO werd het in dit proefschrift beschreven onderzoek verricht. De eerste helft van 1996 is hij, voor 4 dagen in de week, als toegevoegd wetenschappelijk medewerker verbonden geweest aan de sectie Zuivel en Levensmiddelennatuurkunde van de Landbouwuniversiteit Wageningen. Hij heeft zich gedurende dat halve jaar, wederom op NIZO, beziggehouden met een haalbaarheidsstudie met betrekking tot Goudse kaas bereid uit geültrafiltreerde melk. Sinds 15 augustus 1996 is hij als wetenschappelijk medewerker in tijdelijke dienst verbonden aan de afdeling Microbiologie van NIZO.