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Lytr, a phage-derived amidase is most effective in induced lysis of *Lactococcus lactis* compared with other lactococcal amidases and glucosaminidases

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Abstract

In the genome of *Lactococcus lactis* IL1403 five genes encoding peptidoglycan hydrolases are present: four glucosaminidases (*acmA*, *acmB*, *acmC* and *acmD*) and an endopeptidase (*yjgB*). Genes for six prophage lysins have also been identified. The genes *acmB*, *acmC*, *acmD*, *yjgB* and the lysin *lytR* of prophage r1t and the lytic gene of the prophage bIL309 were cloned downstream of the nisin-inducible promoter in *L. lactis* NZ9000. Upon nisin induction, increased lysis of the expressing strain was obtained in all cases. Most lysis was obtained with AcmC and the two prophage lysins. Moreover, lysis by AcmB, AcmD and YjgB depends on the presence of the autolysin AcmA. The genes *lytR, acmD* and *acmD* in an operon-like structure with *acmA* were expressed in a foodgrade manner in a *L. lactis* strain that was used in conjunction with a starter to make Gouda-type cheese. Overexpression of *lytR* or the combination *acmA*/*acmD* resulted in increased cellular lysis in the cheese compared to the reference culture. LytR induced lysis was delayed but eventually resulted in increased lysis *in trans* of the acidifying *L. lactis* strain 13 M.

Keywords: Foodgrade lysis; *Lactococcus lactis*; Phage lysin; Autolysin

1. Introduction

To be able to grow and divide, bacteria express enzymes that hydrolyse the peptidoglycan of their cell wall. The Gram-positive bacterium *Lactococcus lactis* produces AcmA, an *N*-acetylglucosaminidase that is responsible for cell separation and is involved in stationary phase lysis. A deletion mutant of *acmA* did not lyse, showing that AcmA is the major autolysin of *L. lactis* MG1363 (Buist et al., 1995). The autolysin consists of two domains: an N-terminal active site domain and a C-terminal peptidoglycan-binding domain (Buist et al., 1995; Steen et al., 2003, 2005). Three homologues of AcmA are encoded by the genome of *L. lactis* subsp. *lactis* IL1403: AcmB, AcmC and AcmD. All four enzymes have similar active site domains, but there are differences in the modular structures of the proteins (Bolotin et al., 2001; Huard et al., 2003). Next to these (putative) glucosaminidases, a fifth putative lysin is present in the chromosome of *L. lactis* IL1403: *yjgB* (Bolotin et al., 2001). YjgB is homologous to γ-D-glutamyl-L-diamino acid endopeptidases (Bolotin et al., 2001; Smith, Blackman, & Foster, 1996). The enzymes AcmB, AcmC, AcmD and YjgB were overexpressed and purified from *E. coli* and shown to be functional in vitro. Northern analysis revealed that all their genes are transcribed in *L. lactis* MG1363 (Huard et al., 2004).

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AcmB of the closely related *L. lactis* MG1363 has been characterized recently and comprises three domains (Huard et al., 2003): the N-terminal domain resembles a cell wall-binding domain and is followed by the *N*-acetylglucosaminidase active site domain. A third, C-terminal domain has an unknown function but shows homology to known cell wall bound proteins and contains a zinc-binding motif (the modular structures of AcmA and its homologues, and of the other peptidoglycan hydrolases used in this study are summarized in Fig. 1) (Bolotin et al., 2001). Inactivation of *acmB* revealed that AcmB does not influence the chain length of *L. lactis* but may be involved in cellular lysis since the *acmB* mutant lyses to a lesser extent than its parent *L. lactis* MG1363. The effect of *acmB* deletion on cellular lysis is not observed in an *acmA* mutant of MG1363 (Huard et al., 2003). AcmC of *L. lactis* IL1403 has also been studied: the enzyme consists of an *N*-acetylglucosaminidase active site domain without any additional domains. Zymographic analysis of purified AcmC-His revealed that the enzyme is active at pH 4, 5 and 6 (Huard et al., 2004). Analysis of the inferred amino acid sequences of AcmD and YjgB reveals that these enzymes also have a modular structure. AcmD has the same modular organization as AcmA, although its overall pI (4.3) is lower than that of AcmA (10.3) (Bolotin et al., 2001).

Besides the genes for the aforementioned peptidoglycan hydrolases, six prophages are present in the genome of *L. lactis* IL1403 (Bolotin et al., 2001). In contrast to the lactococcal peptidoglycan hydrolases mentioned above, the phage lysins do not have signal sequences for secretion. Holin genes therefore often flank the lytic genes in the prophage DNA. The holins are thought to form pores in the cytoplasmic membrane, enabling release of the phage lysins and subsequent host cell lysis from without (Young, 1992).

L. lactis is widely used in the manufacture of cheese, a process in which cell lysis plays an important role. Recently Lortal and Chapot-Chartier published a review on the importance of cellular lysis of starter cultures in cheese (Lortal & Chapot-Chartier, 2005). In this paper the optimal conditions, methods for measuring and controlling cell lysis and future prospects have been well described mainly for *L. lactis*. Casein in the cheese curd is degraded by proteinases and peptidases expressed by *L. lactis*. The products of proteolysis play an important role in the development of the flavour of cheese. The peptidases and a number of other enzymes of *L. lactis* involved in flavour development are located intracellularly and these enzymes are released in the cheese curd matrix by cell lysis (Law, Sharpe, & Reiter, 1974). Indeed, cheese made with *L. lactis* AM2, which lyses fast, developed higher levels of amino nitrogen and lower levels of bitterness than with the slow lysing strain *L. lactis* NCDO 763 (Chapot-Chartier, Deniel, Rousseau, Vassal, & Gripon, 1994; Tan et al., 1993).

Increasing the rate of lysis of starter strains, thereby improving the release of proteolytic enzymes, can be achieved by selection of fast lysing starter strains, using thermoinducible strains, and by bacteriophage-assisted or bacteriocin-induced lysis (Fox et al., 1996; O'Sullivan, Morgan, Ross, & Hill, 2002). Overexpression of the autolysin AcmA resulted in increased lysis and release of intracellular enzymes in vitro (Buist et al., 1997). Controlled, nisin-induced, expression of the metalloendopeptidase Enterolysin A of *Enterococcus faecalis* in *L. lactis* resulted in almost complete lysis of the Enterolysin expressing strain and in a 27-fold higher lactate dehydrogenase activity in cheese (Hickey, Ross, & Hill, 2004). Mitomycin C induced lysis was observed in *L. lactis* cells carrying a plasmid in which *acmA* was placed behind the mitomycin-inducible promoter of the lactococcal phage r1t (Buist et al., 1997). Induced expression of lysins to increase starter lysis in the cheese is, therefore, an option but requires a foodgrade method of cloning in order to be able to use the strains in the cheese industry in some countries. de Ruyter, Kuipers, Meijer, and de Vos (1997) used a foodgrade nisin-inducible expression system to express lytic genes from the lactococcal bacteriophage phi US3. The lysin and holin of the phage

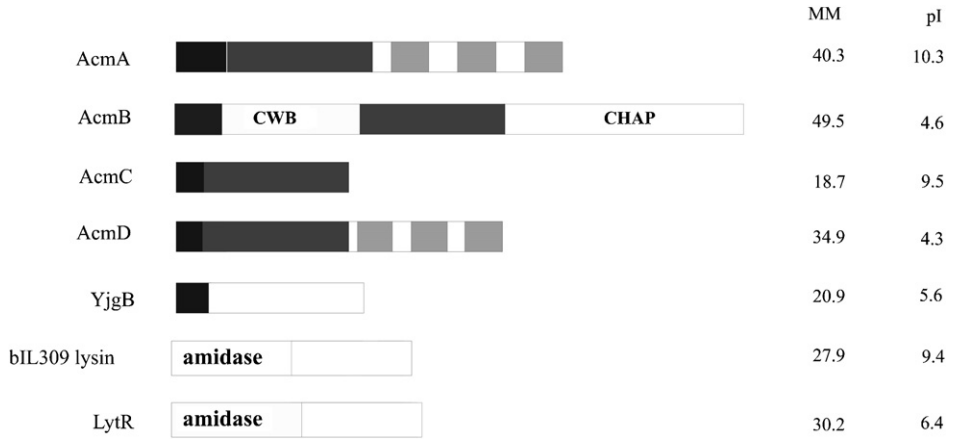

Fig. 1. Modular organization of the lactococcal peptidoglycan hydrolases AcmA, AcmB, AcmC, AcmD, YjgB, the lysin of prophage bIL309 and LytR from phage r1t. Glucosaminidase active site domains are in dark grey, peptidoglycan-binding domains containing LysM domains in light grey. Signal sequences are in black. Putative active site domains, cell wall-binding domains (CWB) and CHAP (amidase) are indicated in white. MM: molecular mass (in kDa); pI: isolelectric point of the proteins.

were expressed, resulting in efficient lysis and concomitant release of the debittering intracellular aminopeptidase N. The most efficient lysis was shown to be dependent on the presence of the phage holin. A chloride-inducible expression system was used to overexpress the holin and lysin of the lactococcal prophage r1t and *acmA* in *L. lactis*. Release of the intracellular marker PepX was highly increased by the addition of salt in vitro, while the presence of NaCl also seemed to stabilize the cells (Sanders, Venema, & Kok, 1997).

In this paper, we describe the separate overexpression in *L. lactis* of the peptidoglycan hydrolases encoded in the lactococcal genome as well as of two phage-derived lysins, which were expressed without their corresponding holins. Expression of all peptidoglycan hydrolases in *L. lactis* resulted in increased cell lysis, although some lysins appeared to be dependent on the presence of the major autolysin AcmA. Moreover, we show that overexpression in *L. lactis* of phage lysins without their corresponding holins can result in efficient cell lysis. AcmD, in combination with AcmA, and the phage r1t lysin LytR were subsequently overexpressed in a foodgrade way and cheeses were made with the cell wall hydrolase-producing strains. In this paper we show that cellular lysis in cheese was accelerated and increased using these strains, which resulted in a slight improvement in overall sensory quality. However, no change in bitterness was observed.

2. Materials and methods

2.1. Experimental procedures

2.1.1. Chemicals and enzymes

All chemicals used were of analytical grade and, unless indicated otherwise, obtained from Merck (Darmstadt, Germany). Enzymes for molecular biology were purchased from Roche Molecular Biochemicals (Mannheim, Germany) and used according to the supplier's instructions.

2.1.2. Bacterial strains, plasmids, media and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1.

L. lactis was grown at 30 °C in M17 broth (Difco Laboratories, Detroit, MI, USA) containing 0.5% glucose (GM17) or 0.5% lactose (LM17). When needed for plasmid selection, chloramphenicol (Sigma-Aldrich, St. Louis, MO, USA) was added (5 μg mL^{-1}).

2.1.3. DNA manipulation and transformation

Molecular cloning techniques were performed essentially as described by Sambrook, Fritsch, and Maniatis (1989). Electrotransformation of *L. lactis* was performed by using a gene pulser (Bio-Rad laboratories, Richmond, CA, USA) as described by Leenhouts and Venema (1992). Minipreparations of plasmid DNA from *L. lactis* were obtained by the alkaline lysis method as described by Seegers, Bron, Franke, Venema, and Kiewiet (1994). PCR products were purified using the Roche High Pure PCR purification kit (Roche Molecular Biochemicals).

2.1.4. Cloning of the peptidoglycan hydrolase genes

The primers used to clone *lytR*, *acmB*, *acmC*, *acmD*, *yjgB* and bIl309 lysin gene are presented in Table 2. DNA isolated from bacteriophage r1t was used as a template to obtain *lytR* and chromosomal DNA of *L. lactis* IL1403 was used as a template to obtain the other peptidoglycan hydrolases. Primers R1tLytR1 and R1tLytR2 were used to amplify *lytR*. The 830-bp PCR fragment was digested with

Table 1
Strains and plasmids used in this study

L. lactis strains	Characteristics	Reference
NZ9000	MG1363 *pepN::nisRK*	Kuipers et al. (1998)
NZ9000*acmAΔ1*	Derivative of NZ9000 carrying a 701-bp *Sac*I/*Spe*I deletion in *acmA*	Lab collection
NZ3900	MG1363 derivative, used as wild-type control (*ΔlacF pepN:nisRnisK*)	de Ruyter et al. (1996)
NZ9700	Nisin-producing transconjugant containing the nisin-sucrose transposon Tn5276	Kuipers, Beerthuyzen, Siezen, and de vos (1993)
13M	Nisin immune transconjugant of *L. lactis* SK110, PrtP +	Meijer et al. (1998)
Plasmids		
pNG8048e	Derivative of pNZ8048 carrying Em^R marker	Lab collection
pNZ8148F	*lacF*$^+$	NIZO
pNG*acmB*	pNG8048e derivative carrying *acmB*	This study
pNG*acmC*	pNG8048e derivative carrying *acmC*	This study
pNG*acmD*	pNG8048e derivative carrying *acmD*	This study
pNG*yjgB*	pNG8048e derivative carrying *yjgB*	This study
PNG*bil*	pNG8048e derivative carrying *bil* from prophage bIL309	This study
pNG*lytR*	pNG8048e derivative carrying *lytR* from prophage r1t	This study
pNZ*acmA*	pNZ8148 derivative carrying *acmA*	This study
pNZ*acmD*	pNZ8148 derivative carrying *acmD*	This study
pNZ*acmDacmA*	pNZ8148 derivative carrying *acmD* and *acmA*	This study
pNZ*lytR*	pNZ8148 derivative carrying *lytR*	This study

*Nco*I and *Eco*RI and cloned into the corresponding sites of pNG8048e. Primers PACMB1 and PACMB2 were used to clone AcmB. The 1440-bp PCR fragment was digested with *Nco*I and *Hin*dIII and cloned into the corresponding sites of pNG8048e, resulting in pNG*acmB*. AcmC was cloned using primers PACMC1 and PACMC2; the 645-bp fragment was digested with *Rca*I and *Hin*dIII and cloned in the *Nco*I and *Hin*dIII sites of pNG8048e. The 1112-bp PCR fragment obtained with primers PACMD1 and PACMD2 was digested with *Rca*I and *Sac*I and cloned into the *Nco*I and *Sac*I sites of pNG8048e, resulting in plasmid pNG*acmD*. YjgB was amplified using primers PYJGB1 and PYJGB2; the 595-bp fragment was digested with *Rca*I and *Hin*dIII and cloned in the *Nco*I and *Hin*dIII sites of pNG8048e. The phage lysin *bil* was cloned using primers BIL1 and BIL2: the 816-bp PCR fragment was digested with *Rca*I and *Pst*I and cloned in the *Nco*I and *Pst*I sites of pNG8048e. To clone *lytR* in a foodgrade way, pNG*lytR* was digested with *Pst*I and *Nco*I and the 832-bp fragment was isolated from an agarose gel. Subsequently the fragment containing *lytR* was ligated in pNZ8148F cut with *Pst*I and *Nco*I.

For foodgrade expression of AcmA and AcmD, the corresponding genes were cloned in plasmid pNZ8148F. The *acmA* gene was amplified by PCR using primers pACMAB and ACMA.rev with *L. lactis* MG1363 DNA as the template. The *acmD* gene was obtained by PCR with primers ACMD1 and ACMD2 using *L. lactis* IL1403 chromosomal DNA as the template. Plasmid pNZ8148F, digested with *Nco*I and *Pst*I, and the PCR containing *acmD* cut with *Rca*I and *Pst*I were ligated; the resulting plasmid, pNZ8148F*acmD,* was digested with *Pst*I and *Xba*I and the PCR fragment containing *acmA* with *Nhe*I and *Pst*I. The digested DNA fragments were ligated to obtain pNZ*acmDacmA*. Plasmid pNZ8148F was digested with *Pst*I and *Xba*I and the PCR fragment containing *acmA,* cut with *Nhe*I and *Pst*I, were ligated, resulting in plasmid pNZ*acmA*.

2.1.5. Nisin induction, enzyme assays and optical density measurements

L. lactis NZ9000 (Kuipers, de Ruyter, Kleerebezem, & de Vos, 1998) and *L. lactis* NZ9000*acmAΔ1* containing pNZ8148e derivatives were grown in GM17 broth containing chloramphenicol, while *L. lactis* NZ3900 (de Ruyter, Kilpers, Beerthuyzen, Alen-Boerrigter, & de Vos, 1996) containing pNZ8148F derivatives were grown in LM17 medium. The cultures were grown until an OD_{600} of 0.5 was reached, after which they were induced with nisin by the addition of 1/1000 volumes of a supernatant of a culture of the nisin-producing strain *L. lactis* NZ9700.

X-prolyl dipeptidyl aminopeptidase (PepX) activity was measured using the chromogenic substrate Ala-Pro-*p*-nitroanilid (BACHEM Feinchemicalien AG, Bubendorf, Switzerland) as described earlier (Buist, Venema, & Kok, 1998). Optical densities were measured in a Novaspec II spectrophotometer (Pharmacia, Uppsala, Sweden) at 600 nm (OD_{600}).

2.1.6. SDS-polyacrylamide gel electrophoresis (SDS-PAGE), AcmA zymograms and Western hybridization

L. lactis cell and supernatant samples were prepared as described previously (Buist et al., 1998). Supernatants were concentrated using phenol and ether as described by Sauve, Ho, and Roberge (1995). AcmA activity was detected by a zymogram staining technique using SDS-PAA (12.5%) gels containing 0.15% autoclaved, lyophilized *Micrococcus lysodeikticus* ATCC 4698 cells (Sigma-Aldrich) or 0.15% autoclaved *L. lactis* MG1363*acmAΔ1* cells, as described previously (Buist et al., 1995). Zymograms were renatured at pH 4, 5, 6 or 7 as described by Huard et al. (2003). The standard low-range and prestained low and high-range SDS-PAGE molecular weight markers (Bio-Rad laboratories) were used as references. Proteins were transferred from SDS-PAA (10%) gels to PVDF membranes (Roche Molecular Biochemicals) as described by Towbin, Staehelin, & Gordon (1992). AcmA antigen was detected with a

Table 2
Nucleotide sequences of primers used to clone the peptidoglycan hydrolase genes used in this study

Name	Sequence 5′–3′*	Gene	Enzyme(s)
PACMB1	CATACCATGGAGGATTAAC	*acmB*	*Nco*I
PACMB2	CGCAAGCTTATTTAGGTTGAATATAAG	*acmB*	*Hin*dIII
PACMC1	CCTGTCATGAAATATAAAACTCGACGAAG	*acmC*	*Rca*I
PACMC2	CGCAAGCTTTTAAATACTTGAACTTTTATC	*acmC*	*Hin*dIII
PACMD1	CCTGTCATGAAACAGAAACATAAATTAGCGC	*acmD*	*Rca*I
PACMD2	CGCAAGCTT*CTGCAG*AGCTCTTAGATTCTAATTGTTTGTCCTGG	*acmD*	*Sac*I/*Pst*I
PYJGB1	CATGTCATGATGAAAAAAATAATTATTTCTGCAGC	*yjgB*	*Rca*I
PYJGB2	CCCGGATCCAAGCTTAATACTCTAATGCAAAATCAGC	*yjgB*	*Hin*dIII
PBIL1	GCGCGAGCTCGGAGGCGAATCATGAGTAGTATTGAAAATATG	*bil*	*Rca*I
PBIL2	CGCAAGCTTCTGCAGTTATAATTTATTCGCATTTAATC	*bil*	*Hin*dIII
R1tLytR1	GAATCCATGGCAATTTACGACAAAACGTTCC	*lytR*	*Nco*I
R1tLytR2	ATATTGAATTCTTATCCTTTTGAAATGTCACG	*lytR*	*Eco*RI
pACMAB	AAAACTGCAGTTAGAAAGGTAATTATTTATGCC	*acmA*	*Pst*I
ACMA.rev	TT*GGATCC*GAATTCGCTAGCGGAATGTCAGAACCGACCCG	*acmA*	*Eco*RI

*Restriction enzyme sites used and listed under enzyme(s) are underlined or in italics.

10,000-fold diluted rabbit polyclonal anti-AcmA antiserum and HRPO-conjugated goat anti-rabbit secondary antibodies (Pharmacia, Uppsala, Sweden) using the ECL chemiluminescent detection system and protocol (Amersham, Piscataway, NJ, USA).

2.1.7. Cheese manufacture and cheese tasting

Gouda-type cheese was manufactured using a standard procedure (Walstra, Noomen, & Geurts, 1987). Milk (200L), eventually resulting in five cheeses) was pasteurized (10 s, 74 °C) and inoculated with 50 mL of a 40-fold times concentrated suspension of *L. lactis* 13 M (CSK food enrichmond, Leeuwarden, the Netherlands) and with 1% *L. lactis* NZ3900 carrying plasmid pNZ8148F, pNZ*acmD*, pNZ*acmDacmA* or pNZ*lytR*. To induce lysin expression from the plasmid nisin was added to the milk (4 μg L^{-1}).

The cheeses were tasted by a certified expert panel at NIZO and subsequently scored for sensory quality and bitterness. The NIZO panel was trained specifically to recognize sensory problems in cheese. Outliers of the scores were not used for calculations.

2.1.8. Analysis of viability and lysis of starter cultures in cheese

Viability of *L. lactis* 13 M and *L. lactis* NZ3900 carrying the lysin-producing plasmids was measured by counting colony forming units (cfu) on GM17 plates containing the chromogenic proteinase substrate Azocoll. *L. lactis* 13 M (proteinase$^+$) colonies are yellow, *L. lactis* NZ3900 (proteinase$^-$) colonies are white.

Total cell lysis in the cheese was measured using two different methods. The amounts of lactate dehydrogenase released in the cheese were measured as described previously (Wittenberger & Angelo, 1970). Numbers of living and dead cells in the cheese were counted using the Live/Dead staining in combination with CSLM as described by (Bunthof, van Schalkwijk, Meijer, Abee, & Hugenholtz, 2001). The viability and lysis tests were all performed at NIZO by trained staff. Errors for cfu counts are standardly set on 35% and for lactate dehydrogenase (LDH) release tests on 5%. For the Live/Dead stains 10 samples were taken and counted separately.

3. Results

3.1. Overexpression of lactococcal peptidoglycan hydrolases in L. lactis results in cell lysis

Sequencing of the genome of *L. lactis* IL1403 revealed that this lactic acid bacterium contains, next to the gene for the autolysin AcmA, at least four other putative peptidoglycan hydrolase genes (Bolotin et al., 2001). *L. lactis* IL1403 also contains prophage sequences in which lytic genes are present. Each prophage lytic gene is accompanied by a holin gene. To examine whether the lactococcal lysins are able to lyse lactococcal cells in vivo, the *L. lactis* IL1403 chromosomal genes *acmB*, *acmC*, *acmD* (encoding glucosaminidases) and *yjgB* (specifying a putative endopeptidase) were cloned downstream of the nisin-inducible promoter P_{nisA} of plasmid pNG8048e in *L. lactis* NZ9000 (see Fig. 1 for an overview of the lysins used in this study). Blast analysis showed that AcmB, besides the glucosaminidase active site domain, also contains a CHAP domain (pfam05257.5) which has recently been shown to encode an *N*-acetylmuramoyl-l-alanine amidase specificity (Llull, Lopez, & Garcia, 2006). In addition, the lysin gene of the lactococcal prophage r1t, *lytR*, and that of the IL1403 prophage *bIL309* (both coding for putative amidases) were cloned in pNG8048e without their respective holin genes. The C-terminal domain of LytR showed considerable similarity to the corresponding regions of lysins encoded by the four closely related prolate-headed temperate lactococcal phages c2, bIL67, NvML3 and Q54. Although the C-terminal cell wall-binding domains of endolysins often consist of repeated regions, such units cannot be discerned within these enzymes. Possibly, cell wall recognition is facilitated by a single domain.

Subsequently, the strains containing the pNG8048e derivatives were grown in GM17 medium and expression of the lysins was induced by the addition of nisin at the mid-exponential growth phase. Cell extracts of cultures expressing the lysins were analysed in zymograms and the results are summarized in Table 3. SDS-PAA gels containing *M. lysodeikticus* cell walls were renatured at pH 4, 5, 6 or 7. At all the tested pH values, LytR activity could be detected. AcmC activity was detected at pH 4, 5 and 6, with highest activity at pH 5. Activity of AcmB, AcmD, YjgB and Bil309 lysin could not be detected on these zymograms. When culture supernatant samples were analysed on zymograms containing *M. lysodeikticus* cell walls, only LytR activity was detected. All samples were also analysed in zymograms containing boiled *L. lactis* cells as cell wall hydrolase substrate. Besides LytR and AcmC, Bil309 lysin activity was detectable on this substrate when the gel was renatured at pH 6. Anti-AcmA antibodies raised against the active site of AcmA were used to try to detect the AcmA homologues AcmB, AcmC and AcmD in Western blots, since these proteins have extensive amino acid sequence similarities with AcmA. Only AcmC could be detected using this assay (date not shown). Using Coomassie Brilliant Blue (CBB) staining, YjgB and AcmD could be detected in the culture supernatant of cells expressing the enzymes. AcmC was only detected in cell extracts and not in the supernatant (Table 3).

Growth and lysis of the strains expressing the lysins were followed by measuring the OD_{600} of the cultures (not shown) and by determining the release of intracellular X-prolyl dipeptidyl aminopeptidase (PepX) activity in the cultures growth medium after 24 h (Fig. 2). Induction of expression of AcmB and AcmD resulted in a small increase in cellular lysis while moderate lysis was obtained by expression of AcmC or YjgB. Surprisingly, most lysis was obtained upon expression of LytR and the lysin of prophage bIl309, although their corresponding holins were

Table 3
SDS-polyacrylamide gel electrophoresis analysis of expression and activity of the *L. lactis* (phage) lysins[a]

Enzyme	pH 4	pH 5	pH 6	pH 7	CBB stain[b]	pI
Control (AcmA)	+	+	+	+	–	10.3
AcmB	–	–	–	–	–	4.6
AcmC	+	+ + +	+	–	+ (cell extract)	9.5
AcmD	–	–	–	–	+ (supernatant)	4.3
YjgB	–	–	–	–	+ (supernatant)	5.6
LytR	+	+	+	+	–	6.4
bIL309 lysin[c]	ND	ND	+	ND	–	9.4

ND: not determined.
[a]Zymograms containing *M. lysodeikticus* autoclaved cells were renatured at pH 4, 5, 6 and 7 in buffer containing 0.1% Triton-X100 and hydrolase activity was scored as clearing zones in the gel. +: clearing zone detectable. + + +: large clearing zone, –: no clearing zone.
[b]Detection of overexpression by Coomassie Brilliant Blue (CBB) staining.
[c]bIL309 lysin was tested on *M. lysodeikticus* cells, but the enzyme is only active in gels with *L. lactis* cell walls as substrate.

not expressed. This is in contrast to earlier studies on expression of phage lysins, which report that cell lysis by phage lysins largely depends on the presence of the holins (de Ruyter et al., 1997).

Cells in which expression of AcmC or LytR was induced stopped growing within 2–3 h after nisin addition. The other strains continued to grow with rates comparable with that of the control strain and reached the same maximal OD_{600} as the control, *L. lactis* NZ9000 (pNG8048e), after which lysis started (data not shown).

AcmA is responsible for stationary phase lysis of *L. lactis* MG1363 in growth medium (Buist et al., 1995). The activity of AcmB has been suggested to be dependent on the activity of AcmA (Huard et al., 2003). The pNG8048e derivatives containing the different lytic genes were introduced in the *acmA* mutant of *L. lactis* NZ9000 to investigate the requirement of AcmA activity for lysis by the other lysins. Only very small amounts of PepX were released after nisin induction of AcmB, AcmD and YjgB in the AcmA-negative background (Fig. 2). Lysis was detected when AcmC, LytR and the bIl309 lysin were expressed in the *acmA* mutant of *L. lactis*, but lower than when expressed in *L. lactis* NZ9000. The highest amount of PepX was released upon induced expression of the phage lysins. From these results we conclude that cell lysis by AcmB, AcmD and YjgB apparently depends on the activity of AcmA.

3.2. Foodgrade overexpression of acmA, acmD and lytR

AcmA, AcmD and LytR were selected for further analysis on the basis of their secreted, relatively high lytic activities, the in trans activity of AcmA (Buist et al., 1997), the low pI of AcmD and the known effective lysis by phage endolysins such as LytR. The genes for these proteins were cloned in a foodgrade way using lactose selection and growth (de Ruyter et al., 1997), to be able to use the lysin-producing strains in cheese manufacture. AcmA is known to be effective in increased lysis of *L. lactis* (Buist et al., 1997). AcmD has the same modular structure as AcmA, i.e., it consists of an N-terminal glucosaminidase domain and a C-terminal peptidoglycan-binding domain (Fig. 1), but its overall pI is 4.3, whereas that of AcmA is 10.3. This suggests that AcmD is active at a lower pH than AcmA, a property that would be beneficial under cheese making conditions. The results described above show that expression of AcmD results in increased lysis in AcmA-expressing cells. To examine whether induced expression of both AcmA and AcmD could enhance the lysis of *L. lactis, acmD* was cloned in an operon-like structure with *acmA*. For proper expression, the ribosome-binding site of a*cmA* was inserted upstream of *acmA*. Since LytR is also very effective in lysing *L. lactis* cells without depending on AcmA (see above), this enzyme was also used in this study to monitor its behaviour in a cheese model.

Cell lysis, as measured by the reduction of the OD_{600} and the release of PepX into the culture medium was followed before and after nisin induction of *L. lactis* NZ3900 containing either of the plasmids pNZ8148F (control), pNZ*lytR*, pNZ*acmD*, pNZ*acmA* or pNZ*acmDacmA*. All cultures were grown in the presence of 0.5% lactose and were induced with nisin at an OD_{600} of 0.5. Induced expression of AcmA, AcmD or the combination of both did not change the growth rates of the cultures (Fig. 3A). As expected, overexpression of AcmA resulted in increased lysis. Surprisingly, overexpression of AcmD led to a decrease in cellular lysis. Stationary phase lysis of the culture overexpressing both AcmA and AcmD was increased compared to the culture overexpressing AcmA alone (Fig. 3B). Interestingly, growth rate and cell lysis were delayed upon overexpression of LytR, but after prolonged incubation an increase in the release of PepX was observed (Fig. 3A and B).

Overexpression of AcmA was evident from zymographic analysis of cell and supernatant samples taken at 2 h after nisin induction (data not shown). LytR activity was detected in cell extracts, not in the supernatant: at the time the samples for zymographic analysis were taken, the LytR expressing strain had not yet lysed (Fig. 3B). AcmD activity could not be detected in cell and supernatant samples of *L. lactis* NZ3900 (pNZ*acmD*) and *L. lactis*

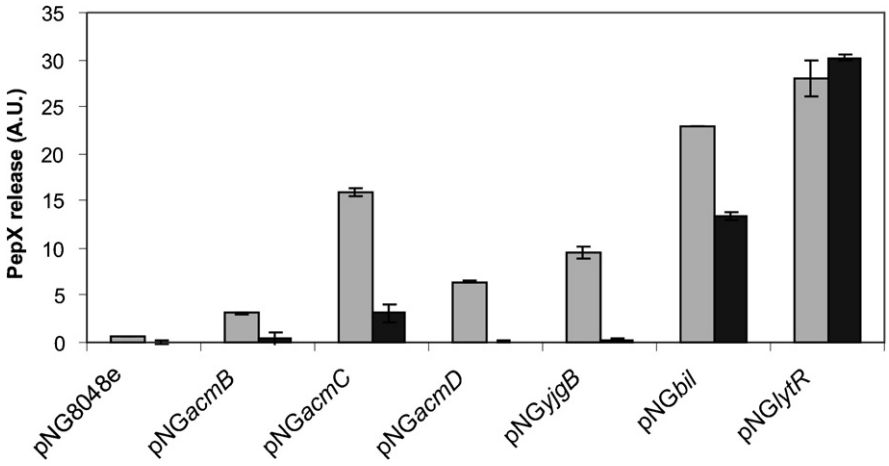

Fig. 2. Determination of cell lysis upon induced expression of lactococcal peptidoglycan hydrolases in *L. lactis*. PepX release 24 h after nisin induction of *L. lactis* NZ9000 (light grey bars) or *L. lactis* NZ9000*acmAA1* (dark grey bars) cultures containing pNG8048e and pNG8048e derivatives in which the peptidoglycan hydrolase encoding genes were cloned downstream of the nisin-inducible promoter (pNG*acmB*, pNG*acmC*, pNG*acmD*, pNG*yjgB*, pNG*bil*, pNG*lytR*). The cultures were grown until an OD_{600} of 0.5 was reached, after which expression was induced by addition of nisin. After 24 h the culture supernatants were analysed for the presence of activity of the intracellular peptidase PepX (in arbitrary units). The means and standard deviations of two parallel experiments are shown, whereas similar patterns were obtained in independent experiments.

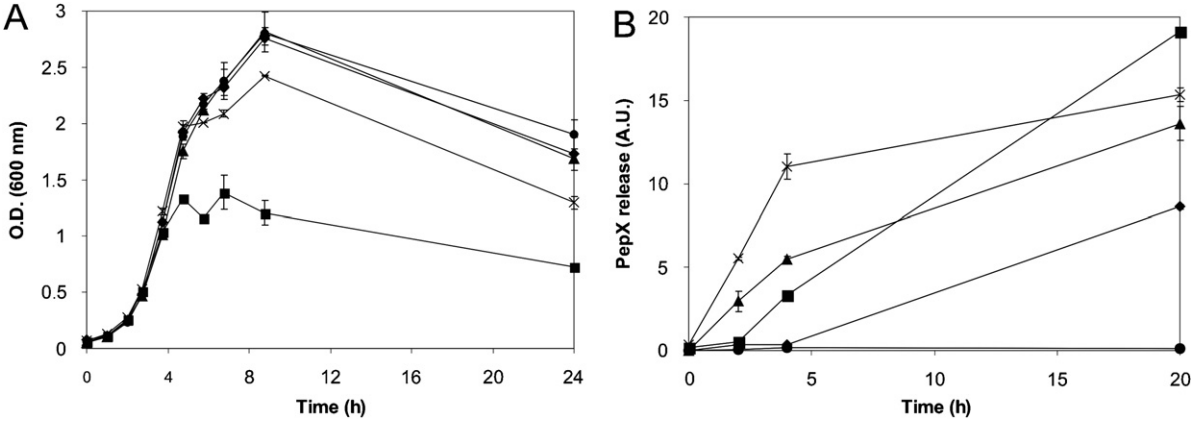

Fig. 3. Foodgrade expression of *acmA*, *acmD* and *lytR*. (A) Growth in LM17 of *L. lactis* NZ3900 containing either pNZ8148F (♦), pNZ*acmD* (×), pNZ*acmA* (▲), pNZ*acmDacmA* (●) or pNZ*lytR* (■). The OD_{600} of the cultures were followed in time; the cultures were induced with nisin at an OD_{600} of ~0.5. The means and standard deviations of two parallel experiments are shown, whereas similar results were obtained in independent experiments. (B): PepX activity (in arbitrary units. per OD_{600}) released into the culture medium after nisin induction of the cultures described in panel A. The means and standard deviations of two parallel experiments are shown, whereas similar results were obtained in independent experiments.

NZ3900 (pNZ*acmDacmA*) by zymographic analysis, not even at pH 4, a pH below the pI of AcmD.

3.3. Lysin-overproducers as starter strains

To examine the behaviour of the lysin-producing strains in cheese, the foodgrade lysin-overproducing strains (except the AcmA overproducer) were each used as starter strains in combination with the nisin-immune acidifying *L. lactis* strain 13 M in cheese making trials. *L. lactis* 13 M is a nisin-immune, non-autolytic derivative of *L. lactis* SK110. *L. lactis* SK110 is autolytic and cheeses made with SK110 are not bitter (Meijer et al., 1998). Nisin (4 μg L^{-1}) was added to the cheese milk for induction of lysin-overexpression in the curd. Overexpression of LytR resulted in a reduced viability of the producer strain compared to the control (Fig. 4A). The viability of the acid-producing strain *L. lactis* 13 M was also measured: only LytR decreased the viability of this strain (Fig. 4B). Lysis of the cells in the cheese matrix was examined by measuring the release of LDH (Fig. 4C) or by using the CSLM live/dead staining technique (Fig. 4D). In the cheese made with the LytR-expressing strain, more lysis was observed than in the control cheese, whereas lysis of the LytR-overproducer resulted in increased lysis of the acidifying culture (lysis in trans). Also, in the cheese made with *L. lactis* NZ3900 (pNZ*lytR*) increased release of intracellular LDH was observed as a result of lysis of the total culture (Fig. 4C) When the cheeses were tested by a certified taste panel, only modest effects on flavour formation were observed (Table 4).

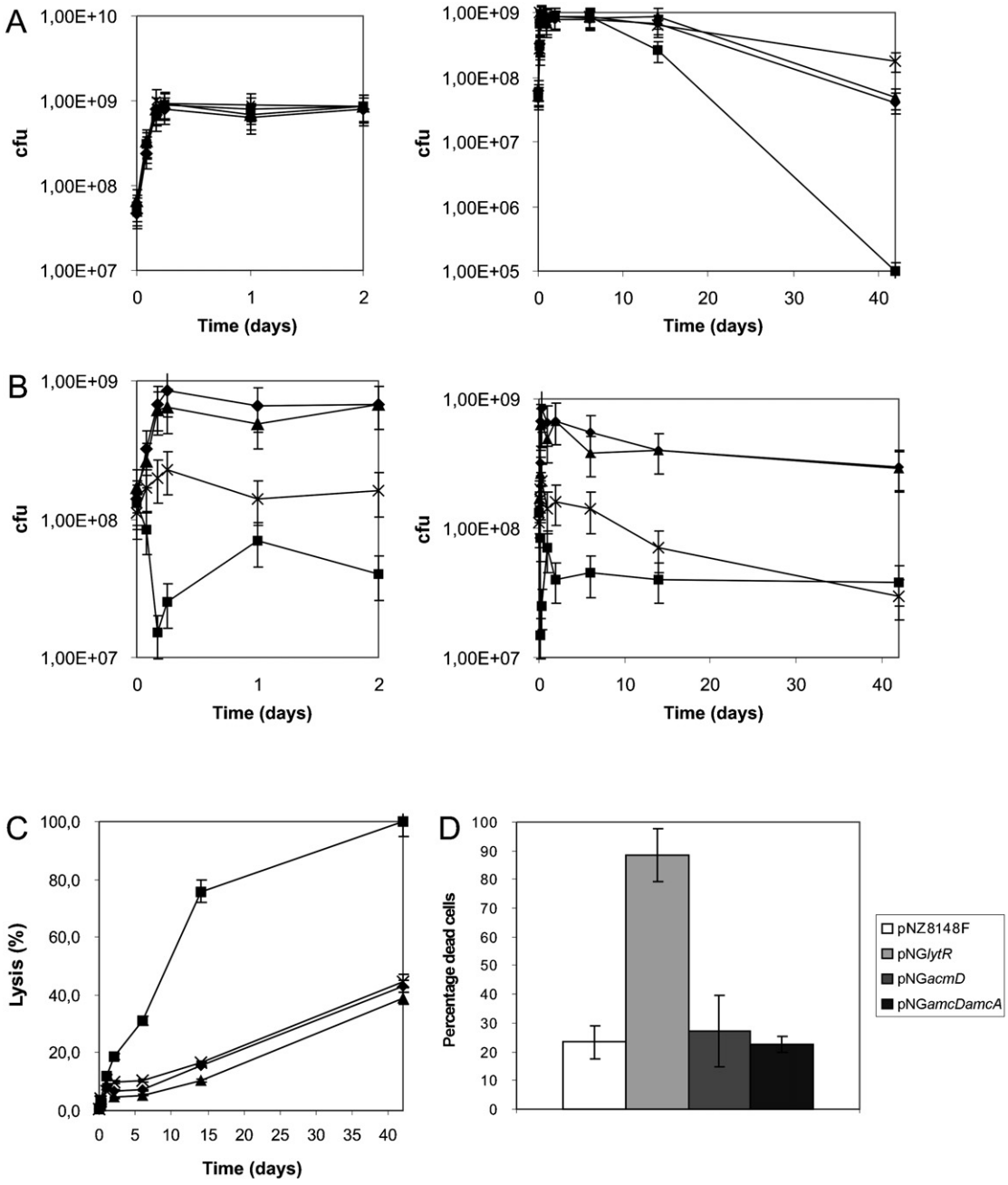

Fig. 4. Viability of lysin- and acid-producing strains during cheese manufacture. (A) Viability of the lysin-producing *L. lactis* NZ3900 strains containing either pNZ8148F (♦), pNZ*acmD* (×), pNZ*acmA* (▲), pNZ*acmDacmA* (●) or pNZ*lytR* (■).Viability is expressed as colony forming units (cfu) per gram of cheese. Left panel: cfu during the first 2 days, right panel: cfu during 42 days of cheese ripening. The standard errors of the cfu counts are set on 35%, based on experience at NIZO. (B) Viability of the acid-producer *L. lactis* 13 M in cheese made in the presence of *L. lactis* NZ3900 carrying the indicated plasmids as described in panel A. Left panel: cfu during the first 2 days, right panel: cfu during 42 days of cheese ripening. The standard errors of the cfu counts are set on 35%, based on experience at NIZO. (C) Lysis cheeses made with the lysin-producing strains: total cell lysis in cheese, made in the presence of *L. lactis* NZ3900 carrying the indicated plasmids as described in panel A, measured by the release of lactate dehydrogenase (LDH). Release of LDH activity after 42 days in the cheese in which *L. lactis* NZ3900 (pNZ*lyt*R) expressing LytR was present as the adjunct strain was set at 100%. The standard error of these experiments was set on 5%, based on experience at NIZO. (D) Percentage dead cells in the cheeses presented in (C) after 42 days by counting living and dead cells using the CSLM live/dead staining technique (Bunthof et al., 2001). Means and standard errors of 10 separate samples are shown.

4. Discussion

We have shown that overexpression of each of the cell wall hydrolases specified by the genome of *L. lactis* IL1403 resulted in increased lysis of the *L. lactis* NZ9000 producer cells compared to the control strain, suggesting that all lysins have cell wall lytic activity in vivo. This confirms the results of Huard et al. (2004) who showed in vitro activity of His-tagged variants of the enzymes produced intracellularly in *Escherichia coli*. In our studies, differences in the

Table 4
Sensory and bitterness scores of cheese made with the foodgrade lytic strains used in this study[a]

Autolysin strain	Sensory score (3–8)		Bitterness score (0–4)	
	6 weeks	12 weeks	6 weeks	12 weeks
PNZ8148F	5.4	6.0	1.4	1.0
pNZ*lytR*	5.8	5.5	1.3	1.3
pNZ*acmD*	5.2	5.8	1.4	1.3
pNZ*acmDacmA*	5.4	6.1	1.6	1.0

[a]The cheeses were tasted by a certified expert panel, which was trained specifically to recognize defects in the sensory quality of cheeses. All scores have been statistically challenged. The sensory score was on a scale of 3 (very poor) to 8 (excellent), the bitterness score was on a scale of 0 (absent) to 4 (very strong).

extent of cellular lysis were observed: overexpression of AcmC and YjgB resulted in high release of intracellular PepX, while overexpression of AcmB and AcmD resulted in low levels of PepX release. Apart from AcmA, the only other enzyme active in zymograms was AcmC, and only so when cell extracts of AcmC expressing cells were analysed. No AcmC activity was detected in supernatant samples. AcmC is active in the range of pH 4–6, with maximum activity at pH 5; no activity was observed at a pH 7 or higher. AcmC has, therefore, an activity pattern different from that of its homologue AcmA, which is active at all pH values tested, with similar activities.

Although activity of AcmD and YjgB was not seen on zymograms, the proteins in concentrated supernatants samples were detectable in CBB-stained PAA gels. Both proteins are, therefore, secreted and released in the culture supernatant, but *trans*- lysing activity is not detectable: when supernatants containing the proteins were mixed with *L. lactis* MG1363*acmAΔ1* cells, the cells did not lyse (data not shown). AcmC activity was present in cell extracts, but the enzyme could not be detected in concentrated supernatants of the overproducing strain, although it has a consensus signal sequence for secretion (von Heijne, 1990). AcmC does not contain a cell wall-binding domain, as is present in AcmA, AcmB and AcmD. The close association of this enzyme with the cell must therefore be caused by a high affinity of the active site domain for peptidoglycan. AcmB was not detectable in zymograms or in CBB-stained gels, although increased lysis was obtained in the strain in which the gene was induced with nisin, presumably because the expression level of AcmB is too low. Huard et al. (2004) have shown activity of the lactococcal lysins in zymograms. However, in their studies, the enzymes were overexpressed in, and purified from, *E. coli*, resulting in high protein concentrations. In our studies the amounts of AcmB, AcmD and YjgB produced were possibly too low to be able to detect their activities in gels.

By expressing the various lysins in an AcmA-negative background it was shown that lysis by AcmB, AcmD and YjgB depends on the presence of AcmA: no cellular lysis was obtained with any of the three enzymes when AcmA is not expressed. For AcmB this phenomenon has been described in an earlier study, in which *acmB* and *acmB acmA* mutants of *L. lactis* were examined (Huard et al., 2003). Why and how AcmB, AcmD and YjgB depend on AcmA is unclear, but probably AcmA changes the peptidoglycan in such a way that it facilitates activity of the other cell wall hydrolases. It is also possible that activity of these cell wall hydrolases results in a peptidoglycan that is more susceptible to hydrolysis by AcmA.

Although we showed here that all the lactococcal lysins known to date are able to lyse lactococcal cells, the precise function of each cell wall hydrolase in *L. lactis* is not clear. Only very low amounts of PepX are released from *L. lactis* NZ9000*acmAΔ1* in stationary phase, indicating that under normal conditions, the lactococcal cell wall hydrolases other than AcmA hardly contribute to cell lysis. Indeed AcmC, for which we have shown that nisin-induced expression in an *acmA* mutant results in lysis, is only expressed early in the exponential phase and not in stationary phase, as was demonstrated by Northern analysis (Huard et al., 2004). Transcripts of *acmB* and *acmD* were detected in stationary phase but since AcmB and AcmD activities depend on the presence of AcmA, expression of these enzymes does not result in lysis in the *L. lactis acmA* mutant. Mutants of AcmC, AcmD and YjgB would provide more evidence on the function of these enzymes during growth of *L. lactis*. AcmB is not necessary for normal growth since *L. lactis acmB* grows normally and has a normal cell morphology (Huard et al., 2003).

Besides the IL1403 chromosomal genes, the lysins of the *L. lactis* R1 prophage r1t and the *L. lactis* IL1403 prophage bil309 were also expressed in this study. These phage-encoded lysins used in this study do not contain a signal sequence for secretion and are not secreted via the secretion apparatus of the cell. In fact, both amidases are expected to be specifically released by their respective holins (Bolotin et al., 2001; van Sinderen et al., 1996). To our knowledge, we show here for the first time that expression of phage lysins in *L. lactis* without their corresponding holins results in cellular lysis. The fact that expression of the lysins in *L. lactis* NZ9000 without their holins results in cell lysis is most easily explained by assuming that the enzymes are released through AcmA-mediated cell lysis. However, lysis by the phage lysins is also obtained in *L. lactis acmA*. This is presumably because in the genome of *L. lactis* MG1363 holin genes are present (Wegmann et al., 2007), which might facilitate the secretion of LytR. It is also possible that the *acmA* mutant is still subject to some residual lysis, enough to release some phage lysin, thereby starting a lysis chain reaction. This would explain why, when LytR is expressed, lysis is slow at first, but increases rapidly after prolonged incubation.

The observation that most cell lysis was obtained upon expression of the phage lysins LytR or the bIl309 lysin in *L. lactis* NZ9000 may be explained by assuming that phage lysin activities are not tightly regulated and are, therefore, responsible for less controlled lysis. AcmA seems to be

regulated via its C-terminal peptidoglycan-binding domain, which binds only at very specific places in the cell wall due to hinderance by other cell wall components, most likely lipoteichoic acids (Steen et al., 2003). LytR and the bIl309 lysin also seem to contain a C-terminal domain that could be involved in cell wall binding, as has also been shown for other phage lysins (Fischetti, 2005). This C-terminal cell wall-binding domain is most likely not targeted to specific loci on the cell surface but will bind all over the cell, thereby increasing the lysis efficiency. This could explain why lysis by phage lysins like LytR is as efficient as shown in Fig. 4. Secondly, LytR and bIL309 lysin are amidases: together with AcmA, a glucosaminidase, the effect on lysis could be bigger than when two glucosaminidases are expressed together.

Earlier studies on foodgrade expression of phage lysins show that the enzymes are useful in cheese manufacture (Chapot-Chartier et al., 1994; de Ruyter et al., 1997). In all cases, efficient lysis was only obtained when the proper holin was also expressed. We have shown here that expression of LytR alone has some desirable effects on lysis: it delays lysis of the expressing strain: this could prevent lysis of starter cells in the curd taking place too rapidly, which would otherwise result in an early loss of total proteolytic activity. Furthermore, the cells are expected to be metabolically active longer, which would increase the stability of enzymes desirable in cheese manufacture. The stability of PepX and a number of intracellular marker enzymes was studied by Wilkinson, Guinee, O'Callaghan, and Fox (1994), who found that PepX activity is quite unstable: only 15% of initial activity remained after 24 h in a cheese slurry system (pH 5.17). The other enzyme activities studied (glucose-6-phosphate dehydrogenase and lactate dehydrogenase) were also relatively unstable. In contrast, Chapot-Chartier et al. (1994) reported that PepX and PepC/N activities were stable in an extract of St. Paulin cheese (pH 5.8).

In addition to causing delayed cell lysis, LytR is able to lyse cells in trans. This is also an important feature of the enzyme, since the LytR-producing strain could be used to lyse other (non-recombinant) starter strains in the cheese matrix. When only lysis of the lysin-producing strain is desired, the AcmB, AcmC, AcmD or YjgB enzymes are more suitable since they are not able to lyse cells in trans (data not shown).

Although the in vitro results with overexpression of LytR were promising, the cheese tasting results were rather disappointing. Only a very small improvement in overall quality of the cheese was obtained. In a study by de Ruyter et al. it was also observed that overexpression of a phage lysin together with its holin resulted in only a minor increase of the release of cytoplasmic enzymes. In this study, the lysin and holin were expressed in the acidifying strain itself. The acidifying strain used in this study is a non-bitter-producing strain and lysis of this strain was expected to improve the taste of the cheese. By choosing other acidifiers we hope to be able to improve the overall quality of cheese with the LytR strain.

5. Conclusions

The *L. lactis* peptidoglycan hydrolases AcmB, AcmC, AcmD, YjgB, LytR and the lysin of prophage bIL309 were overexpressed in *L. lactis*, which resulted in increased lysis of the lysin-producing *L. lactis*. These enzymes are therefore all true lysins. Lysis by AcmB, AcmD and YjgB depended on the presence of the autolysin AcmA. Surprisingly, overexpression of phage lysins without their corresponding holins resulted in efficient lysis of the producing strain.

Cheese trials showed that overexpression of LytR was most suitable to obtain increased cellular lysis in cheese since lysis was delayed in the first stages of cheese manufacture. Moreover, overexpression of LytR resulted in lysis of the producing strain, as well as other *L. lactis* strains (lysis in trans). Usage of the system in the current genetic background or another lactis strain in combination with an acidifier strain with good flavour characteristics might result in faster and improved cheese ripening.

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