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Review

Role, mechanisms and control of lactic acid bacteria lysis in cheese

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Abstract

Lysis of dairy starters is a prerequisite for optimum cheese maturation, since intracellular starter enzymes, particularly peptidases, can then play their role. Here we describe the different methods used to detect starter lysis in situ and current knowledge concerning the impact of lysis on cheese ripening, particularly the increase of free amino acids due to early lysis and the reduction of bitterness by hydrolysis of large hydrophobic peptides. Recent results obtained on the impact of lysis on lipolysis and amino acid catabolism are also described. Then, we present current knowledge regarding the mechanisms involved, focussing mainly on the model most investigated: *Lactococcus lactis*. Recent advances concerning the molecular characterization of peptidoglycan hydrolases are summarized (sequence, structure, regulation) together with current knowledge of the relationship between lysogeny and lysis. Lastly, we review the different approaches proposed to control or induce lysis in situ. In conclusion, we point out unaddressed questions.

Keywords: Lysis; Lactic acid bacteria; Cheese ripening; Peptidoglycan hydrolases

Contents

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1. Introduction

Bacterial autolysis results from the enzymatic degradation of the cell wall peptidoglycan by endogenous peptidoglycan hydrolases (PGHs) named autolysins. Bacterial PGHs are involved in a number of different cellular functions that require modification of the rigid peptidoglycan network during growth and division (Smith, Blackman, & Foster, 2000). Autolysis has been observed in many Gram-negative and Gram-positive bacteria (Shockman & Höltje, 1994). It occurs generally under conditions that result in cessation of peptidoglycan synthesis, for example nutriment starvation or other unfavourable environmental conditions. Under such conditions, hydrolysis of peptidoglycan, which is essential for the cell structural integrity, may cause cellular autolysis and release of intracellular contents.

Autolysis of lactic acid bacteria (LAB) is of special interest regarding their use as starters in dairy fermentations. Controlling and increasing starter LAB lysis is considered as an essential parameter to control and accelerate cheese ripening. Indeed, LAB starters produce intracellular enzymes (peptidases, lipases and enzymes of amino acid catabolism), which play a key role in cheese flavour development during ripening. After the initial breakdown of caseins by rennet, milk endogenous proteases and bacterial cell wall protease, a set of peptidases is able to degrade the resulting peptides into free amino acids (see Kunji, Mierau, Hagting, Poolman, & Konings, 1996, for review). Free amino acids can be subsequently catabolized to volatile aroma compounds by several enzymatic pathways (see Yvon & Rijnen, 2001, for review). Also, esterases and lipases catalyse hydrolysis of triglycerides of milk fat in free fatty acids that are further converted to aroma compounds. They can also synthesize esters from alcohols and glycerides under certain conditions (Liu, Holland, & Crow, 2003). The release of bacterial intracellular enzymes upon cellular lysis allows them to reach substrates present in the cheese matrix. Over the last decade, autolysis of LAB has been the subject of intensive investigation. Numerous studies aimed at measuring its impact on cheese ripening and unravelling the molecular events leading to cellular lysis in order to control it during cheese ripening. Previous reviews dedicated to this topic are those of Crow et al. (1995a), Chapot-Chartier (1996), Lortal, Lemée and Valence (1997a) and Pillidge et al. (2002).

In this review, we present the current knowledge regarding the impact of starter LAB lysis on cheese ripening, the most recent results on the molecular characterization of PGHs in several LAB as well as the various attempts to control *Lactococcus lactis* lysis in cheese.

2. Role of starter LAB lysis in cheese ripening

2.1. Measuring starter lysis in cheese

Although lysis of LAB in cheeses has been suspected for a very long time (Sandberg, Haglund, & Barthel, 1930), it has been unambiguously demonstrated in situ for lactococci only in 1994: in Saint-Paulin by Chapot-Chartier, Deniel, Rousseau, Vassal, and Gripon (1994) and in Cheddar by Wilkinson, Guinee, O'Callaghan and Fox (1994b). Many other examples have been shown, involving varied approaches that are presented below. Due to the inclusion of bacterial cells in the cheese matrix, the demonstration of lysis in situ has to overcome some methodological problems. For this reason, several techniques are generally used in parallel to assess lysis.

Electron microscopy (EM) observations (either transmission EM or scanning EM) can reveal the presence of damaged or lysed cells at different times of ripening. This approach can be used directly on cheese sections. However, the drastic sample preparation can generate artefacts or can damage some fragile cells. Also, it can be regarded as rather subjective, as no quantification can be made. Some new approaches have been proposed more recently by using fluorescence and confocal microscopy avoiding any sample preparation (Bunthof, van Schalkwijk, Meijer, Abee, & Hugenholtz, 2001). However, a quantitative approach in cheese is still not possible by this way.

Classical enumeration of colony forming units measures the decrease of bacterial populations based on the growth of cells on solid media, but does not adequately measure lysis. Some cells may be non-culturable yet still metabolically active, or may be dead (i.e permeabilized) but still not completely lysed as was clearly observed by confocal microscopy (Bunthof et al., 2001).

The release of an intracellular component in cheese remains the principal method of lysis demonstration. DNA and RNA would be excellent lysis markers since they are intracellular and specific to bacteria. However, DNA and RNA are apparently rapidly degraded in cheese (Bie & Sjöström, 1975) making any reliable quantification difficult. By contrast, several cytoplasmic enzymes, including lactate dehydrogenase (LDH), glucose phosphate dehydrogenase, fructose-1,6-biphosphate aldolase (FBP), X-prolyl-dipeptidyl-aminopeptidase (PepX), lysyl-aminopeptidase and dipeptidase, have been used successfully as lysis markers (Wilkinson, Guinee, & Fox, 1994a; Crow et al., 1995a; Pillidge, Govindasamy-Lucey, Gopal, & Crow, 1998; Valence, Richoux, Thierry, Palva, & Lortal, 1998). These free enzymes in cheese are revealed by assaying for enzyme activity, by immunochemical and recently even by proteomic analysis of cheese aqueous extracts (Gagnaire et al., 2004). The immunological detection method has two advantages: (i) it is not influenced by enzyme activity variations due to enzyme instability in cheese and (ii) using specific antibodies, the lysis of one species in a complex ecosystem can be carried out. However, it must be kept in mind that the preparation of the cheese extracts to quantify the lysis markers has to be made in as mild a way as possible to avoid cell damage and/or lysis marker damage.

2.2. Lysis of lactococci in cheese

Law, Sharpe, and Reiter (1974) observed a decrease of starter (*L. lactis* subsp. *cremoris* NCDO924) viability in Cheddar during the ripening and a concomitant release of an intracellular dipeptidase. Since this first observation, lysis of *L. lactis* has been extensively demonstrated in Cheddar and in Saint-Paulin, where it was shown to be highly strain dependant (Chapot-Chartier et al., 1994; Wilkinson et al., 1994b; O'Donovan, Wilkinson, Guinee, & Fox, 1996). By contrast, in cheese made from a retentate of ultrafiltrated milk, the lysis of *L. lactis* species was inhibited, regardless of the strain used (Saboya, Goudedranche, Maubois, Lerayer, & Lortal, 2001; Hannon et al., 2004), indicating that the cheese matrix composition and the technology used can also influence the induction of lysis.

2.3. Lysis of other dairy starter bacteria

Lactobacillus helveticus lysis was demonstrated in experimental and commercial Swiss type cheeses, Emmental, Beaufort and Comté (Valence et al., 1998). In Emmental, lysis occurs early towards the end of the pressing step. It was demonstrated by immunological detection of free peptidase (PepX) as well as D-lactate dehydrogenase (both specific for *Lb. helveticus*). As for lactococci, lysis was shown to have a positive impact on the proteolysis, which is in agreement with the high level of residual activity of free proteolytic enzymes in cheese juice (Gagnaire, Lortal, & Leonil, 1998; Valence, Deutsch, Richoux, Gagnaire, & Lortal, 2000). *Lb. delbrueckii* subsp. *lactis* was shown to lyse in swiss Gruyère (release of free PepX in cheese extracts) (Meyer & Spahni, 1998). Regarding *Streptococcus thermophilus*, lysis was demonstrated in Emmental (Deutsch, Ferain, Delcour, & Lortal, 2002); however, no data exist for yoghurts or for soft cheeses (Camembert, Reblochon), where it is frequently added with the starter to increase the initial acidification rate (Lortal, 2004).

2.4. Impact of starter bacteria lysis on cheese ripening

Proteolysis of caseins, conversion of free amino acids into aroma compounds and lipolysis of milk fat are all known as essential in the cheese ripening process (Fox et al., 1996). When starter lysis occurs readily, proteolysis is increased, in particular the level of free amino acids (2- to 3-fold increase by the end of ripening), and bitterness is reduced, leading to a better flavour (Crow, Martley, Coolbear, & Roundhill, 1995b; Lepeuple, Vassal, Delacroix-Buchet, Gripon, & Chapot-Chartier, 1998b; Meijer, Dobbebbar, & Hugenholtz, 1998). High level of starter autolysis also reduces the propensity of the PI-type cell envelope proteinase in *L. lactis* starter strains to produce bitterness in some cheese types (Pillidge, Crow, Coolbear, & Reid, 2003). Because of this positive impact, several authors have tried to predict the lytic behaviour of starters. Boutrou et al. (1998) used pseudo-curds to test lytic ability and proteolytic activities of various industrial strains of *L. lactis*. However, the correlation between lysis and proteolysis is not easy to establish for a large number of strains.

Recent studies have addressed the question of the impact of LAB lysis on amino acid conversion to aroma compounds in cheese. In the first study, Bourdat-Deschamps, Le Bars, Yvon and Chapot-Chartier (2004) reported that when α-ketoglutarate was not rate limiting, lysis of *L. lactis* AM2 stimulated the catabolism of aromatic amino acids and methionine, and enhanced the formation of benzaldehyde and sulphfur aroma compounds which are considered desirable aroma compounds in cheese. In a second study, Fernandez de Palenzia et al. (2004) induced starter lysis by addition of a bacteriocin-producing strain. This resulted in an increase of isoleucine (Ile) transamination and subsequent decarboxylation of the Ile-derived α-ketoacid, leading to an increase of 2-methyl-butanal formation. Concomitantly, an enhancement of cheese aroma was detected by sensory analysis.

The relationship between the extent of lysis and the level of cheese lipolysis has been even less studied. Collins, McSweeney and Wilkinson (2003) compared two strains exhibiting similar esterasic and lipasic

activity in vitro (as estimated on triolein and *p*-nitrophenylbutyrate substrates), and differing by their extent of lysis in cheese (estimated by LDH release in cheese juice). Levels of individual free fatty acids increased for both strains during the ripening; however, caprylic, myristic, palmitic and stearic acids were significantly higher in cheeses manufactured with the strain highly prone to lysis (*L. lactis* subsp. *cremoris* AM2), suggesting a positive link between lysis and lipolysis.

In an attempt to shorten the ripening period of Cheddar cheese, Hannon et al. (2003) added a highly autolytic strain of *Lb. helveticus* to the main lactococci starter. Lysis was followed by LDH release. Proteolysis was increased and a unique balanced strong flavour was obtained in the presence of this autolytic strain. This is consistent with data from various studies (reviewed by Klein & Lortal, 1999) in which attenuated *Lb. helveticus* were added to increase the amount of bacterial intracellular enzymes in cheese without interfering with the acidification process.

The non-starter lactic acid bacteria (NSLAB) can reach a high biomass at the end of the ripening contributing positively, when selected and inoculated as adjunct, to the final flavour of Cheddar cheese (Crow, Curry, & Hayes, 2001). However, even if a succession of strains is observed, the question of their in situ lysis and its impact has not been addressed until yet.

In conclusion, the rapid release of intracellular enzymes due to autolysis of LAB in the cheese matrix accelerates the ripening process, and in some cases improves the flavour. However, the impact of lysis on amino acid catabolism as well as on lipolysis has to be further explored.

3. Mechanisms of LAB lysis

Bacteria produce PGHs that are capable of hydrolysing bonds in their own protective cell wall peptidoglycan. In Gram-positive bacteria, peptidoglycan constitutes a strong three-dimensional network surrounding the cell. It is a polymer comprised of glycan chains (alternate of *N*-acetyl muramic acid (Mur-NAc) and of *N*-acetyl-glucosamine (Glc-NAc) residues) linked together by short peptide chains (Fig. 1). Depending on the bond they are able to cleave, PGHs have different specificities as indicated in Fig. 1. Several PGHs of identical and/or of different specificities may be present at the same time in the cell (Shockman & Höltje, 1994; Chapot-Chartier, 1996), building a complex enzymatic system of PGHs. Since autolysins are dangerous for cell integrity, their expression and/or activity must be tightly regulated. However, under conditions where peptidoglycan synthesis is arrested, they are thought to cause cellular lysis as a result of uncontrolled activity.

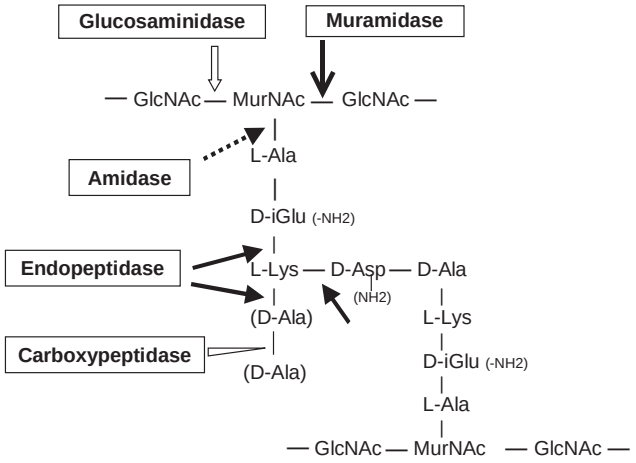

Fig. 1. Peptidoglycan structure of *L. lactis* and hydrolytic bond specificities of peptidoglycan hydrolases. Peptidoglycan is a heteropolymer comprised of linear chains of polysaccharides containing *N*-acetyl-muramic acid (MurNAc) and *N*-acetyl-glucosamine (GlcNAc), cross-linked by short peptidic chains. The cleavage sites of the different peptidoglycan hydrolase classes are indicated.

It is worth noting that numerous strains of *L. lactis* and other LAB species are lysogenic and carry in their genome one or several prophages encoding an endolysin with PGH activity. Prophage induction may result from a mutagenic agent (like mitomycin C) or from an environmental stress (like heat shock). At the end of the phage cycle, lytic proteins are produced, the endolysin and most often a small protein, the holin, allowing the endolysin to access its substate, peptidoglycan, and causing cell lysis.

In this section, we describe the data available on LAB autolysins and prophage-encoded endolysins. Most studies concern *L. lactis*. However, where available, data regarding other dairy LAB species will also be presented.

3.1. Molecular characterisation of autolysins and phage endolysins in LAB

The hydrolytic specificities of PGHs present in a bacterial species can be determined by incubating a bacterial cell wall preparation under conditions allowing self-hydrolysis, and by analysing the bonds that are cleaved. For example, the increase of free reducing groups indicates the activity of a muramidase or a glucosaminidase, and the increase of free amino groups either an amidase or a peptidase (Shockman & Höltje, 1994). Results obtained in this way for several LAB species are summarized in Table 1.

Renaturing SDS–PAGE (zymogram) is an efficient technique to detect peptidoglycan hydrolase activity. Various substrates can be included in the polyacrylamide gel: whole cells, cell walls or purified peptidoglycan of the species under study or of

Table 1
Enzymatic activities detected in vitro following cell wall self-hydrolysis and main peptidoglycan hydrolases detected by renaturing SDS-PAGE in different lactic acid bacteria species

Bacterial species	Enzymatic activities detected in vitro	Renaturing electrophoresis			References
		Substrate	Number of major bands	Apparent mass M_r (kDa)	
Lactococcus lactis	*N*-acetyl-muramidase Glycosidase and [amidase or endopeptidase]	*M. lysodeikticus* *L. lactis*	2	18/45	Buist et al. (1995a)
			1 or 2	45 or 45/46[a]	Østlie et al. (1995) Riepe et al. (1997) Lepeuple et al. (1998a) Mou, Sullivant, & Jago (1976)
Streptococcus thermophilus	ND[b]	*M. lysodeikticus*	0		
		S. thermophilus	1 or 2	51 or 31[a]/51	Husson-Kao et al. (2000a, b)
Lactobacillus helveticus	*N*-acetyl-muramidase	*M. lysodeikticus* *Lb. helveticus*	4	30/42	Lortal et al. (1997a) Valence & Lortal (1995)
		Cells	2	30/42	
		SDS cell walls	3	30/37/42	
Lactobacillus delbrueckii subsp. *lactis*	ND	*M. lysodeikticus*	2	31/44	Lortal et al. (1997b)
Lactobacillus acidophilus	*N*-acetyl-muramidase	*M. lysodeikticus*	4	27/28/30/41	Lortal et al. (1997b)
Leuconostoc citreum	Glycosidase and [amidase or endopeptidase]	*M. lysodeikticus*	2	41/52	Cibik & Chapot-Chartier (2000)
Lactobacillus casei subsp. *casei*	Glycosidase	*Lb. casei*	2	49/55	Cappa & Bottazzi (1996)

[a]Endolysin encoded by a prophage, detected only in some strains.
[b]ND: not determined.

Micrococcus lysodeikticus, a classical PGH substrate. PGH activity is revealed by the presence upon incubation of a translucent band in an opaque background, and the apparent molecular mass of the PGH can be estimated (Fig. 2). This method was used successfully in several LAB species as summarized in Table 1. In general, several lytic bands are detected and the profile is rather stable within a species with characteristic major bands conserved between strains (Østlie, Vegarud, & Langsrud, 1995; Lortal, Valence, Bizet, & Maubois, 1997b; Cibik & Chapot-Chartier, 2004). It is worth noting that this technique allows detection only of enzymes able to renature after SDS–PAGE and that heteromultimeric enzymes cannot be detected.

Several genes encoding PGHs have been identified in LAB (Table 2). The gene encoding the major autolysin AcmA of *L. lactis* MG1363 was the first to be cloned and sequenced (Buist et al., 1995a). It encodes a protein of 46.5 kDa, endowed with an N-terminal putative signal peptide, for exportation across the cytoplasmic membrane. AcmA has a modular structure with two domains: a catalytic domain (identified from sequence homology to the *N*-acetyl-muramidase Mur2 of

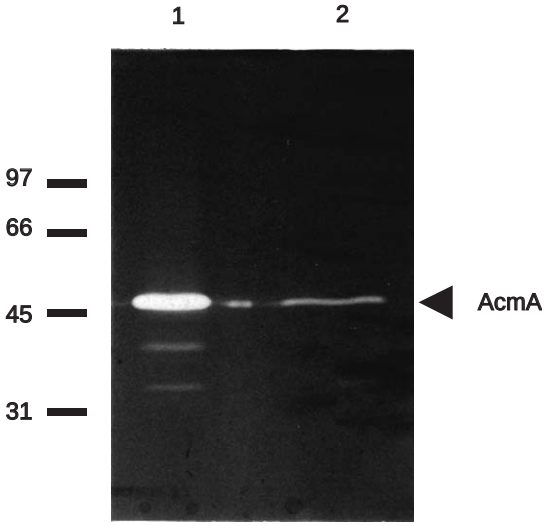

Fig. 2. Detection of peptidoglycan hydrolases in *L. lactis* NCDO763 by renaturing SDS–PAGE with a gel containing 0.2% autoclaved *M. lysodeikticus* cells. Lane 1: cell extract; lane 2: culture supernatant. AcmA is the major autolysin; the weaker bands detected in cell extract could correspond to proteolytic degradation products of AcmA as in *L. lactis* MG1363 (Buist et al., 1995a), or to minor autolysin or endolysin activities.

Table 2
Peptidoglycan hydrolases from lactic acid bacteria characterized at the molecular DNA level

Bacterial species Enzyme name	MM (kDa)	PI calculated	Hydrolytic specificity[a]	Structure	References
Lactococcus lactis (strain MG1363 and/or IL1403)					
AcmA	46.5	10.8	*N*-acetyl-glucosaminidase	Putative signal sequence, two domains N-terminal: catalytic domain[b] C-terminal:peptidoglycan binding domain Three sequence repeats LysM (44 AA)	Buist et al. (1995a) Steen et al. (2001) Steen et al. (2003)
AcmB	52.2	5.0	*N*-acetyl-glucosaminidase	Putative signal sequence, three domains N-terminal: domain rich in S/T/G/P Central region: catalytic domain[b] C-terminal: domain with putative Zn binding site	Huard et al. (2003)
AcmC	23.7	10.2	*N*-acetyl-glucosaminidase	Putative signal sequence, one domain Catalytic domain.	Huard et al. (2004)
AcmD	37.5	4.3	(ND) *N*-acetyl-muramidase or *N*-acetyl-glucosaminidase	Putative signal sequence, two domains N-terminal: catalytic domain[b] C-terminal: three sequence repeats LysM (44 AA)	Huard et al. (2004)
YjgB	20.7	5.8	(ND) Endopeptidase	Putative signal sequence, one domain Catalytic domain[c]	Huard et al. (2004)
Streptococcus thermophilus (strain CNRZ302)					
Mur1	24.7	9.7	(ND) *N*-acetyl-muramidase or *N*-acetyl-glucosaminidase	Putative signal sequence, one domain Catalytic domain[b]	Husson-Kao et al. (2000b)
Leuconostoc citreum (strain 22R)					
Mur	23.8	9.6	(ND) *N*-acetyl-muramidase or *N*-acetyl-glucosaminidase	Putative signal sequence, one domain Catalytic domain[b]	Cibik et al. (2001)

[a]Determined experimentally, or according to sequence similarity (ND).
[b]Sequence similarity with the catalytic domain of *Enterococcus hirae N*-acetyl-muramidase Mur2.
[c]Sequence similarity with the catalytic domain of *Bacillus sphaericus* DL-endopeptidase II.

Enterococcus hirae) and a cell wall binding domain composed of three amino acid sequence repeats (named LysM domains) (Steen et al., 2003). AcmA was shown to correspond to the main activity band detected by zymogram in *L. lactis* MG1363, whereas the minor lower bands in zymogram were shown to be degradation products since they were absent in an *acmA* minus derivative (Buist et al., 1995a).

Four other putative PGHs have been identified by amino acid sequence similarity searching of the whole genome sequence of *L. lactis* IL1403 (Bolotin et al., 2001): AcmB, AcmC, AcmD and YjgB. They were shown to hydrolyse peptidoglycan and their genes are transcribed in *L. lactis* MG1363 during growth in M17 medium (Huard et al., 2003; Huard et al., 2004). AcmB, AcmC and AcmD possess, like AcmA, a catalytic domain homologous to the one of *E. hirae* muramidase Mur-2. The hydrolytic specificity of three of them (AcmA, B and C) was determined on purified *Bacillus subtilis* peptidoglycan substrate by analysis of the

released muropeptides by HPLC and mass spectrometry (Steen et al., 2001; Huard et al., 2003, 2004). These studies indicated that the three enzymes have *N*-acetyl-glucosaminidase activity rather than *N*-acetyl-muramidase activity predicted by sequence similarity. In conclusion, *L. lactis* contains at least three glucosaminidases with different structure, one putative endopeptidase (YjgB), one putative muramidase (AcmD), whereas no amidase is present. Regarding the number of enzymes, the *L. lactis* PGH complement of five enzymes appears of low complexity compared to those of *B. subtilis* and *Escherichia coli*, which contain 35 and 18 PGHs respectively (Smith et al., 2000; Höltje, 1995).

In other LAB species, only two PGHs have been cloned and sequenced: (i) one in *S. thermophilus* exhibiting sequence similarity with the catalytic domain of AcmA but without specific cell wall binding domain (Husson-Kao, Mengaud, Benbadis, & Chapot-Chartier, 2000a) and (ii) its homolog in *Leuconostoc citreum* (Cibik, Taillez, Langella, & Chapot-Chartier, 2001).

In certain LAB lysogenic strains, endolysin activity was detected in cellular extracts, without any prophage inducing factor, suggesting a base level of constitutive expression. This was observed in *L. lactis* subsp. *cremoris* AM2, which is highly prone to lyse in cheese and where the 46-kDa activity detected by zymogram analysis corresponds to the endolysin of the temperate bacteriophage carried by the strain (Lepeuple, van Gemert, & Chapot-Chartier, 1998a). This is also true for strains of *S. thermophilus* where the major activity at 31 kDa in zymogram of bacterial cellular extracts corresponds to the endolysin of the resident temperate phage (Husson-Kao et al., 2000b). Several endolysins have been characterized at the molecular level in temperate phages of *L. lactis* and lactobacilli (Gasson, 1996; Table 3).

3.2. Role of bacterial PGHs in autolysis

The role of AcmA during growth and lysis was studied by constructing an *acmA* deletion mutant in *L. lactis* MG1363 (Buist et al., 1995a). This mutant formed long chains during growth in semi-synthetic medium, indicating that AcmA is involved in the separation of cells after division. This physiological role has been already described for many autolysins (Shockman & Höltje, 1994; Smith et al., 2000). The *acmA* mutant exhibited reduced autolysis during a prolonged incubation after maximal growth in synthetic medium.

However, the involvement of AcmA in lysis observed in cheese has not been clearly established especially when early and extensive lysis is observed. Indeed, strain *L. lactis* subsp. *cremoris* 2250 lyses readily and early in Cheddar cheese but *acmA* knockout in that strain did not change its lysis kinetics (Pillidge et al., 1998). In addition, detection of AcmA using an antiserum to AcmA in several commercial strains indicated differences in levels of AcmA expression that did not correlate with levels of autolysis of the strains grown in milk (Pillidge et al., 2002). All these results suggest that other genes and/or other factors must therefore be implicated in the particular ability of some strains to lyse in cheese.

AcmB of *L. lactis* has been shown to contribute to autolysis in buffered solutions (Huard et al., 2003), but no cheese experiment was carried out with the corresponding mutant. The physiological role as well as the contribution in lysis of the other PGHs of *L. lactis* and other dairy LAB is still not known.

3.3. Regulation of autolysis by proteolysis

The regulation of PGH activity by proteases has been described in several bacteria (Shockman & Höltje, 1994). *L. lactis* produces a cell-wall-associated protease (PrtP also called lactocepin), hydrolysing caseins during growth in milk. PrtP was shown to hydrolyse the major autolysin AcmA. The autolysis of *L. lactis* MG1363 in synthetic broth varies in relation to the presence of this protease and its specificity (Buist, Venema, & Kok, 1998). When the strain MG1363 expressed PI-type protease, its autolysis was strongly reduced compared to the same strain exhibiting PIII-type protease or hybrid PI–PIII protease. However, this result was not confirmed when industrial strains were grown in milk. In this case, lysis was not clearly correlated to the PrtP type and to the degradation of AcmA (Govindasamy-Lucey, Gopal, Sullivan, & Pillidge, 2000). Thus, autolysis of industrial strains after maximal growth in milk appears as a complex multifactorial event. Another extracellular protease, HtrA, was characterized in *L. lactis* IL1403 (a plasmid-free strain devoid of PrtP) that can also contribute to the degradation of the mature form of AcmA (Poquet et al., 2000). Degradation of autolysins or even phage-encoded lysins by cell proteases may contribute to some of the lower bands seen on zymograms.

3.4. Role of prophage-encoded endolysins in bacterial lysis

Early studies correlated the thermolytic response observed in several *L. lactis* subsp. *cremoris* strains to the presence of a thermoinducible prophage (Feirtag & McKay, 1987; Wiederholt & Steele, 1993). More recently, the autolytic phenotype of certain LAB strains was also associated to their lysogenic state. A prophage-cured derivative of the fast-lysing strain *L. lactis* subsp. *cremoris* AM2 was shown to lyse very slowly and to a limited extent in cheese experiments (Lepeuple et al., 1998b). Similarly, the lytic phenotype of several strains of *S. thermophilus* observed through carbon starvation at the end of growth has also been associated to the

Table 3
Main endolysins characterised in temperate bacteriophages of *L. lactis, S. thermophilus* and different lactobacilli

Bacterial species phage name	Hydrolytic specificity[a]	Structure (two domains)	References
Lactococcus lactis			
R1t	Amidase	N-terminal: homologous to the catalytic domain of amidase LytA of *Streptococcus pneumoniae* C-terminal: sequence homology with C-ter− of phages bIL67, ΦvML3, CΦ and bIL170, ΦUS3, ΦSK1	van Sinderen et al., 1996
Tuc2009		N-terminal: homologous to the catalytic domain of endolysins of phages Cp-1, Cp-7 and Cp-9 of *S. pneumoniae*	Arendt, Daly, Fitzgerald, & van de Guchte, 1994
ΦLC3	Muramidase	C-terminal: two sequence repeats LysM	Birkeland (1994)
ΦAM2			Lepeuple et al. (1998a)
BK5-T	Amidase	N-terminal: homologous to the catalytic domain of endolysin of phage Dp-1 of *S. pneumoniae*	Boyce, Davidson, & Hillier (1995)
Φ31			Madsen, Mills, Djordjevic, Israelsen, & Klaenhammer (2001)
bIL285, bIl286, bIL309			Chopin, Bolotin, Sorokin, Ehrlich, & Chopin (2001)
		C-terminal: no sequence homology	
Streptococcus thermophilus			
ΦO1205			
Sfi11, Sfi21, Sfi18, Sfi19	Amidase	N-terminal: homologous to catalytic domain of endolysins of phage DP-1 and BK5-T of *L. lactis*	Sheehan, Stanley, Fitzgerald, & van Sinderen (1999) Lucchini, Desiere, & Brüssow (1999)
		C-terminal: homologous to zoocin A of *Streptococcus zooepidemicus*	
Lactobacillus delbruekii subsp. *bulgaricus*			
mv1	Muramidase	N-terminal: homologous to catalytic domain of endolysins of phages Cp-1, Cp-7 and Cp-9 of *S. pneumoniae*	
mv4		C-terminal: no sequence homology	Boizet, Lahbib-Mansais, Dupont, Rizenthaler, & Mata (1990)
Lactobacillus helveticus			
Φ-0303	Muramidase	N-terminal: homologous to catalytic domain of endolysins of phage Cp-1, Cp-7 and Cp-9 of *S. pneumoniae* C-terminal: no sequence homology.	Deutsch, Guezenec, Piot, Foster, & Lortal (2004)
Lactobacillus gasseri			
Φadh	Muramidase	N-terminal: homologous to catalytic domain of endolysins of phage Cp-1, Cp-7 and Cp-9 of *S. pneumoniae* C-terminal:sequence homology with C-terminal domain of phage endolysins of *Lb. johnsonii* and *Lb plantarum*	Heinrich, Binisshofer, & Bläsi (1995)
Lactobacillus plantarum			
Φgle	Muramidase	N-terminal: homologous to catalytic domain of endolysins of phage Cp-1, Cp-7 and Cp-9 of *S. pneumoniae* C-terminal: no sequence homology.	Kakikawa et al. (2002)
Lactobacillus johnsonii			
Lj965			
Lj928	Muramidase	N-terminal: homologous to catalytic domain of endolysins of phage Cp-1, Cp-7 and Cp-9 of *S. pneumoniae* C-terminal: sequence homology with C-terminal domain of phage endolysins of *Lb. gasseri* and *Lb. Plantarum*	Desiere, Pridmore, & Brüssow (2000)
Lactobacillus casei			
ΦA2	Amidase	N-terminal: homologous to *S. pneumoniae* LytA and endolysin of *L. lactis* phage r1t C-terminal: sequence homology with C-ter− of enterolysin of *E. faecalis*	Moscovo & Suarez (2000)

[a]According to sequence similarity.

presence of a prophage. One hypothesis is that lysis proteins are expressed at a basal non-lethal level, their activity being enhanced by certain environmental conditions like lactose starvation or NaCl addition (Husson-Kao, Mengaud, Gripon, Benbadis, & Chapot-Chartier, 1999). The lytic behaviour of the lysogenic strains of *S. thermophilus* was not tested in cheese.

So in a limited number of LAB strains, a link can be established between the presence of a prophage and fast autolysis. However, although a study based on PCR screening of *L. lactis* strains with primers for conserved integrase gene suggested that this observation could be extended to a higher number of strains (O'Sullivan, Ross, Fitzgerald, & Coffey, 2001), it is likely that a high number of exceptions can be encountered. Indeed, numerous LAB strains contain active or remnant prophages, which does not imply automatically that all of them will be highly autolytic. For example, Pillidge et al. (2002) reported that both autolytic and non-autolytic strains such as 2250 and HP contain the same prophage. Similarly, by PCR screening with primers specific for the endolysin found in *L. lactis* AM2, no correlation could be found between the presence of the lysin gene and high ability to lyse (M.-P. Chapot-Chartier, unpublished data). Similarly, in *Lb. helveticus*, no relationship between lysis and lysogeny was observed in the case of the lysogenic strain CNRZ 303. A prophage-cured derivative of this strain lysed in cheese and also in vitro to the same extent as the mother strain (Deutsch, Neveu, Guezenec, Ritzenhaler, & Lortal, 2003).

3.5. Environmental conditions and induction of autolysis

The effect of culture medium composition and temperature on *L. lactis* autolysis was reported by Vegarud, Castberg and Langsrud (1983). Especially when lactose was replaced by glucose as the carbon source, growth and autolysis rates were increased. Many strains of LAB lyse after maximal growth. Some of them lyse readily as soon as the medium is depleted of a carbon source, as described for some *L. lactis* strains (Riepe, Pillidge, Gopal, & McKay, 1997) and *S. thermophilus* strains (Husson-Kao et al., 1999). Lysis of *S. thermophilus* lysogenic strains with autolytic phenotype could also be induced by other environmental factors like NaCl concentration, presence of low level of ethanol in the medium or heat shock. Also, as already cited above, lysis of thermolytic *L. lactis* strains can be induced by a temperature shift from 38 to 40 °C mimicking Cheddar cheese manufacturing conditions (Feirtag & McKay, 1987; Wiederholt & Steele, 1993; Wilkinson, Guinee, O' Callaghan, & Fox, 1995). These observations suggest that it is possible to induce lysis by changing environmental conditions in a way, which would be compatible with the technology applied. For example, lysis of lysogenic *L. lactis* subsp. *cremoris* SK110 was obtained by a temperature shock applied during cheese manufacturing, which resulted in increased rate of amino acid production (Meijer et al., 1998).

4. Control of LAB lysis during cheese ripening

Several methods have been proposed to control lysis of *L. lactis* starters in cheese: to select natural highly autolytic strains, to construct genetically modified (GM) strains with inducible lysis and to provoke lysis "from the outside" by adding an additional strain producing a bacteriocin in the starter.

4.1. Highly autolytic strains

Various screening assays have been proposed to detect highly autolytic strains: quantification of lysis in buffers, or media mimicking cheese conditions or in milk (Lortal et al., 1997a; Boutrou et al., 1998; Pillidge et al., 1998) or studying the link between lysogeny and lysis (O'Sullivan et al., 2001). However, so far, it is not possible from these results to unequivocally predict for a strain its extent of lysis in cheese.

4.2. Genetically modified strains

GM strains of *L. lactis* with increased or inducible lysis have been constructed. The strategy consisted of placing a gene (encoding the major AcmA autolysin or a phage endolysin) under the control of an inducible promoter. Various such promoters have been isolated, inducible by NaCl, heat or cold shock, acid pH or nisin (reviewed by Sanders, Venema, & Kok, 1999). So far, the latter was tested and shown to be efficient in cheese (de Ruyter, Kuipers, Meijer, & de Vos, 1997). However, the extent of in situ lysis was only four times higher than the mother strain (as measured by LDH release) which was not more than the natural variation between strains. Two patents exist on this approach (Gasson, 1994; Buist, Venema, Kok, & Ledeboer, 1995b). Recently, an inducible lysis system was constructed with the nisin-inducible promoter controlling the expression of enterolysin A, a metalloendopeptidase produced by *Enterococcus faecalis*. This system exhibits high efficiency to lyse *L. lactis* strains since a 27-fold increase of LDH activity release in model cheese was reported upon overexpression of enterolysin A. No biochemical analysis of cheese was reported (Hickey, Ross, & Hill, 2004). Leaky *L. lactis* strains were also constructed by Walker and Klaenhammer (2001) based on the controlled expression of lysin–holin cassettes contained in prophage that were naturally present in the strains. These strains can release β-galactosidase used as a reporter enzyme, during growth without lysis; however, the five

L. lactis peptidases and the two *Lb. helveticus* peptidases tested were not externalized (Tuler, Callanan, & Klaenhammer, 2002). The strains were not tested in a cheese assay.

As the addition of GM starters is not allowed in all countries, this approach is limited by legal barriers. There is also a lack of widespread consumer acceptance. Moreover, in our opinion, because construction of GM starters requires complex molecular techniques, only a limited number of strains can be manipulated, restricting the pool of strains able to be used in cheese making whereas in nature many strains with a wide range of autolytic properties are available.

4.3. Complementation of the starter with bacteriocin-producing strains

The bacteriolytic effect of some bacteriocins has been well documented. Another way to increase in situ lactococcal lysis has been proposed by adding a strain producing a bacteriocin to the main starter (Morgan, Ross, & Hill, 1997; Martinez-Cuesta, Pelaez, Juraez, & Requena, 1997). A strain of *L. lactis* producing the bacteriocins lactococcins A, B and M used together with the non-lytic strain *L. lactis* HP sensitive to these bacteriocins allowed a significant increase of its lysis. The control of this new lytic system was improved by using a three-strain starter: one acidifying strain insensitive to the bacteriocins, one sensitive "sacrificial" strain and one producing the bacteriocin (Morgan, O'Sullivan, Ross, & Hill, 2002). Sensory analysis of the experimental cheeses revealed a significant decrease of bitterness and improvement of cheese flavour. Recently, it was shown that in a mixed starter, a lacticin 3147-producing strain enhanced lysis of two other strains chosen for their sensitivity to the bacteriocin and for their complementary action on Ile catabolism. As a result, an increase of Ile transamination was observed as well as an increase of the volatile derivative 2-methylbutanal. In addition, cheese flavour was enhanced (Martinez-Cuesta, Pelaez, Juraez, & Requena, 2002; Fernandez de Palenzia et al., 2004). A strain producing another bacteriocin, lacticin 481, was used to lyse a non-autolytic starter. This latter bacteriocin presented the advantage to induce the release of intracellular enzymes without complete inhibition of growth thus preserving the acid-producing capability of the starter (O'Sullivan, Morgan, Ross, & Hill, 2002). Also, the use of a nisin-producing *L. lactis* starter to improve autolysis of an adjunct *Lb. delbruekii* subsp. *bulgaricus* strain, with concomitant enhanced proteolysis to improve Cheddar cheese ripening was recently described (Sallami, Kheadr, Fliss, & Vuillemard, 2004).

However, in a complex ecosystem, the presence of a bacteriocin-producing strain can have an effect on the growth of secondary flora depending on its activity spectrum (O'Sullivan, Ross, & Hill, 2003). Moreover, this approach leads to an increase in costs (additional starter). In terms of the mechanism of lysis, endogenous autolysin activity in the target strain is still necessary for bacteriocin-induced lysis to occur (Martinez-Cuesta et al., 2000).

5. Unaddressed questions

Lysis is a complex enzymatic process, resulting from the activity of a mixture of lytic enzymes (autolysins, phage endolysins) on a complex dynamic substrate, the peptidoglycan in the cell wall. In our opinion, several fundamental questions have still to be answered to have a complete view of all the mechanisms involved and to control the lysis in cheese, and of course to manage its impact on cheese ripening.

5.1. What are the physiological and environmental parameters inducing starter lysis in cheese?

In other words, which environmental conditions or which stress linked to the manufacturing process (carbon starvation, bacteriocin production, cooking, salting, etc.) is really inducing lysis in cheese? To answer this question, the molecular as well as biochemical regulations of autolysins and endolysins expression have to be studied. The increasing number of available whole-genome sequences and dedicated transcriptome and proteome analyses will help to address this point.

5.2. Why does the ability to lyse vary so greatly from strain to strain, regardless of the species considered?

In vitro as well as in many cheeses, the ability to lyse was shown to be highly strain dependent. Understanding this would allow to build highly reliable tests to predict efficiently the lysis of a starter. Several hypotheses exist: (i) a more efficient lytic system (autolysins less tightly regulated, basal and sublethal levels of endolysin and holin expression, etc.); (ii) differences in cell wall composition [indeed, the variations of the amount of teichoic acids, the extent of peptidoglycan reticulation at the interpeptidic bridge or its acetylation are well known to modulate lysis in model species. The cell wall composition of LAB was recently reviewed (Delcour, Ferain, Deghorain, Palumbo, & Hols, 1999) but the strain to strain variation was rarely investigated (Gopal & Crow, 1993)] and (iii) the presence of one or several prophages, possibly defective, in the genome, their expression inducing a more or less extended lysis.

These hypotheses do not exclude each other, which is opening a large field of investigations.

5.3. Is starter lysis a general and systematic event in all dairy products?

We do not have an exhaustive view of lysis in the different types of cheese or dairy products. Most studies have been done in experimental hard cheeses, and mainly with *L. lactis* and *Lb. helveticus*. Concerning secondary flora, some data concerning *Propionibacterium freudenreichii* are available (Lemee, Lortal, Cesselin, & van Heijenoort, 1994). Their lysis was shown to be tardy and limited in Emmental (Valence et al., 1998); however, only one strain was tested. As far as we know, no data exist in the literature regarding lysis of surface flora such as corynebacteria, yeast or moulds used to manufacture soft-type cheeses. Taking into consideration the extreme population density in the surface of those types of cheeses, knowledge regarding even partial lysis depending on the ripening conditions would help to control the final cheese quality. Lastly, it is not known whether lysis of one bacterial species can induce, or delay, lysis of another one in the complex cheese ecosystems.

5.4. Apart from an increase of free amino acids and the reduction of bitterness, what is the impact of lysis in the ripening?

Increasing the amount of free amino acids is not sufficient to accelerate ripening, especially in terms of flavour development. Indeed the subsequent step, amino acid catabolism, as well as free fatty acid metabolism probably become the rate-limiting steps of aroma compound formation. In the case of *L. lactis* starter in semi-hard model cheese, transamination, the first step of amino acid conversion to aroma compounds, is the limiting step (Yvon & Rijnen, 2001). This limitation can be removed by addition of α-ketoglutarate (Yvon, Berthelot, & Gripon, 1998). Then, as reported above, recent studies indicate that, when α-ketoglutarate is not in limiting concentration, starter *L. lactis* lysis stimulates amino acid transamination (Bourdat-Deschamps et al., 2004; Fernandez de Palenzia et al., 2004). α-Ketoacids are not aromatic compounds and they have to be degraded further to produce volatile aroma compounds. Their chemical oxidation leading to aldehydes (with two carbon atoms less than amino acid) as well as their enzymatic decarboxylation leading to aldehydes (with one atom less than amino acid) appears also to be favoured by cell lysis. However, the impact of starter lysis on the activity and stability of the other enzymes involved in aroma formation such as α-ketoacid, alcohol and aldehyde dehydrogenases, especially those dependent on cofactors, requires further evaluation.

Moreover, lysis does not release only enzymes but also other intracellular components such as nucleic acids, vitamins, minerals, etc. All these molecules could play a role, either directly or indirectly by stimulating the growth of other flora. Lastly, the role of lysis in texture development has not been studied. One of the practical limitations to fully elucidate the impact of lysis is the difficulty to generate easily various extents of lysis of the same strain in the same cheese matrix.

5.5. What is more suitable for ripening: permeabilized or lysed cells? In other words, is lysis really rate limiting in cheese ripening?

Since certain enzymes are likely to be more active inside the cells (because they require cofactors such as NADH), it seems likely that an extensive early lysis may not be the absolute goal and that a balance of lysed and intact cells is necessary to obtain good quality cheese (Crow et al., 1995a). From several studies, it appears that before lysis (resulting from cell envelope disruption), cells are first permeabilized (Niven & Mulholland, 1998; Bunthof et al., 2001) and stabilized to some extent in the cheese environment (Chapot-Chartier et al., 1994). Cell membrane permeabilization could facilitate the access of the substrates to intracellular enzymes. For instance, a previous study has shown that bacteriocin-induced cell permeabilization increased branched-chain amino acid catabolism (Martinez-Cuesta et al., 2002). It would be of interest to investigate the respective contribution of cell lysis and permeabilization to the acceleration of amino acid catabolism, as well as the products formed since enzyme stability and cofactor availability are likely to be different. We can hypothesize that a well-balanced repartition between permeabilized and lysed cells, or a succession in time of permeabilized state followed by lysis will be more efficient for optimized contribution of bacterial enzymes during ripening.

6. Conclusions

During the last decade, studies of bacterial lysis in internally bacteria-ripened cheese have shown that the major positive effect is a decrease of bitterness resulting from the hydrolysis of casein-derived hydrophobic peptides by released intracellular bacterial peptidases. Also, these studies pointed out that the ability to lyse is highly strain dependant. A great level of knowledge was gained on peptidoglycan hydrolases present in *L. lactis*. As far as mechanisms are concerned, and from the results gained in *L. lactis*, the lysogenic state seems to generate a better ability to lyse. This is apparently not true in other LAB, such as thermophilic lactobacilli. Based on the gained knowledge, several approaches have been proposed to control and induce bacterial lysis during ripening. GM strains with efficient inducible lysis systems have been constructed but their use is still

limited by legal barriers. Strain diversity among a species can be exploited to select highly autolytic strains. Another interesting approach is the use of bacteriocin-producing strains to lyse starter strains.

The main future challenges are (i) to understand the molecular basis of the strain dependency of the ability to lyse and (ii) to explore the link between the physiological state of the cells (viable, non-viable), the cell envelope integrity (permeabilized, lysed cells) and the in situ enzymatic reactions in cheese especially those involved in aroma compounds formation.

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