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Genetics of proteinases of lactic acid bacteria

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Summary — Because it is essential for good growth with concomitant rapid acid production, and for the production of flavorful peptides and amino acids, the proteolytic ability of lactic acid bacteria is of crucial importance for reliable dairy product quality. In view of this importance, considerable research has been carried out to characterize the enzymes involved. The intensified genetic research in the lactic acid streptococci and the development of gene cloning systems for these organisms have resulted in a rapid increase of genetic data on lactic streptococcal proteinases. By now, an evaluation of the biochemical, immunological and genetic data seems to be feasible. These data are discussed and integrated into a working model tentatively explaining some of the characteristics of the proteolytic systems of lactic acid streptococci.

lactic acid bacteria / proteinases / gene cloning / nucleotide sequence

Introduction

The genetics of lactic acid bacteria is a rapidly developing field of research. It was only some 15 years ago that the first evidence was obtained for the existence of plasmids in these organisms. By now, it is well established that a large variety of plasmids is commonly found in strains of lactic streptococci and in lactobacilli, while *Streptococcus thermophilus* and the pediococci in general seem to contain smaller numbers of plasmids [1–7]. With the concomitant discovery that lactose metabolism was linked to plasmid DNA in at least some strains of lactic streptococci [2], the importance of plasmids for dairy practice became evident. This, in turn, initiated an intensive study of plasmids of lactic streptococci. Subsequently, when it was demonstrated that natural gene transfer systems, such as transduction and conjugation, operate in these organisms [8–10], evidence was provided for the plasmid-linkage of a number of additional traits important for dairying, such as proteinase production, resistance to nisin and bacteriophage, production of bacteriocin and diplococcin, and restriction/modification systems [1, 2, 11, 12].

During the 70's, recombinant DNA technology, the methodology to manipulate DNA *in vitro* and to reintroduce it into living organisms in a functional way, was rapidly developing. With a growing realization that this technology of genetic engineering could be of benefit for fundamental research in lactic acid bacteria and, ultimately, also for dairy practice, an urgent need was felt to apply these techniques to these organisms. The ability to use genetic engineering techniques in lactic acid bacteria depended upon the availability of a method to introduce and express free (recombinant) DNA in the cells of these organisms (transformation). The lack of a natural transformation system led a number of laboratories to investigate the possibility of introducing DNA into protoplasts of lactic acid bacteria. Although at first reported as an extremely low-efficiency process [13], in later publications evaluating the system parameters, the efficiency of protoplast transformation systems for lactic acid streptococci was increased considerably [14–16]. The development of plasmid vectors for these organisms which are also replicated and expressed in *Bacillus subtilis* and *Escherichia coli* was of major importance because it directly linked the advanced genetic

engineering protocols available in these organisms to the genetic research of lactic streptococci [17–19]. The first successful gene cloning experiments in the lactic streptococci have been reported (for reviews see [12, 19, 20]) and these are expected to be followed soon by other examples.

In this review, emphasis will be on the recent genetic data concerning lactic streptococcal proteinases and their genes. As reviews on the biochemistry and function of these enzymes have been published regularly ([21, 22] and for a recent review see [23]), we will limit ourselves to a brief summary of these data.

Proteinase plasmids

It has long been known that the production of proteinases by lactic acid streptococci is an unstable trait [24–27]. Proteinase-positive ('fast') strains spontaneously segregate *Prt*[−] variants which show a reduced acid production. Irreversibility of this loss of proteinase activity and the fact that the mutation frequency could be enhanced by treatment of cells with acridine dyes, ethidium bromide or by growth at elevated temperature suggested the involvement of plasmid DNA. The development of improved lysis protocols [28–30], the isolation of plasmid-free strains and the use of agarose gel electrophoresis to examine the plasmid complement have resulted in a rapid increase in the knowledge of plasmids and their functions in the lactic streptococci [1–3, 12].

By now, curing studies have provided evidence for plasmid linkage of proteolytic activity in a number of lactic streptococcal strains; these are summarized in Table I. In *S. lactis* C2, 712, ML3, C10 and M18 the proteinase- and lactose-determinants seem to reside on the same plasmid, ranging from 45 to 67.5 kb in the various strains. The status of the lactose/proteinase plasmid in *S. lactis* C2 is rather confusing and exemplifies the difficulties one can encounter when interpreting plasmid curing data. In a report from McKay *et al.* [31], a 45 kb plasmid was inferred in lactose- and protein-utilization in this strain. Klaenhammer *et al.* [29] obtained contradictory results, however, as the 45 kb plasmid was missing in a *Lac*[−] *Prt*[−] derivative but present in a *Lac*⁺ *Prt*[−] variant. The absence of a 19 and a 27 kb plasmid from the latter strain suggested their involvement in proteinase production, although the *Lac*[−] *Prt*[−] variant lacking the

45 kb plasmid still contained those two plasmids. As discussed by Gasson and Davies [3], this confusion may be explained in terms of structural instability of the lactose/proteinase plasmid, as was observed for the *Lac*⁺/*Prt*⁺ plasmid of *S. lactis* 712 [32]. Frequent deletion formation may cause the appearance of smaller plasmids with a changed genotype or the removal of a determinant from the plasmid through a deletion too small to be detected by agarose gel electrophoresis [32, 33].

A serious fundamental problem further obscuring data of plasmid curing experiments is the loss at high frequency of cryptic plasmids with no relevance to the character sought after. Therefore, the irreversible loss of a trait as a result of plasmid curing may point to plasmid location, but proof of plasmid involvement requires the physical demonstration of the simultaneous transfer of plasmid and trait (see below).

In addition to the data for *S. lactis* and *S. cremoris*, there is one report of a plasmid involved in protein breakdown in *S. lactis* ssp. *diacetylactis* [34]. This concerns the 46.5 kb lactose/proteinase plasmid of strain DRC1. No curing data are available on proteinase plasmids in the other species of lactic acid bacteria.

Proteinase gene transfer

Five gene transfer systems are operative in lactic acid bacteria: transduction, conjugation, protoplast transformation/transfection, protoplast fusion and electroporation (for reviews see [3, 11, 12, 35]). Except for the latter two, these have all been used to provide evidence for plasmid-linkage of proteinase determinants in strains of lactic streptococci (Table I).

In *S. lactis* C2, transduction of plasmid-located lactose genes was observed, together with non-selected cotransfer of proteinase genes in approximately 50% of the cases [36]. In *S. lactis* 712, essentially the same results were obtained and these located the proteinase gene(s) on the 56 kb plasmid pLP712 [1, 11, 32]. Transductional shortening of these plasmids, necessary to fit the plasmids into a phage head, explained the observed incomplete genetic linkage of the lactose- and proteinase-determinants in both strains [31, 32]. In some cases, lactose metabolism was stabilized in the transductants by insertion of the genes into the chromosome [1, 37, 38]. One of the four stabilized lactose trans-

Table I. Localization of proteinase genes in lactic streptococci.

Species	Strain	Size of (suspected) Prt ⁺ -plasmid in kb	Evidence apart from curing	Hybridization with Wg2 probe	Reference
<i>S. lactis</i>	C2	45 ^{1P} , 27, 19	transduction	nd	[29, 31, 36]
	712	56 ^{1P}	transduction conjugation cloning	+	[11, 32, 45]
	ML3	49.5 ^{1P}	conjugation	nd	[3, 80]
	C10	60 ^{1P}		nd	[80]
	M18	67.5 ^{1P}		nd	[80]
	SSL135	— ^a	cloning ^b	— ^c	[46]
<i>S. cremoris</i>	HP	13.5		+	[81, 43]
	ML1	3.3		— ^d	[81]
	Wg2	26	cloning	+	[42, 43]
	SK11	78	conjugation cloning	+	[39, 47]
	UC317	69 ^{1P}	conjugation	+	[38, 40]
	UC205	34.5	conjugation	+	[40]
	UC411	30	conjugation	+	[40]
	P8/2/47	14	conjugation	+	A. Geis, B. Kiefer ^f
	SD8, SD9, SD11	93		nd	H. Neve, A. Geis ^f
	V16	(93, 100) ^{1P}		+(93, 100) ^e	H. Neve, A. Geis ^f
	V18	93 ^{1P}		nd	H. Neve, A. Geis ^f
	1200	21		nd	[3]
	M12R	19.5		nd	[82]
<i>S. lactis diacetyl.</i>	DRC1	46.5 ^{1P}		nd	[34]

^aA plasmid-free derivative was still Prt⁺, suggesting chromosomal linkage of this trait in SSL135 [46].

^bSee text for a discussion of this result.

^cHybridization was done using the *Bgl*II D fragment of pLP712 as the probe (see text).

^dNo hybridization with the 3.3 kb plasmid was observed but, instead, with a high molecular weight plasmid (A. M. Ledeboer, personal communication).

^eHybridization was observed but no discrimination could be made as to which of the two plasmids hybridized (A. Geis and B. Kiefer, personal communication).

^fPersonal communication.

^{1P}: lactose / proteinase plasmid.

nd: not done.

ductants of *S. lactis* C2 was also stabilized for proteolytic activity [37].

Conjugation experiments proved that proteinase genes were located on plasmid DNA in *S. lactis* strains 712 and ML3 [3, 11, 32] and in the *S. cremoris* strains SK11, UC317, UC205, and UC411 [39, 40]. In *S. cremoris* P8/2/47, the proteinase plasmid pKB9 was mobilized by conjugative lactose plasmids (A. Geis and B. Kiefer, personal communication), in the other *S. cremoris* strains, the broad host range erythromycin-resistance plasmid pAM β 1 [41] was used

to first select for Em^r transconjugants which were subsequently screened for proteinase activity. In the *S. cremoris* strains UC205 and UC317, cointegrates were formed between pAM β 1 and the proteinase plasmids [38, 40].

Proteinase gene cloning

The development of a protoplast transformation system [13], its improvement [14–16] and the development of cloning vectors for lactic

streptococci [17–19] provided the possibility to use the promising new recombinant DNA technology in these organisms (for reviews see [12, 19, 20]). Using this fundamentally different way of transferring genes between strains of lactic streptococci, evidence for the plasmid location of the proteinase genes of two *S. cremoris* strains and of one *S. lactis* strain has been obtained (Table I). Localization of the proteinase gene(s) on pWV05 of *S. cremoris* Wg2, suspected by curing experiments to be linked with proteolytic activity [42], was definitely confirmed by insertion of part of the plasmid into a pWV01-based shuttle vector [18] and subsequent transfer by protoplast transformation of the recombinant plasmid into a proteinase-deficient strain of *S. lactis*. Under the growth conditions used, the strain's regained ability to grow rapidly in milk was proof that a gene(s) involved in proteolytic activity was located on the fragment [43]. Gene expression in the heterologous host *B. subtilis* proved that the structural gene(s) for a proteolytic enzyme(s) had been cloned. Crossed immunoelectrophoresis (CIE) experiments showed that in both *B. subtilis* and *S. lactis* the fragment specified the two proteins A and B identified in the proteolytic system of *S. cremoris* Wg2 ([44], see below). Subsequently, other groups have cloned genes for proteinases. De Vos *et al.* found, in a lambda-phage bank of the 78 kb proteinase plasmid pSK111 of *S. cremoris* SK11, plaques in which proteinase was detectable by immunological methods. Subcloning of the specific DNA fragment on a pSH71 derivative allowed the complementation of the proteinase defect of *S. lactis* MG1363 [19]. The lactose/proteinase plasmid pLP712 of *S. lactis* 712 has been mapped in detail, using transductionally shortened derivatives or deleted forms which arose frequently in a strain carrying pLP712 as the single plasmid [32]. In this way, the proteinase gene(s) could be localized on a 4.8 kb *Bgl*II fragment. This fragment was cloned in a pSH71 derivative and partly restored, in strain *S. lactis* MG1363, the ability to grow in milk, again providing evidence for the presence of proteinase gene(s) on the fragment [45].

An extremely important property of the cryptic plasmids used to construct the lactic streptococcal cloning vectors is that they are replicated in *E. coli* and, even more important in this respect, also in *B. subtilis* [17–19]. First, this property has accelerated the genetic research in the lactic streptococci at a time when

direct cloning in lactic streptococci was far from optimal. Second, as it now appears, lactic streptococcal proteinase genes can be expressed in *E. coli* [19]. However, the presence of the genes at a high copy number in this host is deleterious to the cells. The *S. cremoris* SK11 proteinase gene could only be cloned in *E. coli* in low copy number, using a phage lambda derivative. Cloning of the *S. cremoris* Wg2 proteinase gene, on the other hand, was possible in *B. subtilis* on a pWV01 derivative, which has a low copy number in this host [43]. Even in *B. subtilis*, serious problems of deletion formation were encountered during the cloning of the entire proteinase gene of *S. lactis* 712, and only a part of the gene could be cloned [45]. The increased efficiency of protoplast transformation of *S. lactis* enabled von Wright *et al.* [46] to directly clone in *S. lactis* a piece of chromosomal DNA coding for the ability to grow rapidly in milk. They thereby circumvented possible deleterious effects of gene expression in intermediate hosts. These authors were also unable to introduce the recombinant plasmid into *E. coli*. Moreover, subcloning in *S. lactis* of a smaller piece of the fragment on the same cloning vector was impossible, possibly because of a gene dosage effect. The same was observed by De Vos [47], who was unable to clone the proteinase gene of *S. cremoris* SK11 on a high copy number vector in *S. lactis*. Analogous to the situation for the three cloned proteinase genes mentioned above, these results may suggest the presence of a proteinase gene(s) on the chromosomal fragment, but sound proof has still to be provided.

DNA homology

A useful practical expansion of the proteinase gene cloning work for dairy culture genetics is the possibility of using the cloned genes as proteinase gene probes in Southern hybridization experiments. This strategy has been employed in a number of cases. In the first report on proteinase gene cloning, homology of a specific fragment of the *S. cremoris* Wg2 proteinase plasmid pWV05 with the proteinase plasmid of *S. cremoris* HP provided the first indication for the localization of the Wg2 proteinase gene on that fragment [43]. Subsequently, the Wg2 proteinase gene fragment was shown to hybridize to the *S. cremoris* SK11 proteinase plasmid pSK111 (W. M. De Vos, personal communication) and

to the *Bgl*II D fragment of the *S. lactis* 712 lactose/proteinase plasmid pLP712 (M. J. Gasson, personal communication).

The DNA fragment carrying the proteinase gene of *S. cremoris* Wg2 has been used to screen for proteinase plasmids in a number of strains now, and the results are summarized in Table I. Using this probe, the proteinase-specifying region of the 34 kb proteinase plasmid of *S. cremoris* UC205 could be narrowed down to two *Bgl*II fragments of 4.6 and 7.6 kb, and to an 8 kb *Xba*I fragment of the proteinase plasmid of *S. cremoris* UC317 [40]. The 14 kb proteinase plasmid pKB9 of *S. cremoris* P8/2/47 contains two *Hind*III sites. The probe gives a signal only with the smaller (6.5 kb) of the two fragments which is, therefore, likely to carry the proteinase gene of this strain (A. Geis and B. Kiefer, personal communication).

In addition to the examples mentioned in Table I, the Wg2 proteinase gene probe showed strong hybridization with plasmids of the proteolytically active strains (and only these) from two multiple strain starter cultures. Signals were observed with 14, 22.5 and 38 kb plasmids in the various strains. In the slime producer *S. cremoris* V16, isolated from Finnish Viili, loss of proteolytic activity was always observed when isolating lactose negative variants. In all cases, two plasmids of 93 and 100 kb were lost simultaneously. The *S. cremoris* Wg2 proteinase gene probe hybridized with this 93 and 100 kb plasmid complex but, because of the small difference in size between the two plasmids, it was not possible to indicate which of the two actually carried the proteinase gene(s) (A. Geis, H. Neve, and B. Kiefer, personal communication).

A comprehensive study of 24 proteolytically active *S. cremoris* strains showed that, under stringent conditions (*viz.* 42°C, 50% formamide), 21 of these specific plasmids hybridized with two subfragments of the Wg2 proteinase gene. Both probes used, one containing the N-terminal part of the proteinase gene and another one containing only C-terminal sequences, gave a strong signal with the same plasmid. Two *S. lactis* strains included in the test also showed hybridization with both probes. Interestingly, a third probe, containing sequences of an open reading frame (ORF) which is located immediately upstream from the Wg2 proteinase gene (ORF1; see below), strongly hybridized to the same plasmids (A. M. Ledebøer, personal communication).

In all the cases mentioned above, in which the proteinase gene probe has been used to indicate proteinase plasmids, plasmid curing and transfer experiments will have to reveal whether these hybridizing plasmids actually carry proteinase genes.

The homology found in Southern hybridizations between the *S. cremoris* Wg2, SK11 and *S. lactis* 712 proteinase genes is reflected in their restriction enzyme maps (Fig. 1). The maps are very similar and, on the basis of these, the three genes appear to be highly conserved with a calculated homology of 95% [20]. This conservation of restriction enzyme sites is also observed in the proteinase gene regions of the proteinase plasmids of the three *S. cremoris* UC strains (C. Daly and G. F. Fitzgerald, personal communication). Moreover, the 6.5 kb *Hind*III fragment of the proteinase plasmid pBK9 of *S. cremoris* P8/2/47 has a restriction enzyme map

Fig. 1. Physical maps of the proteinase plasmids of *S. lactis* 712 (pLP712, 56 kb), *S. cremoris* SK11 (pSK111, 78 kb) and *S. cremoris* Wg2 (pWV05, 26 kb). The regions are aligned such that maximal overlap is obtained in the region where the proteinase genes are located, as deduced from the nucleotide sequences of the *S. cremoris* Wg2 and SK11 genes [48, 52]. Thick lines indicate the restriction fragments cloned in *S. lactis* and specifying proteinase activity. The bar represents 1 kb of DNA. (For details: see text). B: *Bgl*II; BA: *Bam*HI; C: *Cla*I; E: *Eco*RI; H: *Hind*III; X: *Xho*I. Modified from De Vos [20] (W. M. De Vos and M. J. Gasson, personal communication).

very similar to that of the 6.5 kb *Hind*III fragment carrying the proteinase gene of *S. cremoris* Wg2 (A. Geis and B. Kiefer, personal communication). Most probably, all four regions indicated by Southern hybridization experiments as carrying proteinase determinants actually contain proteinase genes closely related to the Wg2/SK11/712 proteinase gene complex. As yet, there is only one report of the cloning of a piece of DNA involved in protein utilization which does not show homology with the Wg2/SK11/712 proteinase genes and which has a restriction enzyme map differing from that of the other three proteinase genes. This concerns a chromosomal DNA fragment from *S. lactis* SSL135 ([46], S. Tynkkynen and A. von Wright, this issue). If the fragment turns out to carry a proteinase gene(s) (see above), this would be the first indication of a second type of proteinase gene in lactic streptococci.

Nucleotide sequence

The nucleotide sequence of the proteinase gene of *S. cremoris* Wg2 has been determined and it has revealed a number of interesting features of the proteinase it specifies [48]. The coding region contains 1902 codons which could specify a protein with a molecular weight of 200 000. The proteinase gene is flanked by transcription- and translation-regulatory sequences which closely resemble those reported for *B. subtilis*

and *E. coli*, and are in good agreement with the regulatory sequences found in *S. lactis* and *S. cremoris* [20, 49]. On the cloned fragment, a second, incomplete, open reading frame is present immediately upstream from the proteinase gene and directed in opposite orientation (Fig. 2). Surprisingly, the DNA fragment originally cloned did not contain the entire gene but encoded a proteinase lacking the C-terminal 130 amino acids. The truncated proteinase can still complement proteinase deficiency [43]. As it now appears, the three proteinase gene-bearing DNA fragments cloned so far all specify truncated but functional proteinases. These results and those of *in vitro* deletion analysis ([50]; M. J. Gasson, personal communication) show that a large part of the C-terminus of the proteinases can be removed without abolishing enzyme activity. The two *S. cremoris* proteinases belong to different casein breakdown specificity groups ([51]; see below). Both proteinases retained their specificity in the heterologous host *S. lactis*. All three proteinases, moreover, retained their specificity despite the fact that their genes had been cloned only partially [45, 47, 50]. The *S. cremoris* Wg2 proteinase has a signal peptide-like N-terminal amino acid sequence. A protein homology comparison indicated three regions in the streptococcal proteinase which have extensive homology with serine proteinases of the subtilisin family (Fig. 3). Specifically, amino acids involved in the formation of the active center (*viz.* Asp³², His⁶⁴ and Ser²²¹ of the subtilisins) are well conserved in the *S. cremoris* Wg2 proteinase. The homologous sequences are separated by stretches of amino acids which are not found in the subtilisins, most notably, a sequence of approximately 200 amino acids between the His and Ser residues of the active site. Another marked structural feature of the streptococcal proteinase is the presence of a long C-terminal 'tail' which is not present in the subtilisins.

The recent elucidation of the nucleotide sequences of the *S. cremoris* SK11 and *S. lactis* NCDO 763 proteinase genes enabled a comparison of these sequences with that of the *S. cremoris* Wg2 proteinase gene ([52]; P. Vos *et al.*, submitted; M. Shimizu-Kadota, M. Kiwaki and A. Hirashima, personal communication). As expected from the data of Southern hybridizations and restriction enzyme mapping, both *S. cremoris* proteinases appeared to be almost identical with an overall homology of 98% on the nucleotide and amino acid levels (41 differ-

Fig. 2. Detailed restriction enzyme map of the proteinase region of pWV05 as deduced from the nucleotide sequence [48]. The positions of ORF1 and the proteinase gene (ORF2) are indicated by the arrows. The *Hind*III fragment originally cloned in *S. lactis* is shaded [43]. Reprinted from Kok *et al.* [48].

conserved and can encode proteins of 33 kDa. On the basis of the similarities in the restriction enzyme maps of the proteinase plasmids of *S. lactis* 712 and of the *S. cremoris* strains UC205, UC307, UC411 and P8/2/47, this open reading frame also seems to be present in these cases

Deletion of a large part of the C-terminus of the lactic streptococcal proteinases is possible without abolishing their activity (see above). However, such deletions in the *S. cremoris* proteinases result in the secretion of these normally cell wall-associated enzymes into the growth medium ([52]; A. J. Haandrikman *et al.*, sub-

Fig. 4. Comparison of the amino acid sequences of the proteinases from *S. cremoris* Wg2 (Wg2), *S. cremoris* SK11 (SK11) and *S. lactis* NCDO 763 (763), as derived from the nucleotide sequences of the respective genes ([48]; P. Vos *et al.*, submitted; M. Shimizu-Kadota, M. Kiwaki and A. Hirashima, personal communication). The horizontal bar represents the amino acid sequence of the Wg2 proteinase. Differences with the amino acid sequences of the SK11 and 763 proteinases are shown as vertical lines above and below the Wg2 sequence, respectively. When the SK11 and 763 proteinases contain a substitution of the same amino acid, this is indicated by an uninterrupted vertical line. The three regions of homology of the Wg2 proteinase with subtilisin are indicated by the hatched boxes [48]. The amino acids most probably constituting the active site are shown: D: Asp.217; H: His.281; S: Ser.620. Double headed arrows: S: putative signal sequence; PRO: putative prosequence ([52]; Vos *et al.*, submitted). Numbers refer to amino acid residues.

mitted). At the extreme end of the proteinases, in the last 30 amino acids, a typical membrane anchor sequence is present with homology to membrane anchors described for staphylococcal protein A and the streptococcal type 6 M protein (A. J. Haandrikman *et al.*, and P. Vos *et al.*, submitted). Since this sequence will, most probably, be involved in membrane binding, it is more accurate to consider the proteinases to be attached to the cell envelope.

The close proximity of the proteinase gene and the upstream open reading frame (ORF1 in [48] and Fig. 2) suggests involvement of the latter with the proteolytic system. Deletion analysis has shown that this ORF is necessary for the maturation of the proteinase and was consequently (re)named *PrtM* (A. J. Haandrikman *et al.*, and P. Vos *et al.*, submitted). Removal of *PrtM* eliminated proteolytic activity, while synthesis and secretion of an inactive proteinase could still be detected. The inactive proteinase produced in the absence of *PrtM* was larger than that made when *PrtM* was present, suggesting a proteolytic step in proteinase activation, effected by the *PrtM*-gene product.

Proteinase classification and the proteinase degradation model

Strains of lactic streptococci investigated so far characteristically contain a number of different cell envelope-bound proteolytic activities which vary between strains. Multiple cell envelope proteinases have been shown to exist in *S. lactis* [53], but only for the *S. cremoris* proteinases has the complexity been resolved to some extent. The

general impression from biochemical studies is that the enzymes are high molecular weight proteins (with molecular weights ranging from 80 000 to 145 000), with pH optima around 5.5–6.5 and isoelectric points of 4.40–4.55, and which are either activated or stabilized by Ca^{2+} ions. The enzymes are blocked by phenylmethyl-sulfonyl fluoride (PMSF) or diisopropyl fluoro-phosphate (DFP) and are, therefore, serine-type proteinases ([54–59]; for a detailed recent treatise of this subject see [23]). The latter conclusion was confirmed by genetic data (see above). An inconsistency between the biochemical and genetic data concerns the size of the *S. cremoris* Wg2 proteinase, as deduced from the nucleotide sequence of the proteinase gene (200 kDa) [48] and that of the isolated enzyme (140 kDa) [57]. In view of the similarities between the subtilisins and the lactic streptococcal proteinase, we may speculate that the latter is also synthesized as a pre-pro-molecule. At the N-terminus, a signal peptide-like sequence of 33 amino acids is present. If the putative pro-sequence is of substantial length (in the subtilisins it contains 77 amino acids [60, 61]), its removal after the pro-enzyme has reached its final cellular destination would result in a considerable reduction in the molecular weight. The initial synthesis of a pre-pro-enzyme may explain at least part of the difference between the molecular weight of the isolated proteinase and that calculated from the length of the proteinase gene. This idea is confirmed by the work of De Vos *et al.* on the proteinase of *S. cremoris* SK11. They provided evidence for the presence of a pro-sequence preceding the mature enzyme [52]. The difference in size between the proteinase and its gene has to be explained by assuming additional

digestion at the C-terminus. Such a digestion could be involved in further processing of the enzyme, as is the case for the *Serratia marcescens* proteinase, or be part of an excretion process as is the case for the IgA proteinase of *Neisseria gonorrhoeae* [62, 63]. On the other hand, it is well-established that the lactic streptococcal proteinases are quite unstable and that enzymatically active breakdown products are readily observed during proteinase isolation. In one case, excess Ca^{2+} was added to the proteinase preparation to prevent auto-proteolysis [55]. Genetic studies supplement these biochemical data and indicate that a truncated proteinase, specified by a proteinase gene deleted at its 3'-end, is still proteolytically active and can be (partly) inhibited by Ca^{2+} ions [50]. These results offer another possibility to explain the discrepancy between the expected and observed proteinase molecular weight mentioned above. The preferred method to isolate proteinases from lactic streptococci is to incubate cells in a Ca^{2+} -free buffer, resulting in the release of the proteinase from the cell envelope. It is conceivable that, under these conditions of isolation, the release is actually a self-digestion step of a large mature cell envelope-bound proteinase, resulting in the liberation and subsequent purification of a truncated protein of 140 kDa. This 140 kDa proteinase can then digest itself into proteolytically active products of lower molecular weight. Proteolytically active fragments as small as 60 kDa have been isolated from this strain and from *S. cremoris* HP (J. Erkelens, personal communication; [59]). Interestingly, around amino acid residue 1435 of the

proteinase, a stretch of 4 amino acids is present which is identical to one of the digestion sites of the *S. lactis* 763 and *S. cremoris* AC1 proteinases in β -casein ([48, 64]; W. Bockelmann, personal communication). Whether this site is actually used for the release of the proteinase from the cell envelope remains to be established. The postulated self-digestion model predicts that proteinase activity is necessary to release the enzyme from the cell envelope. Preliminary experiments, using monoclonal antibodies to detect the proteinase, indicate that PMSF inhibits the release of proteinase from the cells (H. Laan, personal communication).

Several attempts to clarify the complexity of the lactic streptococcal proteinase systems have been undertaken. Successive studies on a set of *S. cremoris* strains have resulted in their classification on the basis of differences in their proteinase activities, specificities and immunologies. These classifications are summarized in Table II. The first classification was based on the enzyme activity of whole cells and discriminated between two acid activities differing in temperature optimum (30°C for the PIII-activity and 40°C for PI) and a neutral activity at 30°C (PII) [65]. The 30°C neutral activity (PII) was shown to be an artifact of the acid activity at this temperature (PI) [51]. Contrary to PI, the PIII activity was only defined using whole cells. The PI activity from *S. cremoris* HP had the same optimum temperature whether cell envelope-bound or soluble [51, 65]. However, the cell envelope-bound proteinase of *S. cremoris* AC1 [55] had a temperature optimum of 30°C, characteristic of PIII, whereas the soluble, purified

Table II. *S. cremoris* strain classification.

Strain	Classification based on proteinase-							
	activity ^a		specificity ^b			immunology ^c		
Wg2, HP, C13	PI	PII		HP		A		B
E8	PI		(PIII)	HP	AM1	A		C
TR	PI	PII	PIII	HP	(AM1)	A	B	C
FD27	PI	PII	PIII	HP	AM1	A	B	C
AM1, SK11			PIII		AM1	A	A' (B)	C

^{a,b,c}Data derived from [65], [51] and [44], respectively. PIII-activity and AM1-specificity have been added to E8 and TR, respectively on the basis of the consideration [51] that the proteinases from these strains cannot be isolated in a reproducible way. Consequently, E8, TR and FD27 may be considered as one group.

enzyme was most active at 40°C (= PI). These results with the *S. cremoris* AC1 proteinase indicate that a distinction based on a difference in optimum temperature of 10°C may not be conclusive.

A more reliable distinction between the proteolytic systems is their difference in action on different caseins. Using the distinctive breakdown patterns of α_{S1} , β - and κ -casein, two enzyme-specificities could be detected, an HP-type and an AM1-type [51]. The problems mentioned above in distinguishing between PI and PIII activities render the conclusion that the HP-type specificity is caused by PI and the AM1-type specificity by PIII questionable. It is probably more accurate to distinguish the proteinases on the basis of their specificities alone. Twenty-three proteolytically active *S. cremoris* strains isolated from mixed strain starters apparently belong to the HP-type because they only degraded β -casein [56]. Also, five strains of *S. lactis* belong to this group because of specific β -casein degradation [66].

With immunological techniques, different combinations of four proteins, A, A', B and C, have been identified in the partially purified proteolytic systems of several strains of *S. cremoris*. This immunological distinction has been used to divide these strains into four groups [44]. Proteins A and A' share common antigenic determinants, while proteins B and C are immunologically identical [44, 57]. Genetic analysis [50] showed that proteins A and B are both encoded by the proteinase gene of *S. cremoris* Wg2 and these data allow a simplification of the immunological classification of the *S. cremoris* strains. Only one proteinase is isolated from all strains tested. The released proteinase is found in two immunologically distinct and proteolytically active conformations A and B. In view of the fact that protein A is isolated from all strains [44], we envisage that B is either formed from A by a self-digestion step, or may represent a different conformation, due to the absence of Ca^{2+} ions which are known to stabilize several proteins, among which are several proteinases [67–70]. The occurrence of conformation B may also be the result of a combination of both possibilities. B can be further degraded to give smaller products, one of which may be protein C detected in some of the *S. cremoris* strains [44]. If the self-digestion model is correct, only a limited stretch of amino acids is removed from proteinase A to yield conformation B, because proteins A and B have similar

molecular weights [57]. The difference between the conformations A and B will probably be limited, if resulting from either limited self-digestion, or if caused by the absence of Ca^{2+} ions. To reconcile this with the immunological data, one has to assume that a limited number of epitopes for the proteins A and B are recognized by the antibodies present in the rabbit serum used [44]. Possibly, this is caused by the difference in the structure of the proteinase used for raising the antibodies, which may be largely denatured due to its dissolution in Ca^{2+} -free buffer and the presence of Freund's complete adjuvant, and that of the enzyme under native conditions during CIE. If this view is correct, CIE may be insufficiently sensitive to reach the conclusion that all strains tested show a common proteinase. However, increasing evidence in favor of the presence of a common or slightly modified proteinase can be derived from other data. As discussed above, genetic analysis of proteinase plasmids and genes has shown that *S. cremoris* Wg2, SK11, AC1, UC205, UV317 and *S. lactis* 712 and NCDO 763 have a proteinase gene (region) with very similar restriction enzyme maps. A comparison at the nucleotide level revealed that 3 proteinases from two different specificity groups, viz. the *S. cremoris* Wg2 and *S. lactis* NCDO 763 proteinases versus the *S. cremoris* SK11 proteinase, are almost identical. Furthermore, plasmids in various strains of *S. cremoris* and *S. lactis* show a high degree of homology with 5'- and 3'-end fragments of the *S. cremoris* Wg2 proteinase gene in Southern hybridizations (see above). Tentatively we propose a simplification of the classification of the *S. cremoris* strains by assuming that all proteolytically active strains contain a 'basic' proteinase gene, which may be slightly different in the various strains. This difference may well underlie the differences in specificity of casein breakdown and the reproducibly observed differences in the CIE patterns of the various strains. If this view is correct, a specific CIE pattern may be indicative for casein breakdown specificity. Alternatively, the differences in specificity are caused by a difference in the presence of putative 'self-digestion sites', since it is conceivable that part of the casein breakdown-specificity is actually caused by proteinase-specific self-digestion product(s). In both respects, it may be of significance that all strains with an AM1-type specificity contain protein C (Table II). Because of the availability of tools to genetically manipulate the lactic streptococci

and to study their proteinase genes at the molecular level, a distinction between these possibilities and a further clarification of the complexity of lactic streptococcal proteolytic systems may be expected in the near future.

Proteinases in lactic acid bacteria other than lactic streptococci

Data on the presence of proteinases in the other species of lactic acid bacteria (lactobacilli, leuconostocs, pediococci) are very scarce and data on the genetics of these enzymes are not available. Proteinases have been detected in several species of lactobacilli [71–75]. From a strain of *Lactobacillus acidophilus*, a proteinase was isolated, after washing the cells in Ca^{2+} -free buffer, which had a molecular weight of approximately 145 000 (W. Bockelmann, personal communication). It will be interesting to see whether these proteinases show homology with the Wg2/SK11/712 proteinase complex. Southern hybridization experiments with several strains of lactic acid bacteria have been started recently, using Wg2 and SK11 proteinase gene probes as a quick first approach to dissect the genetics of the proteolytic systems in these organisms (A. Mercenier, A. Geis and A. M. Ledeboer, personal communication). From a number of *S. thermophilus* and *L. bulgaricus* strains and a single strain each of *L. lactis*, and *L. helveticus*, total DNA was isolated and cut with *Hind*III. A 6.5 kb DNA fragment of *L. lactis* gave a strong signal with a probe derived from the *S. cremoris* SK11 proteinase gene in Southern hybridization and under stringent conditions of washing (M. O'Regan and A. Mercenier, personal communication).

The gene expression signals from lactic acid bacterial species other than the lactic streptococci are likely to operate in the latter. As long as efficient transformation protocols are not available for the various species of lactic acid bacteria, a fruitful approach to quickly gain knowledge on the genetics of the proteinases of these organisms would be the cloning of the genes concerned in the few 'model' strains of *S. lactis* available for gene cloning, and/or in *B. subtilis*. The cloning vectors constructed for the lactic streptococci appear to be useful in this respect. As recent results indicate that these plasmids replicate and are expressed in the lactobacilli ([35]; B. M. Chassy, personal communi-

cation), the recombinant plasmids can ultimately be transferred back into the original hosts as soon as transformation systems are available. A promising method in this respect seems to be electroporation, which operates in *S. lactis*, *S. cremoris*, *S. lactis* ssp. *diacetylactis*, *S. thermophilus*, *Leuconostoc* and *Lactobacilli* ([35, 76, 77], A. Mercenier, S. David, G. Fitzgerald, A. Harrington and D. van der Lelie, personal communication; G. A. Somkuti, this issue).

Leuconostoc species show a limited ability to grow in milk, which, in part, is caused by the lack of proteolytic activity in these organisms. A 60 kb recombinant between pAM β 1 and the proteinase plasmid of *S. cremoris* UC205 was transferred into *Leuconostoc mesenteroides* X2 via conjugation [78]. The transconjugants showed weak proteolytic activities and improved growth on milk-based agar media as compared to the wild type *leuconostoc* strain. β -Casein breakdown could be demonstrated in SDS-polyacrylamide gel electrophoresis (C. Daly and G. F. Fitzgerald, personal communication). When a *leuconostoc* transconjugant was used as the donor in secondary matings with *S. lactis*, the *S. lactis* transconjugants showed a normal Prt⁺ phenotype, indicating that the *leuconostoc* donor harbored an intact proteinase gene. Via conjugation, the lactose/proteinase plasmid pLP712 of *S. lactis* 712 was transferred into wild (Lac⁻; Prt⁻) strains of *S. lactis* (isolated from frozen peas and a termite's gut; M. J. Gasson, personal communication). Lac⁺ transconjugants from the wild strains were partly proteolytically active and showed enhanced acid production. Transfer of the lactose/proteinase plasmid from a wild strain back into the original *S. lactis* strain resulted in full proteolytic activity in this recipient. In all the cases mentioned above, the weak proteolytic activity of an apparently functional proteinase gene could either be caused by difficulties in (regulation of) gene expression or by one of the steps following initial proteolysis by the proteinase. However, these examples do show that genetic manipulation can be a valuable tool to provide modified lactic acid bacteria which would be more flexible for use in existing or new food fermentations.

Conclusions and future prospects

The knowledge of the genetic basis for the proteolytic activity of lactic streptococci has increased considerably since the discovery that

plasmid DNA governs this trait in many strains. The first indication for the existence of proteinase plasmids, in the early 70's, was rapidly followed by conclusive evidence through the simultaneous transfer of proteolytic ability and specific plasmids *via* conjugation and transduction. Now, some 10 years after the first report on proteinase plasmids, the genes of four strains have been cloned and three of these have been sequenced. The sequences have revealed some interesting features of the proteinases they specify and have clearly demonstrated that the lactic streptococcal proteinases belong to the subtilisin-type of proteinases. Combination of biochemical, immunological and the latest genetic data has given a better insight into the apparent complexity of the proteolytic systems of the lactic streptococci and has resulted in a substantial simplification of this complexity. A further refined classification of the proteinases can be expected in the near future, since monoclonal antibodies against the proteinase of *S. cremoris* Wg2 have been isolated and characterized [79]. The twelve monoclonal antibodies reacted, in Western blotting, with protein bands of different molecular weights, which were most probably proteinase degradation products. One set of monoclonal antibodies reacted only with protein A, while the others reacted with protein B of the *S. cremoris* Wg2 proteolytic system. Results of affinity column chromatography, using monoclonals against protein A or protein B, support the idea that protein B is a product of protein A (H. Laan, personal communication). Combination of genetic work and immunological methods will rapidly lead to a better insight into proteinase processing and has already led to the identification of a gene (*PrtM*) involved in proteinase activation.

The availability of the nucleotide sequences of the proteinase genes of *S. cremoris* Wg2 and SK11 and of *S. lactis* NCDO 763 offers the possibility to apply the powerful strategies of site-directed mutagenesis. Changing the codon for Asp²¹⁷ into an Asn codon by a single base substitution led to the production of an inactive form of the *S. cremoris* Wg2 proteinase, indicating that Asp²¹⁷ is part of the active center of the enzyme. This mutated proteinase will be very useful in studying proteinase maturation (A. J. Haandrikman, personal communication). Moreover, knowledge of the sequences gives the exciting possibility to exchange DNA fragments between the genes. The two *S. cremoris* proteinases belong to different specificity of the hybrid

proteinases on the various types of caseins is an obvious way to determine the functional properties of certain domains and, in fact, is already in progress. Recognition of the regions determining casein breakdown specificity could ultimately lead to the development of strategies aimed at altering and/or improving the proteinase activity and specificity.

DNA probes are now available which can be used to monitor proteinase gene expression, an important but at present underexposed aspect of proteinase synthesis. Furthermore, these proteinase gene probes can be used to investigate whether similar genes are present in the other species of lactic acid bacteria.

Another point of (near) future attention will be the development of strategies to stabilize and, if desired, to amplify the proteinase genes in the chromosome. Since a number of other important dairy functions appear to be plasmid located, and are, therefore, inherently unstable, these strategies will probably be of major importance for future strain improvement programs. The *in vivo* construction, *via* transduction, of a strain of *S. lactis* C2 carrying the proteinase gene stably inserted into the chromosome (see above) has already proven to be of use for dairy practice. *S. lactis* C2 is a bitter strain. In the stabilized derivative, the proteolytic activity was about half that of the parental strain, resulting in the production of a better cheese with less bitter peptides [37].

In conclusion, knowledge of the genetics of the proteolytic systems of lactic acid streptococci should rapidly lead to a better insight into the genetic basis of proteolysis in the lactic acid bacteria as a whole, and to a better understanding of the functioning of this trait of such eminent importance in dairying.

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