Genetics of the proteolytic system of lactic acid bacteria

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Key words: Proteinase; Peptidase; Amino acid/peptide uptake; Plasmid; Gene cloning; DNA sequence

1. SUMMARY

The proteolytic system of lactic acid bacteria is of eminent importance for the rapid growth of these organisms in protein-rich media. The combined action of proteinases and peptidases provides the cell with small peptides and essential amino acids. The amino acids and peptides thus liberated have to be translocated across the cytoplasmic membrane. To that purpose, the cell contains specific transport proteins. The internalized peptides are further degraded to amino acids by intracellular peptidases. The world-wide economic importance of the lactic acid bacteria and their proteolytic system has led to an intensive research effort in this area and a considerable amount of biochemical data has been collected during the last two decades. Since the development of systems to genetically manipulate lactic acid bacteria, data on the genetics of enzymes and processes involved in proteolysis are rapidly being generated. In this review an overview of the latest genetic data on the proteolytic system of lactic acid bacteria will be presented. As most of the work in this field has been done with lactococci, the emphasis will, inevitably, be on this group of organisms. Where possible, links will be made with other species of lactic acid bacteria.

2. INTRODUCTION

Lactic acid bacteria are important components of the microflora of a wide variety of fermented foods and feeds from milk, meat and vegetable origin. Their main function is the rapid conversion of the available sugar to lactate resulting in the low pH which is necessary for good product quality. The proteolytic system of lactic acid bacteria is crucial for the growth of these organisms on protein-rich substrates as they are nutritionally very fastidious. In addition to nucleotides and vitamins, all species of lactic acid bacteria have an absolute requirement for exogenous supplies of various amino acids [1–4]. In natural milk the concentrations of free amino acids and low molecular weight peptides initially present are only sufficient to give growth to cell densities corresponding to 8–16% of the maximum found in coagulated milk and are thus well below the minimum necessary to support rapid acid production [5]. As the cell density increases, milk protein becomes the most important source of nitrogen and a functional set of proteinases and peptidases has to supply the cells with the required amino acids and small peptides. Amino acid and peptide uptake is the next crucial step in the utilization of milk protein by lactic acid bacteria and is mediated by separate systems [6,7]. Lactococci have been shown to possess distinct dipeptide and oligopeptide transport systems [6,7].

A fortuitous but important side effect of the...
action of the proteolytic system is the generation of amino acids and peptides which give the fermentation products their characteristic organoleptic properties. The evident world-wide importance of fermentation processes using lactic acid bacteria has led to a rapid increase in the research devoted to these organisms during the last 20 years. The analyses of the proteolytic system has given new insights in the protein utilization of these organisms and will be reviewed here. The emphasis will be on the recent progress in the knowledge of the genetic basis of the proteolytic system as was largely gained after the second Symposium on Lactic Acid Bacteria, held in The Netherlands in 1987.

3. PROTEINASES

The proteinases of lactic acid bacteria perform the first step in the cascade of reactions leading to the breakdown of casein. Because of their prime importance in fermentation processes, the enzymes are subject to intensive biochemical research for some 15 years now. In this paragraph the knowledge acquired from these studies on the lactococcal proteinases will be summarized and reference will be made to the corresponding enzymes in other species of lactic acid bacteria. As this paper will deal with the genetics of proteolysis, it is beyond its scope to go into details of biochemical aspects of the proteolytic system of lactic acid bacteria. The reader's attention is drawn to recent excellent reviews specifically dealing with this subject [8–10].

3.1. Classification of proteinases

Biochemical studies on lactococcal proteinases show that the enzymes are high molecular weight proteins (the published molecular sizes ranging from 80–145 kilodalton (kDa)), having pH optima around 5.5–6.5 and isoelectric points of 4.40–4.55, and are either activated or stabilized by Ca^{2+}-ions. The enzymes are blocked by phenyl methyl-sulphonylfluoride (PMSF) or diisopropyl fluorophosphate (DFP) and are, therefore, serine-type proteinases. It is generally agreed that the proteinases are located primarily in the cell wall [8, 11–17]; for a detailed review of this subject see Thomas and Pritchard [10]. Although the proteinases from the various lactococcal strains studied so far share these general properties, certain differences do exist and these have been extensively examined.

Lactococci characteristically contain a number of different cell-associated proteolytic activities which vary between strains. Multiple cell wall proteinases have been shown to exist in Lactococcus lactis ssp. lactis [18] and several attempts to

**Table 1**

*L. lactis* ssp. cremoris strain classification

<table>
<thead>
<tr>
<th>Strain</th>
<th>Classification based on proteinase-activity</th>
<th>Specificity</th>
<th>Immunology</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>activity</td>
<td>specificity</td>
<td>Polys</td>
</tr>
<tr>
<td>Wg2, HP, C13</td>
<td>PI PIH</td>
<td>HP</td>
<td>A B</td>
</tr>
<tr>
<td>E8</td>
<td>PI</td>
<td>(PIII) HP AM1</td>
<td>A C</td>
</tr>
<tr>
<td>TR</td>
<td>PI</td>
<td>PIH</td>
<td>HP (AM1)</td>
</tr>
<tr>
<td>FD27</td>
<td>PI PIH</td>
<td>PH</td>
<td>AM1</td>
</tr>
<tr>
<td>AM1, SK11</td>
<td>PI PIH</td>
<td>AM1</td>
<td>A A' (B) C</td>
</tr>
</tbody>
</table>

Polys, monos, polyclonal- and monoclonal antibodies against the purified proteinase of *L. lactis* ssp. cremoris Wg2 [22, 23].

- Polys, monos, polyclonal- and monoclonal antibodies against the purified proteinase of *L. lactis* ssp. cremoris Wg2 [22, 23].
- Data derived from [19], [20], [22], and [23], respectively. PIII-activity and AM1-specificity have been added to E8 and TR, respectively, on the basis of the consideration [20] that the proteinases from these strains cannot be isolated in a reproducible way. Consequently, E8, TR, and FD27 may be considered as one group. Monoclonal antibody groups I, II, and III react with proteinase component A; monoclonal antibody groups IV, V, and VI react with proteinase component B. C13 was not included in the analysis using the monoclonal antibodies [23].
clarify the proteinase complex in a set of strains of the subspecies cremoris have been undertaken. These studies have resulted in the classification of the strains on the basis of differences in their proteinase activity, specificity, and immunology and are summarized in Table 1. 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 P1) and a neutral activity optimal at 30°C (PII) [19]. In a later study, the neutral activity at 30°C (PII) was shown to be an artifact of the acid activity at this temperature (P1) [20]. The P1 activity from L. lactis ssp. cremoris Hp had the same temperature optimum irrespective of being cell wall-bound or soluble [19]. However, the cell wall-bound proteinase of the cremoris strain ACI [13] had a temperature optimum of 30°C, characteristic of PIII, whereas the soluble, purified enzyme was most active at 40°C (=P1). These results suggest that the same proteinase can have different proteolytic activities depending on the state of purification. Since it is difficult to characterize the proteinases by using intact cells, and, since highly purified proteinase preparations are very unstable, in later studies partially purified proteinases were used. A strain classification based on the difference in action of their proteinases on the various casein types present in milk was put forward by Visser et al. [20]; see Table 1 and Fig. 1). Using the distinctive breakdown patterns of αS1-, β-, and κ-casein two enzyme specificities could be detected, an HP-type (P1) and an AM1-type (PIII). Twenty-three proteolytically active cremoris strains isolated from mixed strain starters and five strains of the subspecies lactis apparently belong to the PI-type because they only degraded β-casein [14,21].

Polyclonal antibodies against the proteinases of a number of cremoris strains have been isolated and with immunological techniques four proteins, A, A', B, and C, have been identified in the partially purified proteolytic systems of these strains. Proteins A and B have been shown to be proteolytically active. Component A is present in all strains tested and is always accompanied by at least one of the other three proteins. Proteins A and A' share common antigenic determinants as do B and C ([15,22]; see Table 1). Monoclonal antibodies have also been used to study the proteinases of the same set of strains. The monoclonal antibodies reacted either with protein A or with protein B but never with both components [23]. Monoclonals reacting with protein A reacted with all strains studied, confirming the conclusion reached with the polyclonals that component A is expressed in all strains [22].

3.2. Genetics of lactococcal proteinases

The genetic studies of the proteinases and the corresponding genes from the lactococci have led to a major breakthrough in our understanding of these enzymes and have led to a considerable clarification in the body of biochemical data gathered on the proteinases. In an earlier publication [24] we have dealt with the genetics of the proteinases of lactococci. The availability of the nucleotide sequences of the proteinase loci allowed the detailed analysis of specific mutations in this region and has led to a rapid further
increase in the knowledge of the lactococcal proteinases.

3.2.1. Proteinase plasmids

Production of proteinases by lactococci is an unstable trait [25–28]. Spontaneously and in some cases with considerable frequency, proteinase-negative variants appear in cultures of wild-type Prt+ strains. These mutants show a reduced acid production and are unable to grow in milk to the high cell densities necessary for good fermentation practice ("slow" phenotype). Irreversibility of this loss of proteinase activity and the fact that the mutation frequency could be enhanced by treatment of cells with acridine dyes and ethidium bromide, or by growth at elevated temperatures suggested the involvement of plasmid DNA ([29,30]; see Fig. 2). This genetic methodology to identify proteinase plasmids has been rapidly adopted and, by now, curing studies have provided evidence for plasmid linkage of proteolytic activity in an increasing number of lactococci.

Table 2

Localization of proteinase genes in lactococci

<table>
<thead>
<tr>
<th>Subspecies and strain</th>
<th>Size of (suspected) Prt+ plasmid in kb</th>
<th>Evidence apart from curing</th>
<th>Hybridization with Wg2 probe</th>
<th>Reference</th>
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<tbody>
<tr>
<td><em>lactis</em> C2</td>
<td>45, 27, 19</td>
<td>Transduction</td>
<td>nd</td>
<td>[148–150]</td>
</tr>
<tr>
<td>712</td>
<td>56</td>
<td>Transduction</td>
<td>+</td>
<td>[32,46,137]</td>
</tr>
<tr>
<td>ML3</td>
<td>49.5</td>
<td>Conjugation</td>
<td>nd</td>
<td>[31,151]</td>
</tr>
<tr>
<td>765</td>
<td>55</td>
<td>Cloning</td>
<td>nd</td>
<td>[47]</td>
</tr>
<tr>
<td>C10</td>
<td>60</td>
<td>Cloning</td>
<td>nd</td>
<td>[151]</td>
</tr>
<tr>
<td>M18</td>
<td>67.5</td>
<td>Cloning</td>
<td>nd</td>
<td>[151]</td>
</tr>
<tr>
<td>SSL135</td>
<td>7</td>
<td>Conjugation</td>
<td>nd</td>
<td>[48]</td>
</tr>
<tr>
<td>DRC1</td>
<td>46.5</td>
<td>Conjugation</td>
<td>+</td>
<td>[152]</td>
</tr>
<tr>
<td>UC317</td>
<td>69</td>
<td>Cloning</td>
<td>nd</td>
<td>[155,156]</td>
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<tr>
<td><em>cremoris</em> HP</td>
<td>13.5</td>
<td></td>
<td>+</td>
<td>[44,153]</td>
</tr>
<tr>
<td>ML1</td>
<td>3.3</td>
<td>Cloning</td>
<td>– d</td>
<td>[153]</td>
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<tr>
<td>Wg2</td>
<td>36</td>
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<td>+</td>
<td>[43,44]</td>
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<tr>
<td>E8</td>
<td>&lt;3</td>
<td>Cloning</td>
<td>+</td>
<td>A.J. Haandrikman f</td>
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<td>78</td>
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<td>+</td>
<td>[45,154]</td>
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<td>34.5</td>
<td>Conjugation</td>
<td>+</td>
<td>[156]</td>
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<tr>
<td>UC411</td>
<td>30</td>
<td>Conjugation</td>
<td>+</td>
<td>[155]</td>
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<tr>
<td>P8/2/47</td>
<td>14</td>
<td>Conjugation</td>
<td>+</td>
<td>A. Geis, B. Kiefer f</td>
</tr>
<tr>
<td>H2</td>
<td>63</td>
<td>Conjugation</td>
<td>+</td>
<td>[158]</td>
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<tr>
<td>SD8, SD9, SD11</td>
<td>93</td>
<td></td>
<td>nd</td>
<td>H. Neve, A. Geis f</td>
</tr>
<tr>
<td>VI6</td>
<td>(93 100)</td>
<td></td>
<td>+ (93 100) e</td>
<td>H. Neve, A. Geis f</td>
</tr>
<tr>
<td>VI8</td>
<td>93</td>
<td></td>
<td>nd</td>
<td>H. Neve, A. Geis f</td>
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<tr>
<td>1200</td>
<td>21</td>
<td></td>
<td>nd</td>
<td>[31]</td>
</tr>
<tr>
<td>MI12R</td>
<td>19.5</td>
<td></td>
<td>nd</td>
<td>[157]</td>
</tr>
</tbody>
</table>

a A plasmid-free derivative was still Prt+, suggesting chromosomal linkage of this trait in SSL135 [48].
b,c see reference [24] for a discussion of these results.
d No hybridization with the 3.3-kb plasmid was observed. Instead, hybridization was found with a high molecular weight plasmid (A.M. Ledeboer, personal communication), from which the gene was cloned (P. Vos and W.M. de Vos, personal communication).
e Hybridization was observed but no discrimination could be made as to which of the two plasmids hybridized (A. Geis and B. Kiefer, personal communication).
f Personal communication.
p Proteinase plasmid.
nd, not determined.
strains. Plasmids ranging in size from 13.5 to 100 kilobasepairs (kb) have been implicated in proteinase production. In some strains the genes for proteolytic ability are located on a plasmid which is also involved in lactose metabolism. A summing-up of strains and their proteinase plasmids is given in Table 2.

Structural instability of proteinase plasmids and loss of plasmids which are not involved in proteinase production during curing experiments make interpretation of plasmid curing data difficult [31–33]. Several research groups, therefore, have started to use gene transfer systems to physically link proteolytic ability with certain plasmids. Natural gene transfer systems viz. transduction and conjugation as well as protoplast transformation and electrotransformation have been used to provide evidence for plasmid linkage of proteinase determinants in strains of lactococci (see Table 2).

3.2.2. Proteinase gene cloning

The recently developed cloning vectors and techniques to genetically manipulate lactic acid bacteria ([34–39]; for reviews see [40–42]) have, in a number of instances, been used to localize proteinase genes. In *L. lactis* ssp. *cremoris* Wg2 loss of the 26-kb plasmid pWV05 was accompanied by loss of proteolytic ability ([43]; see Fig. 2). A 6.5-kb HindIII fragment of this plasmid was inserted in a pWV01-based cloning vector [39] and transferred to a proteinase-deficient strain of *L. lactis* by protoplast transformation. The strain thus obtained expressed a functional proteolytic activity and was able to grow normally in milk ([44]; see Fig. 3). With immunological techniques it was shown that in both *Bacillus subtilis* and *L. lactis* carrying the recombinant plasmid the fragment specified the two proteins A and B of the proteolytic system of Wg2 ([22]; see 3.2.1). The fact that the proteinase antigen could be detected in the heterologous host *B. subtilis* proved that the structural gene(s) for a proteolytic enzyme(s) had been cloned. By now, a number of proteinase genes from different lactococcal strains have been cloned and analysed. De Vos et al. identified, in a lambda-phage bank of the 78-kb proteinase plasmid pSK111 of the *cremoris* strain SK11, plaques in which proteinase was detectable with
immunological methods. Subcloning of the specific DNA fragment on a pSH71 derivative allowed the complementation of the proteinase defect of *L. lactis* MG1363 [41,45]. The lactose/proteinase plasmid pLP712 of *L. 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 to a 4.8-kb *BglII* fragment. This fragment was cloned in a pSH71 derivative and partly restored, in strain *L. lactis* MG1363, the ability to grow in milk [46]. The proteinase genes of *L. lactis* ssp. *lactis* 763 [47], of the *cremoris* strains E8 and ML1, and the *lactis* strain UC317 have also been cloned and functionally expressed in *L. lactis* (A.J. Haandrikman, J. Law, P. Vos, W.M. de Vos and G.F. Fitzgerald, personal communication). Until now, it is not clear whether the chromosomal fragment from *L. lactis* SSL135, which encodes the ability to grow rapidly in milk, carries a proteinase gene and/or other genes involved in proteolysis [48–50].

### 3.2.3. Proteinase gene sequences and homology

From a comparison of the restriction enzyme maps of the cloned proteinase genes of the *L. lactis* ssp. *lactis* strains 712 and 763 and the *cremoris* strains Wg2 and SK11 it is obvious that the genes are highly conserved ([24,42,47]; see Fig. 4). The recently cloned genes of the *cremoris* strains E8, ML1 and UC317 also show the same conservation of restriction enzyme sites (A.J. Haandrikman, J. Law, P. Vos, W.M. de Vos and G.F. Fitzgerald, personal communication). Therefore, it appears that many (if not all) lactococci carry essentially the same gene for a cell wall-associated proteinase which has only slightly been subject to change during evolution.

From the genes of three proteinases, those of the *cremoris* strains Wg2 and SK11 and that of the *L. lactis* ssp. *lactis* strain NCDO763, the nucleotide sequences have been elucidated completely [47,51,52]. Three additional genes have been sequenced partially (that from strain E8, in our group, and those from strains ML1 and UC317 (P. Vos, J. Law, W.M. de Vos and G.F. Fitzgerald, personal communication). The conclusions derived from the comparison of restriction enzyme sites of the cloned proteinase genes were fully confirmed by the nucleotide sequences. The genes from the two *cremoris* strains appeared to be almost identical with an overall homology of 98% on the nucleotide and amino acid level (44 amino acid substitutions). Although some amino acid substitutions seem to be clustered, most of them are more or less randomly distributed [52]. The differences with *L. lactis* ssp. *lactis* NCDO763 are

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**Fig. 4.** Physical maps of the proteinase plasmids of *L. lactis* ssp. *lactis* 712 (pLP712, 56 kb), and the *cremoris* strains SK11 (pSK11, 78 kb) and Wg2 (pWV05, 26 kb). The plasmids 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 Wg2 and SK11 genes [51,52]. Thick lines indicate the restriction fragments initially cloned in *L. lactis* specifying proteinase activity. The bar represents 1 kb of DNA. (For details: see text). prtP, proteinase gene; prtM, gene for proteinase maturation protein; IS, insertion sequence; B, *BglII*; A, *BamHI*; C, *ClaI*; E, *EcoRI*; H, *HindIII*; X, *XhoI*. (W.M. de Vos and M.J. Gasson, personal communication).
Fig. 5. Comparison of the amino acid sequences of the proteinases from *L. lactis* ssp. cremoris strains Wg2 and SK11, and *L. lactis* ssp. *lactis* NCDO 763 (763), as derived from the nucleotide sequences of the respective genes [47,51,52]. 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. Identical amino acid substitutions in the SK11 and 763 proteinases are indicated by an uninterrupted vertical line. The 60 amino acid duplication in the C-terminus of the SK11 enzyme is shown by the double arrow above the bar. The membrane anchor is indicated as a small horizontally striped region. The bar at the top-left of the figure represents 100 amino acids. The three regions of homology of the proteinases with subtilisin are indicated by the black boxes. The amino acids constituting the active site are shown: D, Asp.30; H, His.94; S, Ser.433. Double headed arrows: SS (stippled area), signal sequence; PRO (hatched box), pro-sequence. Adapted from [24].

Fig. 6. Homology comparison. A. Sequence homology of the *L. lactis* ssp. cremoris Wg2 proteinase and subtilisins *D. vulgaris*, *D. vulgaris*, and *B. licheniformis*. Only those amino acids differing from the residues in the Carlsberg enzyme are shown; identical residues are boxed. The active site amino acids Asp, His, and Ser are indicated by the vertical dotted lines. B. The homologous regions from A (black boxes) are drawn to scale on a linear map of the whole proteinase and compared with a linear map of subtilisin. Dotted box, signal sequence; hatched box, pro-sequence; striped region, membrane anchor. Numbers refer to amino acid residues. *N*-terminal amino acid of the mature proteinase; D, Asp32/30; H, His64/94; Ser221/423. Adapted from [24].
even smaller: the Wg2 proteinase and that of strain 763 differ in only 18 amino acids \cite{47}. Some of the amino acid differences involve conservative amino acid replacements, while others result in functionally different amino acids. There is a strong bias for more basic amino acid substitutions in the SK11 proteinase, especially in the first 1200 amino acids. The most striking difference between the SK11 proteinase and the other two is the presence in the former of a duplication of 60 amino acids near the C-terminus. The differences in the amino acid sequences between the Wg2 proteinase (PI-type) and the SK11 enzyme (PIII-type) are responsible for the different caseinolytic properties of both enzymes (see sections 3.1. and 3.2.8.). A summary of the homology comparison is given in Fig. 5.

3.2.4. Homology with subtilisins

The nucleotide sequences of the proteinase genes have revealed a number of interesting features of the proteinases they specify \cite{47,51,52}. First, protein homology comparisons indicated three regions in the lactococcal proteinases which have extensive homology with serine proteinases of the subtilisin family (see Fig. 6). Especially, amino acids involved in the formation of the active center (viz. Asp.32, His.64, and Ser.221 of the subtilisins) are well conserved in the lactococcal proteinases. The homologous sequences are separated by stretches of amino acids not found in the subtilisins, most notably, a sequence of approximately 175 amino acids between the His and Ser residues of the active site. Another marked structural feature of the lactococcal proteinases is the presence of a long C-terminal extension not present in the subtilisins.

The coding regions of the Wg2 and 763 proteinase genes contain 1902 codons which could specify proteins with molecular weights ($M_w$) of 200,000. The SK11 proteinase is somewhat larger due to the duplication of 60 amino acids in the C-terminal part of the enzyme. The proteinase genes are flanked by transcription- and translation regulatory sequences which closely resemble those reported for \textit{B. subtilis} and \textit{Escherichia coli} and are in good agreement with the regulatory sequences found in lactococci \cite{42,53}. Like the subtilisins, the lactococcal proteinases are synthesized as pre-pro-proteins. At the N-terminus a typical signal peptide-like sequence of 33 amino acids is present. The N-terminal amino acid sequences of the isolated proteinases have been determined and indicate that in all three cases the mature enzyme starts with the aspartic acid residue at position 188 of the predicted amino acid sequence \cite{47,52,54}.

3.2.5. Proteinase localization

A clear difference between the subtilisins and the lactococcal proteinases is their localization. While subtilisins are fully secreted and found in the culture medium of producing cells \cite{55,56}, the proteinase activity of lactococci is associated with the cells \cite{11-13,15,18,19,22}; for reviews \cite{8-10}. At the extreme C-terminal end of the lactococcal proteinases a region was identified which shows homology with a number of membrane attachment sites of proteins of Gram-positive bacteria \cite{47,52,57}; see Fig. 7). A very hydrophobic $\alpha$-helix of 18 amino acids is flanked by an N-terminal proline residue and a C-terminal hydrophobic region containing three basic amino acids followed by two acidic residues. Upstream of the $\alpha$-helix a proline-rich region with a number of conserved amino acids is located. It follows that the lactococcal proteinases are attached to the cells by anchoring in the cell membrane while the proline-rich region directly upstream of the membrane anchor most probably provides for contacts with the cell wall. Therefore, it is more accurate to refer to the proteinases as cell envelope-attached enzymes. Removal of the C-terminal domain from the proteinases results in the release of the proteins from the cells: all initial proteinase gene clones were truncated at the 3'-end and the C-terminally truncated proteinases (lacking 130 or 403 amino acids) were fully secreted \cite{45,47,57}. Moreover, these results show that C-terminal truncation is possible without abolishing proteinase activity (see also section 3.2.7.).

3.2.6. Proteinase maturation

A second open reading frame (ORF) is present immediately upstream of the proteinase gene and directed in opposite orientation \cite{47,51,52}. It is
found in all the proteinase gene regions sequenced so far and is completely conserved. On the basis of the similarities in the restriction enzyme maps of the proteinase plasmids of \textit{L. lactis} 712 and of other lactococcal strains, this ORF also seems to be present in these cases [24]. Deletion analysis has shown that the ORF is essential for proteolytic activity: it is necessary for the maturation of the proteinase and was consequently (re)named \textit{prtM} ([57,58]; see Fig. 4). In the same two papers it was proposed to use the notation \textit{prtP} for the proteinase gene. In the original clones, \textit{prtM} was truncated and lacked four amino acids at the C-terminus, apparently without interfering with its functioning. The entire gene specifies a protein of 33 kDa. Cells carrying an intact proteinase gene but lacking \textit{prtM} synthesize and correctly locate the proteinase antigen but they are phenotypically proteolytically negative (see Fig. 7). Apparently, the proteinase produced in the absence of \textit{prtM} was larger than that made when \textit{prtM} was present, suggesting a proteolytic step in proteinase activation, effected by the \textit{prtM}-gene product. \textit{PrtM} can exert its effect in trans [58,59]. To examine the roles of \textit{PrtM} and the proteinase itself in the activation process, a mutation in the proteinase was made to destroy the active center of the enzyme. By changing the aspartic acid residue, which is part of the active center of the lactococcal

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<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Proteinase activity</th>
<th>Cell attachment</th>
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<tbody>
<tr>
<td>pWV05</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pGKV502</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pGKV550</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>pGKV1852</td>
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<td>+</td>
</tr>
<tr>
<td>pGKV600</td>
<td>+</td>
<td>-</td>
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</tbody>
</table>

Fig. 7. Effect on proteolytic activity and cell attachment of the proteinase specified by plasmids carrying mutations in the proteinase locus of \textit{L. lactis} ssp. \textit{cremoris} Wg2. Plasmid designations are given on the left. The top line shows the \textit{prt} locus as present on plasmid pWV05 of strain Wg2. \textit{PrtP}: proteinase gene; \textit{prtM}: gene for the proteinase maturation protein. Whether the plasmids specify the maturation protein or (incomplete) proteinase is indicated by the small and long open bar, respectively. The small hatched area at the extreme C-terminus of the proteinase represents the membrane anchor. Asp.30, His.94 and Ser.433 of the active site are shown as is the Asp.30 to *Asn.30 mutation. SS, signal sequence; PRO, pro-sequence. B, H, N, C, M: sites for restriction enzymes \textit{BamHI}, \textit{HindIII}, \textit{NruI}, \textit{ClaI}, and \textit{MluI}, respectively. Fat arrows: promoters for \textit{prtM} and \textit{prtP}. For details: see text. (Taken from A.J. Haandrikman et al., unpublished).
proteinase (at position 30 of the matured enzyme), into asparagine using site directed mutagenesis (SDM) techniques, an inactive proteinase was obtained (Fig. 7; A.J. Haandrikman, personal communication). The SDM-inactivated proteinase had a $M_w$ which was higher than the active enzyme. The elucidation of the role of PrtM in the maturation process, either direct, by performing the activating cleavage step, or indirect, by inducing the pro-proteinase to perform an autocatalytic conversion into the mature active form awaits the determination of the N-terminal amino acid sequences of the inactive enlarged forms of the proteinase.

In a subsequent study, Haandrikman and coworkers overexpressed the prtM gene in E. coli and isolated PrtM-specific antibodies. Using these antibodies in immunogold labelling experiments, it was shown that PrtM is located in the cell envelope of lactococci, while cell fractionation studies showed that the protein was enriched in the membrane fraction. The observation from the nucleotide sequence that PrtM might be a lipoprotein [57,58] was confirmed in experiments with $^3$H-labelled palmitic acid. A 32-kDa $^3$H-labelled protein specifically precipitated with the PrtM di-

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**Fig. 8.** The proteinase model. (1) Precursor forms of the maturation protein and the proteinase are synthesized from a proteinase plasmid carrying the *prtM* and *prtP* genes. (2) Lipomodification at position Cys.24 of pre-maturation protein and subsequent cutting by the lactococcal equivalent of E. coli SPase II (prolipoprotein signal peptidase) results in membrane attachment of the maturation protein. Pre-pro-proteinase is translocated across the cell membrane, cleaved by the equivalent of SPase I (signal peptidase) and remains attached to the cell membrane through the membrane anchor in the extreme C-terminus of the enzyme. (3) The maturation protein effects, in a way not yet understood, the removal of the pro-sequence by cleavage between Thr.187-Asp.188 to give a mature active proteinase (numbers refer to amino acid positions in the pre-pro enzyme). (4) Selfdigestion results in the release of a C-terminally truncated proteinase (which can still be active). Incubation in Ca$^{2+}$-free buffer is thought to stimulate selfdigestion by enhancing proteinase activity or by unmasking of selfdigestion sites in the proteinase molecule.
rected antibodies only in cells carrying the \textit{prtM} gene (A.J. Haandrikman, personal communication).

3.2.7. The proteinase model

The available genetic data presented above on the lactococcal proteinases now allow a reinterpretation of the biochemical and immunological data collected in recent years and their integration in a model for lactococcal proteinase. Fig. 8 schematically depicts the salient features of this model.

The first important conclusion from the genetic information is that the caseinases, the extracellular serine proteinases of lactococci, are all very similar enzymes which differ in only relatively few amino acids. This holds true for the enzymes of both subspecies. The amino acid differences, although few in number, do divide the proteinases in two specificity classes: enzymes which can rapidly degrade \(\alpha\)- and \(\beta\)-casein (PIII-type) and those which primarily act on \(\beta\)-casein but with a specificity differing from that of the PIII enzymes (PI-type). Enzymes with a chimeric specificity are found in certain strains and have been produced by genetic manipulation (see Table 1 and section 3.2.8.).

The second new concept is that the proteinases are attached to the cells via a membrane anchor which is present at the extreme C-terminal end of the molecule. The proline-rich region in the C-terminus directly upstream of the membrane anchor might make contacts with cell wall components and serve as a “spacer” between the C-terminus and the N-terminal proteinase domain. Removal of the anchor sequence results in the release of the proteinase, as was the situation with the initial proteinase gene clones, all of which specified C-terminally truncated secreted proteins.

The third aspect of the model concerns the fact that a second gene, \textit{prtM}, is essential to obtain a functional proteinase. The \textit{prtM} gene product is a membrane located lipoprotein which is, in a way not yet understood, involved in the activation of the proteinase. In this respect, the lactococcal proteinases differ from the subtilisins which perform autocatalytic activation without the need for an additional cellular function [56]. Although no direct proof could be obtained due to the fact that the N-termini of both inactive proteinases, the one produced in the absence of PrtM and the active site mutant, could not be determined ([58]; A.J. Haandrikman, personal communication) it is tempting to speculate that the maturation step involves the removal of the pro-region from the inactive pro-proteinase, a step effected by PrtM.

We originally postulated a proteinase degradation model to reconcile biochemical and immunological work with genetic data indicating that a discrepancy existed between the \(M_w\) of the proteinase isolated from \textit{L. lactis} ssp. \textit{cremoris} Wg2 and the size of the product predicted from the proteinase gene of this strain [24, 60]. The principals of this model still hold true and, actually, have been substantiated by the new data. In the new model shown in Fig. 8 the data on PrtM have been incorporated. As is the case for subtilisin, the lactococcal proteinase is synthesized as a pre-proprotein. The size of the proteinase of strain Wg2 as deduced from the nucleotide sequence of its gene is 200 kDa [51]. The putative signal peptide contains 33 amino acids [51, 52], while the pro-region consists of 154 amino acids [47, 51, 54]. Removal of this N-terminal stretch of amino acids would result in a mature enzyme of 1715 amino acids with a molecular size of 187 kDa. Additional proteolytic cleavage was postulated to account for the difference between this calculated size and that of the isolated enzyme (140–165 kDa) [22, 51, 54]. As the purified enzyme starts with Asp.188 [47, 51, 54], the cleavage occurs at the C-terminus of the proteinase. Apparently, large C-terminal deletions can occur without abolishing proteinase activity. This observation was confirmed by in vitro deletion analysis of the proteinase gene: large 3’-end deletions resulted in the production of smaller but still active proteinase molecules ([60], M.J. Gasson, personal communication). Proteolytically active degradation products of the proteinase have been isolated which were approximately 60–80 kDa ([17, 61]; J. Erkelens, personal communication). The 80-kDa proteinase initially isolated from the \textit{lactis} strain 763 [16] must be considered to be such a degradation product as in a later study a 145-kDa proteinase was purified from the same strain. Interestingly, the 80-kDa proteinase fragment was not only active, but also showed the same specificity towards...
casein as the larger enzyme ([62–64]; see below). Recently, it was shown that an 87-kDa degradation product of the Wg2 proteinase still had the same N-terminal amino acid (Asp.188) as the largest proteinase band of 165 kDa present in this preparation, indicating that extensive C-terminal degradation had taken place [54].

In the model presented in Fig. 8, Ca\(^{2+}\) is thought to play an important role in proteinase degradation as it can reduce proteolytic activity at concentrations higher than 10 mM or enhance activity at lower concentrations. The preferred method of proteinase isolation, the “release” of the enzyme from the cells in a Ca\(^{2+}\)-free buffer, is considered to result in autocatalytic degradation. This can either be envisaged by an increase in proteinase activity or by assuming that at low Ca\(^{2+}\) concentrations a putative cleavage site in the C-terminus of the proteinase becomes accessible to proteolytic cleavage. In both cases the result is the liberation of the mature cell envelope-bound proteinase as a large C-terminally truncated protein of 140–165 kDa. This large proteinase fragment can then digest itself to proteolytically active products of lower molecular weight. Several putative “selfdigestion sites” with similarities to digestion sites of lactococcal proteinases in β-casein have been identified in the C-terminus of the Wg2 proteinase [60,64]. Whether one of these sites is actually used for the release of the proteinase from the cell wall remains to be established. The postulated selfdigestion step predicts that proteinase activity is necessary to release the enzyme from the cell envelope. It has already been shown that the inactive proteinase, produced in the absence of PrtM, can not be released from the cells ([58]; A.J. Haandrikman, personal communication). One possibility could be that PrtM performs the C-terminal cleavage step. It appears, however, that the active center of the proteinase itself is involved in proteinase release. The active center mutant of the proteinase (Asp.30 to Asn.30) was normally produced and could be detected on the outside of the cells with monoclonal antibodies. The inactive proteinase, however, could not be released from the cells by incubation in Ca\(^{2+}\)-free buffer (A.J. Haandrikman, personal communication). Evidence in favour of the selfdigestion model was obtained in proteinase inhibition studies [54]. Cells producing proteinase were incubated in the presence of the serine proteinase inhibitor PMSF prior to the incubation step in Ca\(^{2+}\)-free buffer. By using monoclonal antibodies it was shown that the proteinase antigen could not be released from these cells. The inhibited proteinase could only be released by the addition of other proteinases. These observations make a model in which the proteinase is thought to be C-terminally processed, after which the enzyme stays attached to the membrane-bound C-terminus via Ca\(^{2+}\)-bridges, less likely [58,61].

The proteinase degradation model was also used to explain the occurrence of four immunologically distinct proteins, A, A', B and C, in the proteolytic system of several \textit{L. lactis} ssp. \textit{cremoris} strains ([15,22,24,60]; see section 3.1). 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. Autoproteolytic cleavage is assumed to lead to an irreversible conformational change resulting in the exposure of the antigenic sites of conformation B and, at the same time, in the masking or loss of the antigenic sites for conformation A. With the aid of monoclonal antibodies it has been shown that component A can be converted to component B, but not vice versa [65]. B can be further degraded to give smaller products, one of which may be protein C found in some \textit{cremoris} strains [22].

### 3.2.8. Engineering of the proteinase specificity

As discussed in section 3.1., two main proteinase substrate specificity classes exist: PI enzymes mainly hydrolyse β-casein whereas PIII proteinases degrade β-casein with a different specificity and, moreover, α-casein. The genes of a representative of each class have been cloned and sequenced: that of the \textit{cremoris} strain Wg2 (PI; [44,51]) and that of \textit{cremoris} strain SK11 (PIII; [45,52]). Both proteinases had the original specificity in the heterologous host \textit{L. lactis} ssp. \textit{lactis}. Moreover, they retained their specificity despite the fact that their genes had been cloned only partially, resulting in C-terminally truncated enzymes. Three \textit{L. lactis} ssp. \textit{cremoris} strains seem
to have a mixed PI- and PIII-type specificity: TR, FD27 and E8 [20]. To examine whether this was due to the presence of two proteinases with different specificities in one cell, or to a proteinase with a mixed specificity, the proteinase gene of strain E8 was cloned (A.J. Haandrikman et al., unpublished). *L. lactis* ssp. *lactis* MG1363 carrying the E8 gene produced a proteinase with the original E8 specificity, indicating that the mixed specificity of strain E8 is caused by a single proteinase.

The knowledge of the sequences of the two proteinase genes of the different specificity classes was the basis for a joint Dutch project in which hybrid PI/PIII proteinase genes were constructed to investigate which of the 44 amino acid substitutions contributed to the differences in casein cleavage specificity (P. Vos et al., unpublished). DNA fragments were reciprocally exchanged between the two proteinase genes. The proteinases produced in *L. lactis* host strains carrying the various hybrid genes thus constructed were isolated and their caseinolytic properties were examined. In this way, two regions in the lactococcal proteinase were identified which are involved in cleavage specificity. One region showed homology to the substrate binding region of subtilisin and comprises seven amino acid differences. The other region is located in the C-terminal part of the lactococcal proteinase which is not present in subtilisin. Two amino acids in this part of the proteinase, residues 747 and 748 of the mature enzyme, appeared to be important for cleavage specificity. Interestingly, some of the hybrids had a specificity of casein degradation which was different from either of the wild-type enzymes. With simple genetic techniques it is therefore possible to create proteinases with new caseinolytic specificities. Such "new" proteinases may also be generated by single amino acid substitutions using SDM techniques, when the two regions essential for cleavage specificity have been further delineated.

### 3.2.9. Proteinases in lactic acid bacteria other than lactococci

Only few data are available on the presence of proteinases in species of lactic acid bacteria other than lactococci (lactobacilli, leuconostocs, pediococci) and information on the genetics of these enzymes is lacking completely. Proteinases have been detected in *Lactobacillus helveticus*, *Lb. lactis*, *Lb. bulgaricus*, *Lb. casei*, *Lb. acidophilus* and *Lb. plantarum* [66–71]. The enzymes from *Lb. acidophilus* and *Lb. helveticus* have been isolated and characterized [70,71]. Both proteinases could be liberated from whole cells by repeated washing in a Ca²⁺-free buffer. In the case of the *Lb. helveticus* enzyme the level of LDH activity in the crude extract was 3% indicating that liberation of intracellular enzymes was limited. Using polyacrylamide gel electrophoresis, the molecular size of the proteinase of *Lb. acidophilus* was estimated to be approximately 145 kDa and is therefore in the range of that of the lactococcal proteinases. Both lactobacillus enzymes were severely inhibited by DFP and PMSF and not by ethylenediaminetetraacetic acid (EDTA), indicating that they are serine proteinases. The *Lb. acidophilus* proteinase resembles the lactococcal enzymes in having a rather acid pH-optimum (pH₉₀ around 5.7) while the optimum pH of the *helveticus* enzyme was 7.5–8.0. The digestion sites of the proteinase from *Lb. helveticus* on a₅- and β-casein have been determined and show that the enzyme has a broad cleavage specificity.

Leuconostoc species show a limited ability to grow in milk, which, in part, is caused by the lack of proteolytic activity in these organisms. Via conjugation, lactococcal proteinase plasmids have been introduced in *Leuconostoc* and lactose/proteinase deficient wild strains of *L. lactis* ([72]; C. Daly, G.F. Fitzgerald and M.J. Gasson, personal communication). The resulting transconjugants were weakly proteolytically active. Transfer of the proteinase plasmid from the transconjugants back into the original *L. lactis* strain resulted in full proteolytic activity in this recipient. Apparently, the weak proteolytic activity was not caused by alterations in the proteinase gene but was the result of inferior gene expression or poor utilization of the products resulting from initial proteolysis.

### 4. PEPTIDASES

The second step in casein utilization involves the degradation of the relatively large peptide
fragments produced by the proteinases to smaller products by peptidases. Owing to their importance, research on this set of enzymes has intensified since the early 70s, which is reflected in the publication of several reviews on the subject in recent years [8–10,65,73,74]. The study of peptidases has involved the isolation and biochemical characterization of the (partially) purified enzymes and by now a number of different peptidase activities has been described. Aminopeptidases, di- and tripeptidases, an aryl-peptidyl amidase, aminopeptidase P, proline iminopeptidase, prolactase and prolidase, X-prolyl-dipeptidyl aminopeptidases, endopeptidases, and carboxypeptidases have all been found in various species of lactic acid bacteria. A summary of the available data is presented below. As the first genetic data on peptidases are emerging, the importance of this development in relation to the biochemical analyses will be discussed.

4.1. Peptidase isolation and characterization

A number of factors complicate the analysis of peptidases in lactic acid bacteria. These have been recognized and thoroughly discussed in recent literature [8–10]. There seem to be a large number of different peptidases present in any one strain,

### Table 3

<table>
<thead>
<tr>
<th>Peptidase</th>
<th>Strain</th>
<th>$M_w$ ($\times 10^3$)</th>
<th>Type*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>General AP</td>
<td><em>Lb. acidophilus</em></td>
<td>38+/156 *</td>
<td>metallo</td>
<td>[81]</td>
</tr>
<tr>
<td>General AP</td>
<td><em>L. cremoris</em> AC1</td>
<td>36 *</td>
<td>metallo</td>
<td>[13]</td>
</tr>
<tr>
<td>General AP</td>
<td><em>Lb. lactis</em> 1183</td>
<td>78+/81 *</td>
<td>metallo</td>
<td>[82]</td>
</tr>
<tr>
<td>General AP</td>
<td><em>L. cremoris</em> Wg2</td>
<td>95+</td>
<td>metallo</td>
<td>[85]</td>
</tr>
<tr>
<td>General AP</td>
<td><em>L. lactis</em> 267</td>
<td>85 *</td>
<td>metallo</td>
<td>[83]</td>
</tr>
<tr>
<td>General AP</td>
<td><em>L. bulgaricus</em></td>
<td>95 *</td>
<td>metallo</td>
<td>[114]</td>
</tr>
<tr>
<td>General AP</td>
<td><em>L. cremoris</em> AM2</td>
<td>50+/300 *</td>
<td>–</td>
<td>[84]</td>
</tr>
<tr>
<td>Ap A</td>
<td><em>L. cremoris</em> HP</td>
<td>43+/130 *</td>
<td>metallo</td>
<td>[86]</td>
</tr>
<tr>
<td>X-PDAP</td>
<td><em>Lb. lactis</em></td>
<td>90+/165 *</td>
<td>serine</td>
<td>[90]</td>
</tr>
<tr>
<td>X-PDAP</td>
<td><em>S. thermophilus</em></td>
<td>80+/165 *</td>
<td>serine</td>
<td>[90]</td>
</tr>
<tr>
<td>X-PDAP</td>
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<td>90+/160–180 *</td>
<td>serine</td>
<td>[91]</td>
</tr>
<tr>
<td>X-PDAP</td>
<td><em>Lb. bulgaricus</em> 397</td>
<td>82 *</td>
<td>serine</td>
<td>[93]</td>
</tr>
<tr>
<td>X-PDAP</td>
<td><em>L. lactis</em> 763</td>
<td>85+/190 *</td>
<td>serine</td>
<td>[92]</td>
</tr>
<tr>
<td>X-PDAP</td>
<td><em>Lb. helveticus</em> 32</td>
<td>72 b</td>
<td>serine</td>
<td>[94]</td>
</tr>
<tr>
<td>Prolidase</td>
<td><em>L. cremoris</em> H61</td>
<td>43 *</td>
<td>metallo</td>
<td>[88]</td>
</tr>
<tr>
<td>Proline imino peptidase</td>
<td><em>L. cremoris</em> HP</td>
<td>45+/130 *</td>
<td>metallo</td>
<td>[95]</td>
</tr>
<tr>
<td>Dipeptidase</td>
<td><em>L. cremoris</em> H61</td>
<td>100 *</td>
<td>metallo</td>
<td>[96]</td>
</tr>
<tr>
<td>Dipeptidase</td>
<td><em>L. cremoris</em> Wg2</td>
<td>49 *</td>
<td>metallo</td>
<td>[98]</td>
</tr>
<tr>
<td>Tripeptidase</td>
<td><em>L. lactis</em> 267</td>
<td>75 *</td>
<td>metallo</td>
<td>[83]</td>
</tr>
<tr>
<td>Tripeptidase</td>
<td><em>L. cremoris</em> Wg2</td>
<td>52+/103 *</td>
<td>metallo</td>
<td>[104]</td>
</tr>
<tr>
<td>Endopeptidase</td>
<td><em>L. cremoris</em> H61</td>
<td>98 *</td>
<td>metallo</td>
<td>[108]</td>
</tr>
<tr>
<td>Endopeptidase</td>
<td><em>L. cremoris</em> H61</td>
<td>40+/80 *</td>
<td>metallo</td>
<td>[109]</td>
</tr>
<tr>
<td>Aryl peptidyl amidase</td>
<td><em>Lb. casei</em> 151</td>
<td>ND</td>
<td>–</td>
<td>[105]</td>
</tr>
</tbody>
</table>

* metallo, serine: enzymes are inhibited by EDTA or PMSF, respectively.

+ determined on both native- and SDS-polyacrylamide gels.

+ determined by SDS-polyacrylamide gel electrophoresis.

* determined by gel filtration.

AP, aminopeptidase; X-PDAP, X-prolyl dipeptidyl aminopeptidase.

ND, not determined.
which, of course, might reflect the complexity of the peptidase system itself. However, part of this complexity is caused by the choice of substrates used to characterize the enzymes while the differences in the substrates used by the various authors make it difficult to compare the peptidases under study. Furthermore, the use of cell-free extracts, even when they are fractionated, may result in the failure to resolve distinct peptidase activities.

Fractionated cell extracts have been used in many studies to estimate the number of different peptidase activities present in one or only a few strains [75–80]. In a recent study [78], 11 strains of lactococci were classified into three groups on the basis of peptidase activity profiles towards a wide range of substrates (including amino acyl para-nitroanilides (-pNAs), di-, tri-, and tetrapeptides and carbobenzoxy peptides). After DEAE cellulose fractionation of cell-free extracts, three to four different peptidases were detected and characterized on the basis of substrate specificity, pH dependency and effect of inhibitors and metal ions. This and other work strengthens the view [10] that the peptidase complex of lactic acid bacteria consists of only a relatively small number of exopeptidases. Recent literature shows that the emphasis in this field of research is changing towards the isolation and purification of single peptidases to allow a detailed analysis of the enzymes. A summary of the peptidases purified so far is given in Table 3.

### 4.1.1. General aminopeptidases

An aminopeptidase with a broad substrate specificity has been detected in *L. lactis* ssp. *cremoris* nTR [78]. Apart from amino acyl-pNAs, the enzyme was able to hydrolyse certain di-, tri- and tetrapeptides. In this respect, the enzyme is similar to the purified aminopeptidases from *Lb. acidophilus* R26 [81], *Lb. lactis* [82], the AP I enzyme from *L. lactis* ssp. *lactis* CNRZ 267 [83] and the aminopeptidases from the *L. lactis* ssp. *cremoris* strains AM2 [84] and Wg2 [85] (see Table 3). The aminopeptidase from strain Wg2 does not hydrolyse dipeptides containing N-terminal alanine or phenylalanine, while the enzyme from *Lb. lactis* is able to degrade such dipeptides, provided that the second amino acid is basic or aromatic. Ala-Ala (Ala₂) is not hydrolysed by the Wg2 aminopeptidase whereas Ala₃ to Ala₆ are substrates, suggesting that the enzyme preferentially hydrolyses peptides with more than two amino acids. The aminopeptidase from *cremoris* AC1 possibly falls in the same class of broad substrate specificity general aminopeptidases as it reacts not only with amino acyl-pNAs and dipeptides but also with the larger peptides produced from β-casein by the action of lactococcal proteinase [13,70]. All the aminopeptidases mentioned above, with the exception of that from strain AM2, are metalloenzymes as they are inhibited by metal-complexing agents such as EDTA. The molecular size of the AM2 aminopeptidase is different from that of the Wg2 enzyme (50 kDa versus 95 kDa) and both enzymes have different N-terminal amino acid sequences [84,85]. All enzymes, including the one from strain AM2, are inhibited by sulfhydryl-blocking reagents and do not show carboxypeptidase or endopeptidase activity. It appears that aminopeptidases with a broad substrate specificity are common in lactic acid bacteria, and it is of interest to see how many different enzymes constitute this class of general aminopeptidases.

In *L. lactis* ssp. *lactis* 527 an aminopeptidase activity of much more confined specificity has been detected. It only hydrolyses amino acyl-pNAs and Leu₃ but none of the other di-, tri- and tetrapeptides tested [78]. This enzyme, therefore, does not belong to the broad-substrate-specificity-class. A completely different aminopeptidase was purified from *L. lactis* ssp. *cremoris* HP. This enzyme appears to be highly specific for glutamyl- and aspartyl peptides and thus resembles mammalian aminopeptidase A. It is a trimeric metalloenzyme with identical subunits and has a native molecular size of 130 kDa [86].

### 4.1.2. Proline-specific peptidases

Proline-containing dipeptides and oligopeptides containing an N-terminal proline residue are not hydrolysed by the general aminopeptidases nor by the broad specificity di- and tripeptidases (see below). In order to be able to degrade the proline-rich oligopeptides liberated from casein by
the action of the proteinase, lactic acid bacteria depend on proline-specific peptidases. Amino-
peptidase P, proline iminopeptidase, imino-
dipeptidase (prolinase), imidodipeptidase (pro-
lidase) and X-prolyl dipeptidyl aminopeptidase
(X-PDAP) have all been detected in the various
species of lactic acid bacteria [70,75,78,87–95].
Several of these enzymes have been purified to
homogeneity and characterized in detail ([88,90–
92,94,95]; see Table 3). All X-PDAPs specifically
degrade peptides of the structure X-Pro-Y… (X
and Y can be any amino acid) but the rate of
hydrolysis depends on the type of amino acids
surrounding proline. Some differences exist be-
tween the X-PDAPs with respect to the substrate
which is hydrolysed most efficiently. Slight activ-
ity with Ala-Ala-pNA is detected but no amino-
peptidase activity is observed. The lactococcal en-
zymes have been shown to split β-casomorphin
(Tyr-Pro-Phe-Pro-Gly-Pro-Ile) starting from the
amino terminus into the successive X-Pro di-
peptides and Ile [91,92]. The native enzymes are
dimers of identical subunits and have molecular
sizes of 160–180 kDa. The published monomeric
sizes range from 80–90 kDa [90–93]. The enzymes
are inhibited by the serine proteinase inhibitors
PMSF and DFP and not by EDTA. The X-PD
from Lb. helveticus is somewhat different in hav-
ing a native monomeric size of 72 kDa [94].

The prolidase of L. lactis ssp.cremoris H61 is
perfectly suited to degrade the products of X-PDP
activity as it is highly specific for X-Pro di-
peptides. The prolidase is completely inhibited by
the metal-chelators EDTA and 1,10-phenantro-
line, and has a $M_w$ of 43 000 ([88]; see Table 3).

Recently, a proline iminopeptidase from L. lac-
tis ssp. cremoris HP has been purified to homo-
genieny. The metalloenzyme is a 130-kDa multi-
tomer of 45 kDa subunits with a preference for tripeptides containing proline as the amino termi-
nal residue ([95]; see Table 3).

4.1.3. Dipeptidases

Dipeptidases of broad substrate specificity have
been purified and characterized from two strains of L. lactis ssp. cremoris ([96–98]; see Table 3).
Both enzymes are metallopeptidases as they are
severely inhibited by EDTA and 1,10-phenantro-
line. The dipeptidase from strain H61 has a $M_w$
which is markedly higher than the enzyme from
strain Wg2 (100 000 versus 49 000, respectively).
Both enzymes hydrolyse various dipeptides but
not tri- and tetrapeptides. Both enzymes fail to
hydrolyse dipeptides having glycine or proline as
the N-terminal amino acid. Differences in $M_w$,
turnover numbers, metal dependencies and sub-
strate specificities indicate that both enzymes are
distinctly different. Dipeptidase activities showing
a broad substrate specificity have been detected in
several other lactic acid bacteria [78,80,99–103].
The general dipeptidase peak detected in Lacto-
coccus [78] is contaminated with a prolidase as,
most probably, are the dipeptidase peaks observed
by Kolstad and Law [80]. Even when the substrate
specificity profiles are reinterpreted on the basis
of this assumption, the remaining dipeptidase ac-
tivity in the peaks is of the broad-substrate-
specificity-type, indicating that these types of di-
peptidases may be widely distributed among lactic
acid bacteria.

4.1.4. Tripeptidases

Various authors have described the existence of
tripeptidase activity in lactic acid bacteria
[75,77,78,83,95,103,104]. To date, there are three
reports on the purification of a tripeptidase. One
concerns the proline-specific tripeptidase de-
scribed above [95]. The specificity of this enzyme
clearly differs from the partially purified tripepti-
dase (AP II) from L. lactis ssp. lactis CNRZ 267
and a tripeptidase which has been purified to
homogeniety from the cremoris strain Wg2
([83,104]; see Table 3). All three enzymes are
inhibited by EDTA. EDTA-inactivated AP II can 2+ be reactivated by Co ions while the Wg2 enzyme
can be reactivated by Zn$^{2+}$, Mn$^{2+}$ and to a lesser
extent by Co$^{2+}$. The Wg2 tripeptidase is inhibited
by reducing agents such as dithiothreitol and β-
mercaptoethanol and has a broad specificity for
tripeptides only. It does not attack tripeptides
containing proline in the second position. Like the
Wg2 enzyme, AP II is active on a number of
different tripeptides. Both tripeptidases differ in
their molecular size which is 103–105 kDa for the
Wg2 enzyme and 75 kDa for AP II. Both similari-
ties (EDTA inactivation and reactivation) and dif-
ferences ($M_w$ and sensitivity to β-mercaptoethanol) exist with the intracellular tripeptidase activities analysed by Law [77] in cell-free extracts of different lactococcal strains. These results suggest that a broad substrate specificity tripeptidase may be a common enzyme in the peptidase complex of lactococci. As yet, no data are available on the possible existence of such broad substrate specificity tripeptidases in other species of lactic acid bacteria.

4.1.5. Carboxypeptidases

The peptidases described above hydrolyse their substrates starting from the N-terminus. The presence in lactic acid bacteria of carboxypeptidases, attacking substrates from the C-terminal end, has been examined in a number of studies. The conclusion from this work seems to be that this activity is not present in lactococci, *S. thermophilus*, *Lb. helveticus* and *Lb. bulgaricus* [78,87,105,106]. Apparently, casein derived peptides are degraded by these organisms from the N-terminus only. Two studies, however, show that *Lb. casei* and *Lb. plantarum* do contain an activity on N-terminally blocked dipeptides: a carboxypeptidase which was highly specific for carbobenzoxyglycyl arginine was found in *Lb. casei* 151 [101,107]. This is in contrast with a later study in which it was shown that the same strain contained a carboxypeptidase activity with a broad substrate specificity. A similar activity was found in *Lb. plantarum* [103]. *Lb. lactis* appears to produce a carboxypeptidase hydrolysing the substrates N-benzoyl-Gly-Arg and N-benzoyl-Gly-Phe [82].

4.1.6. Endopeptidases

The peptidases described so far are exopeptidases, enzymes which remove one or, in the case of the X-prolyl dipeptidyl aminopeptidase, two amino acids from a peptide. Recently, two studies describe the purification and characterization of two endopeptidases, LEP I and LEP II ([108,109]; see Table 3). Both enzymes were purified from *L. lactis* ssp. cremoris H61 and are metallopeptidases with unique substrate specificities. They were isolated using αs1-CN(1–23) as the substrate. This peptide, liberated from αs1-casein by chymosin, and αs1-CN(91–100) are hydrolysed by both peptidases (but at different peptide bonds). β- or κ-casein are not degraded, no more than αs1-casein, αs1-CN(1–54) and αs1-CN(61–123) although the latter three substrates do contain the susceptible peptide bonds. Apparently, the spatial conformation of the substrates plays a role in enzyme specificity [108,109]. LEP II has a broader substrate specificity than LEP I and hydrolys various small polypeptides (up to a size of 3.5 kDa) at peptide bonds involving the amino groups of hydrophobic amino acids. In the other reports on endopeptidolytic activity, either N-glutaryl- or succinyl-phenylalanine-pNA or N-acetyl-alanine-pNA were used as the substrate [78,79,101,105–107,110]. As shown by El Soda and Desmazeaud [105], interpretation of results using this kind of substrates should be done with great care as hydrolysis does not necessarily mean that the enzyme involved is an endopeptidase. The activity in *Lb. casei* on succinyl-Phe-pNA appeared to result from a ribosomal aryl-peptidyl amidase (E.C.3.5.1). Recently, an intracellular endopeptidase was partially purified from an *L. lactis* ssp. lactis 763 mutant which lacked the cell envelope-bound proteinase (caseinase) [111]. The enzyme had a $M_w$ of 93000 by gel filtration, was not inhibited by serine or thiol enzyme inhibitors but was affected by EDTA. The enzyme only slowly degraded β-casein but readily cleaved oxidized insulin B-chain with a specificity similar to other intracellular metalloproteinases, isolated from *L. lactis* and *S. thermophilus* [109,112,113].

4.2. Peptidase localization

Despite many attempts none of the above-mentioned peptidases has been unequivocally assigned a cellular location. Determination of the true cellular location of peptidases using fractionated cell extracts is hampered by cross contamination of the fractions. Although both intracellular and extracellular peptidase activities have been described, conclusive evidence on their in vivo location is lacking.

As many of the primary breakdown products of casein are too large to enter the cell (the size exclusion being approximately six amino acid residues [2,6,7], it follows that the cells need (an)
extracellular peptidase(s) to reduce the peptides liberated by the proteinases to a manageable size. To assign an extracellular status to peptidases in the presence of highly active intracellular peptidases requires careful estimation of contamination of the “extracellular” fraction with cytoplasmic enzymes such as aldolase or lactate dehydrogenase. When applying the rigorous criteria needed evidence for the existence of extracellular enzymes in lactic acid bacteria is scanty. Several of the general aminopeptidases are thought to be extracellular [13,82,85,114]. Only for the cremoris Wg2 and the Lb. bulgaricus enzymes intracellular marker enzymes have been used to assess cell lysis [85,114]. The aminopeptidase from cremoris AM2 appears to be intracellular [84] while for the aminopeptidase AP I from L. lactis CNRZ 267 an intracellular location was also claimed [83]. With respect to dipeptidases both intracellular [77,80,98] and extracellular activities have been described. Lactococcal protoplast supernatants devoid of intracellular marker enzymes contained an apparently cell wall-located dipeptidase distinct from the intracellular dipeptidase liberated upon osmotic lysis of the protoplasts [77]. The observation that most of the LEP I endopeptidase activity was present in the cell wall fraction was not substantiated [108]. Except for one report [91], all publications concerning X-prolyl dipeptidyl aminopeptidase agree on an intracellular location for this enzyme ([90,92,93,94]; see below). The location of the prolidase from L. lactis ssp. cremoris H61 has not been determined [88]. The fact that proline is an essential amino acid for cremoris [1] and the observation that proline is not taken up by L. lactis as a free amino acid but, instead, is very efficiently transported as a dipeptide ([115]; see section 5.2.) justify the speculation that this enzyme (and, in fact, all peptidases liberating free proline from a peptide) is intracellular in L. lactis ssp. cremoris.

4.3 Peptidase mutants

The elucidation of the complexity of the peptidase system, of the number of different peptidases involved and the way peptidase synthesis is regulated, awaits the isolation of peptidase mutants. The first examples of such mutants have now become available. Atlant et al. [114] isolated peptidase mutants from Lb. bulgaricus by classical mutagenesis techniques: cells were treated with nitrosoguanidine and mutants were screened for their inability to hydrolyse chromogenic substrates by a direct enzymatic assay on bacterial colonies. In this way, mutants were obtained which lack a lysyl-aminopeptidase (AP II). Arginyl- and leucyl-aminopeptidase activities are reduced in the mutants and the remaining activity was attributed to the presence of three other aminopeptidases. In a subsequent study [93], mutants for the X-prolyl-dipeptidyl aminopeptidase of the same strain of Lb. bulgaricus were obtained. Both types of mutants are proteinase positive and have the wild-type amino acid requirements and have the wild-type amino acid requirements and sugar fermentation patterns. Both peptidases were shown to be non-essential for growth in milk. The mutants have the wild-type doubling time in rich (MRS) medium but are characterized by a shorter exponential growth phase leading to a significantly lower biomass. Lack of X-prolyl-dipeptidyl aminopeptidase results in an enhancement of proteolytic activity while mutants devoid of AP II display an increase in X-PDAP activity when cells are grown in milk. These data are indicative of the presence of a common regulatory mechanism controlling the biosynthesis of the three enzymes [93,114]. Mutants in X-PDAP activity have already proved useful for the cloning of X-PDAP structural genes.

4.4 Cloning and sequence of X-prolyl dipeptidyl aminopeptidase

To date, the only peptidase genes cloned and characterized at the nucleotide level are those for X-prolyl dipeptidyl aminopeptidase from two strains of Lactococcus. The X-PDAP gene of L. lactis ssp. cremoris P8/2/47 was isolated in our laboratory by screening an E. coli plasmid bank of chromosomal DNA of P8/2/47 for X-PDAP activity using a chromogenic substrate (B. Mayo et al., unpublished). Expression of the gene in this heterologous host and in B. subtilis proved that the structural gene for X-PDAP had been cloned. The chromosomal piece of DNA was subcloned
on a pWV01-derived cloning vector [39] and with the resulting recombinant plasmid the X-PDAP deficiency of an *L. lactis* mutant obtained after methylimethane sulphonate treatment could be restored. In an independent study, the X-PDAP gene of *L. lactis* ssp. *lactis* 763 was cloned by constructing a chromosomal DNA bank from strain 763 in an isogenic X-PDAP-negative mutant (obtained by nitrosoguanidine mutagenesis). Subsequently, this bank was screened using an enzymatic plate assay and a chromogenic substrate (M. Nardi et al., personal communication). In this way, a plasmid was obtained which not only restored the X-PDAP deficiency in the lactococcal host, but also conferred the X-PDAP phenotype to *B. subtilis*, indicating that the entire structural gene had been cloned. In both cases, the plasmids carrying the X-PDAP structural genes had copy numbers of approximately 5 to 10 per chromosome equivalent. However, the increase of the number of copies of the X-PDAP gene in *L. lactis* did not result in a significant increase in X-PDAP activity, suggesting regulation of (expression of) the X-PDAP gene. Both genes have been sequenced completely and contain 763 codons. An amino acid comparison indicated an almost complete homology between the two peptidases. The size of the protein deduced from the inferred amino acid sequence (88 kDa) corresponds well with that of the purified enzyme (approximately 90 kDa; [91,92]). The N-terminus of the predicted protein does not contain a signal peptide-like sequence. Rather, the N-terminal amino acid sequence of purified X-PDAP of *cremoris* P8/2/47 (W. Bockelmann, personal communication) was identical to the N-terminal stretch of amino acids deduced from its gene. These results indicate that X-PDAP is not processed at the N-terminus and that the cellular location of the enzyme awaits careful reexamination of the localization studies using specific antibodies.

5. AMINO ACID AND PEPTIDE UTILIZATION

During the first stages of multiplication in milk and, subsequently through the combined action of proteinases and peptidases on milk proteins, lactic acid bacteria are confronted with small peptides and free amino acids which serve as substrates for growth. Many amino acids are either essential or stimulatory for growth of these multiple auxotrophic organisms. Most lactic acid bacteria can utilize amino acids and peptides to satisfy their growth requirements [6-8,116]. Lactococci possess separate transport systems for amino acids, di- (and tri-), and oligopeptides [6,7,115–117]. Until recently, only limited information was available on the mechanisms by which the amino acid and peptide transport systems operate. The recent development of a procedure for the incorporation of functional proton-motive-force (pmf)-generating systems into bacterial membranes has led to a rapid increase in the knowledge of the bioenergetics of amino acid and peptide transport by lactococci. The intention of this review allows only a brief summary of these data. Excellent reviews on the bioenergetics of amino acid and peptide transport have appeared recently [118–120].

5.1. Amino acid transport

Three types of amino acid transport systems have been identified in lactococci:

(A) pmf-driven transport, in which the uptake is primarily coupled to the proton motive force. The uptake of L-methionine and the branched amino acids L-leucine, L-isoleucine and L-valine, of the neutral amino acids L-serine, L-threonine, L-alanine and glycine, and of the basic amino acid L-lysine is pmf-driven [121–126]. The uptake of L-histidine, L-cysteine, L-tyrosine and L-phenylalanine might also be coupled to the pmf [120,125,127]. L-proline is not taken up in a free form ([115]; see below);

(B) phosphate-bond-linked transport, which is driven by the high-energy phosphate bond of ATP or an ATP-derived metabolite. The uptake of glutamate, glutamine, asparagine and, possibly, aspartate have been shown to be phosphate-bond-driven in lactococci [120,128,129];

(C) exchange transport, which is exemplified by the arginine/ornithine antiporter present in *L. lactis* ssp. *lactis*. The driving force for arginine uptake and ornithine excretion is supplied by the
concentration gradients of the two amino acids. The arginine taken up is metabolized via the arginine deiminase pathway [130–131].

5.2. Peptide transport

Different mechanisms of utilization of peptides with up to six amino acid residues can be envisaged [132,133]. The intact peptide can be transported into the cell where it is subsequently hydrolysed, the peptide uptake can be directly coupled to peptide hydrolysis by a transmembrane peptidase which releases the liberated amino acids inside the cell, or the peptide is first hydrolysed on the outside of the cell, after which the free amino acids are taken up. The latter mechanism has been ruled out for most of the various peptides studied since high extracellular concentrations of the amino acids constituting the peptides did not inhibit the uptake of the peptides [6,7,134]. In a study to discriminate between the first two mechanisms, evidence was presented that peptide transport is not directly coupled to peptide hydrolysis [117]. Dipeptide transport was studied in L. lactis membrane vesicles which were fused with liposomes containing beef heart cytochrome c oxidase as a pmf-generating system. In this way, peptide transport could be studied in the absence of peptidase activity. Accumulation of dipeptides was only observed upon addition of an electron donor to the system and was thus shown to be driven by the pmf. Therefore, dipeptide utilization takes place by a two-step process: first, the peptide is translocated across the cytoplasmic membrane via a specific peptide transport system, while in the second step the peptide is hydrolysed by an intracellular peptidase. The dipeptide transport system has a broad substrate specificity [117]. Slow uptake of dipeptides by de-energized cells via the secondary peptide transport system is driven by the inwardly directed chemical gradient of the peptide which is maintained by intracellular peptidase activity. The latter mechanism of dipeptide utilization is thought to play an important role during cheese ripening [117]. In a subsequent study, Smid et al. isolated spontaneous mutants of L. lactis ML3 deficient in dipeptide transport, by selecting for resistance to the toxic chlorated dipeptide L-alanyl-β-chloro-L-alanine. With this mutant they showed that the dipeptide transport system can also transport tripeptides [115]. The mutant has full proteinase- and peptidase activity and the uptake of amino acids and tetra-, penta-, and hexapeptides is unaffected. Nevertheless, the mutant is unable to grow on media containing casein as the sole source of nitrogen, indicating that during casein degradation one or more essential or stimulatory amino acids mainly become available as di- or tripeptides. Because L. lactis can not take up L-proline, this amino acid, most likely, enters the cell in a peptide-bound configuration. Apart from a functional set of proteolytic enzymes, dipeptide transport is therefore also an essential component in the process of casein utilization by lactococci.

Interestingly, the uptake rate of various amino acids is significantly increased in the dipeptide transport mutant, suggesting that dipeptide transport plays a role in the regulation of some of the amino acid transport systems. The possible involvement of peptides in the expression of amino acid transport systems was noted earlier [135]. The 10-fold reduced expression of the branched-chain amino acid carrier in cells grown in complex broth, as compared to cells cultivated in a chemically defined medium supplemented with amino acids, was postulated to be attributable to a (partially) repressed synthesis of the carrier by the presence of peptides.

6. CONCLUSIONS AND FUTURE PROSPECTS

The knowledge of the genetic basis for the proteolytic activity of lactococci has increased considerably since clear proof was furnished in the 70s that plasmid DNA governs this trait in many strains. Now, some 10 years after the first report on proteinase plasmids, proteinase genes of several strains have been cloned and three of these have been sequenced. The sequences have convincingly demonstrated that the lactococcal proteinases belong to the serine proteinases. By combining biochemical, immunological, and genetic data a substantial simplification of the proteinase complex
was obtained. Only one cell-envelope-bound proteinase is produced by lactococci from a “basic” proteinase gene. Because the enzyme is extremely unstable, smaller active degradation products may be formed and it is this instability which has caused the confusion about the apparent complexity of the proteinase system. The proteinase synthesized may be slightly different in the various lactococcal strains as a result of a small number of mutations in the proteinase gene. These mutations may lead to amino acid substitutions and to the production of a proteinase with an altered caseinolytic specificity.

With molecular genetic techniques two regions in the proteinase have been identified which are involved in enzyme specificity. This knowledge was used to manipulate the specificity of the proteinase and has led to the production of proteinases with new caseinolytic specificities. Ultimately, site directed mutagenesis strategies might result in the construction of proteinases with improved properties such as a higher stability or an altered casein breakdown specificity such that the liberation of bitter peptides from casein is reduced or even abolished.

In the future, by using the homology with subtilisin as a guide-line, knowledge-based alterations in the proteinase will lead to the unravelling of the function of the various regions in the enzyme. One such alteration, the change of the active site aspartate (residue 30 of the mature enzyme) into asparagine has led to the production of an inactive form of the proteinase. This mutation has already helped in gaining more insight in the process of proteinase maturation. Maturation of the inactive precursor-form of the lactococcal proteinase into the active mature enzyme involves a digestion step for which a second protein is indispensible. This maturation process is unique for prokaryotes and the next goal will be the elucidation of the precise mechanism.

As DNA probes of the proteinase system are available now, an important field of study in the future will be the regulation of expression of the genes for proteinase and the proteinase maturation protein, an at present underexposed aspect of proteinase synthesis. Furthermore, the proteinase gene probes are used at the moment to investigate whether similar genes are present in other lactic acid bacteria. The availability of synthetic DNA primers for the proteinase gene opens the possibility to use the Polymerase Chain Reaction to speed up the cloning of such genes.

An explanation for the similarity in the organization and nucleotide sequences of the proteinase loci in lactococci is provided by the occurrence of insertion elements on proteinase plasmids [136–141]. Immediately downstream of \( \text{prtM} \) an insertion sequence is present in the proteinase plasmids of strains Wg2 and SK11 (see Fig. 4). The IS elements have been sequenced [141] and both are similar to the two IS elements found on the lactose/proteinase plasmid pSK08 (which is probably identical to pLP712 shown in Fig. 4). It is conceivable that through cointegratc formation with conjugative plasmids the genes have been rapidly disseminated within strains of lactococci.

Compared to the genetic knowledge of the proteinase system, that of the peptidases of lactic acid bacteria is lagging far behind. This is partly due to the lack of mutants in peptidase activity. The genes for the various peptidases are, most probably, located on the chromosome, which makes their analysis more complicated. Using classical mutagenesis techniques, the first peptidase mutants have been isolated and characterized. With these mutants the first data on regulation of peptidase synthesis have been obtained. Moreover, they enabled the detection of peptidase activities which were partly masked in the wild-type strain. The availability of X-prolyl-dipeptidyl aminopeptidase mutants facilitated the cloning and analysis of the structural gene of this peptidase. These studies are the first to illustrate the way to tackle the next important challenge: the elucidation of the complexity of the peptidase system of lactic acid bacteria. In particular, the important issue of peptidase localization needs attention. For this, the isolation to homogeneity of the various enzymes is a prerequisite and is presently undertaken by several groups. Undoubtedly, this will lead in the (near) future to the availability of specific antibodies so that the question of the cellular distribution can be properly addressed with immunological techniques. In this respect, the availability of antibodies against a strictly
membrane-bound protein (the proteinase maturation protein PrtM) may prove of great help as they could be used to determine the degree of contamination of cell extracts with membranes.

The know-how on the mechanisms and energetics of amino acid and peptide transport in lactococci is rapidly increasing. This is largely due to the development of a procedure for the functional incorporation of proton-motive-force-generating systems into bacterial membranes. Three distinct mechanisms of amino acid transport are operative in lactococci, while a pmf-dependent dipeptide transport system has recently been identified. The isolation of a mutant in di- and tripeptide transport enabled the detailed analysis of peptide uptake and indicated that it is essential for growth of Lactococcus lactis in milk. Again, the isolation and characterization of mutants and of genes involved in amino acid and peptide uptake will be inevitable for a complete understanding of these uptake systems.

The development during the 80s of techniques which enabled the application of recombinant DNA methodology to lactic acid bacteria stood at the basis of the current understanding of the proteinase system of lactococci. Continued fundamental research devoted to the development of genetic engineering techniques to effectively manipulate lactic acid bacteria is a guarantee that new insights will be rapidly gained. Such new developments which have already proved useful are the construction of expression vectors and vectors which can integrate in the chromosome of lactic acid bacteria [42,142–147]. With the first type of vectors it is now possible to express homologous genes and to increase the expression of homologous genes in lactic acid bacteria. By using an expression vector the neutral proteinase of B. subtilis has been expressed in L. lactis, resulting in an increased proteolytic ability of the new strain (M. van de Gachte, personal communication). The integration vectors have been helpful in two ways. First, they have been used to stably integrate one or multiple copies of the proteinase gene of cremoris strain Wg2 into the chromosome of L. lactis resulting in new strains of which the properties are under investigation (K.J. Leenhouts, personal communication). Second, one of the vectors was used to make a strain in which the chromosomal X-prolyl dipeptidyl aminopeptidase gene had been inactivated (K.J. Leenhouts, personal communication). The fact that by using the integration vectors we can now make specific mutations in the chromosome will be of major importance for the analysis of genetics and regulation of proteolysis in the lactic acid bacteria.

In conclusion, we can be confident that we will see a rapid increase in knowledge of the genetic basis of proteolysis in lactic acid bacteria and in our understanding of the functioning of this trait which is of such eminent importance in food fermentations.

ACKNOWLEDGEMENTS

I would like to thank Dr. Gerard Venema for critical reading of the manuscript, Alfred Haandrikan, Tjakko Abee, Harry laan and Eddy Smid for helpful discussions throughout this work and Henk Mulder for preparation of the figures. I am grateful to my colleagues for communicating their results prior to publication.

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