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Genetic Manipulation of the Peptidolytic System in Lactic Acid Bacteria

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ABSTRACT

Due to their presumed involvement in product flavour the peptidases of lactic acid bacteria have been subject to extensive research. A major breakthrough was the isolation and purification of the various enzymes to homogeneity. This allowed a re-evaluation of the number of different enzymes in one species by careful investigation of their specificities. Moreover, the purified proteins offered novel ways to the genetic analysis of the peptidolytic system. N-terminal amino acid sequences of, and antibodies against, the purified peptidases were used to clone the majority of peptidases described for Lactococcus lactis. Some peptidase genes, especially those from a number of lactobacilli, were obtained by complementation of peptidase-negative cloning hosts. Thus, by deduction, the amino acid sequences of these peptidases is known and all belong to known families of peptidases. The recently developed chromosomal integration systems for LAB have been used to construct (multiple) peptidase-negative mutants in L. lactis. The growth characteristics of these strains are now under study. As a number of the mutants were made in a food grade way their performance in cheesemaking can be evaluated. The review presented will focus on the latest developments in the genetic analysis of peptidases in LAB and will evaluate our current understanding of peptidolysis by LAB and its importance in dairy fermentations.

INTRODUCTION

Many strains of lactic acid bacteria contain proteolytic systems that allow them to liberate essential and growth stimulatory amino acids and small peptides from the protein-rich substrates such as milk, meat and vegetables in which they are

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primarily found. Centuries of selection on these and other properties of lactic acid bacteria have resulted in the availability of strains very well suited for the production of fermented foods and feed. Since the emergence of large scale industrial fermentation technologies, considerable stress has been placed on the reliable performance of starter strains resulting in an urge to know more about the molecular biology of these economically important microorganisms. The realisation that proteolysis by certain strains of lactic acid bacteria is an unstable trait, has led to a considerable research effort to try and understand the underlying mechanism, and has resulted in a detailed knowledge of the components involved in protein hydrolysis by these organisms. It is clear now that the system used to break down milk protein encompasses a proteinase that performs the initial cleavage of casein, a number of uptake systems that internalize the liberated peptides and amino acids, and an array of peptidases with different cleavage specificities degrading the large peptides into smaller peptides and, ultimately, into free amino acids.

The first step in proteolysis, the degradation of casein by the proteinases of lactic acid bacteria, has been dealt with extensively in recent reviews (Kok, 1990; Pritchard & Coolbear, 1993; Kok & de Vos, 1994). Only salient new aspects of this process will be dealt with in this overview. New insights into the processes of peptide uptake by lactococci have recently accumulated and will be reviewed here. The main body of this work, however, will be the evaluation of the peptidolytic activity of these organisms with emphasis on the genetic analysis of the enzymes involved. Moreover, the possibilities of genetically manipulating the system once the genes and regulatory elements are known will be discussed. As the latter aspect can at present only be fruitfully addressed in the lactococci, the peptidolytic system of these organisms will be dealt with most extensively. Space requirements preclude full citation of all the original literature. Most of these can be found in the review by Kok & de Vos (1994).

**PROTEINASES**

Proteinases from lactococci and lactobacilli used in milk fermentations are closely related. In both groups of organisms the proteinase involved in casein degradation is a cell envelope-located molecule that is initially produced as a preprotein. The precursor is subject to self-cleavage resulting in the mature, active enzyme, a process for which the lipoprotein PrtM is essential. PrtM may be considered as an extracellular chaperone and shows similarities to a protein, PrsA of *Bacillus subtilis*, for which a comparable function has been postulated (for review, see Kok & de Vos, 1994).

Although it is an important component of yoghurt starter cultures used in dairy fermentations, the proteolytic system of *Streptococcus salvarius* ssp. *thermophilus* (*S. thermophilus*) has received much less attention than that of lactococci and lactobacilli. In general, *S. thermophilus* is proteolytically not very active. Among 97 strains of *S. thermophilus* tested only three possessed proteinase activity levels comparable to that of proteinase-positive strains of *L. lactis* (Shahbal et al., 1991). The enzymes of two of these strains appeared refractory to purification (Shahbal et al., 1993). Proteinase activity was not released from the cell wall by incubation of the cells in a Ca\(^{2+}\)-free buffer, a standard technique used to liberate lactococcal
proteinases, indicating that the enzyme is either tightly coupled to the cell wall or subject to rapid denaturation or (self-) digestion during the extraction procedure. The streptococcal proteinases are serine-type enzymes as they are inhibited by PMSF and DFP. The enzymes may be quite different from the lactococcal proteinase as a DNA probe of the lactococcal prt genes did not hybridise with chromosomal DNA of *S. thermophilus*. Moreover, antibodies specific for the *L. lactis* proteinase did not react with proteins from *S. thermophilus*.

The casein-degrading enzymes of Lactococcus and Lactobacillus are serine proteinases, the substrate specificity of which determines the nature of the degradation products liberated from casein and, thus, the growth of the organisms in milk and the generation of flavour compounds in the ultimate fermentation product. The importance in flavour development has resulted in a detailed analysis of the substrate specificity of the *L. lactis* proteinase. Two main specificities have been identified by analysing casein breakdown products by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE). Proteinases of the P<sub>i</sub>-type preferentially degrade β-casein and not, or only slightly, α<sub>j</sub>-casein. P<sub>III</sub>-type proteinases degrade α<sub>j</sub>-and β-casein and the latter with a specificity that is distinct from that of the P<sub>i</sub>-type proteinases (Visser *et al.*, 1986). The specificity of κ-casein breakdown has also been used to distinguish P<sub>i</sub>-and P<sub>III</sub>-type proteinases (Reid *et al.*, 1994). Studies addressing the peptide bond cleavage specificity of representatives of the two proteinase classes using SDS–PAGE have revealed that the lactococcal proteinases are able to liberate relatively small peptides from casein (Reid *et al.*, 1991; Visser *et al.*, 1991; Monnet *et al.*, 1992). A detailed analysis using reverse-phase high performance liquid chromatography (HPLC) combined with mass spectrometry indicated the liberation of numerous small peptides from casein by a P<sub>i</sub>-type proteinase (Juillard *et al.*, 1995). About a hundred different peptides were identified but release of free amino acids other than phenylalanine, or di- and tripeptides was not observed. From this work it is clear that the P<sub>i</sub>-type proteinase has a relatively broad specificity. About 17% of the peptides were composed of four to eight residues. These results emphasize the fact that, apart from the proteinase, *L. lactis* does not need extracellular peptidases to be able to grow in milk. The proteinase produces peptides that contain all the amino acids the cells need and a subset of the peptides are of the size that can be taken up by the oligopeptide transport system (see below). However, a strain producing the P<sub>i</sub>-type proteinase is in short supply of the essential amino acids leucine and histidine. The results of Reid *et al.* (1994) suggest that at least the latter amino acid is provided by the histidine-rich peptides released early from κ-casein.

**PEPTIDASES**

The peptidolytic properties of lactic acid bacteria, most notably those of *L. lactis* and a number of Lactobacillus species, have been studied extensively. Starting with a biochemical analysis of the number of different peptidases in a single strain or species, the purification of the various enzymes to homogeneity and their detailed enzymatic characterization, the emphasis in this field has more recently shifted to the dissection of the genetic organization of the peptidolytic system.
Peptidases of lactobacillus

Table 1 lists the peptidases of various species of Lactobacillus for which the corresponding genes have been cloned and sequenced. Also indicated are those peptidases for which a defined mutation in the peptidase gene is available. It is clear from this compilation that our knowledge of the genetics of the peptidolytic system of lactobacilli is rapidly increasing. In the following, a brief description of the genetic data will be given.

Proline iminopeptidases
The genes of three proline iminopeptidases have been cloned and sequenced to date. The Lactobacillus delbrueckii ssp. bulgaricus (Lb. bulgaricus) proline iminopeptidase PepIP is a proline-specific peptidase capable of hydrolysing di- and tripeptides with proline at the N-terminal position, but not longer peptides

### Table 1

Peptidases from Lactobacilli and Streptococcus thermophilus for which the proteins have been purified or the gene sequenced

<table>
<thead>
<tr>
<th>Organism</th>
<th>Enzyme</th>
<th>Monomer M&lt;sub&gt;c&lt;/sub&gt; (×10&lt;sup&gt;-3&lt;/sup&gt;)</th>
<th>Class</th>
<th>Substrate&lt;sup&gt;d&lt;/sup&gt;</th>
<th>e</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. salivarius ssp. thermophilus</td>
<td>St-PepN</td>
<td>97</td>
<td>metallo</td>
<td>Lys-pNA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>st-pepC</td>
<td>50</td>
<td>thiol</td>
<td>Lys-pNA, Phe-βNA</td>
<td>+</td>
</tr>
<tr>
<td>Lb. delbrueckii ssp. lactis</td>
<td>PepN</td>
<td>95</td>
<td>metallo</td>
<td>Lys-βNA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PepC</td>
<td>51</td>
<td>(thiol)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Gly-Ala-βNA</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>PepL</td>
<td>34</td>
<td>serine</td>
<td>Leu-βNA</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>PepL</td>
<td>35</td>
<td>metallo</td>
<td>Pro-βNA</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>PepV</td>
<td>52</td>
<td>metallo</td>
<td>β-Ala-His</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>PepX</td>
<td>88</td>
<td>serine</td>
<td>Ala-Pro-pNA</td>
<td>+</td>
</tr>
<tr>
<td>Lb. delbrueckii ssp. bulgaricus</td>
<td>PepIP</td>
<td>33</td>
<td>serine</td>
<td>Pro-pNA</td>
<td>+</td>
</tr>
<tr>
<td>Lb. helveticus</td>
<td>PepC</td>
<td>49</td>
<td>(thiol)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Lys-pNA, Y+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PepN</td>
<td>93</td>
<td>metallo</td>
<td>Lys-pNA, Y+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pepXP</td>
<td>88</td>
<td>(serine)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Ala-Pro-pNA, Y+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PepPN</td>
<td>35</td>
<td>(serine)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Pro-Leu</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>PepDI</td>
<td>54</td>
<td></td>
<td>Arg-Leu, Y+</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Literature references are given in the text.

<sup>b</sup> The genetic abbreviation is given where appropriate.

<sup>c</sup> Calculated from the genes when available.

<sup>d</sup> Routinely used substrate.

<sup>e</sup> +, gene cloned and sequenced; Y, knock-out mutant available.

<sup>f</sup> Based on amino acid sequence similarity.
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(Atlan et al., 1994; Gilbert et al., 1994). PepIP has a deduced molecular mass of 33 kDa and forms trimers of Mₖ 100,000. The N-terminal amino acid sequence of the enzyme purified from an E. coli overproducer was the same as that of the primary translation product of pepIP indicating that PepIP does not undergo N-terminal processing in this host. Most probably, the enzyme has an intracellular location in Lb. bulgaricus. A very similar enzyme was found in Lb. delbrueckii ssp. lactis (Lb. lactis). The gene of this protein, pepI, has been cloned and sequenced recently (Klein et al., 1994a). Only eight out of 294 and 295 amino acids that constitute PepI and PepIP, respectively, are different. The proline iminopeptidase from Lb. helveticus, designated PepPN, shows 32% similarity with the iminopeptidases of the two Lb. delbrueckii subspecies (Dudley & Steele, 1994). All three enzymes contain the motif GXSXGG specific for the active site of prolyl oligopeptidases and, accordingly, all three are inhibited by serine proteinase inhibitors.

Aminopeptidases

The pepN gene of Lb. lactis has been cloned by complementation of E. coli for the ability to hydrolyse His-β-naphthylamide (His-βNA) and Phe-βNA (Klein et al., 1993). The deduced molecular mass of the lactobacilicar PepN is 95 kDa and the enzyme is active as a monomer. It is a metallopeptidase belonging to the aminopeptidase N family and is most similar to PepN of L. lactis (62% similarity). Of a number of p-nitroanilide (pNA) substrates tested, Lys-pNA was cleaved best. pepN of Lb. helveticus, encoding the 97-kDa metalloaminopeptidase PepN, has been cloned and sequenced, and overexpressed in L. lactis (Nowakowski et al., 1993; J.L. Steele, pers. comm.). The enzyme from Lb. helveticus showed 72% and 49% amino acid similarity to the PepN aminopeptidases from Lb. lactis and L. lactis, respectively.

Enzymes with similarity to the general aminopeptidase PepC of L. lactis have also been identified in lactobacilli. Recently, the nucleotide sequences of the pepC genes of Lb. lactis and Lb. helveticus became available (Klein et al., 1994b; Fernández et al., 1994). The deduced molecular masses of both enzymes are 50.9 and 48.8 kDa, respectively, and both show high (75%) amino acid sequence identity. The overall identities with lactococcal PepC are 50 and 54%, respectively. Two regions probably involved in catalysis were identified on the basis of homologies with the cysteine proteinase family exemplified by papain and, based on these similarities, the two Lactobacillus PepC's are also taken to be thiol aminopeptidases. Using chromogenic dipeptide- and amino acid substrates in an enzymatic plate assay, PepC from Lb. lactis was shown to be a broad-specificity aminopeptidase, hydrolysing any amino acid from the N-terminal except proline (Klein et al., 1994b).

The gene of an enzyme from Lb. lactis with leucyl-aminopeptidase activity, PepL, has recently been cloned in a multiple peptidase-negative E. coli strain using Leu-βNA as the chromogenic substrate (Klein et al., 1994c). Two peptidase genes were thus isolated: pepN described above and pepL, encoding a protein of 35 kDa. PepL showed homology to the prolinase PepPN of Lb. helveticus (46% identity) and the proline iminopeptidases of both Lb. delbrueckii subspecies (25% identity). Moreover, significant similarities were observed with iminopeptidases of Bacillus subtilis (22% identity) and Neisseria gonorrhoeae (16% identical residues).
Dipeptidases

*Lb. lactis* can hydrolyse unusual β-alanyl-dipeptides such as β-Ala-His (carnosine). The gene encoding this activity, *pepV*, has been isolated from a gene bank of *Lb. lactis* in *E. coli* UK197 (Vongerichten et al., 1994). This strain lacks *pepD* and *hisG* and, therefore, depends on a (cleavable) extracellular histidine source. *pepV*-positive clones were identified by their ability to grow on minimal plates supplemented with carnosine. The deduced enzyme, 470 amino acids with a calculated molecular mass of 52 kDa, showed similarity only with the dipeptidase PepV of *L. lactis* (47% identity over their entire lengths). Distant relationship-predicting computer programmes revealed homology of both enzymes to the ArgE/DapE/CPG2/YscS family of proteins that share the common characteristic of hydrolysing amide bonds in substrates with similar structure and a dependence on cobalt or zinc for activity. Interestingly, PepV was shown by inhibition studies to be a metallopeptidase.

The gene encoding the peptidase activity DPI identified by Nowakowski et al. (1993) has recently been sequenced (J.L. Steele, pers. comm.). The deduced amino acid sequence of DPI, with a calculated molecular mass of 54 kDa, did not show significant similarity to other known proteins.

**X-prolyl dipeptidyl aminopeptidase**

The gene for PepX of *Lb. lactis* was identified by a chromogenic plate assay in a plasmid bank of this species in *E. coli* (Meyer-Barton et al., 1993). The nucleotide sequence revealed that PepX contains 792 amino acids with a calculated molecular mass of 88 kDa. The enzyme is not N-terminally processed, does not seem to contain transmembrane sequences, and is most probably an intracellular enzyme. PepX is 36% identical to the lactococcal PepXP's and shows 70% identity with PepXP of *Lb. helveticus*, for which the gene has recently been cloned and sequenced (Nowakowski et al., 1993; J.L. Steele, pers. comm.)

**Peptidases of Streptococcus thermophilus**

Table 1 lists the enzymes that have been purified to homogeniety from *S. thermophilus*. There are indications that *S. thermophilus* contains a peptidolytic system that may be as complex as that of the lactococci (Chapot-Chartier et al., 1994 and references therein). Genetic data on the system are now emerging.

**Aminopeptidases**

Two aminopeptidases, the streptococcal equivalents of PepN and PepC (st-PepN and st-PepC), have been analysed in quite some detail recently.

st-PepN has been independently isolated to homogeniety by two groups (Midwinter & Pritchard, 1994; Rul et al., 1994). Both groups used Lys-pNA as the substrate during purification. The enzyme is a metallopeptidase with a broad substrate specificity towards pNA amino acids derivatives. Di- and tripeptides are also hydrolysed, with a preference for hydrophobic or basic amino acids at the N-terminal position, but the enzyme shows a preference for longer peptides. It successively removed amino acids from the N-terminal of various oligopeptides but did not cleave peptide bonds on the N-terminal side of a proline residue. The molecular size of st-PepN is 97 kDa and, despite the slight discrepancy in the molecular masses, the PepN activity isolated by Tsakalidou & Kalantzopoulos...
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(1992) may in fact represent the same enzyme. The enzyme is most probably located intracellularly. The N-terminal sequence of 21 amino acids of the mature enzyme shows a fairly high degree of similarity with that of PepN of L. lactis.

The only peptidase gene of S. thermophilus cloned and sequenced thus far is that of st-PepC (Chapot-Chartier et al., 1994). The gene has been cloned by complementation of an E. coli strain for the ability to degrade Phe-βNA. st-PepC is highly similar to PepC from L. lactis (70% identity) and, like the latter, contains a region with strong similarity to the active site of cysteine proteinases. The bacterial PepC-type enzymes and the eukaryotic bleomycin hydrolase appear to constitute a new family of thiol aminopeptidases. st-PepC has a molecular mass of 50.4 kDa and seems to be an intracellular enzyme considering the fact that it is not N-terminally processed. Overproduction in E. coli allowed purification and biochemical characterization of the enzyme. St-PepC exists in a hexameric structure with a mass of 300 kDa and has a broad substrate specificity.

Peptidases from Lactococcus lactis

In Table 2, the latest details of the peptidases purified and/or cloned from Lactococcus are compiled. All enzymes listed in this table attack peptides from the N-terminal end. To date, no carboxypeptidase activity has been described in L. lactis. In total some fourteen peptidases have been studied in detail and will be discussed briefly here. For a more detailed description the reader is recommended to read the original literature given in the reference list or the recent review by Kok & de Vos (1994).

Aminopeptidases

Aminopeptidases are common in lactococci and have been purified to homogeneity from various strains of L. lactis. They can be roughly divided into two classes, the general aminopeptidases PepA, PepC, PepN, and PCP, and the proline-specific peptidases such as PepXP, prolidase, and proline iminopeptidase. Apart from the latter two, the genes of all of the other peptidases have been cloned and sequenced. All general aminopeptidases have neither endopeptidase nor carboxypeptidase activity. All are inhibited by sulfhydryl-blocking agents and EDTA and are, thus, metalloenzymes. PepC is an exception as it is blocked by chemicals reacting with thiol groups.

General aminopeptidases

The gene of PepN, an aminopeptidase with broad substrate specificity, has been sequenced from two strains of L. lactis ssp. cremoris and the two sequences were shown to be almost identical (Tan et al., 1992; Strøman, 1992). PepN was given its name because of its similarities to aminopeptidase N of both eu- and prokaryotic origin. The enzyme has a deduced molecular mass of 95.4 kDa. Of six regions in the amino acid sequence of L. lactis PepN which show similarity to other aminopeptidase N sequences, one region (comprising residues 281 to 301) shows significantly higher sequence similarity and may contain the active site of PepN. This region shows homology to a segment in Zn-dependent neutral proteinases of the thermolysin family that is part of the active site and contains an essential Zn-ion binding site.
TABLE 2
Lactococcal peptidases<sup>a</sup>

<table>
<thead>
<tr>
<th>Enzyme&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Purified</th>
<th>Monomer Class</th>
<th>Substrate&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Gene sequenced</th>
</tr>
</thead>
<tbody>
<tr>
<td>PrtP neutral proteinase</td>
<td>Y&lt;sup&gt;c&lt;/sup&gt;</td>
<td>200</td>
<td>serine</td>
<td>casein</td>
</tr>
<tr>
<td>PepA</td>
<td>Y</td>
<td>39</td>
<td>metallo</td>
<td>Glu/Asp-pNA</td>
</tr>
<tr>
<td>PepC</td>
<td>Y</td>
<td>48</td>
<td>thiol</td>
<td>Leu/Lys-pNA</td>
</tr>
<tr>
<td>PepN</td>
<td>Y</td>
<td>95</td>
<td>metallo</td>
<td>Leu/Lys-pNA</td>
</tr>
<tr>
<td>PepXP</td>
<td>Y</td>
<td>88</td>
<td>serine</td>
<td>X-Pro-pNA</td>
</tr>
<tr>
<td>amino-peptidase P</td>
<td>Y</td>
<td>44</td>
<td>metallo</td>
<td>bradykinin</td>
</tr>
<tr>
<td>PCP</td>
<td>—</td>
<td>25</td>
<td>serine</td>
<td>pyroGlu-pNA</td>
</tr>
<tr>
<td>PepV</td>
<td>Y</td>
<td>49</td>
<td>metallo</td>
<td>Leu-Leu</td>
</tr>
<tr>
<td>PepT</td>
<td>Y</td>
<td>52</td>
<td>metallo</td>
<td>tripeptides</td>
</tr>
<tr>
<td>proline peptidase</td>
<td>Y</td>
<td>43</td>
<td>metallo</td>
<td>X-Pro</td>
</tr>
<tr>
<td>proline imino peptidase</td>
<td>Y</td>
<td>50</td>
<td>metallo</td>
<td>Pro-X-(Y)</td>
</tr>
<tr>
<td>PepF&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Y</td>
<td>70</td>
<td>metallo</td>
<td>bradykinin</td>
</tr>
<tr>
<td>PepO&lt;sup&gt;x&lt;/sup&gt;</td>
<td>Y</td>
<td>70</td>
<td>metallo</td>
<td>Metenkephalin</td>
</tr>
</tbody>
</table>

<sup>a</sup>Literature references are given in the text.
<sup>b</sup>The genetic abbreviation is given where appropriate.
<sup>c</sup>Calculated from the genes when available.
<sup>d</sup>Routinely used substrate.
<sup>e</sup>Y = yes.
<sup>f</sup>PepF is identical to LEP I.
<sup>x</sup>PepO and NOP are the same enzymes.

PepC is a general aminopeptidase present in various strains of lactococci, albeit at different levels of activity. The gene was cloned from *L. lactis* ssp. *cremoris* strain AM2 by complementation of *E. coli* (pepN) (Chapot-Chartier et al., 1992). As with PepN, PepC was given its name on the basis of the genetic study, indicating similarity of the enzyme to cysteine proteinases such as papain and aleurin. A marked difference between PepC and the other cysteine proteinases is that the latter are about half the size of PepC. Two regions encompassing the amino acids of the active centre of papain are especially well conserved and may in fact form the active site of PepC. This would agree with the observation that PepC is inhibited by thiol-reacting reagents.

The genes of two narrow specificity aminopeptidases, glutamyl aminopeptidase (PepA) and pyrrolydonyl carboxylpeptidase (PCP), have recently been isolated and characterized. The *pepA* gene was cloned by employing the N-terminal amino acid sequence of the purified enzyme (M.J. Gasson, pers. comm.). The deduced protein has a molecular mass of 38.1 kDa and is, thus, somewhat smaller than that determined previously by biochemical techniques. The enzyme resembles mammalian aminopeptidase A in that it is highly specific for glutamyl-and
aspartylpeptides (hence the name PepA). It shows homology with an endoglucanase of *Clostridium thermocellum*.

The gene for PCP was cloned by polymerase chain reaction techniques using conserved amino acid sequences of the PCPs of *Bacillus subtilis* and *Streptococcus pneumonia* (A.J. Haandrikman, pers. comm.). The deduced molecular size of lactococcal PCP is 25 kDa and, not surprisingly, the enzyme shows similarity to its bacillar and streptococcal counterparts.

**Proline-specific peptidases**

PepXP, X-prolyl dipeptidyl aminopeptidase, is, as its name indicates, able to cleave X-Pro dipeptides from the N-terminal of oligopeptides. It is the only proline-specific peptidase of Lactococcus to date for which the gene has been cloned and sequenced. The deduced amino acid sequences of the genes cloned from two *L. lactis* ssp. cremoris strains were almost identical and confirmed earlier biochemical data on the size of the enzymes: 88 kDa or 763 amino acids (Mayo *et al*., 1991; Nardi *et al*., 1991). The enzyme does not show significant similarity to other proteins. Recently, the active site of PepXP has been identified by 

\[ ^{3}H\text{DFP} \]

labeling (Chich *et al*., 1992). The active site of PepXP showed only limited homologies with other serine proteinases but the motif GXSYXG was present in all three known X-prolyl dipeptidyl aminopeptidases (PepXP, mammalian DPPIV and yeast DPAB). The presence of a reactive serine residue in the active site fits with inhibitor studies showing that PepXP is inactivated by serine proteinase inhibitors.

Prolidase of *L. lactis* H61 has a specificity that would perfectly complement the action of PepXP: it only degrades X-Pro dipeptides (Kaminogawa *et al*., 1984). The protein has a molecular size of 43 kDa.

A proline iminopeptidase of approximately 50 kDa has been purified from *L. lactis* HP (Baankreis & Exterkate, 1991). It was shown to be present in all lactococcal strains tested but not in lactobacillus species. The enzyme hydrolyzes almost all di- and tripeptides with Pro as the amino terminal residue but does not cleave X-Pro bonds or peptides longer than four residues.

Recently, aminopeptidase P was purified to homogeneity from *L. lactis* NCDO 763 by using bradykinin (RPPGFSPFPR) as the substrate (Mars & Monnet, 1994). The aminopeptidase is a metalloenzyme with a molecular size of 44 kDa. It is highly specific for X-Pro-Pro N-terminal sequences and does not exhibit prolidase-like activity on X-Pro dipeptides. The optimal size of the substrate seems to be near five residues.

**Dipeptidases and tripeptidases**

Recently, dipeptidase genes have been cloned and sequenced from two *L. lactis* ssp. cremoris strains (P. Strøman & F. Mulholland, pers. comm). The deduced amino acid sequences of the enzymes, approximately 52 kDa in size, differed in 18 out of the 473 amino acid residues. PepV, as the enzyme was designated, showed similarities to the carnosinase of *Lb. lactis* described above and to *E. coli* succinyl-diaminopimelate desuccinylase and acetylornithine deacetylase, two enzymes that cleave a peptide bond-like site in their respective substrates. Moreover, these enzymes are similar to carboxypeptidase G2, a Zn-metallopeptidase.

The gene of the tripeptidase PepT of *L. lactis* ssp. cremoris Wg2 has been cloned by reverse genetics (Mierau *et al*., 1994). The pepT gene may be in an
operon with two ORFs of unknown function surrounding it, as no promoter nor terminator structures could be identified immediately upstream or downstream of pepT. PepT contains 413 amino acids and the deduced molecular size, 46 kDa, is somewhat smaller than that determined biochemically (52 kDa). PepT shows significant amino acid sequence similarity with PepT of *Salmonella typhimurium* and carboxypeptidase G of *Pseudomonas putida*.

**Endopeptidases**

Several reports exist on the purification of oligopeptidases from lactococci but, on the basis of their substrate specificities, only two different enzymes seem to be present in these organisms (Monnet, 1994). PepO, purified to homogeneity using Met-enkephalin as the substrate, was the first endopeptidase for which the gene was isolated and sequenced (Mierau *et al.*, 1993). The gene is immediately downstream of the oligopeptide permease operon, opp (see below) and is transcribed from a promoter upstream of oppA, the last gene of opp. PepO contains 627 amino acid residues and has a calculated molecular mass of 71.5 kDa. The enzyme shows striking similarities to the mammalian neutral endopeptidase, enkephalinase, especially in a segment of 42 amino acids containing the putative active site of the protein and a motif (HEXXH) characteristic for zinc-dependent metalloproteinases.

PepF was purified using the fact that it specifically cleaves the F—S bond in bradykinin as a distinctive marker (Mars & Monnet, 1994). The enzyme cleaves peptides containing seven to seventeen amino acid residues, not smaller or larger peptides, while the cleavage site should be at least three residues from the C-terminal of the substrate. The pepF gene was located on the lactose-proteinase plasmid of *L. lactis* NCDO 763 and was cloned by reverse genetics and PCR strategies. PepF contains 601 amino acids with a deduced molecular size of 70 kDa (Monnet *et al.*, 1994). The characteristic Zn-dependent metalloproteinase motif HEXXH was present in position 387 to 391 of the enzyme. PepF shows homology with a number of bacterial metallopeptidases sharing this motif, of which dipeptidyl carboxypeptidase of *E. coli* shows the highest identity. A second region of 33 amino acids shows similarity with creatine kinases from vertebrates and with arginine kinase from lobster.

**AMINO ACID AND PEPTIDE TRANSPORT SYSTEMS**

To date, none of the amino acid carriers described in detail biochemically (for review, see Konings *et al.*, 1989) have been characterized genetically. A system devoted to the internalization of di- and tripeptides and a carrier involved in oligopeptide uptake have been analysed biochemically and genetically relatively recently and will be discussed here.

**Di- and tripeptide uptake**

Di- and tripeptide uptake by lactococci is a proton-motive-force-driven process and the system has a broad substrate specificity (Smid *et al.*, 1989a). It has a high specificity for proline-containing dipeptides, a property which was used to clone the gene involved by complementation of a dipeptide transport-negative proC
mutant of E. coli (Hagting et al., 1994). The di- and tripeptide transporter, DtpT, appeared to be specified by a single gene encoding an integral membrane protein with 12 putative transmembrane sequences. It contains 463 amino acid residues and has a molecular size of 50.6 kDa. So far, DtpT of L. lactis is the only proton-linked peptide transport system described in bacteria.

**Oligopeptide transport**

Oligopeptides are taken up by L. lactis via an ATP-driven transport system (Opp) that is capable of transporting peptides of four to at least eight amino acid residues (Kunji et al., 1993; Tynkkynen et al., 1993). As discussed above, β-casein is degraded by PrtP to peptide fragments of four to thirty residues, of which 17% are smaller than nine residues and, thus, potential substrates for Opp. Indeed, all essential and growth-stimulating amino acids for L. lactis are released from β-casein in the form of peptides that are internalized exclusively by Opp at rates high enough to meet the growth requirements. Only His and Leu had to be added to a chemically defined medium containing β-casein as the protein substrate to allow good growth (Kunji et al., 1995). Inactivation of Opp led to a complete block in oligopeptide uptake and growth in milk or on β-casein.

The five genes encoding the oligopeptide transport system of L. lactis have been cloned and sequenced (Tynkkynen et al., 1993). Opp belongs to the superfamily of ABC transporters. This family encompasses, among others, all bacterial peptide transport systems described so far, with the exception of the lactococcal DtpT (see above). Two genes, oppB and oppC, encode the transmembrane components of the system, oppD and oppF specify the two proteins that are thought to deliver the energy for transport, while oppA codes for the lipoprotein OppA, the substrate-binding protein.

**MANIPULATING THE PEPTIDOLYTIC SYSTEM**

There are several reasons to manipulate the proteolytic and peptidolytic systems of lactic acid bacteria. Firstly, there is a fundamental interest as the availability of mutants in these systems would make it possible to decipher their complexity. Then, the mutants would be very helpful in assessing the importance of the product of a mutated gene(s) in industrial processes. Ultimately, this knowledge would permit the engineering of biochemical properties and product levels of individual components of the proteolytic system in a single strain and in a mixture of strains used in starter blends.

**Manipulation of prt**

The first step in casein breakdown by L. lactis has been manipulated in various ways. As this has been reviewed recently (Kok & de Vos, 1994), it will only be discussed briefly here. Strains lacking proteinase PrtP activity were obtained either spontaneously, as a consequence of proteinase plasmid loss or deletion formation, or have been made by in vitro genetic mutation of active site residues (for reviews, see Kok, 1990; Pritchard & Coolbear, 1993; Kok & de Vos, 1994). These studies have revealed that PrtP is essential for lactococci to grow in milk.
Also, the copy number of the \textit{prt} genes has been steered at will by combining them on a plasmid with a copy number higher than the original proteinase plasmid or by placing them on the chromosome of \textit{L. lactis}. In the latter case, the stability of the trait was shown to be dramatically increased (Leenhouts \textit{et al.}, 1991). The specificity of the lactococcal proteinase has been engineered by constructing hybrids between \textit{P1} and \textit{P}_{III}-type proteinases (Vos \textit{et al.}, 1991). This and subsequent studies have identified regions determining the caseinolytic specificity of the enzyme and, moreover, led to the production of proteinases with novel specificities. In an entirely different approach, Van de Guchte \textit{et al.} (1990) expressed the gene for neutral proteinase of \textit{B. subtilis} in \textit{L. lactis}.

\textbf{Mutations in peptide uptake}

A di- and tripeptide transport deficient mutant has been isolated by selection of \textit{L. lactis} ML3 for resistance to the toxic dipeptide L-alanyl-L-chloro-L-alanine (DiACA), a substrate of this transport system (Smid \textit{et al.}, 1989b). The mutant had normal proteinase and peptidase activities, was unaffected in the accumulation of peptides larger than three residues, but did not grow on a chemically defined medium with casein as the sole nitrogen source. The mutants still possessed residual transport activity for some di- and tripeptides. Recently, a defined mutant in di- and tripeptide uptake was made. DtpT was deleted from the chromosome via homologous recombination (Hagting \textit{et al.}, 1994).

A defined integration mutant in \textit{oppA} was constructed and used to show that the oligopeptide transport system is essential for growth of \textit{L. lactis} on peptides longer than three residues (Tynkkynen \textit{et al.}, 1993). In this study it was shown that without a functional Opp system lactococci are not able to grow in milk. Moreover, the studies of Tynkkynen \textit{et al.} (1993) and Kunji \textit{et al.} (1993) clearly show that peptidases hydrolysing the casein breakdown products delivered by PrtP are all located intracellularly in \textit{L. lactis}.

\textbf{Construction of peptidase mutants}

Peptidase-negative mutants of lactic acid bacteria have been obtained by mutagenizing strains with chemicals such as ethyl methane sulphonate and nitrosoguanidine. Studies in \textit{Lb. bulgaricus} using two of such mutants, one affected in \textit{PepXP} activity, the other one mutated in lysyl-aminopeptidase, have shown that neither is essential for growth of \textit{Lb. bulgaricus} in milk (Atlan \textit{et al.}, 1990). Some evidence was obtained for the existance of a common regulatory mechanism controlling the biosynthesis of the two peptidases and proteinase activity.

A drawback of chemical mutagenesis is that one can never exclude the possibility that, apart from the peptidase gene under study, other parts of the chromosome have been mutated which may or may not directly influence the properties of the strain as far as performance in milk fermentations is concerned. Moreover, only a limited number of peptidases, namely those for which a good selection for mutants exists, will be easily accessible for analysis. This is reflected in the fact that until now chemical mutants in Lactobacillus have been isolated only for \textit{PepXP} and lysyl-aminopeptidase activity. To circumvent these problems a number of groups have used the availability of the genes of several peptidases to make defined chromosomal knock-out mutations. The first example of such a
mutant was *L. lactis* (*pepXP*) (Mayo et al., 1993). Analysis of this mutant showed that PepXP is not essential for growth in milk. Altered degradation of a peptide substrate was observed by an extract of the *pepXP* strain, suggesting that changing the expression of PepXP in a starter strain may have an impact on the peptide composition of fermented milk. In *Lb. helveticus* single and double mutants in *pepC*, *pepN* and *pepXP* have been constructed as well as a *pepC-pepN-pepXP* triple mutant. Moreover, a deletion was made in the chromosomal gene for DPI. These strains are now being analysed with respect to growth in milk and rate of acid production (Bhowmik et al., 1993; J.L. Steele, pers. comm.).

A two-step recombination procedure specifically developed for the easy selection of mutants in any gene, even ones of which the products are difficult to assay, has recently been developed. The strategy, depicted in Fig. 1, ultimately results in a gene replacement mutant that is devoid of foreign DNA (Leenhouts & Venema, 1993). Using this method, a series of isogenic peptidase mutants have been made in *L. lactis* MG1363 (I. Mierau, pers. comm.). A total of sixteen mutants including single, double, triple, quadruple mutants and one quintuple *pep* mutant are available (see Table 3). The *prt* genes of the parental strain *L. lactis* NCD0712 were introduced in each mutant in order to be able to study its growth and acidification rates in milk. Also, as a preliminary characterization the peptidase profiles of all strains have been examined. All strains except for two of the three four-fold mutants and the one quintuple mutant were able to grow in milk (Table 3). In the case of two mutants (*pepC-pepN* and *pepO-pepN*) a slight reduction of approximately 25% was observed in the final pH and optical density of the cultures after 24 h of growth at 30°C. The three strains that did not grow in milk performed even worse than a genuine Prt' strain i.e. a strain lacking active proteinase. From these studies a number of conclusions can be drawn. First, they show that the peptidase system of *L. lactis* is a complex of peptidases with overlapping specificities. Moreover, it is likely that the diversity of degradation products generated by the proteinase and internalised by Opp allows various combinations of peptidases to liberate the essential amino acids. Only if every possible degradation pathway is blocked, as is apparently the case in the five-fold mutant, is growth in milk impeded. The fact that the growth of the quadruple and quintuple mutants is even less than a Prt' strain indicates that residual growth of the Prt' strain is for a large part attributable to the presence of free peptides in milk.

CHEESE TRIALS USING MUTANT OR RECOMBINANT LACTIC ACID BACTERIA

Experimental cheeses using either foodgrade (chemical) mutants of LAB or strains of lactococci that had been engineered genetically have recently been made and scrutinized.

The effect of two chemically-induced mutations, *pepN* and *pepXP*, on flavor development in Gouda-type cheeses has been examined by Baankreis (1992). The amount of mutant cells in the starter blend of 10% bitter-producing Prt' and 90% Prt− cells was varied and the organoleptic quality and amino acid and salt-soluble nitrogen were examined. Increasing the concentration of the *pepXP* strain did not affect bitterness but resulted in a decrease in organoleptic quality. Raising
Fig. 1 Replacement recombination strategy using pORI280, a vector that cannot replicate in a host strain without the plasmid replication protein. ORI\(^+\): plus origin of replication; A,B: 5'- and 3'-ends of gene to be mutated; Δ: internal deletion in gene A/B. For details, see Leenhouts & Venema (1993), from which this figure is an adaptation.

The concentration of the pepN mutant led to a pronounced increase in bitterness which was attributed to the accumulation of low molecular weight bitter peptides.

A Lb. casei ssp. casei DAP (PepXP) mutant has been used to study the role of the enzyme in cheddar cheese ripening (El Abboudi et al., 1992). To cheese milk which had been inoculated with a commercial cheese starter freeze-shocked Lb. casei was added just prior to renneting. No differences in cheese ripening and flavor development were observed between the wild-type and DAP-negative Lb. casei strains.

The effect on ripening and flavor development in cheddar cheese of genetically modified L. lactis has been recently assessed (McGarry et al., 1994). Three-fold overproduction of PrtP did not enhance the quality of the cheese nor did it accelerate ripening. Cheeses made with strains producing the B. subtilis neutral
Peptidolytic system of lactic acid bacteria

TABLE 3
Growth in milk of genetically defined L. lactis MG1363 peptidase mutants

<table>
<thead>
<tr>
<th>Peptidase mutation</th>
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</table>

*a X, T, O, C, N: PepXP-, PepT-, PepO-, PepC, PepN-negative, respectively. All strains carry the lactose/proteinase plasmid pLP712.

*b Measured after 24 h at 30°C.

proteinase scored very poorly with respect to body and texture, although their flavor was satisfactory. αs1- and β-casein in these cheeses were degraded very rapidly and were almost completely hydrolysed after one month of ripening. Clearly, such a powerful proteolytic activity is not desirable. By carefully controlling the amount of recombinant Npr-producing lactococcal cells in a blend with a wild-type starter strain a superior cheese was obtained that had fully ripened after two months (Fitzgerald, pers. comm.).

CONCLUSIONS AND PROSPECTS

The genes of many components of the proteolytic system of lactic acid bacteria have been cloned and analysed at the nucleotide sequence level. The last few years have seen a rapid increase in the number of peptidase genes cloned and sequenced from both lactococci and lactobacilli. In the lactococci, moreover, two peptide uptake systems, one for di-and tripeptides and the other one for oligopeptides, have been biochemically described and genetically dissected. Due to the development of genetic strategies that allow access to the chromosome of lactic acid bacteria it is now possible to specifically target and mutate the various genes involved in proteolysis in order to answer the following questions:
— is the gene under study essential for growth in milk?
— What is the impact of the mutation(s) on cheese ripening and flavor development?

Through the analysis of mutants we have now learned that the proteolytic system of lactococci is an intricate system in which the cell envelope-associated proteinase degrades casein into oligopeptides of four and more residues. The oligopeptide transport system is able to take up peptides with four to at least eight residues and a selection of the oligopeptides produced extracellularly by PrtP are internalized by Opp. These peptides are then further degraded by a complex of intracellular peptidases. Most probably as a result of the overlapping specificities of these peptidases and because essential amino acids will be available as components of different peptides in the diversity of internalized oligopeptides, all of the single, double, and triple pep mutants are able to grow in milk. The latter observation is very important as this will make it possible to analyse the effect of the mutations and, thus, by inference, the impact of the corresponding peptidases on cheese ripening and flavor development. This is the next crucial step to be taken in the process of understanding the intricacies of the proteolytic system of lactic acid bacteria to such an extent that rational modification of this system for the improvement of food products will ultimately be feasible. The set of \textit{L. lactis} mutants compiled in Table 3 provides a good tool to start such an analysis and is especially interesting in this respect as the mutants have been constructed in such a way that they do not contain any foreign DNA and should, therefore, be considered ‘foodgrade’.

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