Bacteriocins from lactic acid bacteria
Venema, Konraad

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CHAPTER I

INTRODUCTION

Koen Venema, Gerard Venema, Jan Kok.
Chapter I

CHAPTER I

INTRODUCTION

1. General introduction.

Lactic acid bacteria (LAB) are of eminent economic importance because of their widespread use in food and feed fermentations and their potential to produce a number of different substances with antimicrobial activity which can be used as biopreservatives. The ability of LAB to inhibit the growth of other bacteria has been known for many years (98). Substances responsible for this antagonistic activity include organic acids, diacetyl, hydrogen peroxide, enzymes, defective phages, lytic agents and bacteriocins (16,76,96).

The past several years have seen an explosion of research activity on bacteriocins produced by LAB (18,50,56,57,65,67,82,95). This field continues to expand, and our knowledge about LAB and their bacteriocins is increasing rapidly, owing to detailed genetic and biochemical studies of the peptides, their mechanism of action, immunity-, processing and secretion systems.

Bacteriocins, as defined by Tagg et al. in 1976 (110), are proteinaceous compounds that kill closely related bacteria. This definition is true for the majority of bacteriocins investigated, but gradually it has become evident that certain bacteriocins may also elicit bactericidal activity against species that are more distantly related to the bacteriocin producer. Moreover, there are examples of bacteriocinogenic complexes of a protein and another heterogeneous moiety that is required for activity, which may include lipid or carbohydrate.

Biochemical and genetic studies led to the classification of bacteriocins of LAB into four distinct classes (65):

(I) lantibiotics; small membrane-active peptides containing the unusual amino acids lanthionine, β-methyl lanthionine, dehydro-alanine and/or dehydro-butyrine;
(II) small (< 5 kDa) heat-stable, non-lanthionine-containing membrane-active peptides characterized by a double glycine processing site in the bacteriocin precursors. This site is not restricted to this class: it is also present in some lantibiotics and in colicin V (see section 2.1.a). Most of the bacteriocins characterized to date, including the lactococcins, belong to this class (see section 2);
(III) large (> 30 kDa) heat-labile proteins;
(IV) complex bacteriocins, composed of protein plus one or more non-proteinaceous moieties (lipid, carbohydrate) required for activity.

Work in this thesis deals with class II bacteriocins, which will be detailed below (for a detailed description of the other bacteriocin classes, the reader is referred to recent excellent reviews: 50,57,65,82).

2. Class II bacteriocins produced by LAB.

This class of membrane active peptide-bacteriocins (Table 1) is characterized by the absence of unusual amino acids and the presence of a double glycine processing site in the bacteriocin precursors (see section 2.1.a). The leader sequence of the precursor bacteriocin is cleaved off by the ABC-transporter involved in secretion of the bacteriocins (see section 2.3.a). Most of the bacteriocins characterized to date, including the lactococcins and pediocin PA-1, the subjects of investigation of this PhD study, belong to this class which can be divided into four subclasses (see also Table 1):

(a) bacteriocins that are active on Listeria; the so-called pediocin-like bacteriocins (83);
(b) complexes that consist of two peptides required for activity;
(c) thiol-activated bacteriocins. The only example so far known is lactococcin B; and
(d) the bacteriocins that can not be grouped in the first three subclasses.

This class of bacteriocins will be discussed with focus on the lactococcins and pediocin PA-1. However, some features of the other subclasses will also be briefly reviewed.

Table 1. Bacteriocins produced by LAB.
### Bacteriocin | Producer | References |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Class IIa bacteriocins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pediocin PA-1/AcH</td>
<td><em>Pediococcus acidilactici</em> PAC1.0/ H</td>
<td>79/6</td>
</tr>
<tr>
<td>sakacin A/curvacin A</td>
<td><em>Lactobacillus sake</em> Lb706/ <em>Lactobacillus curvatus</em> LTH1174</td>
<td>42/111</td>
</tr>
<tr>
<td>sakacin P</td>
<td><em>Lactobacillus sake</em> LTH673</td>
<td>111</td>
</tr>
<tr>
<td>leucocin A-UAL187/ leucocin B-Ta11a</td>
<td><em>Leuconostoc gelidum</em> UAL187/ <em>Leuconostoc carnosum</em> Ta11a</td>
<td>37/24</td>
</tr>
<tr>
<td>mesentericin Y105</td>
<td><em>Leuconostoc mesenteroides</em> Y105</td>
<td>77</td>
</tr>
<tr>
<td>bavarianin A</td>
<td><em>Lactobacillus bavaricus</em> MI401</td>
<td>75</td>
</tr>
<tr>
<td>acidocin A</td>
<td><em>Lactobacillus acidophilus</em> TK9201</td>
<td>59</td>
</tr>
<tr>
<td>carnobacteriocin BM1</td>
<td><em>Carnobacterium piscicola</em> LV17</td>
<td>93</td>
</tr>
<tr>
<td>carnobacteriocin B2</td>
<td><em>Carnobacterium piscicola</em> LV17</td>
<td>93</td>
</tr>
<tr>
<td>sakacin 674</td>
<td><em>Lactobacillus sake</em> Lb674</td>
<td>44</td>
</tr>
<tr>
<td><strong>Class IIb bacteriocins</strong></td>
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<tr>
<td>lactococcin G</td>
<td><em>Lactococcus lactis</em> LMG2081</td>
<td>86</td>
</tr>
<tr>
<td>lactococcin M</td>
<td><em>Lactococcus lactis</em> 9B4, WM4, LMG2130</td>
<td>113,48,109</td>
</tr>
<tr>
<td>lactacin F</td>
<td><em>Lactobacillus johnsonii</em> VPI11088</td>
<td>2</td>
</tr>
<tr>
<td>plantaricin S</td>
<td><em>Lactobacillus plantarum</em> LCPO10</td>
<td>J.L. Ruiz Barba, pers. comm.</td>
</tr>
<tr>
<td>plantaricin A</td>
<td><em>Lactobacillus plantarum</em> C-11</td>
<td>16,87</td>
</tr>
<tr>
<td><strong>Class IIc bacteriocins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lactococcin B</td>
<td><em>Lactococcus lactis</em> 9B4, WM4, LMG2130</td>
<td>Chapter III, this thesis</td>
</tr>
<tr>
<td><strong>Class IIId bacteriocins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lactococcin A/diplococcin</td>
<td><em>Lactococcus lactis</em> 9B4, WM4, LMG2130/346</td>
<td>113,48,109/17</td>
</tr>
<tr>
<td>carnobacteriocin A</td>
<td><em>Carnobacterium piscicola</em> LV17</td>
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</tr>
<tr>
<td>piscicolin LV61</td>
<td><em>Carnobacterium piscicola</em> LV61</td>
<td>43</td>
</tr>
<tr>
<td>colicin V</td>
<td><em>Escherichia coli</em> ATCC14763</td>
<td>33</td>
</tr>
<tr>
<td><strong>sec-dependent secreted bacteriocins.</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>divericin A</td>
<td><em>Carnobacterium divergens</em> LV13</td>
<td>121</td>
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</table>

#### 2.1 Genetics of lactococcin production.

In the most thoroughly characterized examples studied...
so far, the genes specifying lactococcin production, immunity and secretion are plasmid located (48,105,112,115). As an example p9B4-6 from \textit{L. lactis} subsp. \textit{cremoris} 9B4 is shown in Figure 1. From nucleotide sequence and mutational analyses it became clear that this particular plasmid (and probably also pNP2 of \textit{L. lactis} subsp. \textit{lactis} biovar. \textit{diacetylactis} WM4 and the 55-kb bacteriocin plasmid of \textit{L. lactis} subsp. \textit{cremoris} LMG2130) contains three bacteriocin operons, specifying three different bacteriocins, lactococcin A, B and M, and their corresponding immunity proteins. In addition it carries two identical copies of the genes specifying the proteins LcnC and LcnD, required for lactococcin secretion and processing.

2.1.a Lactococcin structural and immunity genes.

Van Belkum \textit{et al.} (112,115) determined the nucleotide sequences of three different bacteriocin determinants from plasmid p9B4-6, specifying low (lactococcin M), high (lactococcin A) and intermediate (lactococcin B) bacteriocin activity. In detailed mutational analyses the lactococcin structural and immunity genes were identified (Fig. 1). Both genes of 69 (\textit{lcnM}) and 77 (\textit{lcnN}) codons of the lactococcin M determinant were implicated in the production of the bacteriocin (112). Separate expression of \textit{lcnM} and \textit{lcnN} and mixing of the supernatants of these strains, gave full bacteriocin activity, whereas the separate supernatants were inactive (K. Venema, unpublished results). This indicated that lactococcin M is a member of the Ib class of bacteriocins, in which a complex of two peptides is required for

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**Figure 1:** Plasmid p9B4-6 and location of the three operons for lactococins A, B and M (66). The linear map represents a reconstruction based on nucleotide sequence data and restriction enzyme analyses (48,106,113,115). The baseline represent the DNA sequence and is shown with restriction enzyme cutting sites. Where the line is broken, no nucleotide sequence data is available. The direction of transcription is indicated by arrowheads in the open reading frames. The right/left turn arrows indicate promoters. Lollipops are presumed terminator structures. Below the baseline the size of the ORF’s in codons is indicated. C, D, A, iA, B, iB, M, N, iM: \textit{lcnC}, \textit{lcnD}, \textit{lcnA}, \textit{lciA}, \textit{lcnB}, \textit{lciB}, \textit{lcnM}, \textit{lcnN} and \textit{lciM}, respectively. EV, HIII: \textit{EcoRV} and \textit{HindIII}, respectively.
activity. In the case of the high- and intermediate-activity determinants, the ORFs of 75 (lcnA) and 68 (lcnB) codons were shown to be involved in the production of lactococcin A and B, respectively. This extensive genetic analysis convincingly illustrated that the bactericidal action of a strain may, in fact, reflect the activity of more than one bacteriocin. Conclusive evidence that lcnA was indeed the structural gene of lactococcin A came from Holo et al. (1991). These authors cloned and sequenced a DNA fragment from the 55-kb bacteriocin plasmid from strain LMG2130 that reacted with a DNA probe designed from the N-terminal amino acid sequence of purified lactococcin A. The nucleotide sequence appeared to be identical to that of the high-activity determinant of p9B4-6. By comparing the amino acid sequence determined from the purified bacteriocin with that deduced from the structural gene, it was evident that lactococcin A was synthesized as a 75-amino-acid-precursor with an N-terminal extension of 21 amino acids. Processing of this precursor takes place behind a glycine doublet (Fig. 2). The N-terminal extension, or leader peptide, and the processing site do not follow the rules of Von Heijne (117) for genuine signal peptides required for sec-dependent protein export, which suggests that the leader in prelactococcin A is not a signal sequence in the classical sense (48). LcnB, M and N all contain N-terminal sequences with homology to the lactococcin

| Lactococcin A | MK----------NQLNFIIVSDDEELSEANGG |
| Lactococcin B | MK----------NQLNFIIVSDDEAVNGG |
| Lactococcin M | MK----------NQLNFIIVSDDEQINGG |
| Lactococcin N | MKDEDEATFKEYSSSFAPDTEDEELING |
| Lactococcin Ga | MK----------ELSEKELRECVVG |
| Lactococcin Gb | MK----------NNNFFKGMEEIEDGELVSITGG |
| Pediocin PA-1 | MK----------KEIKTEKEMANIIGG |
| Plantaricin A | MK----------IQIKGMLSNKEKMXIIVG |
| Lactacin Fa | MK----------QFNYLHKLAVVVG |
| Lactacin Fx | MK----------LNDKELSKIVGG |
| Carnobacteriocin A1 | M----------NNVKEKLSKEMQWTVGG |
| Carnobacteriocin B2 | M----------NSVKEKLNKMQVHLHG |
| Carnobacteriocin B4 | M----------SVKELNKKEMQVING |
| Carnobacteriocin B3 | M----------NKEFSLNVEKMKING |
| Curvacin A/Sakacin A | M----------NNVKEKLSMEQTITGG |
| Leucocin A | MK----------PTSEXQLDNSALEQVVG |
| Leucocin B | M----------NNMKSAADNYQLDNSNALEQVVG |
| Sakacin P | M----------EKFIELSEKVTAITGG |
| Sakacin 674 | M----------EKFIELSEKVTAITGG |
| Acidocin A | M----------ISMISSHQTLTDFKELALISG |
| Mesentericin Y105 | M----------TNMSKVEAYQQLDNSQNIKVVVG |
| ORF4 Sakacin A-operon | M----------LNYIEKQLTNKQLIKIIGG |
| Piscicolin LV61 | M----------NNVKEKLSKEMQVTGG |
| Salvarcin A | MK----------NSKDLNNAEVESEKELMEVAG |
| Streptococcin A-FF22 | M----------EKNNEVINSIQEVSLDELDQIIAG |
| Streptococcin A-M49 | M----------TKEIEIINSIQEVSLDELDQIIAG |
| Lacticin 48L | MK----------EQNSFNLQEVTESEDILIGD |
| Cytolyisin A1 | M----------ENLSVVPSEELSEEMEATQG |
| Cytolyisin A2 | ..NYYSNKLELVGPSFEELSEEMEAIQGS |
| Colicin V | MR----------TLTLNLEDSVSGG |

Figure 2: Alignment of double glycine type leaders of bacteriocins. The sequences were taken from references 2,4,20,21,24,27,32,33,37,42,43,44,48,53,54,59,79,87,89,93,99,109,111,113,115,120. The sequences from mesentericin Y105 and carnobacteriocins B3 and B4 were obtained from Christophe Fremaux and Luis Quadri as personal communications, respectively.
A leader peptide (Fig. 2). For lactococcin B it has recently been shown that it is processed after the double glycine (Chapter II, this thesis), and, although LcnM and LcnN have not been purified, it is tempting to assume that they are also processed behind the glycine doublet (or glycine-serine sequence in the case of LcnN). The glycine processing site is found in numerous class II peptide bacteriocins of LAB, and even in some lantibiotics and colicin V, an Escherichia coli class II peptide bacteriocin of LAB, and even in some LcnN). The glycine processing site is found in numerous class II peptide bacteriocins of LAB, and even in some lantibiotics and colicin V, an Escherichia coli bacteriocin (Fig. 2).

Van Belkum et al. (112,115) made deletion or frameshift mutations in either of the three larger genes, expected to encode immunity (the genes of 154, 98 and 91 codons for the lactococcin M, A and B determinants, respectively, (Fig. 1)) without disturbing the corresponding bacteriocin structural genes. Unexpectedly, L. lactis transformants carrying the various constructs were viable, although the colonies were initially small and grew poorly. After serial transfers in fresh medium the cells gradually started to grow more rapidly. If lactococcin (50% (vol/vol) of a supernatant of a lactococcin producing strain) was added to the plates on which the initial transformants were to be selected, no transformants were obtained. When the presumed immunity genes were cloned separately from the bacteriocin genes under the control of a separate promoter, they provided immunity to the transformed lactococcal strain. These results indicated that the genes indeed specified bacteriocin immunity, and that cells can, apparently, overcome the lethal action of lactococcin at a stage when little bacteriocin is produced. The number of transformants obtained with a plasmid carrying lcnB and a mutated immunity gene was the same as that obtained with a plasmid carrying functional lcnB and lciB (Chapter IV, this thesis). Apparently, the absence of a functional immunity gene does not interfere with the viability of these transformants. It is unlikely that each of the viable transformants would carry a mutation leading to lactococcin resistance. A more likely explanation is that the viability of these transformants is due to a non-genetic change in membrane-lipid composition, such that the lactococcin cannot form pores in the altered membranes. Because of the frequent occurrence of the acquisition of tolerance towards lactococcin, we envisaged this change in lipid composition to be an reversible adaptation rather than a mutation. Here we will restrict the term immunity to lactococcin-insensitivity caused by the lci product. We propose to denote the reversible acquisition of insensitivity to bacteriocins as tolerance, whereas the term resistance will be reserved for permanent acquisition of insensitivity to bacteriocins.

2.1.b LcnC and LcnD are needed for lactococcin production.

Nucleotide sequence analysis of a 5.2-kb DNA fragment derived from plasmid pNP2 of L. lactis subsp. lactis biovar. diacetylactis WM4 revealed that this plasmid also specifies lactococcin A (109). Moreover, it was shown that two additional genes were required for bacteriocin activity. These genes, designated lcnC and lcnD, are located in an operon immediately upstream of the lactococcin A structural and immunity genes (Fig. 1). An identical copy of this operon is present upstream of the lactococcin M operon (K. Venema, unpublished data). Tn5 insertions in either lcnC or lcnD eliminated lactococcin production without negating immunity (109). A promoter immediately upstream of lcnA drives expression of both lactococcin A activity and immunity (112). Therefore, loss of lcnC or lcnD functions were reasoned to impact the maturation and/or secretion processes (109).

L. lactis strain IL1403 carries genes for LcnC and LcnD on its chromosome, explaining why plasmids carrying only the bacteriocin structural and immunity genes still produced active extracellular bacteriocin (48,112,115). Using a DNA probe encompassing lcnC and lcnD in Southern hybridizations, a signal with the chromosome of IL1403 was obtained. When probed with the various bacteriocin structural and immunity genes, no chromosomal signal was observed (Chapter VI, this thesis). Also, PCR products encompassing almost the entire operon were obtained using primers designed from the plasmid encoded lcnC an lcnD genes, indicating that the chromosomally located lcnC and lcnD of IL1403 are highly homologous to their plasmid encoded counterparts. Part of the chromosomally encoded lcnC was cloned and sequenced. Several nucleotide substitutions were found compared to the plasmid encoded lcnC, but none of these led to amino acid changes in the translation product (Chapter VI, this thesis). The reduced production of lactococcin in IL1403 carrying a plasmid with only the bacteriocin operon, as compared to the wild type strain carrying all of the essential genes on a plasmid, can be explained by assuming that the lower copy number of the chromosomally located genes (most probably one) results in a lower secretion efficiency. When lcnC and lcnD, originating from p9B4-6, were introduced on a plasmid in IL1403, this lead to a drastic increase in the production of lactococcin A.

2.2 Secretion of class II bacteriocins.
All class II bacteriocins are secreted. They are produced as prepeptides with a leader sequence that is not of the classical type as required for sec-dependent secretion. It is now well established that for extracellular lactococcin activity two membrane located proteins, LcnC and LcnD, are required (see section 2.1.b). Also for other bacteriocin systems genes homologous to lcnC and lcnD have been identified (see section 2.3.b).

### 2.2.a LcnC and LcnD are essential for lactococcin secretion.

Comparisons of LcnC and LcnD with protein database sequences revealed that these proteins share significant similarities to Gram-negative proteins implicated in signal sequence-independent secretion pathways. Stoddard et al. (109) showed that LcnC is a member of the HlyB-like family of ATP-binding cassette (ABC) transporters (23,41, see section 2.3.b). The similarity is most pronounced in the C-terminal stretch of 200 amino acids of LcnC, which contains the ATP-binding cassette. Protein structure-predicting computer programs indicate six transmembrane sequences (TMS) in the N-terminus of LcnC; a situation similar to HlyB (31, see section 2.2.b). LcnD is largely hydrophilic, with the exception of the N-terminal 43 amino acids. This region in LcnD is a transmembrane domain and, thus, LcnD seems to be a structural homolog of HlyD (see section 2.2.b, Chapter VII, this thesis; 107). LcnD does not show amino acid sequence similarity with HlyD. These results strongly suggested that LcnC and LcnD are required for secretion of the lactococcins via a system dedicated to bacteriocin export (66,109). Similar dedicated secretion systems have now been proposed for other LAB bacteriocins (see section 2.3.b).

### 2.2.b The hemolysin A secretion machinery.

The prototype sec-independent secretion system, the E.coli hemolysin A system (45,47,72), is now understood in considerable molecular detail. The hly determinant is composed of four contiguous genes, hlyCABD. These genes encode a cytoplasmic 20 kD HlyC protein, required for post-translation activation of the protoxin (55), a 110-kD product of hlyA (the actual toxin) and two membrane proteins, HlyB (79 kD) and HlyD (53 kD), specifically required to translocate hemolysin A (119). Additional genes have been identified elsewhere on the chromosome which are involved in regulation of hly expression, toxin activation or late stages in secretion of the toxin. However, all these functions are recruited from the host and have distinct, house keeping functions. They are beyond the scope of this introduction.

HlyA is secreted extremely rapidly and can reach external levels of 2 to 3 mg protein/ml. The toxin is synthesized and activated before secretion. The folding state of the protein during the secretion process is not known. Hemolysin A is not processed during secretion (25) and no periplasmic intermediate can be detected (34). The key protein in hemolysin A secretion is HlyB, a so called ‘traffic ATPase’, or ABC transporter. It is the prototype of a very large family of membrane ATPases (7,23,41). Members of this super family of highly conserved proteins, which include CFTR (involved in cystic fibrosis), Mdr and P-glycoprotein (involved in multidrug resistance), secrete an extremely wide range of substrates. They are composed of an N-terminal (transport) domain and a cytoplasmic ATPase which apparently regulates the activity of the channel in response to the presence of the substrate. HlyD, the second membrane protein involved in secretion of HlyA, is believed to span the inner and outer membrane, and to make contact with the minor outer membrane protein ToIC (118), thereby preventing hemolysin A to enter the periplasmic space (107). The topology of both HlyB and HlyD in the E.coli membrane has been investigated using LacZ, PhoA and/or β-lactamase fusions (31,107). HlyB has six transmembrane sequences (TMS) in the N-terminal transport domain. HlyD has one TMS located in the N-terminal moiety of the protein.

HlyA contains an essential secretion signal of 50 to 60 amino acids located in the extreme C-terminus of the molecule (34,62) that is not removed during secretion (25). Fusion of this secretion signal region to a variety of bacterial and mammalian proteins, including the normally intracellularly located β-galactosidase, allowed specific and efficient secretion by E.coli of the hybrid proteins in the presence of HlyB and HlyD (46,61).

### 2.2.c Topology of LcnD in the lactococcal cytoplasmic membrane.

LcnC and LcnD may form a dedicated secretion machinery for lactococcins. As a first characterization of this putative secretion machinery, the LacZ and PhoA fusion strategy was used to determine the topology of LcnD in the cytoplasmic membrane (see also Chapter VII, this thesis). Fusions of LacZ, a cytoplasmic protein, to sequences that are normally located in the cytoplasm result in active β-galactosidase. Fusions between LacZ and parts of a protein that are normally extracellular result in highly reduced β-galactosidase activities. For PhoA, a periplasmic enzyme, the reverse is true.
Hydrophobicity analysis of LcnD indicates that the stretch of amino acids from residue 22 to 38 might form a transmembrane $\alpha$-helix. The rest of the protein is rather hydrophilic. With this computer prediction as a guide, four LacZ and four PhoA fusions were constructed in front of, in, and behind the predicted transmembrane helix, and at the very C-terminal end of LcnD (Chapter VII, this thesis). The analysis showed that, indeed, the N-terminus of LcnD is in the cytoplasm, the stretch of amino acids between residue 22 to 38 cross the membrane, while the remainder of the protein is located outside of the cell.

### 2.3 Processing of class II bacteriocins.

As discussed above, class II bacteriocins are synthesized as prebacteriocins, containing a leader peptide that is cleaved behind a glycine doublet before or during transport. Until recently, it was unclear whether the presumed leader peptidase activity was also present in this operon. Mutations in the structural gene ($pedA$), or during transport. Until recently, it was unclear whether the presumed leader peptidase activity was also present in this operon. Mutations in the structural gene ($pedA$), or whether the conserved leader peptidase activity was also encoded by the bacteriocin operon, or whether it was a more generally acting peptidase.

#### 2.3.a Processing of bacteriocins; involvement of the ABC-transporters in processing.

The first indication that a protein encoded by the bacteriocin operon was involved in this process came from our studies (Chapter VIII, this thesis) on the pediocin operon.

As for lactococcins, production of active pediocin requires the structural gene, $pedA$, and two additional genes ($pedC$ and $pedD$) that show homology to the proteins involved in sec-independent secretion of polypeptides (79). PedC shows structural homology to LcnD, whereas PedD has considerable amino acid sequence homology to LcnC (49% identity with an overall homology of 67%). The homology of the latter two proteins is not restricted to the ABC-box, but extends over the entire polypeptide. This is also the case with several other ABC-transporters involved in secretion of bacteriocins that contain the double glycin motif in the leader peptide. Notably, a region in the N-terminal 190 amino acids is conserved in these proteins (Chapter VIII, this thesis; Fig. 3).

$E. coli$ cells containing the complete $ped$ operon ($pedABCD$) produce active, secreted pediocin. Apparently, all the information for both secretion and processing is present in this operon. Mutations in the structural gene ($pedA$), $pedC$, or $pedD$ abolish production of extracellular pediocin activity by $E. coli$. The primary translation product of $pedA$ contains 62 amino acids encompassing the 18-amino-acid-leader peptide and the 44 amino acids that constitute mature pediocin (79). $pedA$ was expressed in $E. coli$ behind the strong T7 RNA polymerase promoter. When lysates of this strain were run on an SDS-PAA gel and overlayered with pediocin sensitive cells, a faint halo of activity was observed, corresponding to a peptide with a lower electrophoretic mobility than that of mature pediocin produced by $E. coli$ containing the complete $ped$ operon. Apparently, the primary translation product of $pedA$ is biologically active albeit at a very reduced level. No extracellular activity was found in the strain carrying $pedA$ alone. To determine which of the genes in the operon was responsible for pediocin secretion and/or processing, $pedC$ and $pedD$ were expressed separately or together in the strain producing prepediocin from the T7 promoter. When both $pedC$ and $pedD$ were present, active extracellular pediocin was produced. However, when only $pedC$ or $pedD$ were present, no extracellular activity was found. Lysates of the strain carrying $pedA$ and $pedC$ contained prepediocin activity only. However, lysates of the strain carrying $pedA$ and $pedD$ showed, in addition to prepediocin activity, an activity on the gel that was indistinguishable from that of mature pediocin. These results conclusively show that both PedC and PedD are involved in secretion of active pediocin and that PedD is capable of processing the pediocin precursor. Since mature pediocin was found intracellularly in the strain containing only $pedA$ and $pedD$, it seemed likely that the conserved 190-amino-acid-domain in the N-terminus of the protein, which is shown to be intracellular by topology studies (Eric Emond, pers. commun.), is involved in processing of the leader peptide. When this domain was expressed separate from and in the absence from the remainder of the protein, the pediocin was processed (Chapter VIII, this thesis). These results showed that bacteriocin processing can be uncoupled from secretion.

We predict that, similar to PedD, LcnC will be the processing enzyme for the lactococcins. Since cloning of $lcnC$ in $E. coli$ is lethal (K. Venema, unpublished results) we have not yet been able to verify this prediction.
Figure 3: Alignment of the first 190 amino acids of PedD with the equivalent domains in the ABC-transporters implicated in the transport of lactococcins A, B, and M (LcnC; 109), carnobacteriocin B2 (CbnT; Luis Quadri, pers. comm.), cytolysin of Enterococcus (CylB; 32), colicin V of Escherichia coli (CvaB; 23), lactococcin DR (LcnDR3; 97), lactococcin α and Gß (LagD; 81), sakacin B (SapT; 4), and mesentericin Y105 (MesD; Christophe Fremaux, pers. comm.). ComA, involved in competence of Streptococcus pneumoniae (51) and putatively involved in secretion of the competence factor, has been included as well. Below the sequences the similarity is indicated: (*), identical amino acid; (.), conserved amino acid.

Recently, Havarstein et al. (39) reported a similar study on the ABC transporter (LagD) of the two
component bacteriocin lactococcin G. These authors have used an in vitro assay for proteolytic cleavage of the substrate. Using PCR, the proteolytic domain of LagD, followed by an affinity tag of six histidine residues at the C-terminus, was cloned in an expression vector and purified. Also the substrate, the prelactococcin Gα peptide, one of the two peptide required for lactococcin G activity, was purified making use of the histidine affinity tag. This prepeptide did not have antimicrobial activity when mixed with lactococcin Gß, whereas the mature lactococcin Gα had. By incubating this prepeptide with the purified N-terminal domain of LagD, and subsequent mixing with lactococcin Gß, antimicrobial activity was observed, indicating that cleavage of the prepeptide had occurred.

2.3.b ABC-transporters of other LAB bacteriocins.

The lantibiotic nisin is also transported by an ABC-transporter, NisT (73). It is the only transporter that has been cloned from LAB that secretes a bacteriocin that does not contain a double glycine leader. The other ABC-transporters, putatively involved in secretion of bacteriocins that have the double glycine motif in the leader peptide, carry a conserved region in the N-terminal 190 amino acids. This domain is absent in NisT. These domains are aligned in Fig. 3 and include ABC-transporters of the non-lantibiotics, the E.coli bacteriocin colicin V, and the lantibiotics lacticin 481 and cytolysin. Also included in this figure is ComA, involved in competence of Streptococcus pneumoniae (51). We predict that the substrate for this ABC-transporter, the competence factor (CF: a small proteinaceous substance), also contains a double glycine motif in its leader. Several other genetic components of secretion systems have been cloned as well, but their sequences have not yet been published. These include the secretion systems for leucocin A (Marco van Belkum, pers. comm.) and divergicin 750 (Askild Holck, pers. comm.).

The N-terminal moieties of the ABC-transporter share three conserved regions (Fig. 3): one from Q13 to I27 (numbering as in PedD): QVDENDCGLAALNMI; the second from H98 to V102: HYYVV; the third encompassing D114 and P115. Conceivably, these regions compose the active site of the leader peptidase.

Most of the bacteriocin secretion systems described above also contain a protein structurally similar to PedC: LcnD (109), SapE (4), ComB (51), LagE (39), LcaD (Marco van Belkum, pers. comm.), MesE (Christophe Fremaux, pers. comm.), CbnE (Luis Quadri, pers. comm.), the product of an incomplete ORF located upstream of the laf- operon (27) and CvaA (33) all contain one TMS in the N-terminus, suggesting that a common mechanism exists for secretion of these small bacteriocin molecules (and CF). Notably, this accessory protein is not encoded in the operons of lactacin 481 or cytolysin, suggesting that secretion of these lantibiotics is different from that of the non-lantibiotics.

2.3.c Leader exchange and sec-dependent secretion of bacteriocins.

The leader peptide is probably required for maintaining the prebacteriocin in an inactive state. Whether it also directs the bacteriocin to the secretion machinery remains to be established. An indication that this might be so was provided by studies on the exchange of the leader of lactococcin A and pediocin PA-1 and vice versa (Eric Emond, pers. comm.). In this study, four constructs were made: i) lactococcin A containing the lactococcin A leader peptide, ii) lactococcin containing the pediocin PA-1 leader, iii) pediocin containing the pediocin leader, and iv) pediocin containing the lactococcin leader. All constructs were used to transform L.lactis IL1403, containing the lactococcin secretion machinery on its chromosome (see section 2.1.b; Chapter VI, this thesis). All bacteriocins were secreted and active, indicating that LcnC, the lactococcin ABC transporter, recognized the leader of pediocin and was able to cleave this leader from the molecules tested. Preliminary results indicate that also the pediocin secretion machinery is capable of processing some, but not all, of these hybrid bacteriocin molecules (Eric Emond, pers. comm.).

Allison et al. (3) reported on the heterologous expression of the lactacin F peptides (a two component bacteriocin) in Carnobacterium piscicola LV17. This strain produces the three carnobacteriocins A, B2 and BM1 with leader sequences similar to those of the two lactacin F peptides (Fig. 1). The ability of C. piscicola LV17 to produce lactacin F activity in the absence of the lactacin F secretion machinery demonstrates that the machinery for the carnobacteriocins is capable of both processing and export of the lactacin F peptides.

Cloning of only the structural and modification genes of the lantibiotic lactacin 481 in L. lactis IL1403 (carrying lcnC and lcnD on its chromosome) resulted in extracellular activity (97), indicating that LcnC was capable of processing and secreting lactacin 481. Thus it seems that the LcnCLcnD machinery is not only capable of processing and secretion of different non-lantibiotic bacteriocin (several lactococcins and pediocin PA-1), but also of a lantibiotic. As the mature parts of
these molecules are very different, recognition of their leaders seems to be the first step in the secretion-processing process.

Recently a bacteriocin secreted by the general sec-system has been described (121). Divergicin A is a narrow-spectrum non-lantibiotic bacteriocin produced by *Carnobacterium divergens* LV13. Both the structural and the immunity genes are located on a 3.4-kb plasmid. The structural gene (*dvaA*) encodes a prepeptide of 75 amino acids, consisting of a 29-amino-acid N-terminal extension and a mature peptide of 46 amino acids. The signal sequence is cleaved behind a consensus sec-dependent Ala-Ser-Ala processing site. Heterologous production of divergicin A was observed in hosts (including *L. lactis* MG1363) containing only the structural and immunity genes. Fusion of the divergicin signal sequence to alkaline phosphatase resulted in the secretion of this enzyme into the periplasm of *E. coli*.

Conversely, the N-terminal extension of leucocin A has been used to secrete divergicin A, that is normally secreted through the general sec-dependent secretion pathway, in *Leuc. gelidum*, using the leucocin A secertion apparatus and in *L. lactis*, using the lactococcin secertion machinery (Marco van Belkum, pers. comm.). Similarly, divergicin production was also observed when fused to the lactococcin A leader (Marco van Belkum, pers. comm.).

2.4 Mode of action of class II bacteriocins.

Although over 15 class II bacteriocins have been characterized at the genetic level, of only a few (notably lactococcin A, lactacin F and pediocin PA-1) has the mode of action been investigated in detail. Of several other bacteriocin systems, only the effect on survival and lysis of an indicator strain has been assessed. Here, we will discuss only those reports that have provided details addressing the working mechanism of these bacteriocins.

2.4.a Introduction on the working mechanisms of lactococcin A and lactococcin B.

Lactococccins A and B specifically inhibit the growth of lactococci. Both lactococccins belong to a broader group of antimicrobial cationic, hydrophobic peptides permeabilizing membranes, including some of completely different origin: the lantibiotics Pep5 from *Staphylococcus aureus* (69), epidermin from *Staphylococcus epidermidis* (49), subtilin from *Bacillus subtilis* (71), gallidermin from *Staphylococcus gallinarum* (60) and nisin from *Lactococcus lactis* (52), and the eukaryotic peptides cecropins (14), alamethicin (35), melittin (36,116), and magainins (58,122). In spite of their different sources these peptides have some properties in common: a molecular mass of 3-6 kDa and a high isoelectric point. The peptides also differ in certain aspects: most act voltage-dependently (8,71,101,104) whereas the lactococcins act in a voltage-independent way (114) (see below). Some act on liposomes or planar lipid bilayers (14,28,29,58,116), whereas the lactococcins have no effect on liposomes and need, in all likelihood, a protein receptor for activity. However, a common feature of these peptides seems their capacity to form pores, composed of several molecules of the same peptide, in the cytoplasmic membrane (28,29,69,70,104).

2.4.b Mode of action of lactococcin A and B.

In analogy to the mode of action of the lantibiotics and a number of eukaryotic non-bacteriocin peptides (for reviews see: 57,100), the possible target for the action of lactococcin A and lactococcin B might be the cytoplasmic membrane of sensitive cells. Van Belkum *et al.* (114) investigated this possibility in detail for lactococcin A. The mode of action of purified lactococcin A was studied on whole cells and membrane vesicles of sensitive and immune lactococcal strains, and on liposomes obtained from lactococcal phospholipids. Similar studies were done with partially purified lactococcin B on whole cells (Chapter III, this thesis). At lactococcin concentrations that did not affect immune cells, both bacteriocins rapidly dissipated the membrane potential (and in the case of lactococcin B also the pH-gradient across the membrane) of glucose-energized sensitive cells. A non-metabolizable alanine analogue (AIB, 2-α-amino isobutyric acid), taken up in symport with a proton such that the uptake is driven by the proton motive force (pmf) (68), was rapidly lost from sensitive whole cells treated with lactococcin (114; Chapter III, this thesis). To show that efflux was a direct consequence of permeability changes in the cytoplasmic membrane, and not caused by the absence of a pmf, uptake of L-glutamate was studied. Glutamate uptake by *L. lactis* is driven by a phosphate bond-linked unidirectional process (90) and dissipation of the pmf should, therefore, prevent the efflux of accumulated glutamate. However, addition of either lactococcin to sensitive cells that had accumulated glutamate, resulted in the immediate efflux of the amino acid, even when the pmf in the cells was dissipated prior to the addition of the bacteriocin (114; Chapter III, this thesis). These
results indicate that both lactococcins are able to permeabilize the membrane in a voltage-independent manner.

Lactococcin A inhibited leucine uptake in cytoplasmic membrane vesicles from sensitive lactococcal cells, but not from vesicles derived from Bacillus subtilis, Clostridium acetobutylicum or Escherichia coli membranes (114). Liposomes derived from lactococcal phospholipids were not affected by lactococcin A. From these data, and the observation that lactococcin A specifically inhibits lactococcal strains, Van Belkum et al. (114) concluded that lactococcin A permeabilizes the cytoplasmic membrane of sensitive cells and that a Lactococcus-specific receptor protein is required.

A 21-amino-acid-sequence between residues Ala30 and Phe50 in lactococcin A (Fig. 4A) could possibly form a membrane-spanning α-helix (66). Conceivably, lactococcin A inserts into the cytoplasmic membrane of sensitive cells by this hypothetical transmembrane helical segment. A large number of pore-forming toxins are known to create channels through a 'barrel stave' mechanism (63,88,103). Pore formation requires the molecules to aggregate like barrel staves surrounding a central water-filled pore. It is assumed that lactococcin A and lactococcin B form pores by this mechanism, although in the case of lactococcin B the method of Kyte and Doolittle (74) does not predict a membrane spanning helix. The size of the pore would be determined by the number of molecules involved in pore formation (Fig. 5). An indication that different pore sizes can exist was derived from the observation that low concentrations of lactococcin B allow leakage of protons and ions, whereas for leakage of glutamate 150 times more bacteriocin was needed (Chapter III, this thesis).

The effect of purified diplococcin from L. lactis subsp. cremoris 346 on sensitive cells has been studied as early as 1981 (17). Addition of 8 arbitrary units (AU) of diplococcin to sensitive cells completely abolished DNA and RNA synthesis within 2 minutes, possibly causing the immediate but incomplete cessation of protein synthesis as a secondary effect. Now we know that diplococcin is identical to lactococcin A (81) and that these effects are caused by the increase in permeability of the cytoplasmic membrane.

Lactococcin B activity requires that its only cysteine residue is in the reduced state (Cys24, Chapter III, this thesis). Partially purified lactococcin B was almost inactive on whole cells. Only after addition of small amounts of dithiothreitol (DTT; 5 mM) was lactococcin B capable of dissipating the membrane potential. Oxidation of Cys24 in lactococcin B by HgCl₂ led to inactivation of the bacteriocin, while HgCl₂-oxidized lactococcin B could be reactivated by DTT.

Cys24 of lactococcin B was replaced via site directed mutagenesis by all other possible 19 amino acids (Chapter IV, this thesis). All mutants were active except those in which the cysteine had been replaced by a positively charged amino acid (Cys24His, Cys24Lys and Cys24Arg; Chapter IV, this thesis). Apparently, Cys24 is not essential for activity of lactococcin B but when it is present, oxidation leads to inactivation of lactococcin B. In this regard lactococcin B resembles a group of thiol-activated toxins in which the reduced state of a cysteine residue appears to be essential for the generation of functional lesions in toxin-treated membranes (9). Also in these molecules the cysteine can be replaced without loss of activity. The reason for inactivation of lactococcin B by oxidation of Cys24 is unclear.

Lactococcin B treated with HgCl₂ is inactive, apparently because reaction of this chemical with Cys24 leads to a positive charge at this position. Cys24 could be oxidized without loss of bacteriocin activity, when oxidative chemicals were used that did not leave a positive charge at position 24 (Chapter IV, this thesis). The only other oxidative chemical that inactivated lactococcin B was peroxodic acid. Apart from reacting with cysteine residues, this compound also reacts with methionine residues, two of which are present in the mature lactococcin B. No positive charge is left at position 24 of lactococcin B treated.

Figure 4: Single letter amino acid sequence of mature lactococcin A (a), lactococcin B (b) and the immunity protein against lactococcin A (c) and helical wheel presentations of the hypothetical transmembrane helix in lactococcin A and the putative amphiphilic α-helices in lactococcin B and LciA. Amino acids in the helices are underlined in the amino acid sequence.
Chapter I

Introduction

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**Lactococcin A**

LcnA residues 30-50

```
K L T F Ì Q S T A A G D L Y Y
N T N T H K Y V Y Ì Ì Q T Q A
F G A A A N T I V N G W M G S
A A G G F G L H H
```

Hydrophobicity

\[
\text{Hydrophobicity} = 0.52
\]

---

**Lactococcin B**

LcnB residues 25-46

```
S L Q Y V M S A G P Y T W Y K
D T R T G K T I C K Q T I D T
A S Y Z F G V M A E G W G K T
F H
```

Hydrophobic Moment

\[
\langle \mu H \rangle = 0.35
\]

---

**Lci A**

LciA residues 29-47

```
M K A K I E F F E N E L R S M
L A T A L E K D I S Q E E H N
A L N I A E K A L D N S E Y L
P K I I L N L K A L T P I A
I N R T L N H D L S E L Y K F
I T S S K A S N K N L G G G L
I M S W G R L F
```

Hydrophobic Moment

\[
\langle \mu H \rangle = 0.52
\]
with performic acid, so it seems that inactivation is due to oxidation of one (or both) of the methionine residues. The most likely candidate is Met38 as this residue is part of the (putative) amphiphilic α-helix and oxidation would destroy the amphipaticity.

2.4.3 Mode of action of pediocin PA-1, mesentericin Y105 and lactacin F.

Treatment of sensitive cells with pediocin resulted in loss of intracellular K⁺-ions and U.V.-absorbing materials (6). Some of the strains investigated lysed upon addition of pediocin, others did not. Treatment of cell walls to remove lipoteichoic acid prevented pediocin binding, suggesting that this is (one of the) binding sites for pediocin (6). Pediocin dissipates the transmembrane electrical potential and inhibits amino acid transport in sensitive cells (12). It acts in a voltage-independent manner. Pediocin also interfered with amino acid uptake in cytoplasmic membrane vesicles derived from sensitive cells. In liposomes fused with cytoplasmic membrane vesicles from *Pediococcus*, pediocin elicited
efflux of small ions and, at higher concentrations, also of a fluorescent probe with a molecular mass of 9400. As for lactococcin B it was concluded that the size of the pore depends on the number of molecules contributing to the pore.

Mesentericin Y105, a bacteriocin produced by Leuconostoc mesenteroides has a similar spectrum of inhibition as pediocin PA-1. The two bacteriocins are homologous in their N-terminal moieties and belong to the 'pediocin-like' subclass IIa (see section 2.8.a). Mesentericin Y105 dissipates the membrane potential of Listeria monocytogenes and inhibits the transport of leucine and glutamic acid (77). In addition, the bacteriocin uncouples the energy state of rat liver mitochondria through inhibition of ATP synthesis and adenine nucleotide translocase. The authors suggest that this is caused by pore formation in the energy-transducing membranes.

Addition of lactacin F to Lactobacillus delbruckii cells resulted in efflux of K⁺-ions from these cells (1). E. faecalis cells started to lose K⁺ after a lag-time of one minute. The lag-time was a function of lactacin F concentration and ionic strength. High concentration and low ionic strength resulted in the best efflux. The lag-time increased with lower temperatures. Lactacin F was optimally active at low pH. Lactacin F induced efflux of K⁺ was much more rapid than efflux mediated by transport of K⁺ after dissipation of the proton motive force, indicating that lactacin F forms pores in the cytoplasmic membrane. Activity was inhibited by di- and trivalent cations (1). The rate of K⁺ efflux decreased with decreasing temperature, whereas at temperatures below 10 °C no loss was observed. Since decreasing the temperature increases the ordering of the lipid molecules in the membrane, it was concluded that a higher degree of ordering prevents insertion of the lactacin F molecules in the membrane (1). This is the first report on the mode of action of a two-component bacteriocin which seems to act similarly to that of the other (one component) class II bacteriocins. LafA and LafX both contain two cysteine residues in the mature peptide, which may be important for the structure or mode of action of lactacin F (27). Substitution of Cys30 of LafA by Tyr resulted in loss of lactacin F activity (27).

2.5 Immunity to class II bacteriocins.

Although the mode of action of class II bacteriocins has been studied in considerable detail, much less is known about the nature of immunity against these bacteriocins. In some cases the immunity gene has been identified. For several bacteriocin systems the immunity genes have not been cloned at all, and no information about immunity is available. Only LciA, the lactococcin A immunity protein, has been studied in some detail (Chapter V, this thesis) as will be discussed below.

2.5.a Mode of action of the immunity protein LciA.

Since lactococcin A is active on membrane vesicles derived from sensitive cells, but not on those derived from immune cells, Van Belkum et al. (114) concluded that the lactococcin A immunity protein, LciA, was present in the membrane. To study the mode of action of the immunity protein, monoclonal antibodies were raised against the protein (Chapter V, this thesis). One of the antibodies localized LciA to the membrane fraction of fractionated cells of Lactococcus lactis (Chapter V, this thesis). However, similar amounts of the protein were also present in the cytosolic and membrane-associated fractions. LciA was not detectable in the supernatant or soluble cell wall fraction. Deletion analysis revealed that the epitope in LciA is located in the C-terminus between amino acids 60 and 80. By performing ELISA (enzyme-linked-immunosorbent assay) using the monoclonal antibody on right-side-out and inside-out vesicles it was shown that the C-terminus of LciA is located at the outside of the cytoplasmic membrane (Chapter V, this thesis). The epitope in LciA can be prevented from reacting with the monoclonal antibody by externally added lactococcin A. Proteinase K-treated sensitive vesicles became insensitive to the bacteriocin suggesting that the proteinase had digested the bacteriocin receptor, previously inferred by van Belkum et al. (114) on the basis of the lactococcin A mode of action studies, rendering the membrane vesicles insensitive to the action of lactococcin A.

LciA could prevent the action of lactococcin A by binding and, thus, neutralizing the bacteriocin, or by interacting with and blocking the bacteriocin receptor. To distinguish between these two possibilities, right-side-out vesicles isolated from immune and sensitive cells were fused and the ability of the fused membranes to accumulate leucine was examined (Chapter V, this thesis). In case LciA would bind and neutralize lactococcin A, the fused vesicles would still be immune. If LciA would block the bacteriocin receptor, the fused vesicles would be sensitive because of unblocked receptor molecules contributed by the sensitive vesicles. As leucine uptake in the fused vesicles was completely blocked in the presence of lactococcin A, it was concluded that LciA directly interacts with the lactococcin A receptor.
Nissen-Meyer et al. (85) have purified the LciA protein and have shown that it is not post-translationally modified. Protein structure computer programs predict that the stretch of amino acids from residue 29 to 47 in LciA could form an α-amphiphilic helix with a strong hydrophobic moment of 0.52 (Fig. 4C). The results presented above were united in a model for LciA topology, in which the C-terminus, from residue 48 to 98 and containing the epitope, is at the outside of the cell. Residues 29 to 47 span the cytoplasmic membrane as an α-amphiphilic helix presumably by interaction with another transmembrane protein, possibly the lactococcin A receptor. The N-terminus of LciA resides on the cytoplasmic face of the membrane. A model visualizing the immunity mechanism is presented in Figure 5D. It provides an explanation for the observation that only a fraction of the synthesized immunity protein molecules is present in the cytoplasmic membrane. Apparently, this fraction is sufficient to prevent the receptors from interaction with the bacteriocin. The cytoplasmic and membrane-associated fractions may constitute a reservoir from which molecules can be drawn when new receptors are synthesized and inserted into the membrane, thus resulting in immediate blocking of the receptor (Chapter V, this thesis).

2.5.b Immunity to pediocin PA-1 and carnobacteriocin B2.

*pedB* encodes the immunity protein against pediocin PA-1 and increased expression of this gene leads to a higher level of immunity to pediocin PA-1 (Chapter VIII, this thesis). In mode of action studies with pediocin PA-1, Chikindas et al. (12) showed that this protein is associated with the cytoplasmic membrane. CbiB2, the protein conferring immunity against carnobacteriocin B2 has been purified. The majority of this protein was located in the cytoplasm, with a smaller proportion being associated with the cytoplasmic membrane (94).

2.5.c Immunity to class Ic bacteriocins.

Interestingly, immunity proteins against the two peptide bacteriocins (class Ic) all contain several transmembrane sequences (usually 4). This is true for LciB (the lactococcin M immunity protein, 113), the ORFY product (the putative lactacin F immunity protein, 2) and LagC (the putative lactococcin G immunity protein, 81). No doubt the mode of action of these immunity proteins will differ considerably from that of LciA, and might resemble that of the immunity proteins against pore-forming colicins (11,91,92). Cai (the colicin A immunity protein) and ImmE1 (the colicin E immunity protein) contain three and four membrane spanning α-helices, respectively (30,108) and directly interact with their bacteriocins (123).

2.6 Regulation of class II bacteriocin expression and lactacin B.

Lactococcin production by *L. lactis* only occurs in rich media (Chapter II, this thesis). It seems, therefore, that production is regulated. Holo et al. (48) defined a 19-bp inverted repeat (IR) overlapping with the -10 sequence of the lactococcin A promoter. This IR was suggested to be a binding site for a regulatory protein required for *lcnA* transcription. However, no evidence for inducibility has been obtained for the lactococcins.

Production of amylovorin L471 has been reported to be inversely correlated to growth rate of *Lb. amylovorus* (Luc de Vuyst, pers. comm.), suggesting that non-optimal growth conditions favour the synthesis of this bacteriocin.

In the operons of some of the other class II bacteriocins genes have been identified that encode proteins similar to two-component regulatory systems. Diep et al. (20) reported that the gene encoding plantaricin A is located on the same transcriptional unit as *plnB, plnC* and *plnD*. These genes encode a histidine kinase and two response regulators, respectively. Their role in regulation of the synthesis of plantaricin A has not been determined, but seems evident from the genetic organization. Also, the nature of the extracellular signal triggering regulation is unknown. Of the genes involved in production of sakacin A by *Lb. sake*, *sapK* (previously designeted *sakB*) and *sapR* presumably encode a histidine kinase and a response regulator, respectively (4). Northern analysis revealed that the SapKR system probably acts by transcriptional activation, since deletion of *sapK* resulted in loss of transcription of the structural gene. A 35-bp sequence upstream of the *sapA* promoter and a similar sequence upstream of *sapK* were both necessary for production of sakacin A, and thus seem to be the target for the response regulator. The signal triggering activation of the operon has not been determined yet. Also genes homologous to two-component signal transduction systems have been identified for carnobacteriocin B2 (CbnK and CbnR; Luis Quadri, pers. comm.).

2.7 Application of class II bacteriocins.

Factors that have contributed to the increase in applied
research on bacteriocins and bacteriocinogenic LAB are: i) the safe use of nisin for over 30 years and its approval by the FDA as a GRAS (generally regarded as safe) substance; ii) consumer resistance to, and concern about the safety of traditional preservatives; iii) realization that bacteriocinogenic strains have been isolated from food and, therefore, can be considered safe; and iv) the availability of molecular biology tools to engineer genetic variants of bacteriocins to alter stability, host range or other properties. Most reports involve the use of pediocin PA-1. This bacteriocin has been used to prevent or reduce growth of spoilage bacteria in fermented dry sausages (5,26), vacuum-packed wiener (19) and fresh meat (84). Recently, in a report on the use of LAB bacteriocins for biopreservation of brined shrimp, the 'pediocin-like' bacteriocin bavaricin A extended the shelf life by 6 days to 16 days (22). A review on food biopreservatives of microbial origin has recently been published (95).

2.8 Miscellaneous features.

2.8.a Class IIa bacteriocins.

The class IIa bacteriocins (Table 1), defined by Nieto Lozano et al. (83) and termed 'pediocin-like' by Nes, are characterized by a consensus amino acid sequence in the N-terminal region of the mature peptides: ..-Tyr-Gly-Asn-Gly-Val-Xaa-Cys (where Xaa is His, Thr, Ser or Tyr). Except for acidocin A (59), all these bacteriocins contain also a second conserved cysteine residue in the consensus sequence Cys-Xaa-Val-Asn/Asp (Where Xaa is Thr, Ser or Trp). Pediocin PA-1 contains four cysteine residues (40). For some of these bacteriocins it has been reported that the first two cysteines form a disulfide bridge in the mature molecule necessary for activity (37,40,93). So far, this subgroup contains 10 different bacteriocins. They are active against Listeria and their overall spectrum of inhibition is very similar. The role of the consensus sequences is not understood, but it may be of fundamental importance for bacteriocin activity. The sequence may be directly involved in antimicrobial activity or, since all bacteriocins more or less affect the same indicators, may be important in determining target-cell specificity. Some of the bacteriocins in this class differ by only two amino acids and have a somewhat different spectrum of inhibition. This subgroup of class II bacteriocins, therefore, forms an interesting subject for structure-function relationships.

2.8.b Class IIb bacteriocins.

The class IIb bacteriocins are characterized by the fact that two peptides are required for bacteriocin activity. For four of the five bacteriocins in this group the two peptides are encoded by separate genes (lactococcin M, 113; lactacin F, 2; lactococcin G, 86; plantaricin T, Jose Luis Ruiz Barba, pers. comm.). Alone these peptides do not have significant activity. Mixing of the separately purified peptides (lactococcin G; Nissen) or complementation of the crude peptides in vitro (lactococcin M; K.Venema, unpublished data) or in vivo (lactacin F, 2) restored activity. The fifth bacteriocin of this group, plantaricin A, is encoded by a single gene (20,87). The two peptides, α and β, differ by one amino acid: the β-peptide contains an extra alanine residue at the N-terminus. The prebacteriocin contains a leader sequence with the consensus double glycine processing site. However, the sequences of the α and β peptides start 4 and 3 amino acids downstream of this processing site, respectively. It seems that apart from processing by the ABC transporter, these peptides are prone to further processing as well, or that unspecific proteolytic degradation occurred during purification.

2.8.c Expression of bacteriocins in heterologous species.

Lactococcin A has been expressed in P. acidilactici using its own promoter and secretion machinery (13). Expression of the operon in E.coli was not observed (112). The plasmid containing the lactococcin secretion machinery is structurally highly unstable (K. Venema, unpublished data). Pediocin PA-1 could only be expressed in L.lactis when cloned behind a strong lactococcal promoter (13). Expression of pediocin in E.coli has been achieved under control of the ped promoter (79). Again, expression was unstable, although the plasmid profile of the producing and non-producing clones did not differ (Michael Chikindas, unpublished data). Possibly a point mutation was the result of loss of pediocin production in E.coli. Lactacin F has been expressed in C. piscicola. In this case secretion of the two lactacin peptides was accomplished by the secretion machinery for carnobacteriocins (3). Mesentericin Y105 has been produced in Lactobacillus using the lactacin F processing and export mechanism (Christophe Fremaux, pers. comm.).

3. Conclusions and perspectives.

The past few years has seen significant progress in our understanding of class II bacteriocins. Apart from
the cloning and sequencing of the structural and immunity genes and of genes encoding the secretion and processing machinery, we now begin to understand the mode of action of several bacteriocins, and the way the lactococcin A immunity protein LciA works. Although the lactococcin A receptor has not yet been identified, it is likely to be the site of action of the immunity protein. Processing of the bacteriocin precursors occurs by the N-terminal domain of the bacteriocin ABC-transporter. This knowledge, combined with structure-function studies of the bacteriocins, will allow the construction of molecules with enhanced or altered activities and broader specificities in the future.

4. Outline of this thesis.

As outlined in this introduction, the past years have seen an explosion on research of class II bacteriocins from LAB, their mode of action, the nature of immunity, the characterization of their secretion and processing machineries, and the regulation of their synthesis. In this thesis, most of these issues were addressed for the lactococcins and pediocin PA-1.

- Chapter II describes the purification of lactococcin B and pediocin PA-1 using a general, high yield, and rapid method, that consists of precipitation, isoelectric focusing and ultrafiltration.
- In Chapter III the mode of action of purified lactococcin B is described. These studies indicated that the bacteriocin forms pores in the cytoplasmic membrane in a voltage-independent manner. Lactococcin B activity required that its single cysteine residue (Cys24) is in the reduced state.
- Using site-directed mutagenesis, Cys24 was replaced by each of all other amino acids. These experiments are described in Chapter IV. In addition, the lactococcin B molecule was treated with several oxidative chemicals. Only the introduction of a positive charge at the position of Cys24 resulted in inactivation of the bacteriocin.
- Chapter V describes the mode of action of the lactococcin A immunity protein, LciA. The results indicated that the immunity protein blocks the bacteriocin receptor such that the bacteriocin can no longer insert into the cytoplasmic membrane.
- In Chapter VI evidence is presented that important genetic components of the lactococcin secretion/maturation machinery in Lactococcus lactis II.1403 are located on the chromosome.
- In Chapter VII the topology of LcnD, one of the proteins required for lactococcin secretion was examined. These studies indicated that LcnD is a membrane protein containing one transmembrane sequence near its N-terminus.
- Chapter VIII describes the experiments in which PedD, the pediocin PA-1 ABC-transporter, was shown to be the prediocin processing enzyme. The processing domain is situated in the N-terminus of the ABC-transporter.

References


110. Tagg, J.R., Dajani, A.S. and Wannamaker, L.W.
Chapter I

Introduction


