CHAPTER V

Localization of epitope-tagged derivatives of the bacteriocin lactococcin A from *Lactococcus lactis*

SUMMARY

This chapter describes the mutational analysis, and the cellular localization of epitope tagged derivatives of the lactococcal bacteriocin lactococcin A (LcnA). LcnA is produced as a precursor of which the N-terminal leader peptide is processed behind a conserved glycine doublet. This leader peptide is removed by the N-terminal part of the membrane protein LcnC which, together with LcnD, forms the dedicated secretion apparatus for LcnA. Mutagenesis of various amino acids in both the leader peptide or the mature part of the bacteriocin, all showed drastic effects on the (extracellular) antimicrobial activity of the respective mutated bacteriocins, even in case of just one single amino acid substitution. Cell fractionation studies proved that the precursor of LcnA has an intrinsic property to insert into the cytoplasmic membrane, as both in the presence or the absence of the secretion apparatus preLcnA was present in the membrane fraction. The same was observed for the various epitope-tagged derivatives constructed in this study. Our results suggest that this intrinsic property of the bacteriocin to insert into the cytoplasmic membrane can be ascribed to the presence of a C-terminal putative α-helix. This α-helix is believed to be involved in the antimicrobial activity of the bacteriocin by the formation of pores in sensitive cells. In addition to the α-helix, the leader peptide strongly influenced the cellular localization of the bacteriocin in the presence of the secretion apparatus. As it has been shown that the leader peptide directs the secretion of the bacteriocin, this effect may be explained by an interaction of the leader peptide with the secretion apparatus. Moreover, the results suggest that, in addition to the leader, also the putative α-helix may be involved in (efficient) secretion of the bacteriocin.
INTRODUCTION

All species of lactic acid bacteria are able to produce bacteriocins, which are proteinaceous compounds with antagonistic activity. One group of bacteriocins is formed by the small heat-stable peptides known as Class II bacteriocins (18). During the last years, knowledge concerning the mode of action of and immunity against these bacteriocins has increased rapidly (1,4,23,29,39,43,46 and 2,16,26,30,48,49, respectively), but as to how these molecules are secreted little is known. Secretion of Class II bacteriocins most commonly involves a dedicated system consisting of two membrane proteins. One protein belongs to the family of ATP-binding cassette (ABC) transporters, while the other is an accessory protein. Both proteins are encoded by the same operon and are essential for bacteriocin activity (2,11,25,38,44). In a number of cases, the general sec-dependent pathway of protein secretion is utilized for bacteriocin secretion (22,51).

In this study we examined lactococcin A (LcnA), a bacteriocin produced by certain strains of Lactococcus lactis (16,38,42). LcnA belongs to the Class II bacteriocins and is synthesized as a precursor protein containing an N-terminal leader peptide of the double-glycine type. The precursor of LcnA consists of 74 amino acid residues, of which the first 20, composing the leader, are removed concomitantly with secretion. LcnA acts on sensitive cells by increasing the permeability of the cytoplasmic membrane, thereby dissipating the membrane potential and leading to cell death (43). It has been assumed that LcnA multimers are able to form pores in the cytoplasmic membrane. Hydrophobicity analyses indicate that the C-terminal part of LcnA contains a stretch of amino acid residues that could form an α-helix possibly involved in pore formation (50). Immunity towards LcnA is effected by LciA, the lactococcin A immunity protein. The genes of both LcnA and LciA are encoded within the same operon, which is in close proximity to the lactococcin secretion genes (42,50).

The secretion apparatus of LcnA consists of the two proteins LcnC (the ABC transporter) and LcnD (the accessory protein). Recently, we studied the membrane topology of both proteins (Chapters II and IV of this thesis) and have shown that the N-terminal part of LcnC is involved in the processing of preLcnA into its mature, active form (this thesis Chapter IV). It has also been shown previously that homologous copies of lcnC and lcnD are chromosomally located in L. lactis IL1403 (47) and that this strain carries all the genetic information necessary to secrete active LcnA (42).

In this study, preLcnA was mutagenized and an antigenic epitope was introduced into the peptide at various positions. The localization and antimicrobial activities of (pre)LcnA and its derivatives were subsequently examined in L. lactis. All amino acid alterations in preLcnA had drastic effects on the (extracellular) antimicrobial activities of the respective derivatives.
Moreover, our results suggest that both the leader peptide and the putative C-terminal α-helix are involved in the cellular localization of the bacteriocin. In addition, both these domains of the prebacteriocin may be involved in (efficient) secretion.

**MATERIAL & METHODS**

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. *L. lactis* was grown as standing cultures at 30°C in 2-fold diluted M17 broth (41) supplemented with 0.5% glucose (G½M17). For *L. lactis*, chloramphenicol and erythromycin were used at final concentrations of 4 and 5 µg/ml, respectively. *E. coli* was grown in TY medium (32) at 37°C with aeration. Chloramphenicol and ampicillin were used at final concentrations of 10 and 100 µg/ml for *E. coli*, respectively.

**Molecular cloning, transformation and nucleotide sequencing.** General molecular cloning techniques were performed essentially as described by Sambrook *et al.* (33). Enzymes were purchased from Boehringer Mannheim GmbH (Mannheim, Germany) and used according to the instructions of the supplier. DNA was isolated as described before (5,35). Electrottransformation of *L. lactis* was performed with a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.), as described earlier (15) with the following modifications. Glycine was used at a concentration of 0.5% (for IL1403) or 1.8% (for NZ9000). After mixing with DNA, the cells were exposed to one pulse of 10 kV cm⁻¹, at a capacitance of 25 µF. *E. coli* was transformed as described before (9,24). Automated nucleotide sequencing was performed on a Vistra DNA Labstation 625 in combination with a Vistra DNA sequencer 725 (Amersham International, Little Chalfont, UK). Manual sequencing was done as described previously (34). Oligonucleotides for manual sequencing and for PCR mutagenesis were synthesised on an Applied Biosystems 381A DNA synthesizer (Applied Biosystems, Inc., Foster City, Calif.) or purchased from Isogen Bioscience BV (Maarsen, the Netherlands). Texas Red-labeled primers for automated sequencing were purchased from Amersham International or Eurogentech (Seraing, Belgium).

**Construction of plasmids for the overexpression of LcnA and its derivatives.** The ScaI/HindIII fragment of pKV4 was cloned in pMc, digested with *Sma*I and *Hind*III, resulting in plasmid pCF20. Subsequently, the *Hind*III/EcoRI cassette of pCF20 was transferred to pUC19, giving plasmid pCF19A. To delete the 5‘ part of lcnB on this plasmid, a PCR fragment was made using the M13/pUC universal primer (Boehringer) in combination with primer KOV4 (48). After digestion of the resulting PCR fragment with *Eco*RI and *Hind*III, the PCR fragment was introduced into pUC19 (pCF19A00). By means of two-step mutational PCR on the latter plasmid, *Bam*HI restriction enzyme sites were introduced at various positions in lcnA. The PCR fragments in step 1 were obtained using the M13/pUC universal primer in combination with one of the following mutational primers (*Bam*HI restriction enzyme sites are indicated in bold):

CMF6 : 5'-CCA GCC GCT GTG GAT CCA ATA AAT GTT AAT TTT CC
CMF7 : 5'-GCG CCA CCC ATG GAT CCA TTA ACA ATG G
SvH1 : 5'-GTT AAT TTT CCT CCG TTG GAT CCT TCT GAA AGT TCT TC
SvH2 : 5'-GTT TTG AGT TTG TTG GGA TCC ATA TTT GTG TGT ATT AG
SvH3 : 5'-CAT ATC TTA ACT CA G GAT CC A TGG TGC AAC CCG AAC C

Subsequently, in step 2, these PCR fragments were used as megaprimer in combination with the M13/pUC reverse primer. The resulting PCR products were inserted as EcoRI/HindIII fragments between the corresponding sites in pUC19. The plasmids with a BamHI restriction enzyme site introduced at the proper position in lcnA by the primers SvH1, CMF6, SvH2, CMF7 and SvH3 were named pCF19A1, pCF19A2, pCF19A3, pCF19A4 and pCF19A5, respectively. The plasmids pCFA00 to pCFA5 were restricted with HpaI and HindIII and the lcnA/lciA containing fragments were cloned in pMG36c digested with SmaI and HindIII, giving rise to the plasmids pMGCA0 to A5, respectively. These latter plasmids were then digested with BamHI and a synthetic oligonucleotide linker encoding either one or two epitopes was introduced. This linker was made by annealing the primers SPRALADS (5'-GATCTTCTCCACGTGCTTTAGCTGATTCTG-3') and SPRALADSinv (5'-GATCCAGAATCAGCTAAAGCACGTGGAGAA-3') or 2XS (5'-GATCTTCTCCACGTGCTTTAGCTGATTCTG-3') and 2XSinv (5'-GATCCAGAACGACGTGGAGAGGATCCAGAATCAGCTAAAGCACGTGGAGAA-3'). The resulting plasmids were labeled by extending the name of the original plasmids by S or 2S, respectively.

To express lcnA and its derivatives together with lciA under control of the inducible nisin promoter PnisA, an RcaI restriction enzyme site, incorporating the ATG start codon of lcnA, and an XbaI restriction enzyme site downstream of lciA were introduced by PCR with the mutational primers CMF60III (5'-AATTCGAATTC TCATGA AAAATCAATTAAATTTTAATATTG-3') and CMF61 (5'-CTAGATCTAGAAAGCTTACAGTCGTCCCCACGACATAAT-3') using plasmids pMGCA0 to 5 or pMGCA12S to 52S as templates (the RcaI and XbaI restriction enzyme sites in the primers CMF60III and CMF61, respectively, are indicated in bold). The PCR fragments were restricted with RcaI and XbaI and cloned into pNZ8048 digested with NcoI and XbaI. The resulting plasmids were named pNA00 to pNA5 and pNA12S to pNA52S, respectively.

To overexpress lcnA and its derivatives in L. lactis IL1403, the EcoRI fragment of pNZ9573 containing nisK and nisR was inserted into pIL253 restricted with EcoRI, resulting in pCF9B. All (PCR-derived) inserts used to construct the above described plasmids were sequenced to verify their integrity.

Nisin-induced overexpression of the lcnA(derivative)/lciA operons in L. lactis. Overexpression of genes under control of the nisA promoter was carried out in L. lactis NZ9000 or in L. lactis IL1403 (pCF9B). Overnight cultures were diluted 100-fold into fresh medium and grown at 30 °C until an OD at 600 nm of 0.4 to 0.5 was reached. Subsequently, the cultures were induced with nisin at a final concentration of 0.5 ng/ml and incubated for another four hours. Purified nisin A was kindly provided by the NIZO (Ede, The Netherlands).

Table 1. Bacterial strains and plasmids
Localization of LcnA derivatives

<table>
<thead>
<tr>
<th>Strains/plasmids</th>
<th>Relevant properties/genotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td><em>L. lactis</em> subsp. <em>lactis</em> IL1403</td>
<td>Plasmid free, secretion/maturation machinery for LcnA</td>
<td>(7)</td>
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<tr>
<td><em>L. lactis</em> subsp. <em>cremoris</em> NZ9000</td>
<td>Plasmid free, carrying nisK and nisR integrated into the chromosome</td>
<td>(19)</td>
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<td><em>E. coli</em> MC1000</td>
<td>araD139 Δ lacX74 Δ ara,leu,7697 galU galK strA</td>
<td>(6)</td>
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<tr>
<td><em>E. coli</em> JM101</td>
<td>supE thi Δ (lac-proAB) N80 lacZ Δ M15</td>
<td>(27)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pKV4</td>
<td>Em’, carrying lcnC, lcnD, lcnA, lciA</td>
<td>(47)</td>
</tr>
<tr>
<td>pIL253</td>
<td>Em’, cloning vector</td>
<td>(36)</td>
</tr>
<tr>
<td>pMG36c</td>
<td>Cm’, cloning vector</td>
<td>R. Kiewiet*</td>
</tr>
<tr>
<td>pMC</td>
<td>Cm’, cloning vector</td>
<td>(37)</td>
</tr>
<tr>
<td>pUC19</td>
<td>Amp’, cloning vector</td>
<td>(52)</td>
</tr>
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<td>pNZ8048</td>
<td>Cm’, carrying the promoter of nisA</td>
<td>(19)</td>
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<td>pNZ9573</td>
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<td>this study</td>
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<td>this study</td>
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<td>pCF19A5</td>
<td>Amp’, pUC19 encoding LcnA5/LciA</td>
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</tr>
<tr>
<td>pCF20</td>
<td>Cm’, pMc derivative carrying lcnA and lciA</td>
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<td>Cm’, like pCFA00 to 5, but pMC36c based</td>
<td>this study</td>
</tr>
<tr>
<td>pNA00 to 5</td>
<td>Cm’, like pCFA00 to 5, but pNZ8048 based</td>
<td>this study</td>
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<tr>
<td>pMGCA00-5 containing either once</td>
<td>(S) or twice (2S) the epitope SPRALADS</td>
<td>this study</td>
</tr>
<tr>
<td>pMGCA00-5 containing twice the epitope SPRALADS</td>
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<tr>
<td>pNA(00 to 5)2S</td>
<td>Cm’, like pNA00-5 containing twice the epitope SPRALADS</td>
<td></td>
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Em: erythromycin; Cm: chloramphenicol; Amp: ampicillin; a.a. amino acid; * laboratory collection

**Cell-fractionations.** Cell-fractionation on cells from 90 ml of culture was carried out as described earlier (3), with the following modifications. Spheroplasts were disrupted using a French-pressure cell (SLM AMINCO, Urbana, IL). Subsequently, samples were centrifuged for 10 minutes at 4°C (1.000 g) to remove intact cells, cell debris and possible inclusion bodies. The supernatant was collected and centrifuged at 135.000 g for 30 minutes at 4°C and the resulting supernatant was designated...
cytoplasmic fraction. The fractions obtained by this procedure were not dialyzed. Culture supernatants were concentrated 20 times by lyophilization using the Labconco Lyph-lock 4.5 Freeze Dry System (Labconco, Kansas City, MO). Concentrated samples were dialyzed twice, for one and 16 hours, respectively, against water at 4°C in dialysis membranes with a cut-off value of 1 kDa (Spectrum, Houston, TX). The dialysed samples were concentrated 50-fold in an Eppendorf Concentrator 5301 (Eppendorf, Hamburg, Germany).

**Bacteriocin assays.** Cells from *L. lactis* cultures in the end-logarithmic phase of growth were harvested by centrifugation, washed twice in the buffer used for cell fractionation, and resuspended to the original volume of the same buffer. Aliquots of the supernatant fraction or the resuspended cells were then boiled for 10 minutes. The bactericidal activity of LcnA or its derivatives in the samples was determined by the dilution assay essentially as described earlier (10), except that microtiter plates with wells containing 200 µl of GM17 broth were used. In each microtiter plate well, 100 µl of a 10^{-4} diluted overnight culture of *L. lactis* IL1403 (pNZ8048, pCF9B), containing approximately 10,000 colony forming units, was used as indicator.

**Protein electrophoresis and Western hybridization.** After harvesting and washing in cell-fractionation buffer (3) the cells were resuspended in the same buffer. Protein samples for SDS polyacrylamide gel electrophoresis (SDS-PAGE) were prepared by 2-fold dilution of aliquots of resuspended cells, concentrated supernatants or samples obtained by cell-fractionation in 2x sample buffer (21) supplemented with SDS to a final concentration of 2.5 %. Samples were boiled for 10 minutes. SDS-PAGE was carried out as described earlier (21). Protein samples were run on either SDS-(15%)PAA or tricine-SDS-(16%)PAA gels. Proteins were blotted onto Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore Corp, Bedford, MA) according to standard protocols (20). Proteins were detected using the ECL or ECL+ detection kit (Amersham). Polyclonal antibodies against the di- and tripeptide transport system (DtpT) and LcnA were kindly provided by the Department of Microbiology, University of Groningen, and H. Holo of the Agricultural University of Norway, respectively. Monoclonal antibodies against the aminopeptidase PepC and anti-CroLac I were kindly provided by M.-P. Chapot-Chartier (INRA, Jouy-en-Josas, France), and Innogenetics (Ghent, Belgium) respectively. Horseradish peroxidase-conjugated mouse antirabbit and rabbit antimouse immunoglobulins (Amersham) were used as secondary antibodies. Immunoblots were stripped as described in the ECL Western blotting protocols manual (Amersham) and reprobed. To ensure that blot-stripping did not affect the relative intensities of the bands, the first antibody of a blot was used again after several strippings.

**Quantification of protein bands and peptidase activity measurements.** The intensities of protein bands on the immunoblots were determined using the Ultrascan XL Enhanced Laser Densitometer (LKB, Bromma, Sweden). Enzymatic activities of PepN and PepC were measured essentially as described before (28) using the substrate Lys-p-nitroanilid (Sigma Chemical Company, St. Louis, MI).
Localization of LcnA derivatives

Fig. 1. Amino acid sequence of prelactococcin A. Amino acid substitutions generated by the introduction of BamHI restriction enzyme sites in lcnA are indicated above the sequence. The names of the corresponding LcnA derivatives are given between brackets. The nucleotide sequences encoding the epitopes were introduced into the BamHI sites. The amino acids are numbered relative to the first amino acid (+1) of mature LcnA. The arrow indicates the maturation cleavage site in preLcnA. The putative α-helix is underlined.

RESULTS

The introduction of a BamHI restriction enzyme site at various positions in lcnA led to amino acid substitutions in the leader peptide of preLcnA at position -4 (resulting in LcnA1), in the N-terminus of mature LcnA at position 6 (LcnA2), at positions 23 and 24 upstream of the putative α-helix (LcnA3), and in the α-helix at position 42 (LcnA4) and to an extension of the polypeptide by two amino acids at the C-terminus (position 54; LcnA5) (Fig 1). The introduction in the lcnA derivatives of the nucleotide linker encoding the epitope SPRALADSGSSPRALADS resulted in a duplication of Gly and Ser residues flanking the epitopes. All lcnA derivatives obtained in this way were expressed under the control of the strong lactococcal promoter P32. The BamHI derivatives and those with the linker encoding the epitope SPRALADSGSSPRALADS were overexpressed using the inducible nisA promoter. As for induction of the latter promoter the presence of NisK and NisR is required, plasmid pCF9B was constructed to introduce the genes nisK and nisR into L. lactis IL1403. All mutations led to either a strong decrease (LcnA1, LcnA2 and LcnA5) or to a complete loss (LcnA3 and LcnA4) of antimicrobial activity. In all cases in which the sequence SPRALADSGSSPRALADS was introduced, antimicrobial activity was completely lost (data not shown).

Table 2. Distribution of DtpT, PepC and PepN over cell-fractions of L. lactis IL1403 (pCF9B) expressing LcnA or one of its derivatives

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Localization of LcnA and its derivatives in \textit{L. Lactis}. \textit{L. lactis} IL1403 strains containing the various P32 expression plasmids were grown to end exponential phase and samples of both cells and concentrated supernatants were examined on Coomassie Brilliant Blue-stained gels after tricine-SDS-(16\%) PAGE. In none of the samples additional bands were observed when compared to samples of a strain carrying the cloning vector pMG36c (data not shown). In Western hybridizations using the monoclonal antibody anti-CroLacI, which specifically recognizes the amino acid sequence SPRALADS, only the mature form of the protein LcnA4 with the twin epitope insertion (LcnA42S) could be detected in the cell sample (data not shown). In all other cases no specific band of the right size was observed, neither in the cells nor in the concentrated supernatants. The same was true for \textit{E. coli} carrying the various constructs: no bands of the expected sizes were observed (data not shown).

Nisin-induced overexpression of (pre)LcnA and its derivatives carrying the twin epitope was observed in Coomassie Brilliant Blue-stained SDS-(15\%)PAA gels when \textit{L. lactis} IL1403 (pCF9B) and \textit{L. lactis} NZ9000 were used as the hosts (Fig. 2). In addition, another band of slightly lower mobility appearing upon nisin induction suggests that \textit{lciA}, present in all constructs and part of the bacteriocin operon, is also overexpressed. LciA has a molecular weight of 11.1 kDa (48).

Next, the nisin-induced strains were subjected to cell-fractionation studies. As a control for proper cell-fractionation, the distribution over the various fractions of the cytoplasmic enzyme PepC (40) and the membrane protein DtpT (12) was determined (Table 2). As

<table>
<thead>
<tr>
<th>cell-fraction</th>
<th>DtpT (^b)</th>
<th>PepC (^b)</th>
<th>PepN / PepC (^c)</th>
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<tr>
<td></td>
<td>IL1403 NZ9000</td>
<td>IL1403 NZ9000</td>
<td>IL1403 NZ9000</td>
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<tr>
<td>Supernatant</td>
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<td>0 (0) 0 (0) 0 (0)</td>
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<tr>
<td>Membrane</td>
<td>87 (4) 86 (3) 2 (1)</td>
<td>2 (1) 2 (1) 1 (0)</td>
<td>1 (0) 1 (0)</td>
</tr>
<tr>
<td>Membrane associated</td>
<td>12 (4) 10 (3) 6 (3)</td>
<td>6 (3) 6 (3) 10 (1)</td>
<td>9 (2)</td>
</tr>
</tbody>
</table>
| Cytoplasm          | 0 (0) 4 (1) 92 (3) | 92 (3) 89 (2) 90 (0) |}

\(^a\) Values are percentages of the total amount and are the average of seven independent cell-fractionation studies. Standard deviations are shown in brackets.

\(^b\) Densitometric measurement of the corresponding bands on immunoblots using anti-DtpT or anti-PepC antibodies.

\(^c\) Enzymatic measurements using the substrate Lys-p-nitroanilid.
expected, PepC was detected mainly in the cytoplasm, whereas DtpT was mainly present in the
cytoplasmic membrane and, in minor quantities, in the membrane-associated fraction. The
enzymatic activities of the cytoplasmic peptidases PepN and PepC were also measured in the
different fractions (Table 2) and since these results were comparable with the immunological
data obtained for PepC, we conclude that the relative intensity of bands on the immunoblots is
a reliable measure for the relative amounts of the corresponding protein in the different
fractions.

Fig. 2. Coomassie Brilliant Blue-stained SDS-(15%) PAA gel containing extracts of
sonicated nisin-induced cells of
L. lactis IL1403 (pCF9B) carrying, in
addition, pNZ8048 (lane 1), pNA00 (lane 2), pNA12S (lane 3), pNA22S (lane 4),
pNA32S (lane 5), pNA42S (lane 6), pNA52S (lane 7). The sizes (in kDa) of the
proteins in the Rainbow molecular weight
marker in lane M (Amersham) are shown
in the left margin. The solid arrow in the
right margin indicates the position of the
overproduced bacteriocin (derivatives).
The dashed arrow indicates the band of
LciA.

The results of the cell-fractionation studies on nisin-induced cells of L. lactis IL1403
(pCF9B) expressing the LcnA derivatives with the twin epitope are shown in Fig. 3. The
quantified data are summarized in Table 3A. As expected, no bands were observed in the
LcnA-containing samples (Fig. 3A). Only in cells expressing LcnA42S (Fig. 3E), two bands
reacting with the anti-Crolac I antibody were present in the cytoplasm. NZ9000 (pNA42S),
lacking the LcnC/D secretion system, only produced the upper of the two bands (data not
shown). As we have previously shown that LcnC is capable of cleaving the precursor of LcnA,
these results show that the upper band represents preLcnA42S and the lower band the mature
form, LcnA42S. PreLcnA42S was also observed in the membrane and membrane-associated
fractions. The minor band of preLcnA42S in the cell wall was only observed when the LcnC/D
proteins were present (Table 3B). The precursors of LcnA12S and LcnA32S were detected in
equal amounts in the cytoplasm and membrane fraction. Very little was present in the
membrane-associated fraction (Fig. 3B,D). By contrast, preLcnA22S and preLcnA52S were
almost exclusively located in the cytoplasmic membrane (Fig. 3C,F); only minor amounts of
the unprocessed forms were detectable in the cytoplasm. Only in the case of LcnA52S, upon
long exposure times, a tiny amount of the mature protein (LcnA52S) was observable in the cell.
wall fraction (Table 3A). None of the LcnA derivatives could be detected with the anti-CroLac I antibodies in the supernatants of the producing strains.

The same filters were also examined with a polyclonal antibody raised against LcnA (data not shown). Unprocessed LcnA was present exclusively in the membrane fraction. The results for LcnA12S, LcnA32S and LcnA42S using this antibody confirmed those obtained with the anti-CroLac I antibody, except that the processed form of LcnA42S was not detectable with the anti-LcnA antibodies. LcnA22S and LcnA52S, on the other hand, were hardly detectable with the anti-LcnA antibody, precluding reliable localization of these proteins.

**Fig. 3.** Immunoblots of tricine-SDS-(16%)PAA gels of cell fractions of end exponential growing *L. lactis* IL1403 cultures. In each lane the equivalent of 500 µl of culture was loaded. Cells carried plasmid pCF9B in combination with a plasmid encoding LcnA (A), LcnA12S (B), LcnA22S (C), LcnA32S (D), LcnA42S (E) or LcnA52S (F). In all cases the anti-CroLac I antibody was used as primary antibody. P; precursor form, M; mature form. Fractions: SUP; supernatant, CW; cell-wall, MEM; membrane, MA; membrane-associated, CYT; cytoplasm.

**Table 3.** Distribution of LcnA and its derivatives over the various cell-fractions of (A), nisin-induced *L. lactis* IL1403 (pCF9B; *lcnC*, *lcnD*) and (B), NZ9000 (*lcnC*, *lcnD*) carrying the
indicated plasmids. The values represent percentages of the total of the protein bands reacting on immunoblots with the anti-Crolac I as determined by densitometric measurements. P: precursor form, M: processed form, SUP: supernatant, CW: cell-wall, MEM: membrane, MA: membrane associated, CYT: cytoplasm, -: negative control.

A

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>SUP</th>
<th>CW</th>
<th>MEM</th>
<th>MA</th>
<th>CYT</th>
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<tbody>
<tr>
<td>pNA00</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>pNA12S</td>
<td>0</td>
<td>0</td>
<td>52</td>
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**Effects of LcnC and LcnD on the localization of (pre)LcnA and its derivatives.** To examine the effect of the LcnC/D secretion system on the localization of LcnA and its derivatives, the distribution of the bacteriocin variants over the various cell-fractions of *L. lactis* NZ9000 was examined. Strain NZ9000 is an MG1363 derivative (19) that does not carry the genes for the lactococcin secretion apparatus. Accordingly, when LcnA00 was overexpressed in this strain, no antimicrobial activity was detectable in the culture supernatant. The localization of the LcnA derivatives in this host is presented in Table 3B. This table shows
that in the absence of the secretion apparatus the precursors are still present in the cytoplasmic membrane fraction, indicating that they have an intrinsic capacity to insert into the membrane. In the absence of LcnC and LcnD (Table 3B), relatively more of the preforms of LcnA32S, LcnA42S and LcnA52S were observed in the cytoplasmic fraction (compare with Table 3A). LcnA22S was the only derivative of which the localization was independent of the presence or absence of LcnC and LcnD. As expected, in all cases only the unprocessed forms were observed, due to the absence of the bacteriocin secretion apparatus in L. lactis NZ9000.

**DISCUSSION**

In this study we mutagenized the bacteriocin LcnA from *L. lactis* by amino acid substitutions and insertions and examined the cellular location of (pre)LcnA and its derivatives. Even the amino acid changes due to the insertion of the *Bam*HI site in *lcnA* already had drastic effects on the (extracellular) bacteriocin activity. When either one or two copies of the SPRALADS epitope were introduced in LcnA, bacteriocin activity was completely abolished in all cases. Due to the fact that we were not able to detect LcnA nor its modified derivatives in supernatant fractions by immunological techniques, we can not distinguish whether the decrease in bacteriocin activity is caused by lowered activity of the bacteriocin on the sensitive indicator cell or by impaired bacteriocin secretion and/or maturation.

All preLcnA variants were capable of insertion in the cytoplasmic membrane in the absence of the secretion apparatus, indicating that the prebacteriocin has the intrinsic property to become membrane-inserted. The same was previously observed for haemolysin (HlyA), which carries an amphiphatic helix in its C-terminus and is secreted by a similar ABC transporter system (31). In the case of wildtype LcnA and LcnA22S, in which the twin epitope neither interrupts the leader sequence nor the putative α-helix, the majority of the precursor was present in the membrane. In contrast, when the α-helix was interrupted, considerable quantities remained cytoplasmic (LcnA42S). This was also the case when the twin epitope was inserted close to the α-helix (LcnA32S and LcnA52S), which might have interfered with the (proper) formation of this helix. These results suggest that the α-helix facilitates the insertion of the prebacteriocin into the membrane, and would agree with the proposed role of this helical structure in pore formation in the membrane of bacteriocin-sensitive cells.

Of the bacteriocins with an uninterrupted leader sequence, in general more precursor accumulated in the membrane when the secretion apparatus is present. As we have previously shown that LcnC and LcnD, which form the secretion apparatus, are integral membrane proteins (Chapters IV and II of this thesis, respectively), it is likely that this increase in the relative amount of membrane-bound prebacteriocin is caused by initial binding of the leader to the secretion apparatus followed, if not precluded by the mutation, by the initiation of the
translocation process. This assumption is supported by the fact that the leader of LcnA contains the information for proper secretion and maturation by LcnC/D (17,45) and that the N-terminus of LcnC is able to process preLcnA (Chapter IV of this thesis). Only in the case of LcnA12S, a decrease in the relative amount of the precursor form in the membrane was observed when the secretion apparatus was present. LcnA12S carries the twin epitope in the leader, downstream of a highly conserved region that may be involved in binding to the processing domain of LcnC (13,14). Competition between insertion of preLcnA12S in the membrane via its α-helix and the supposedly weak interaction of the mutated leader with LcnC may lead to release of the prebacteriocin in the cytoplasm.

The fact that the processed form of LcnA42S was detected in the cytoplasmic fraction is another indication that bacteriocin secretion and processing can be uncoupled. Previously, this has been shown in vitro for LcnG and in vivo for pediocin PA-1 and LcnA (13,49, this thesis Chapter IV). If we assume that accumulation of the processed form of LcnA42S in the cytoplasm is due to impaired secretion, this would imply a role of the putative α-helix in, at least facilitating, secretion. Interestingly, studies on the secretion of colicin V (ColV), an E. coli bacteriocin with a double-glycine-leader, showed that the presence of a putative α-helix improved the efficiency of recognition and/or secretion of the protein (11). The fact that only little of the processed form of LcnA52S was observed in the cell wall and nothing in the supernatant implies that secretion of this mutant is not efficient. This may be due to the increased size of the molecule and/or because of the close proximity of the epitope to the α-helix. However, as some LcnA52S was secreted, the lactococcin secretion apparatus should, in principle, be able to translocate the other LcnA derivatives of identical size across the cytoplasmic membrane. As neither processing nor secretion of LcnA12S, LcnA22S and LcnA32S was observed, this suggests that the epitope insertions interfere with processing and secretion of these LcnA derivatives. It is not clear at this point why the preform of LcnA42S was observed in the cell-wall fraction.

Summarizing, our results indicate the importance of both the putative α-helix and the leader peptide in the localization of the prebacteriocin in the cell. As shown previously, the leader peptide directs the secretion of the bacteriocin, probably due to the specific interaction of the leader with the N-terminal part of the ABC transporter component of the dedicated secretion machinery (17,45). Our results, however, suggest that also the putative α-helix may be involved in secretion. This may be mediated either by improving the kinetics of secretion due to concentration of the prebacteriocin in the membrane (although our results do not rule out the possibility that the prebacteriocin can also interact directly with the secretion apparatus from the cytoplasm) or it may be a direct effect by interaction of this hydrophobic part of the bacteriocin with (one of) the secretion proteins during secretion. Indeed, all heterologous proteins described to have been secreted by the secretion apparatus of LcnA are bacteriocins
and all contain hydrophobic stretches in their C-terminal parts either in the form of a putative α- helix or an amphiphatic helix.

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REFERENCES


sequences with leaderpeptides that are common among peptide bacteriocins produced by Gram-positive bacteria. Microbiology **140**:2383-2389.


