Structure, Organization, and Expression of the \textit{ict} Gene for Lacticin 481, a Novel Lantibiotic Produced by \textit{Lactococcus lactis}*

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The structural gene for the lactococcal lantibiotic lacticin 481 (\textit{ict}) has been identified and cloned using a degenerated 20-mer DNA oligonucleotide based on the amino-terminal 7 amino acid residues of the purified protein. The transcription of the \textit{ict} gene was analyzed, and its promoter was mapped. DNA sequence analysis of the \textit{ict} gene revealed an open reading frame encoding a peptide of 51 amino acids. Comparison of its deduced amino acid sequence with the amino-terminal sequence and the amino acid composition of lacticin 481 indicates that the 51-residue peptide is pre-lacticin 481, containing a 27-residue carboxy-terminal propeptide and a 24-residue amino-terminal leader peptide which lacks the properties of a typical signal sequence and which is significantly different from the leaders of other lantibiotics. The predicted amino acid sequence of prolacticin 481 contains 3 cysteines, 2 serines, and 2 threonines which were not detectable in amino acid analyses of mature lactocin 481. Based on these results and on characterization by two-dimensional NMR techniques, a structural model is proposed in which 2 cysteine residues are involved in lanthionine and one in \(\beta\)-methylanthionine formation, and a 4th threonine residue is dehydrated. This model predicts a molecular mass for lacticin 481 of 2,901, which is in excellent agreement with that obtained from mass spectrometry.

Lactic acid bacteria are Gram-positive bacteria that are frequently able to produce proteins with antimicrobial activity, designated bacteriocins (Piard and Desmazeaud, 1992). The bacteriocins of lactic acid bacteria which have been biochemically characterized include two types of peptides. Those containing the common 20 amino acids residues, such as lactacin F produced by \textit{Lactobacillus acidophilus} (Muriana and Klaenhammer, 1991a, 1991b), lactococcin A from \textit{Lactococcus lactis} (Hol et al., 1991; van Belkum et al., 1991), and leucocin A-UAL 187 from \textit{Leuconostoc gelidum} (Hastings et al., 1991), containing 57, 54, and 37 amino acids, respectively. A second class of peptides, called lantibiotics, is characterized by the presence of dehydrated amino acids (dehydroalanine and dehydrobutyrine) and thioether amino acids lanthionine and \(\beta\)-methylanthionine (Schnell et al., 1988). The lantibiotics nisin A (Hurst, 1981; Buchman et al., 1988), nisin Z (Mulders et al., 1991), and lactocin 481 (Piard et al., 1990, 1992), are produced by \textit{L. lactis}, and lactocin S, by \textit{Lactobacillus sake} (Mortvedt et al., 1991). Lantibiotics have also been isolated from other Gram-positive organisms, such as \textit{Bacillus subtilis} (subtilin), \textit{Staphylococcus epidermidis} (epidermin and Pep6), and \textit{Staphylococcus gallinarum} (gallidermin) (Jung, 1991). In contrast to most antibiotics, the lantibiotics are synthesized via a ribosomal pathway as prepeptides which are subsequently modified. The serine and threonine residues are dehydrated to dehydroamino acids which can react with the thiol group of cysteine to form the thioether bridges of lanthionine or \(\beta\)-methylanthionine, respectively. Nisin is the best characterized lantibiotic, and its spatial structure has been studied by two-dimensional NMR (van de Ven et al., 1991; Lian et al., 1992). However, extensive structure-function studies are necessary to understand the molecular basis of lantibiotic action and to be able to construct mutants with an improved spectrum of activity and physico-chemical properties. To achieve this, two approaches are presently followed. One is to generate by random or site-directed mutagenesis new lantibiotics and study their properties. The other is to characterize the structure and action of naturally occurring lantibiotics. The feasibility of both approaches has been shown in studies of nisin, by engineering dehydrated and other residues (Kuipers et al., 1992), and analyzing the biological activities of the two natural variants nisin A and nisin Z (de Vos et al., 1993). Here we continue with the last approach and focus on the lantibiotic lactocin 481, which is a broad spectrum bacteriocin exhibiting bactericidal activity against a wide range of lactic acid bacteria and against \textit{Clostridium tyrobutyricum} (Piard et al., 1990). We describe the structure, organization, and transcription of the \textit{ict} gene encoding lactocin 481. In addition, we propose a structural model for lactocin 481 based on the deduced amino acid sequence of pre-lacticin 481, its amino acid composition and characterization by two-dimensional NMR spectroscopy.

**EXPERIMENTAL PROCEDURES**

Bacterial Strains, Plasmids, and Media—Lactocin 481-producing \textit{L. lactis} subsp. \textit{lactis} (further designated as \textit{L. lactis}) CNZR 481 was obtained from the CNRZ culture collection (INRA, Jouy-en-Josas, France). The nisin producer \textit{L. lactis} NIZO R5 harboring the \textit{nisA} encoding transposon Tn916 (Rausch and de Vos, 1992) was from NIZO. A lactocin 481-producing (\textit{Lct}+) transconjugant, \textit{L. lactis} JC17, was obtained by conjugal matings between \textit{L. lactis} 481 (\textit{Lct}+) and \textit{L. lactis} IL1441 (\textit{Lct}). Microbial Genetics Laboratory, INRA as described elsewhere. \textit{Escherichia coli} JM38 (Vieira and Messing, 1982) and TG1 (Gibson, 1984) were used as hosts in cloning experiments.
cilline (100 pg/ml) and erythromycin (5 pg/ml) were added. Isopropyl-
containing 0.5% glucose at 30 °C. If appropriate, antibiotics carbeni-
tosis-Total DNA from
E. coli and L. lactis, respectively.
For expression studies L. lactis MG1614 (Gasson, 1983), IL1441 and
NIZO R520 (Rauch et al., 1991) were utilized. Plasmids pUC19 (Vieira
and Messing, 1982) and pIL253 (Simon and Chopin, 1988) were used
as cloning vectors in L. lactis (Vos and Gasson, 1989). Subsequent manipulations
were performed as described (Sambrook et al., 1989). DNA was extracted from
E. coli by the alkaline lysis method (Sambrook et al., 1989). For plasmid extraction
in L. lactis, this procedure was slightly modified as described by de
Hong van Kooij and de Vos (1992). Plasmid DNA was extracted from
E. coli by the alkaline lysis method (Sambrook et al., 1989) or by the
alkaline extraction method (Sanger et al., 1977) using M13 universal primers and primers
P28 (5'-CTTGAAGAAGATTAAAAGAG-3') and P29 (5'-GATTT-
TACTTGCTGCTC-3'), complementary to position 844-824 and
829-824, respectively (see Fig. 1). Computer analysis of DNA and amino acid sequences was performed with the programs
of PC/GENE (Genofit).

**DNA Isolation, Northern Blot Analysis, and Primer Extension
Studies—** L. lactis strains were grown to A600 = 0.6. Cells were
pelleted, resuspended in 0.5 ml of TE (10 mM Tris-HCl, pH 7.5, 1
mM EDTA) and kept on ice. Subsequently, the suspension was
transferred to a microcentrifuge tube containing 0.6 g of zirconium
glass beads (Biospec Products), 0.17 g of 4% Macaloid (Rhex, for
preparation see below), 0.5 ml of phenol, pH 7.5, and 0.05 ml of 10%
sodium dodecyl sulfate. The tubes were shaken at maximal setting
in a Biospec homogenizer at room temperature for 1 min. The
supernatant was stored at -20 °C, centrifuged, and resuspended before each use.
Macaloid 4% was prepared as follows. Two grams of Macaloid were
suspended in 100 ml of TE, boiled for 5 min, and sonicated with a
stereilized probe sonicator (Heat Systems) until a gel formed (about
10 min). The gel was collected and extracted successively with
phenol/chloroform and chloroform. RNA was precipitated with
ethanol at -20 °C, centrifuged, and resuspended before each use.

RNA probes for the L. lactis 481 Lacticin gene were hybridized to 10
μg of total RNA, which was isolated from L. lactis MG1614 by the method
(Sanger et al., 1977) using M13 universal primers and primers
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supernatant was stored at -20 °C, centrifuged, and resuspended before each use.

**DNA Hybridization, Southern Hybridization, and Sequence Analysis—** Total DNA from L. lactis was isolated as described by Rauch and de Vos (1992). Plasmid DNA was extracted from E. coli by the
alkaline lysis method (Sambrook et al., 1989). For plasmid extraction
in L. lactis, this procedure was slightly modified as described by de
Hong van Kooij and de Vos (1992). Subsequent manipulations in vitro and in E. coli were performed as described (Sambrook et al., 1989). L. lactis was
transformed by electroporation using a GenePulser (Bio-Rad) (Vos et al., 1989). For Southern hybridization, DNA was electrophoresed in an agarose gel, depurinated for 20 min in 0.25 M HCl, and blotted for 1 h onto a nylon GeneScreen Plus membrane (Du Pont) using a
blotting device (Vacugene, Pharmacia LKB Biotechnology Inc.) and
sterilized probe sonicator (Heat Systems) until a gel formed (about
10 min). The gel was allowed to cool, washed with TE, centrifuged, and separated from the supernatant. This allowed us to recover about 50
μg of gel.

**For Northern blot analysis, RNA was glyoxylated, fractionated on
a 1.0% agarose gel, and blotted and hybridized as described previously

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1 R. Raya, personal communication.
FIG. 2. Northern blot analysis of RNA isolated from lane 1, L. lactis CNRZ 481 (Lct*); lane 2, L. lactis JC17 (Lct*); lane 3, L. lactis IL1441 (Lct*). Hybridization was performed with a single-stranded probe for the lct gene and upstream region (see “Experimental Procedures”). The size of the transcripts is indicated on the left, in bases (b).

TABLE I

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<tr>
<td>Total</td>
<td>23</td>
<td>27</td>
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* Lan, lanthionine; β-CH3Lan, β-methylanthionine.

† Obtained from amino acid analyses.

‡ Deduced from the DNA sequence of lct.

(Fromega). This mixture was heated for 5 min at 65 °C and subsequently allowed to cool at room temperature. Subsequently, 1.6 μl of dNTP solution (1 mM dCTP, dGTP, and dTTP, and 100 μM dATP) was added, and the final volume was adjusted to 16 μl with 0.2 μl of α-32P-dATP and 0.2 μl (20 units) of avian myeloblastosis reverse transcriptase. After a 30-min incubation at 42 °C, the reaction mixture was extracted successively with phenol/chloroform and chloroform, and then precipitated with ethanol. The pellet was washed with 70% ethanol and dissolved in 3 μl of water and 3 μl of sequencing dye. The samples were heated for 3 min at 98 °C and loaded on an acrylamide sequencing gel. Controls that consisted of RNA to which no primer was added were treated similarly.

Preparative Purification of Lacticin 481—Fifteen liters of lacticin 481-containing supernatant was purified according to the previously reported procedure (Piard et al., 1992) except that the gel filtration step was omitted. Preparative reverse phase chromatography was performed using C18 Sep-Pak cartridges equilibrated with 25 mM ammonium acetate. Lacticin 481 activity was eluted with 50% 2-propanol in 20 mM ammonium acetate. Additional and final purification was achieved by reverse phase high performance liquid chromatography (Waters, model 510) using a 250 × 20-mm Hi-Pore RP-318 (Bio-Rad) column at 30 °C. Running buffers (10 ml/min) were 10% acetonitrile in 25 mM ammonium acetate (buffer A) and 90% acetonitrile in 20 mM ammonium acetate (buffer B). A linear gradient of 55% buffer A plus 45% buffer B, to 45% buffer A plus 55% buffer B run within 35 min was used. Absorbance was monitored at 220 nm, and a data acquisition system (Waters Maxima 820) was used. The active eluting fractions were pooled, and ammonium acetate was removed by consecutive washes with distilled water and freeze-drying.

Amino Acid Analysis of Lacticin 481—Pure lacticin 481 was hydrolyzed for 24 and 96 h in 6 M HCl at 110 °C; the 96-h hydrolysis was used for the complete hydrolysis of Val-Ile bonds. The hydrolysates were concentrated by lyophilization, dissolved in 0.2 mM sodium citrate, pH 2.2, and analyzed on an amino acid analyzer (LKB, type 4151). The sum of the lanthionine and β-methylanthionine residues was determined using d,L-lanthionine as a standard.

Mass Spectrometry—Lacticin 481 was analyzed as described by van Dorsselaer et al. (1990) using a VG Biotech BioQ mass spectrometer (VG Biotech Ltd., Altrincham, U.K.) consisting of an electrostatic spray ion source operating at atmospheric pressure, followed by a quadrupole mass analyzer with a mass range of 4,000. The electrospray potential was about 4,000 V. The voltage of the extracting cone was adjusted to 100-200 V. Lacticin 481-containing samples were prepared in methanol/acetic acid, 99:1 (v/v), and 10 μl was introduced into the ion source at a flow rate of 3 μl min⁻¹. Results were processed using a data system and quoted as a mean molecular mass derived from several multiply charged ion peaks plus the standard deviation.

NMR Spectroscopy—One- and two-dimensional NMR spectra were taken on Bruker AM400 and AM600 spectrometers operating at 400.13 and 600.13 MHz, respectively. The spectra were referenced to

Fig. 3. Primer extension products of RNA transcribed from lct. The relevant DNA sequence (complementary to lct sequence) is indicated, and the determined transcription start site is marked by an asterisk. Lanes A, C, G, T are sequencing reactions carried out as described under “Experimental Procedures”; lanes 1 and 2, L. lactis CNRZ 481 (Lct*) with and without primer, respectively; lanes 3 and 4, L. lactis JC17 (Lct*) with and without primer, respectively; lane 5, L. lactis IL1441 (Lct*) with primer.

(van Rooljen and de Vos, 1990). 32P-Labeled single-stranded DNA obtained by primer extension of oligonucleotide P28 was used as hybridization probe.

In primer extension studies, oligonucleotide P28 (10 ng) was hybridized to 10 μg of RNA in a total volume of 14 μl containing 70 mM Tris-HCl, 10 mM MgCl₂, 15 mM dithiothreitol, and 40 units of RNasin

Amino acid analysis of lacticin 481

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The resulting recombinant transformants were screened by the position of lacticin 481 (see below) revealed that the 51-kilobase(s) copy; NOESY, nuclear Overhauser enhancement spectroscopy; L. lactis, that was subcloned in M13mp18 and M13mp19 for sequencing. EcoRI-linearized pUC19, and introduced into E. coli JM83. Southern hybridization of pLCT8 revealed that the oligonucleotide P25. One of the colonies showed a reproducible strong colony blot and Southern hybridization using the oligonucleotide based on the amino-terminal amino acid sequence of lacticin 481. Hybridization was found with a 9.5-kb signal was detected with DNA from L. lactis IL1441. Transcripts of larger size (7-9 kb) were also detected in both lacticin 481 producers and may represent either untranslated transcript was present in the nonproducer L. lactis JC17 was determined by primer extension mapping of RNA, using oligonucleotide P28 as primer. The same sized primer extension product was detected in both lacticin 481 producer

RESULTS

Isolation and Sequence Analysis of Lacticin 481 Structural Gene, lct, and Its Flanking Regions—Southern blots of EcoRI-digested total DNA from L. lactis 481 (Lct*), JC17 (Lct*), and IL1441 (Lct-)) were hybridized with the degenerated P25 oligonucleotide based on the amino-terminal amino acid sequence of lacticin 481. Hybridization was found with a 9.5-kb fragment of L. lactis 481 DNA and a 4.5-kb fragment DNA of L. lactis JC17 (data not shown). In contrast, no hybridization signal was detected with DNA from L. lactis IL1441. The 4.5-kb EcoRI fragments of L. lactis JC17 were isolated, ligated to EcoRI-linearized pUC19, and introduced into E. coli JM83. The resulting recombinant transformants were screened by colony blot and Southern hybridization using the oligonucleotide P25. One of the colonies showed a reproducible strong signal and contained the plasmid pLCTS. Restriction analysis and Southern hybridization of pLCT8 revealed that the lct gene was present on a 1.3-kb BamHI-HindIII subfragment that was subcloned in M13mp18 and M13mp19 for sequencing.

Analysis of the 1.3-kb DNA fragment sequence showed an open reading frame that could encode a peptide of 51 amino acids (Fig. 1). Comparison of the deduced amino acid sequence with the amino-terminal sequence and the amino acid composition of lacticin 481 (see below) revealed that the 51-residue peptide is prelacticin 481 consisting of a 27-residue carboxyl-terminal propeptide and of a 24-residue amino-terminal extension. The calculated molecular mass for prolacticin 481 is 2973 Da. A potential Shine-Dalgarno sequence (GGAG) is located 7 base pairs upstream of the ATG start codon of the lct gene (ΔG value of complementarity to the L. lactis 3' 16 S rRNA sequence was -9.4 kcal mol⁻¹). Twenty-seven base pairs downstream of the lct stop codon an inverted repeat was identified which could act as a rho-independent terminator with a ΔG value of -20.6 kcal mol⁻¹ (D'Aubenton Carafa et al., 1990).

Analysis of the DNA sequence upstream of the lct gene showed the end of a putative open reading frame. A data base search revealed that the deduced protein sequence had significant similarity (26% identity within a stretch of 88 amino acids) with the transposase from the E. coli IS4 insertion element (Fig. 1).

Analysis of the DNA sequence downstream of the lct gene showed the start of a putative open reading frame with a GTG start codon preceded by a potential ribosome binding site (AAGGA). However, no clear promoter consensus sequence was identified. Data base searching did not show similarity with other DNA sequences including those flanking structural genes of other lantibiotics.

Transcriptional Analysis of the lct Gene—Transcripts of the lct gene in L. lactis strains 481 and JC17 were detected by Northern blots by hybridization using a radiolabeled cDNA obtained with the P28 oligonucleotide as probe. The Northern blot (Fig. 2) reveals the presence of a small transcript of 280 bases in both lacticin 481 producers. In contrast, no hybridizing transcript was present in the nonproducer L. lactis IL1441. Transcripts of larger size (7-9 kb) were also detected in the lacticin 481 producers and may represent either unprocessed polycistronic mRNA containing the lct gene or transcription products of the region upstream of lct.

The transcription start site of lct in strains L. lactis 481 and JC17 was determined by primer extension mapping of RNA, using oligonucleotide P28 as primer. The same sized primer extension product was detected in both lacticin 481 producer
PLCT9 or PLCT10 did not result in lacticin 481 production and immunity. In contrast, promoter and shows that increasing the copy number of the was approximately four times more than that produced by transcription initiation site and consisting of the canonical hexanucleotide sequences TTGCAT (-35) and TATAAT (-10) which are separated by 17 nucleotides.

Expression Studies—To test the functionality of the promoter in vivo, the 4.5-kb EcoRI fragment from pLCT8 containing the lct gene was cloned in both orientations in pIL253 to yield pLCT9 and pLCT10. L. lactis 481 harboring pLCT9 or pLCT10 produced similar amounts of lacticin 481, which was approximately four times more than that produced by L. lactis 481. This indicates that lct was under control of its own promoter and shows that increasing the copy number of the lct gene results in increased production of lacticin 481. Transformation of L. lactis IL1441, MG1614, and NIZO R520 with pLCT9 or pLCT10 did not result in lacticin 481 production and immunity. In contrast, L. lactis R520 transformed with pNZ9100 containing the nisA gene and flanking regions did yield nisin-producing strains (Kuipers et al., 1991). This indicates that the systems allowing nisin maturation and secretion are not efficient for the processing of lacticin 481.

Amino Acid Content and Mass Spectrometry of Lacticin 481—Prerequisite to prediction of the structure of lacticin 481 is an accurate determination of the amino acid composition. The amino acid composition deduced from the DNA sequence differed from that reported previously (Piard et al., 1992). We therefore performed extended acid hydrolysis of lacticin 481. These analyses resolved additional isoleucine and valine residues and two additional lanthionine or β-methylthionine residues that had not been detected in previous analyses (Table I). Twenty-three amino acids are identified in mature lacticin 481. Any dehydrated amino acid residues would not be detected in these analyses. Lacticin 481 contains a relatively high content of glycine (13%) and a high proportion of hydrophobic or apolar amino acids. As predicted by PC/GENE, prolacticin 481 has an isoelectric point of 7.1 (7.7 if we consider that the 3 cysteines form thionine rings) because of the presence of 2 histidines, 1 lysine, and 1 glutamic acid residue.

Mass spectral measurements of lacticin 481 using electrospray ionization are shown in Fig. 4. From the series of multiple charged ions an average molecular mass of m/z = 2,901.14 ± 0.28 Da was found.

NMR Spectroscopy—In the one-dimensional NMR spectrum of lacticin 481 (Fig. 5) some unusual features were observed. An appreciable number of amide resonances, particularly those from the residues which form part of the three cyclic structure (see below), show a more or less severe line broadening. An increase in temperature reduces this line broadening (Fig. 5, a and b). On the other hand a comparison of 400 and 600 MHz NMR spectra (Fig. 5, a and c) does not show the improvement in resolution expected at the higher spectrometer frequency. These observations indicate the presence of chemical exchange: i.e. the molecule is exchanging between two (or more) states (most probably conformational states).

Using standard techniques (amino acid pattern recognition from TOCSY spectra and sequential assignment based on NOE data) an almost complete assignment of the NMR spectrum of lacticin 481 was achieved (Table II). The NOESY spectrum showed sequential connectivities (NH-NH+1, CαH-CαH-NH+1, or other) for the segments 3–10, 11–14, 15–19, and 22–26. The resonances of Lys1, Gly2, Phe31, and Ser27 were assigned by elimination. In the case of Alas1, Phe26, and Phe38 a complete assignment was not possible, most probably because of a severe broadening of their NH resonances.

The β-methylthionine Abu-S-Ala14 was readily identified by its relatively strong β-β and β-γ contacts in the NOE spectrum. However, the β-β contacts of the remaining two lanthionines were not observed, leaving two possibilities for the structure of lacticin 481 i.e., thioether bridges between residues 11 and 25, and 18 and 26 or between residues 11 and 26, and 18 and 25.

The NMR data showed unequivocally that a dehydrobutyrine residue is located at position 24. From the higher intensity of the NH-γ NOE as compared with that of the NH-β cross-peak, it can be concluded that the dehydrobutyrine residue has the Z conformation (i.e. CαH directed toward the NH group), which has also been observed for several dehydrobutyrine residues in nisin and nisin mutants (Chan et al., 1989; Kuipers et al., 1992).

DISCUSSION

DNA sequence analysis of the lct gene reveals lacticin 481 as a new lacticoccal lantibiotic that is ribosomally synthesized.
Structure of the L. lactis lct Gene for Lacticin 481

TABLE II

1H chemical shifts of lacticin 481

<table>
<thead>
<tr>
<th>Residue</th>
<th>Chemical shift</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N\textsuperscript{\textdegree}H</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Lys\textsuperscript{1}</td>
<td>4.12</td>
</tr>
<tr>
<td>Gly\textsuperscript{2}</td>
<td>8.83</td>
</tr>
<tr>
<td>Gly\textsuperscript{2}</td>
<td>8.47</td>
</tr>
<tr>
<td>Ser\textsuperscript{2}</td>
<td>8.39</td>
</tr>
<tr>
<td>Gly\textsuperscript{2}</td>
<td>8.54</td>
</tr>
<tr>
<td>Val\textsuperscript{9}</td>
<td>8.26</td>
</tr>
<tr>
<td>His\textsuperscript{8}</td>
<td>8.63</td>
</tr>
<tr>
<td>Abu\textsuperscript{9}</td>
<td>8.29</td>
</tr>
<tr>
<td>Ile\textsuperscript{8}</td>
<td>7.85</td>
</tr>
<tr>
<td>Ala\textsuperscript{11}</td>
<td>ND\textsuperscript{*}</td>
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<tr>
<td>His\textsuperscript{12}</td>
<td>9.19</td>
</tr>
<tr>
<td>Glu\textsuperscript{13}</td>
<td>8.95</td>
</tr>
<tr>
<td>Ala\textsuperscript{14}</td>
<td>7.13</td>
</tr>
<tr>
<td>Asn\textsuperscript{15}</td>
<td>7.80</td>
</tr>
<tr>
<td>Met\textsuperscript{16}</td>
<td>8.09</td>
</tr>
<tr>
<td>Asn\textsuperscript{17}</td>
<td>8.40</td>
</tr>
<tr>
<td>Ala\textsuperscript{18}</td>
<td>8.17</td>
</tr>
<tr>
<td>Trp\textsuperscript{19}</td>
<td>7.82</td>
</tr>
<tr>
<td>Gln\textsuperscript{20}</td>
<td>7.82</td>
</tr>
<tr>
<td>Phe\textsuperscript{21}</td>
<td>ND</td>
</tr>
<tr>
<td>Val\textsuperscript{22}</td>
<td>7.79</td>
</tr>
<tr>
<td>Phe\textsuperscript{23}</td>
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<tr>
<td>Dhb\textsuperscript{24}</td>
<td>9.37</td>
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<td>Ala\textsuperscript{26}</td>
<td>8.01</td>
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<tr>
<td>Ala\textsuperscript{26}</td>
<td>8.41</td>
</tr>
<tr>
<td>Ser\textsuperscript{27}</td>
<td>8.17</td>
</tr>
</tbody>
</table>

\*ND, resonance could not be identified.

FIG. 6. Two potential structures for lacticin 481. Ala-S-Ala, lanthionine; Abu-S-Ala, \(\beta\)-methyllanthionine.

as a prepeptide of 51 amino acids. The lct gene appears to be organized as single transcriptional unit since the size of the transcript (290 bases) corresponds to that predicted from the positions of the transcriptional start site and the putative rho-independent terminator. However, we cannot rule out the possibility that a large transcript is processed. Increased production of lacticin 481 when the lct gene is cloned in a high copy number vector suggests that expression of the lct gene is constitutive.

Structure of Lacticin 481—Analysis of the hydropathy of the 51 residue prelacticin 481 shows that it shares the same profile as that of other prelantibiotics (Jung, 1991) i.e. it contains a hydrophobic carboxyl-terminal prepeptide (prolacticin 481) and a rather hydrophilic amino-terminal extension. The deduced amino acid composition of prolacticin 481 compared with the amino acid content of lacticin 481 shows that 2 serine, 2 threonine, and 3 cysteine residues that should be present based on the DNA-deduced protein sequence are not found in mature lacticin 481 (Table I). On the other hand, 3 additional (\(\beta\)-methyl)lanthionine residues were detected in lacticin 481. We consider it likely that 3 of the 4 additional serine and threonine residues detected in prolacticin 481 are dehydrated and condensed with the 3 cysteines to form the 3 \(\beta\)-methyl)lanthionine residues of mature lacticin 481 and that the 4th additional serine or threonine is a dehydrated residue. This structural model for lacticin 481 predicts a molecular mass of 2,901 Da, which is in excellent agreement with that estimated by electrospray mass spectrometry.

The NMR data show clearly the presence of a thioether bridge between residues 9 and 14 and a dehydrobutyrine residue at position 24. The presence of two lanthionines could be inferred from the NMR spectra, but the positions of the two thioether bridges could not be established. Therefore, two structural models can be proposed for lacticin 481, in which Ser\textsuperscript{14} and Ser\textsuperscript{18} are, respectively, involved in lanthionine formation with Cys\textsuperscript{9} and Cys\textsuperscript{19} in one case (Fig. 6A) and with Cys\textsuperscript{8} and Cys\textsuperscript{23}, in the other case (Fig. 6B). In both models, the rigidity of lacticin 481 would be high because of the presence of cyclic structures in the peptide chain of lacticin 481. Such rigidity, in the absence of other secondary structural elements, could be important in the ability of lantibiotics to form pores in membranes.

Since in lacticin 481 the lanthionine moieties originating from dehydrated residues are situated on the amino-terminal side of their respective "cysteine" partners, lacticin 481 might be classified as a type A lantibiotic (Jung, 1991). However, the low net charge of lacticin 481 (+1) and the intramolecular
Structure of the L. lactis lct Gene for Lacticin 481

TABLE III
Alignment of prolantibiotics

<table>
<thead>
<tr>
<th>Lantibiotic</th>
<th>Prolantibiotic sequence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>nisin A1</td>
<td>I S L L T P G C - K T A</td>
</tr>
<tr>
<td>nisin Z2</td>
<td>I S L L T P G C - K T A</td>
</tr>
<tr>
<td>subtilin3</td>
<td>W K S L T P G C - V T A</td>
</tr>
<tr>
<td>epidermin4</td>
<td>I A S K P C T P G C A R T G S F N S Y C C</td>
</tr>
<tr>
<td>gallidermin5</td>
<td>I A G K L C T P G C A K T G S F N S Y C C</td>
</tr>
<tr>
<td>pep5</td>
<td>T A G P A I R A V E Q C Q F I L A R A T R L P T V S C K G K N G G K</td>
</tr>
<tr>
<td>lacticin 481</td>
<td>K G G S G V I H T S H C M N N S W Q F V F T C C S</td>
</tr>
</tbody>
</table>

* The processed residues are underlined; identical amino acids are boxed; for streptococcin A-FF22, the sequence reported is that of the mature peptide obtained from Edman degradation that stopped at position 23; dots indicate blank cycles. Sequence references: 1, Buchman et al. (1988); 2, Mulders et al. (1991); 3, Banerjee and Hansen (1988); 4, Allgaier et al. (1986); 5, Kellner et al. (1988); 6, Kellner et al. (1991); 7, Jack and Tagg (1991).

TABLE IV
Alignment of lacticin 481 leader sequence with those of lantibiotic-type and non-lantibiotic bacteriocins

<table>
<thead>
<tr>
<th>Bacteriocin*</th>
<th>Leader sequence</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Lantibiotics</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>nisin A and Z1, 2</td>
<td>M S T K - - D F P N L D L - V S V S K K - D S G A S P R T</td>
</tr>
<tr>
<td>subtilin3</td>
<td>M S K P D D P D L D V - V K V S K Q - D S K I T P Q W</td>
</tr>
<tr>
<td>pep5</td>
<td>M K N N K N - L F D L E K K E - T S Q N T D E L E P O T</td>
</tr>
<tr>
<td>lacticin 481</td>
<td>M K E O N - S F N L - L Q - E V T E S E L D L I L G A K</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Other bacteriocins</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>lactococcin A7</td>
<td>M K N Q L N F P N I V - - - S D E B L E S A N G G K</td>
</tr>
<tr>
<td>lactococcin B8</td>
<td>M K N Q L N F P N I V - - - S D E B L A E V N G G S</td>
</tr>
<tr>
<td>lactococcin Ma9</td>
<td>M K N Q L N F P I L - - - S D E B L O G I N G I</td>
</tr>
<tr>
<td>lactacin F10</td>
<td>M K - - - K I B K E - - - S H K D L A V V V V G G R</td>
</tr>
<tr>
<td>pediococin PA-111</td>
<td>M K - - - K I B K E - - - T E K E M A N I I G G K</td>
</tr>
<tr>
<td>leucocin A12</td>
<td>M M N M M K P T P S Y E Q L - - - D N S A L E Q O V V G G K</td>
</tr>
</tbody>
</table>

* 1, 2, 3, 4, 5, 6, same references as in Table III; 7, van Belkum et al. (1991) and Holo et al. (1991); 8, van Belkum et al. (1992); 9, van Belkum et al. (1991); 10, Muniana and Klaenhammer (1993); 11, Marrug et al. (1992); 12, Hastings et al. (1991).

distance between the ring-forming residues are more reminiscent of type B lantibiotics. Comparison of prolacticin 481 with other type A prolantibiotic sequences is shown in Table III. Prolacticin 481 appears significantly different from those prolantibiotics in that the homology in the position of the processed residues in pronisin, prosubtilin, proepidermin, progallidermin, and propep5 was not found in prolacticin 481. However, a high level of sequence similarity was observed between lactacin 481 and streptococcin A-FF22 from Streptococcus pyogenes that contains three lanthionine or β-methylanthionine residues (41% identity). In addition, the positions of putative processed residues in lactacin 481 correspond to those of the residues blank cycles after Edman degradation in streptococcin A-FF22 (Table III). Since Edman degradation provides blank cycles for lanthionine or β-methylanthionine residues and stops when it meets a dehydroamino acid, the residues in positions 8, 10, 13, and 17 in streptococcin A-FF22 might be involved in lanthionine or β-methylanthionine formation, and residue in position 23 might be a dehydroamino acid. Therefore, lactacin 481 and streptococcin A-FF22 appear to share a similar structure. Further comparison awaits DNA sequence or structural data on A-FF22. In previous studies on lantibiotics, it was suggested that there was a common lantibiotic ancestor which would have diverged in different organisms leading to the occurrence of various structural variants (Buchman et al., 1988). Although this hypothesis appears to be justified with respect to the homology between nisin A, nisin Z, subtilin, epidermin, and gallidermin, it seems that lactacin 481 and...
streptococcin A-FF22 form a new class of the type A lantibiotics.

**Leader Sequence of Lactacin 481**—Typical signal sequences have a hydrophobic core and conform to the −1, −3 rule of von Heijne (1988). Analysis of the amino-terminal extension of prelactacin 481 shows that it lacks these properties. Alignment of the 21-residue amino-terminal extension from the leader sequences in prelantibiotics is still unknown, although the conform to the consensus sequence found in other lantibiotics (X+1-X-3-Pro-X-1-X+n, where residues X+1, X-3, and X+n are hydrophobic, residue X-1 is negatively charged or polar, and residue X+1 is large and positively charged or polar) (Jung, 1991). This indicates that the leader peptides involved in cleavage of the lactacin 481 leader sequence differs from the leader sequences of prelantibiotics. Although the function of leader sequences in prelantibiotics is still unknown, the conservation in some regions of these sequences suggested that they may be crucial in the molecular recognition of the prepeptide by the maturation enzyme(s) or that they could protect the precursor from the premature lethal effect of lantibiotics before secretion (Jung, 1991).

The low similarity of the leader of prelactacin 481 to those of prelantibiotics as well as a higher similarity to those of non-lantibiotics also points to other possible functions, such as interaction with the translocation and processing proteins, leading to secretion of active lactacin 481.

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**REFERENCES**


