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Structure, Organization, and Expression of the *lct* Gene for Lacticin 481, a Novel Lantibiotic Produced by *Lactococcus lactis**

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The structural gene for the lactococcal lantibiotic lacticin 481 (*lct*) 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 *lct* gene was analyzed, and its promoter was mapped. DNA sequence analysis of the *lct* 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 prelacticin 481, containing a 27-residue carboxyl-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 lacticin 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 β-methyllanthionine 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 *Lactobacillus acidophilus* (Muriana and Klaenhammer, 1991a, 1991b), lactococcin A from *Lactococcus lactis* (Holo *et al.*, 1991; van Belkum *et al.*, 1991), and leucocin A-UAL 187 from *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 β-methyllanthionine (Schnell *et al.*, 1988). The lantibiotics nisin A (Hurst, 1981; Buchman *et al.*, 1988), nisin Z (Mulders *et al.*, 1991), and lacticin 481 (Piard *et al.*, 1990, 1992), are produced by *L. lactis*, and lactocin S, by *Lactobacillus sake* (Mørtvedt *et al.*, 1991). Lantibiotics have also been isolated from other Gram-positive organisms, such as *Bacillus subtilis* (subtilin), *Staphylococcus epidermidis* (epidermin and Pep5), and *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 β-methyllanthionine, 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 lacticin 481, which is a broad spectrum bacteriocin exhibiting bactericidal activity against a wide range of lactic acid bacteria and against *Clostridium tyrobutyricum* (Piard *et al.*, 1990). We describe the structure, organization, and transcription of the *lct* gene encoding lacticin 481. In addition, we propose a structural model for lacticin 481 based on the deduced amino acid sequence of prelacticin 481, its amino acid composition and characterization by two-dimensional NMR spectroscopy.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Media—Lacticin 481-producing *L. lactis* subsp. *lactis* (further designated as *L. lactis*) CNRZ 481 was obtained from the CNRZ culture collection (INRA, Jouy-en-Josas, France). The nisin producer *L. lactis* NIZO R5 harboring the *nisA* encoding transposon Tn*5276* (Rauch and de Vos, 1992) was from NIZO. A lacticin 481-producing (Lct$^+$) transconjugant, *L. lactis* JC17, was obtained by conjugal matings between *L. lactis* 481 (Lct$^+$) and *L. lactis* IL1441 (Lct$^-$, Microbial Genetics Laboratory, INRA) as described elsewhere. *Escherichia coli* JM83 (Vieira and Messing, 1982) and TG1 (Gibson, 1984) were used as hosts in cloning experiments.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) X71410.

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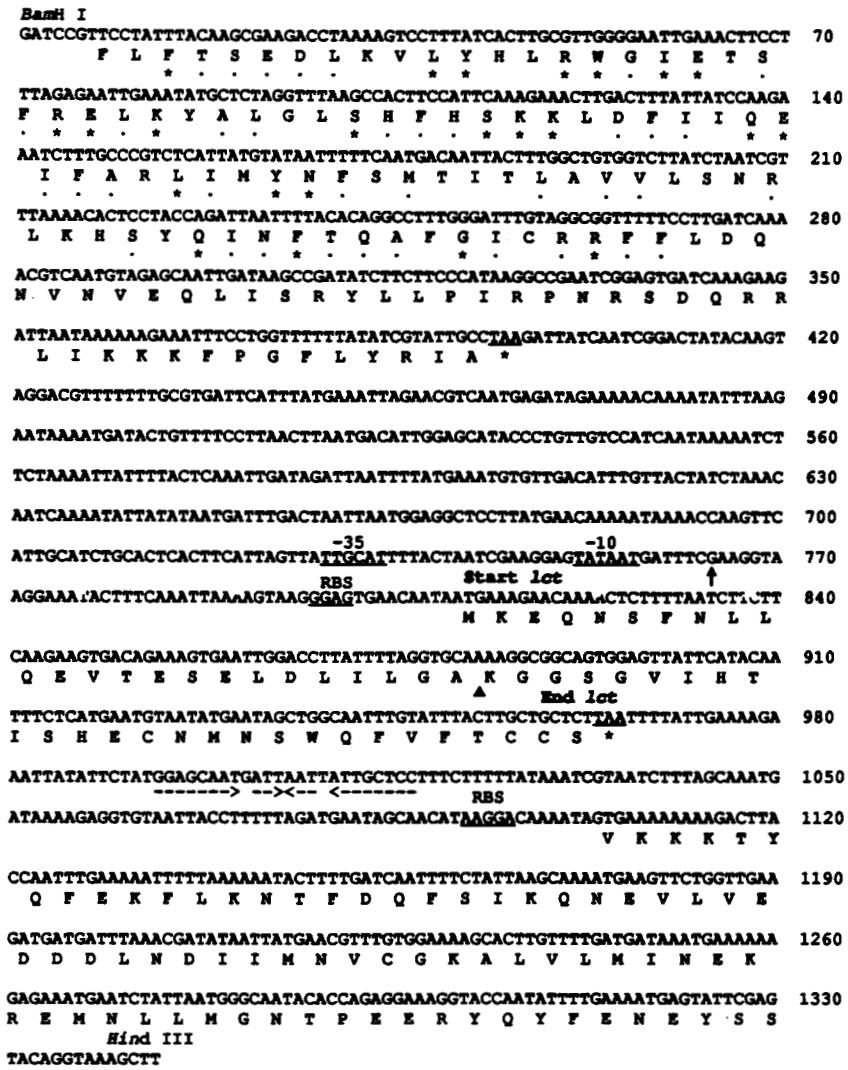

FIG. 1. **Single-stranded DNA sequence of the region containing *lct* gene and deduced amino acid sequence.** The processing site in prelacticin 481 is indicated (▲). The promoter consensus sequences (−35, −10) and potential ribosome binding site (*RBS*) are *underlined*. The transcription start site of *lct* is indicated by an *arrow*. Termination codons are *underlined*. The *horizontal dashed arrows* below the nucleotide sequence indicate inverted repeats. Identical and functionally related amino acids between the deduced residues from the upstream open reading frame and IS*4* transposase are indicated by an *asterisk* and a *black dot*, 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 *E. coli* and *L. lactis*, respectively.

E. coli strains were grown in L-broth (Sambrook *et al.*, 1989) at 37 °C and *L. lactis* strains in M17 broth (Terzaghi and Sandine, 1975) containing 0.5% glucose at 30 °C. If appropriate, antibiotics carbenicilline (100 μg/ml) and erythromycin (5 μg/ml) were added. Isopropyl-1-thio-β-D-galactopyranoside and 5-bromo-4-chloro-3-indolyl β-D-galactoside were used at 0.025%.

DNA Methodology, 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 Vos and Gasson (1989). 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 1 M NaOH as a transfer buffer. Colony hybridization was performed with the same type of membrane (Sambrook *et al.*, 1989). DNA probes were end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. A degenerated 20-mer, P25 (5′-ATNACNCC(A/G)CTNCCNCC(T/C)TT-3′) was synthesized on a Biosearch Cyclone DNA Synthesizer (New Brunswick Scientific) and used to identify the *lct* gene in both Southern and colony blot hybridizations. Hybridizations and washing were performed at 52 and 37 °C, respectively.

DNA sequencing was performed by the dideoxy chain termination method (Sanger *et al.*, 1977) using M13 universal primers and primers P28 (5′-CTTGAAGAAGATTAAAAGAG-3′) and P29 (5′-GTATTTACTTGCTGCTC-3′), complementary to position 844–824 and 946–963 of the *lct* gene, respectively (see Fig. 1). Computer analysis of DNA and amino acid sequences was performed with the programs of PC/GENE (Genofit).

RNA Isolation, Northern Blot Analysis, and Primer Extension Studies—*L. lactis* strains were grown to $A_{600\ nm}$ = 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 (Rheox, for preparation see below), 0.5 ml of phenol, pH 7.5, and 0.05 ml of 10% sodium dodecyl sulfate.[1] The tubes were shaken at maximal setting in a Biospec homogenizer at room temperature for 3 × 1 min with 1-min intervals. The microtubes were subsequently centrifuged at 12,000 rpm for 15 min. Four layers were visible; the upper one, containing RNA, was collected and extracted successively with phenol/chloroform and chloroform. RNA was precipitated with ethanol and dissolved in 100 μl of TE. For storage, RNA was kept in ethanol at −20 °C, centrifuged, and resuspended before each use. Macaloid 4% was prepared as follows. Two grams of Macaloid was suspended in 100 ml of TE, boiled for 5 min, and sonicated with a sterilized probe sonicator (Heat Systems) until a gel formed (about 1 min). The gel was allowed to cool, washed with TE, centrifuged, and separated from the supernatant. This allowed us to recover about 50 ml of gel.

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

[1] R. Raya, personal communication.

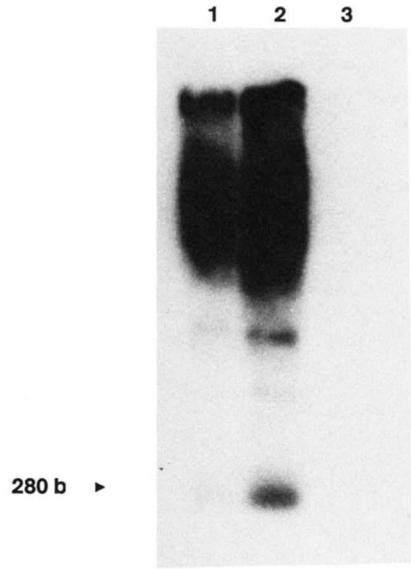

FIG. 2. **Northern blot analysis of RNA isolated from *lane 1*, *L. lactis* CNRZ 481 (Lct$^+$); *lane 2*, *L. lactis* JC17 (Lct$^+$); *lane 3*, *L. lactis* IL1441 (Lct$^-$).** Hybridization was performed with a single-stranded probe for the *lct* gene and upstream region (see "Experimental Procedures").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

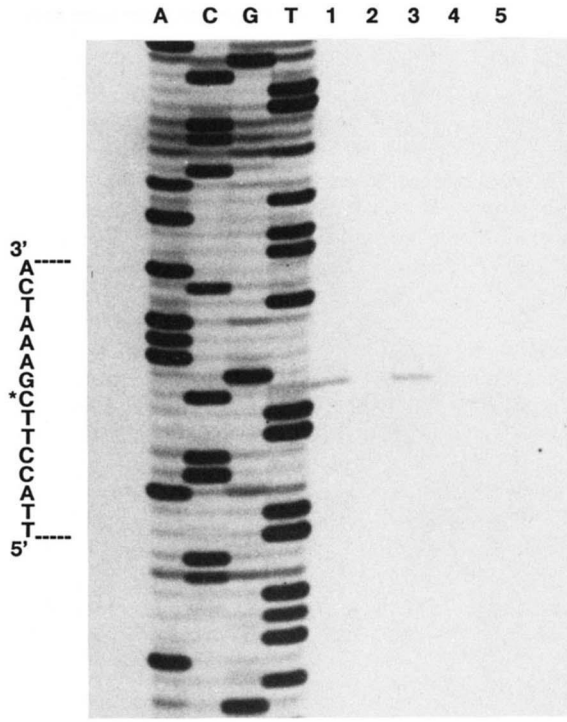

FIG. 3. **Primer extension products of RNA transcribed from *lct*.** The relevant DNA sequence (complementary to *lct* sequence) is indicated, and the determined transcription start site is marked by an *asterisk*. *Lanes A*, *C*, *G*, *T* are sequencing reactions carried out as described under "Experimental 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 Rooijen and de Vos, 1990). ^{32}P-Labeled single-stranded DNA obtained by primer extension of oligonucleotide 28 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 $MgCl_2$, 15 mM dithiothreitol, and 40 units of RNasin (Promega). 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-^{35}S-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.

TABLE I
Amino acid analysis of lacticin 481

Amino acid[a]	No. of residues/molecule in		Most probable no. of processed residues
	Lacticin 481[b]	Prolacticin 481[c]	
Asx	2	2	
Cys	0	3	3
Glx	2	2	
Gly	3	3	
His	2	2	
Ile	2	2	
Lan + β-CH_3Lan	3		
Lys	1	1	
Met	1	1	
Phe	2	2	
Ser	2	4	2
Thr	0	2	2
Trp	1	1	
Val	2	2	
Total	23	27	

[a] Lan, lanthionine; β-CH_3Lan, β-methyllanthionine.
[b] Obtained from amino acid analyses.
[c] Deduced from the DNA sequence of *lct*.

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 C_{18} 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 60% 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 M sodium citrate, pH 2.2, and analyzed on an amino acid analyzer (LKB, type 4151). The sum of the lanthionine and β-methyllanthionine 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 spectrophotometer (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

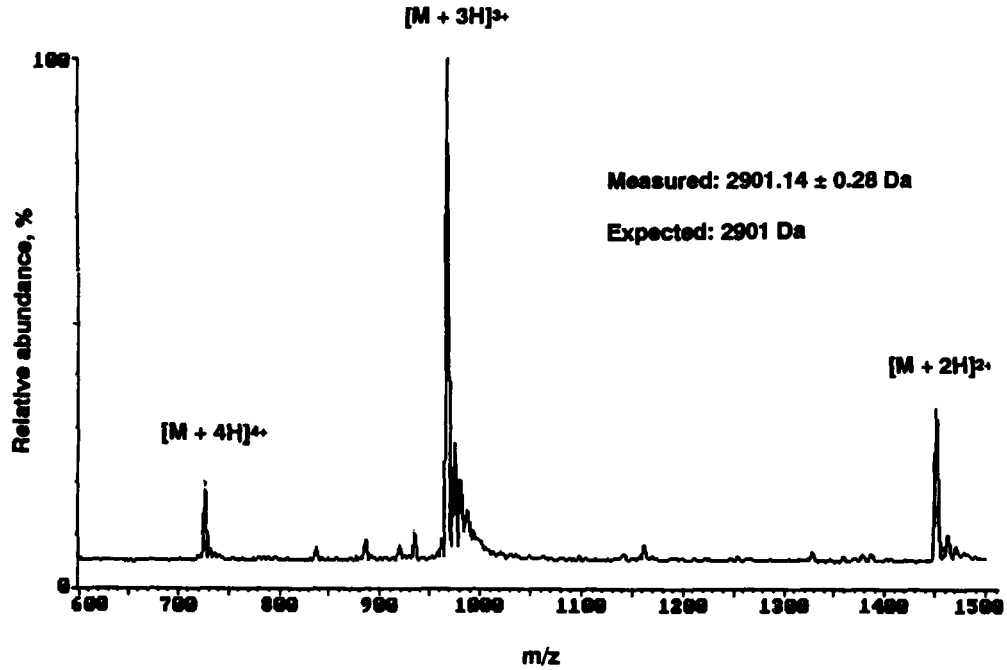

FIG. 4. **Electrospray mass spectrum of lacticin 481 representing the relative abundance as a function of mass to charge ratio (*m/z*) of the multiply charged ions.**

external 3-(trimethylsilyl)-2,2,3,3-tetradeuteropropionic acid. For the TOCSY[2] (mixing times 15 and 70 ms) and NOESY (mixing time 500 ms) experiments, approximately 5 mM solutions of lacticin 481 in 10% D_2O, 90% H_2O, pH 3.6, 25 °C were used. The solvent resonance was suppressed by presaturation during the relaxation delays. In the NOESY experiment the water resonance was also irradiated during the mixing time. The two-dimensional NMR spectra were acquired in the phase-sensitive mode using time proportional phase incrementation.

RESULTS

Isolation and Sequence Analysis of Lacticin 481 Structural Gene, lct, and Its Flanking Regions—Southern blots of *Eco*RI-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 *Eco*RI fragments of *L. lactis* JC17 were isolated, ligated to *Eco*RI-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 pLCT8. Restriction analysis and Southern hybridization of pLCT8 revealed that the *lct* gene was present on a 1.3-kb *Bam*HI-*Hin*dIII 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* IS*4* 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

[2] The abbreviations used are: TOCSY, total correlated spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy; kb, kilobase(s).

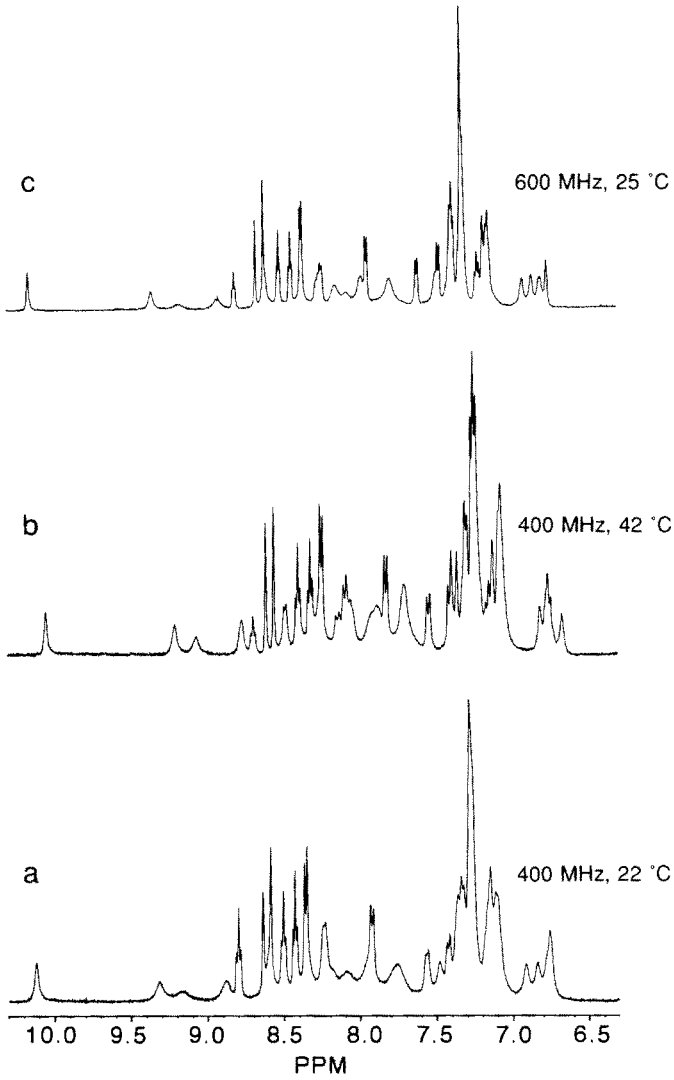

FIG. 5. **Amide-aromatic region of the ^{1}H NMR spectrum of lacticin 481.** Spectra were taken at 22 °C and 400 MHz (*a*), at 42 °C and 400 MHz (*b*), and at 25 °C and 600 MHz (*c*). Experimental conditions: approximately 5 mM peptide in 10% D_2O, 90% H_2O, pH 3.6.

strains. The deduced transcription start site was at the G residue at position 764 (Figs. 1 and 3). Inspection of the sequence upstream of the 5′-end of the transcript revealed the presence of a consensus lactococcal promoter (van de Guchte *et al.*, 1992) located 6 base pairs upstream of the 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 *Eco*RI 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-methyllanthionine 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 lanthionine 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 NOESY data) an almost complete assignment of the NMR spectrum of lacticin 481 was achieved (Table II). The NOESY spectrum showed sequential connectivities (NH_i-NH_{i+1}, $C^{\alpha}H_i$-NH_{i+1} or other) for the segments 3–10, 11–14, 15–19, and 22–26. The resonances of Lys^1, Gly^2, Phe^{21}, and Ser^{27} were assigned by elimination. In the case of AlaS^{11}, Phe^{21}, and Phe^{23} a complete assignment was not possible, most probably because of a severe broadening of their NH resonances.

The β-methyllanthionine Abu^9-*S*-Ala^{14} was readily identified by its relatively strong β-β and β-γ contacts in the NOESY spectrum. However, the β-β contacts of the remaining two lanthionines were not observed, leaving two possibilities for the strucure 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.* -$C^{\gamma}H_3$ 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 lactococcal lantibiotic that is ribosomally synthesized

TABLE II

^1H chemical shifts of lacticin 481

Spectra were taken at 400 MHz in 10% D_2O, 90% H_2O, pH 3.6, 25 °C. Amino acid nomenclature is according to IUPAC-IUB recommendations (*Eur. J. Biochem.* (1984) **138**, 9–37). Ala* and Abu* represent the alanyl- and β-methylalanyl-moieties of lanthionines. Dehydrobutyrine is denoted by Dhb.

Residue	Chemical shift			
	$N^{\alpha}H$	$C^{\alpha}H$	$C^{\beta}H$	Other
			ppm	
Lys[1]		4.12	1.98	$C^{\gamma}H_2$ 1.52 $C^{\delta}H_2$ 1.75 $C^{\epsilon}H_2$ 3.05 $N^{\epsilon}H_3^+$ 7.58
Gly[2]	8.83	4.09		
Gly[3]	8.47	4.08		
Ser[4]	8.39	4.53	3.93	
Gly[5]	8.54	4.01		
Val[6]	7.97	4.09	2.00	$C^{\gamma}H_3$, $C^{\gamma'}H_3$ 0.83, 0.91
Ile[7]	8.26	4.16	1.72	$C^{\gamma}H_2$ 1.44 $C^{\beta'}H_3$ 1.14 $C^{\delta}H_3$ 0.83
His[8]	8.63	4.98	3.17, 3.27	$C^{2}H$ 8.64 $C^{5}H$ 7.35
Abu*[9]	8.29	4.64	3.65	$C^{\gamma}H_3$ 1.12
Ile[10]	7.85	4.49	1.84	$C^{\gamma}H_2$ 1.34 $C^{\beta'}H_3$ 1.03 $C^{\delta}H_3$ 0.77, 0.84
Ala*[11]	ND[a]	4.44	3.02	
His[12]	9.19	4.48	3.36	$C^{2}H$ 8.70 $C^{5}H$ 7.43
Glu[13]	8.95	4.17	2.23	$C^{\gamma}H_2$ 2.48
Ala*[14]	7.13	4.67	3.05	
Asn[15]	7.80	4.70	2.69, 2.89	$N^{\gamma}H_2$ 6.95, 7.52
Met[16]	8.09	4.37	1.91, 2.07	$C^{\gamma}H_2$ 2.36, 2.50 $C^{\epsilon}H_3$ 2.01
Asn[17]	8.40	4.66	2.62	$N^{\gamma}H_2$ 6.89, 7.43
Ala*[18]	8.17	4.42	2.88, 3.22	
Trp[19]	7.82	4.58	3.29	$C^{2}H$ 7.21 $C^{4}H$ 7.63 $C^{5}H$ 7.17 $C^{6}H$ 7.24 $C^{7}H$ 7.49 $N^{1}H$ 10.17
Gln[20]	7.82	4.19	1.74	$C^{\gamma}H_2$ 1.91 $N^{\delta}H_2$ 6.79, 7.20
Phe[21]	ND	4.58	2.97, 3.02	$C^{2,6}H$ 7.17 $C^{3,4,5}H$ 7.33
Val[22]	7.79	4.05	1.91	$C^{\gamma}H_3$, $C^{\gamma'}H_3$ 0.86
Phe[23]	ND	4.49	3.18, 3.26	$C^{2,6}H$ 7.40 $C^{3,4,5}H$ 7.35
Dhb[24]	9.37		6.84	$C^{\gamma}H_3$ 1.31
Ala*[25]	8.01	4.63	3.05	
Ala*[26]	8.41	ND	2.83, 3.05	
Ser[27]	8.17	4.41	3.89	

[a] ND, resonance could not be identified.

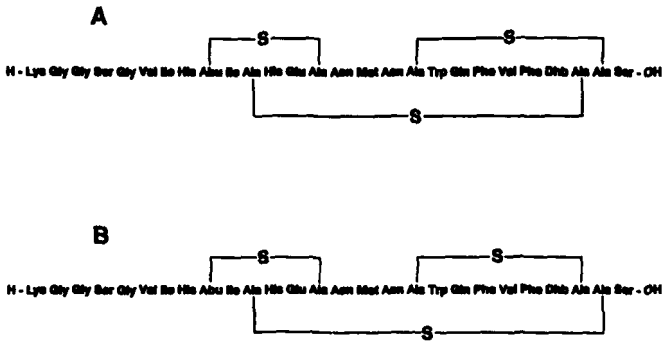

FIG. 6. **Two potential structures for lacticin 481.** Ala-*S*-Ala, lanthionine; Abu-*S*-Ala, β-methyllanthionine.

as a prepeptide of 51 amino acids. The *lct* gene appears to be organized as single transcriptional unit since the size of the transcript (280 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 propeptide (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 (β-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 (β-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^{11} and Ser^{18} are, respectively, involved in lanthionine formation with Cys^{25} and Cys^{26} in one case (Fig. 6*A*) and with Cys^{26} and Cys^{25}, in the other case (Fig. 6*B*). 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

TABLE III

Alignment of prolantibiotics

Lantibiotic	Prolantibiotic sequence*
nisin A[1]	I T S I S L C T P G C - K T G A L M G C N M K T A T C H C S I H V S K
nisin Z[2]	I T S I S L C T P G C - K T G A L M G C N M K T A T C N C S I H V S K
subtilin[3]	W K S E S L C T P G C - V T G A L Q T C F L Q T L T C N C - - K I S K
epidermin[4]	I A S K F I C T P G C A K T G S F N S Y C C
gallidermin[5]	I A S K F L C T P G C A K T G S F N S Y C C
pep5[6]	T A G P A I R A S V K Q C Q K T L K A T R L P T V S C K G K N G C K
lacticin 481	K G G S G V I H T I S H E C N M N S W Q F V P T C C S
streptococcin A-FF22[7]	G K N G V F K . I . H E . H L N . . A F L A . . . . .

* 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

Bacteriocin*	Leader sequence
Lantibiotics	
nisin A and Z[1, 2]	M S T K - - D F N L D L - V S V S K K - D S G A S P R↓I
subtilin[3]	M S K F D D F D L D V - V K V S K Q - D S K I T P Q↓W
epidermin[4]	M E A V K E K N D L F N L D V K V N A K E S N D S G A E P R↓I
gallidermin[5]	M E A V K E K N E L F D L D V K V N A K E S N D S G A E P R↓I
pep5[6]	M K N N K N - L F D L E I K K E - T S Q N T D E L E P Q↓T
lacticin 481	M K E Q N - S F N L - L Q - E V T E S E L D L I L G A↓K
Other bacteriocins	
lactococcin A[7]	M K N Q L N F N I V - - - S D E E L S E A N G G↓K
lactococcin B[8]	M K N Q L N F N I V - - - S D E E L A E V N G G↓S
lactococcin Ma[9]	M K N Q L N F E I L - - - S D E E L Q G I N G G↓I
lactacin F[10]	M K - Q - - F N Y L - - - S H K D L A V V V G G↓R
pediocin PA-1[11]	M K - - - K I E K L - - - T E K E M A N I I G G↓K
leucocin A[12]	M M N M K P T F S Y E Q L - - - D N S A L E Q V V G G↓K
[lacticin 481]	M K E Q N S F N L L Q E V T E S E L D L I L G A↓K

* 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, Muriana and Klaenhammer (1991b); 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 lacticin 481 and streptococcin A-FF22 from *Streptococcus pyogenes* that contains three lanthionine or β-methyllanthionine residues (41% identity). In addition, the positions of putative processed residues in lacticin 481 correspond to those of the residues providing blank cycles after Edman degradation in streptococcin A-FF22 (Table III). Since Edman degradation provides blank cycles for lanthionine or β-methyllanthionine 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-methyllanthionine formation, and residue in position 23 might be a dehydroamino acid. Therefore, lacticin 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 lacticin 481 and

streptococcin A-FF22 form a new class of the type A lantibiotics.

Leader Sequence of Lacticin 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 prelacticin 481 shows that it lacks these properties. Alignment of the 21-residue amino-terminal extension from prelacticin 481 with other leader peptides in prelantibiotics (Table IV) reveals no homologies, apart from the F(N/D)L consensus sequence. Unexpectedly, the amino-terminal extension of prelacticin 481 showed significant similarity to leader sequences of non-lantibiotic bacteriocins. In addition, the proteolytic cleavage site in the prelacticin leader shows similarity to non-lantibiotic processing sites and does not conform to the consensus sequence found in other lantibiotics (X^{-4}-X^{-3}-Pro^{-2}-X^{-1}-X^{+1}, where residues X^{-4}, X^{-2}, and X^{+1} are hydrophobic, residue X^{-3} is negatively charged or polar, and residue X^{-1} is large and positively charged or polar) (Jung, 1991). This indicates that the leader peptidase involved in cleavage of the lacticin 481 leader sequence differs from the serine protease that was recently found to be involved in processing of the precursor of the other lactococcal lantibiotic nisin (van der Meer *et al.*, 1993). 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 producer organism from the premature lethal effect of lantibiotics before secretion (Jung, 1991).

The low similarity of the leader of prelacticin 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 lacticin 481.

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