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Lactococcin A, a New Bacteriocin from *Lactococcus lactis* subsp. *cremoris*: Isolation and Characterization of the Protein and Its Gene

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A new bacteriocin, termed lactococcin A (LCN-A), from *Lactococcus lactis* subsp. *cremoris* LMG 2130 was purified and sequenced. The polypeptide contained no unusual amino acids and showed no significant sequence similarity to other known proteins. Only lactococci were killed by the bacteriocin. Of more than 120 *L. lactis* strains tested, only 1 was found resistant to LCN-A. The most sensitive strain tested, *L. lactis* subsp. *cremoris* NCDO 1198, was inhibited by 7 pM LCN-A. By use of a synthetic DNA probe, *lcnA* was found to be located on a 55-kb plasmid. The *lcnA* gene was cloned and sequenced. The sequence data revealed that LCN-A is ribosomally synthesized as a 75-amino-acid precursor including a 21-amino-acid N-terminal extension. An open reading frame encoding a 98-amino-acid polypeptide was found downstream of and in the same operon as *lcnA*. We propose that this open reading frame encodes an immunity function for LCN-A. In *Escherichia coli lcnA* did not cause an LCN-A$^+$ phenotype. *L. lactis* subsp. *lactis* IL 1403 produced small amounts of the bacteriocin and became resistant to LCN-A after transformation with a recombinant plasmid carrying *lcnA*. The other lactococcal strains transformed with the same recombinant plasmid became resistant to LCN-A but did not produce any detectable amount of the bacteriocin.

A number of strains of *Lactococcus lactis* produce bacteriocins. In an extensive survey by Geis et al. (15), it was found that about 5% of the 280 lactococcal strains tested were bacteriocin producers. These workers divided the bacteriocins into eight different classes, but none of them was characterized in detail. Despite numerous reports of lactococcal bacteriocins, little is known about their chemical composition and structure, mode of action, or genetics. Nisin is the only bacteriocinlike compound from *L. lactis* that has been studied in detail. The molecular structure and genetic determinant of nisin have been identified and, to some extent, its mode of action has been elucidated (5, 11, 16, 21, 22, 36). Another bacteriocin, termed diplococcin, produced by *L. lactis* subsp. *cremoris*, has also been purified and its amino acid composition has been determined (10). Davey (9) showed that the gene coding for diplococcin was located on a 54-MDa conjugative plasmid. Conjugal transfer of bacteriocin plasmids in *L. lactis* has also been observed by others (30, 38). Harmon and McKay (19) identified a *BclI* DNA fragment carrying a bacteriocin determinant from a conjugative plasmid. Recently, two bacteriocin genes from another conjugative lactococcal plasmid, previously described by Neve et al. (30), were cloned by van Belkum et al. (46). The clones also carried the immunity factors of the two bacteriocins. The inhibitory spectra of the different lactococcal bacteriocins described vary but are generally more narrow than that of nisin (15). It is therefore plausible that many of the lactococcal bacteriocins described are very different from nisin and thus do not belong to the lanthibiotic family (39) of bacteriocinlike compounds.

In this report, we describe the isolation and characterization of a new bacteriocin, termed lactococcin A (LCN-A), which is produced by a strain of *L. lactis* subsp. *cremoris*. The gene encoding the bacteriocin has been cloned and sequenced.

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MATERIALS AND METHODS

Bacterial strains, media, plasmids, and enzymes. The bacterial strains, plasmids, and phages used in this study are listed in Table 1. All lactococcal strains were grown in M17 broth (44) and maintained as frozen stocks at −80°C in M17 broth containing 10% glycerol. *Escherichia coli* DH5α was used for propagating pUC18 and its derivatives. M13 vectors and clones were propagated in 2× YT (2a) with *E. coli* JM101 as the host.

Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, and DNA molecular weight standards were purchased from Bethesda Research Laboratories, Inc. (Gaitherburg, Md.). Calf intestinal alkaline phosphatase, sequence-grade trypsin, and endoprotease glu-C were purchased from Boehringer GmbH (Mannheim, Germany). Sequenase was obtained from United States Biochemical Corp. (Cleveland, Ohio).

Plasmid curing. *L. lactis* subsp. *cremoris* LMG 2130 was grown in M17 broth supplemented with 1% glucose at 38°C in the presence of 0.1 μg of novobiocin per ml. Diluted aliquots from this culture were spread on M17 broth–1% glucose plates and incubated at 30°C. Colonies were scored for bacteriocin production.

Bacteriocin assays. Three methods were used to determine bacteriocin activity. (i) Colonies of possible bacteriocin-producing bacteria were grown on agar plates overnight. A lawn of 3 ml of M17 soft agar (0.7%) containing 100 μl of a fresh culture of the indicator organism was poured over a plate. After incubation overnight at 30°C, the colonies were examined for zones of growth inhibition. (ii) In M17 agar plates, wells with a diameter of 4 mm were made and filled with bacteriocin solutions. After the liquid had been completely absorbed by the gel, M17 soft agar containing the indicator organism was overlaid on the plates to demonstrate bacteriocin activity as described above. (iii) Bacteriocin activity was quantified as described by Geis et al. (15), except that microtiter plates with wells containing 200 μl of M17 broth were used. One unit of bacteriocin activity (BU)

TABLE 1. Strains, plasmids, and phages used in this study

Strain, plasmid, or phage	Relevant phenotype	Source or reference
Strains		
L. lactis subsp. *cremoris*		
LMG 2130	LCN-A-producing strain	G. Vegarud
LMG 2131	*lcnA* derivative of LMG 2130	This study
IMN C18		D. Lillehaug
BC 101		51
L. lactis subsp. *lactis*		
NIZO 4-25	Biovar diacetylactis	J. Narvhus
IL 1403		6
E. coli		
DH5α		17
JM101		31
Plasmids and phages		
pIL253		41
pUC18		53
M13mp18		31
M13mp 19		31
pON1	pUC18 with 4-kb *Hin*dIII fragment containing *lcnA*	This work
pON2	pUC18::pIL253 with 4-kb *Hin*dIII fragment containing *lcnA*	This work
pON7	pUC18::pIL253 with 1.2-kb *Rsa*I-*Hin*dIII fragment containing *lcnA*	This work

was arbitrarily defined as the amount of bacteriocin required to produce 50% growth inhibition (50% of the turbidity of the control without bacteriocin) of *L. lactis* subsp. *cremoris* IMN C18 in this assay.

Purification of LCN-A. The bacteriocin was purified from 1-liter cultures of *L. lactis* subsp. *cremoris* LMG 2130. The various steps of the purification procedure were carried out at 4°C unless otherwise stated. The cells were grown to the early stationary phase, and the bacteria were removed by centrifugation at 10,000 × *g* for 10 min. The bacteriocin was precipitated from the culture supernatant by the addition of 280 g of ammonium sulfate per liter. Following centrifugation at 10,000 × *g* for 30 min, the pellet was dissolved in water and adjusted to pH 7.3 by the addition of 0.5 M Na_2HPO_4. This solution was applied to a 10-ml CM-Sepharose column (Pharmacia, Uppsala, Sweden) equilibrated with 20 mM sodium phosphate (pH 7.3). The column was washed with 40 ml of 20 mM sodium phosphate (pH 7.3) before the bacteriocin was eluted with 20 ml of the same buffer containing 0.3 M NaCl. The bacteriocin was subjected to reverse-phase liquid chromatography at room temperature with fast protein liquid chromatography equipment (Pharmacia). The eluate from the cation exchanger was applied to a 1-ml phenyl-Superose column (Pharmacia) equilibrated with 10 mM sodium phosphate (pH 7.3). Following washing with 10 mM sodium phosphate (pH 7.3), elution was carried out with a linear gradient of 0 to 60% ethanol at a flow rate of 0.3 ml/min. Purified LCN-A was stored in 60% ethanol–2.5 mM sodium phosphate (pH 7.3) at −20°C. Protein concentrations were determined spectrophotometrically at 280 nm.

Amino acid sequencing. An Applied Biosystems (Foster City, Calif.) 477A sequencer was used for amino acid sequencing (8). The phenylthiohydantoin-derivatized amino acid residues were determined on-line with an Applied Biosystems 120 phenylthiohydantoin analyzer. The C-terminal part of the sequence was obtained after cleavage of the Asn-Gly bond with hydroxylamine at pH 9 as described by Bornstein and Galian (3).

DNA isolation, analysis, and manipulations. Plasmid DNA was isolated from *L. lactis* as described by Klaenhammer (23). Small-scale preparation of *E. coli* plasmid DNA was performed with GeneClean (BIO 101, La Jolla, Calif.). Large-scale isolation of plasmids from *E. coli* was performed by the alkaline lysis method described by Maniatis et al. (25). The M13 plus-strand DNA template for sequencing was prepared from infected 1.5-ml cultures as described previously (2a).

Enzymes for DNA manipulations were used in accordance with manufacturer specifications. Plasmid DNA from strain LMG 2130 used for cloning was purified by CsCl isopycnic centrifugation (33).

Restriction fragments of the desired size for cloning were isolated and purified from 0.7% agarose gels with GeneClean.

DNA cloned in *E. coli* was subcloned in lactococci as follows. pUC18 plasmids with inserts were fused to pIL253 by *Eco*RI digestion and ligation. The resultant constructs were transformed into *E. coli*. Clones were obtained by selection for erythromycin (300 μg/ml) and ampicillin (50 μg/ml) resistance. Plasmid DNA extracted from the clones was used to transform lactococci by electroporation as described by Holo and Nes (20).

Transformation of *E. coli* was performed by the method of Hanahan (17).

Nucleic acid hybridizations and nucleotide sequencing. On the basis of the sequence extending from amino acid 25 in LCN-A, the following 64-fold-degenerated synthetic oligodeoxynucleotide probe was made (with an Applied Biosystems 381A DNA synthesizer): 3′-ATIGT(T/C)GT(T/C)TG(I/C)TG(T/C)TTICG(I/C)AAICC-5′. Colony hybridization was performed as described by Hanahan and Meselson (18). Southern blots were made by vacuum transfer (2016 Vacugene; Pharmacia) of restriction endonuclease-digested DNA (fractionated on 0.7% agarose gels) to GeneScreen Plus membranes (NEN Research Products, Dupont, Boston, Mass.) (42). Hybridization with the 26-mer oligodeoxynucleotide was performed as described by Church and Gilbert (7). Nucleotide sequencing by the dideoxynucleotide method (37) was carried out on restriction fragments cloned into M13mp18 and M13mp19. [α-^{35}S]dATP (600 Ci/mmol; Amersham International, Amersham, United Kingdom) was used for labeling. Computer analyses of nucleotide and amino acid sequences were performed with PCGENE (IntelliGenetics, Mountain View, Calif.) microcomputer software. The search for DNA sequence homology was carried out with the FASTA program of the EMBL Nucleotide Sequence Data Library (32). The search for amino acid sequence similarity in the Swiss-Prot (A. Bairoch, Department of Medical Biochemistry, University of Geneva, Geneva, Switzerland) and the NBRF (Biotechnology Center, University of Wisconsin, Madison) protein data bases was carried out with the FSTP SCAN program (IntelliGenetics).

TABLE 2. Purification of LCN-A

Fraction	Vol (ml)	A_{280}	Total activity (10^5 BU)	Sp act (BU/ml/A_{280})	Purification (fold)	Yield (%)
Culture supernatant	1,000	14.6	15	102.8	1	100
Ammonium sulfate precipitate	100	5.35	13	2,428	23	87
Cation-exchange chromatography	12	0.17	9.6	4.6×10^5	4,485	64
Reverse-phase chromatography	2	0.51	2.4	2.4×10^5	2,281	16

Nucleotide sequence accession number. The nucleotide sequence presented in this article has been assigned EMBL accession number M63675.

RESULTS

Purification of LCN-A. *L. lactis* subsp. *cremoris* LMG 2130 was found to produce a bacteriocin constitutively during growth in M17 medium. A procedure for purifying the bacteriocin from the culture supernatant was developed. The purification scheme is shown in Table 2. The chromatogram in Fig. 1 shows the elution of the bacteriocin from a phenyl-Superose column. The bacteriocin was eluted at about 40% ethanol. The overall purification procedure resulted in about a 2,000-fold purification of the bacteriocin, with a recovery of 16%. Despite both a higher yield and a higher specific activity at the cation-exchange chromatography step, a last step was required to obtain a pure bacteriocin to sequence. The protein was about 95% pure, as judged by amino acid sequence analysis. The amino acid sequence of the purified bacteriocin is shown in Fig. 2. The bacteriocin was found to contain 54 amino acid residues with a calculated molecular weight of 5,778. No significant sequence similarity was found to any protein or putative gene product in the Swiss-Prot or NBRF data bases.

We have named the new bacteriocin LCN-A. As shown in Fig. 2, the protein was rich in alanine residues (8 of 54) and glycine residues (8 of 54) and contained only three charged amino acid residues. Unusual amino acids were not found. The calculated isoelectric point of the bacteriocin was 9.2. The extinction coefficient of LCN-A at 280 nm was estimated to be 1.2×10^4 cm^{-1} M^{-1} from its content of tryptophan and tyrosine (4). Thus, pure LCN-A had a specific activity of about 4.9×10^5 BU/mg. Assuming that the activity of LCN-A was not reduced during purification, strain LMG 2130 produced about 3 mg of LCN-A per liter. By comparison, *L. lactis* subsp. *cremoris* 346 was found to produce 6 mg of diplococcin per liter (10). The pure bacteriocin was not very soluble in water. Upon storage in aqueous buffers at 4°C, the bacteriocin formed an inactive precipitate. Pure LCN-A could, however, be stored longer than 6 months at −20°C in 60% ethanol containing 2.5 mM sodium phosphate (pH 7.3) without a detectable loss of activity.

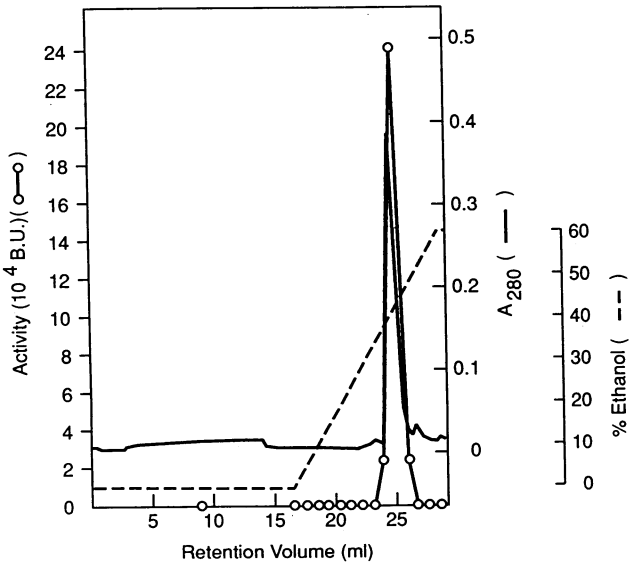

FIG. 1. Purification of LCN-A by reverse-phase chromatography on phenyl-Superose. The sample applied to the column was from the NaCl eluate from the CM-Sepharose column. Fractions (0.9 ml) were collected and assayed for bacteriocin activity.

Effect of proteases. LCN-A lost its activity when exposed to various proteases, including the highly specific endoprotease glu-C and trypsin. In phosphate buffer (pH 7.8), endoprotease glu-C could cleave the bacteriocin at one site, between amino acid residues 12 (Asp) and 13 (Leu) (Fig. 2); trypsin could cleave the bacteriocin at the carboxyl side of its two lysine residues (1 and 21) (Fig. 2).

Inhibitory spectrum and mode of action. By means of the agar diffusion assay, more than 120 strains of *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* were found to be sensitive to purified LCN-A. Sensitive strains were rapidly killed by the bacteriocin. The viable count of an exponentially growing culture of strain IMN C18 dropped from 2×10^8/ml to 7×10^5/ml after 5 min of exposure to 200 BU/ml in M17 medium at 30°C.

Table 3 shows the sensitivities of various lactococcal strains to LCN-A. Wide variations in sensitivity were found. The most sensitive strains tested appeared to be more sensitive to the bacteriocin when grown in lactic broth (14) than in M17 medium. In lactic broth, 50% growth inhibition of strain NCDO 1198 was observed at a calculated LCN-A concentration of 40 pg/ml, or 7 pM. This amount corresponds to about 400 molecules of LCN-A per CFU in the assay.

Of the strains tested, only two, the bacteriocin producer itself (LMG 2130) and *L. lactis* subsp. *lactis* biovar diacetylactis NIZO 4-25, were resistant. This latter strain, however, was not found to produce the bacteriocin. The nisin (*L. lactis* subsp. *lactis* NCDO 496 and NCDO 1403)- and diplococcin (*L. lactis* subsp. *cremoris* NCDO 893)-producing strains tested were all sensitive to LCN-A and were inhibitory to LMG 2130. In addition, the bacteriocin showed weak inhibition of *L. garvieae* NCDO 2155 (Table 3). We were unable to demonstrate the sensitivity of *L. raffinolactis* NCDO 617 to LCN-A in any of the assays used. The bacteriocin was also tested against a selection of bacteria outside the *Lactococcus* genus. Sensitive strains were not found.

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1     N- lys  - leu - thr - phe - ile - gln - ser - thr - ala- ala-
11       gly  - asp - leu - tyr - tyr - asn - thr - asn - thr- his-
21       lys  - tyr - val - tyr - gln - gln - thr - gln - asn- ala-
31       phe  - gly - ala - ala - ala - asn - thr - ile - val- asn-
41       gly  - trp - met - gly - gly - ala - ala - gly - gly- phe-
51       gly  - leu - his - his -C
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FIG. 2. Amino acid sequence of LCN-A.

Identification and cloning of the genetic determinant for LCN-A. An oligodeoxynucleotide probe based on the amino acid sequence of LCN-A was used in Southern hybridization analysis to localize the gene. When plasmid DNA from strain LMG 2130 was probed, one signal, corresponding to a 55-kb plasmid, was observed (Fig. 3, lane 1). Strain LMG 2130 was exposed to plasmid curing. One isolate, LMG 2131, which did not produce LCN-A, was found both to be deprived of the 55-kb plasmid and to give no signal on a Southern blot (Fig. 3, lane 3). Furthermore, Southern analysis of LMG 2130 plasmid DNA digests revealed signals from a 4-kb *Hin*dIII fragment (Fig. 3, lane 2), a 1.2-kb *Hin*dIII-*Rsa*I fragment (data not shown), and a 0.6-kb *Dra*I fragment (data not shown). The 4-kb fraction of *Hin*dIII-digested LMG 2130 plasmid DNA was cloned in *E. coli* with pUC18 as the vector. Of 1,400 clones, 10 were found to be positive after screening with the oligodeoxynucleotide probe. The recombinant plasmid (pON1) from one of these 10 clones was further restricted with *Dra*I and *Rsa*III (Fig. 3A). The fragments that hybridized to the probe, the 4-kb *Hin*dIII fragment, the 1.2-kb *Hin*dIII-*Rsa*I fragment, and the 0.6-kb *Dra*I fragment (Fig. 3B), were subcloned into M13mp18 and M13mp19 to yield inserts in both orientations.

TABLE 3. Sensitivities of some lactococcal strains to LCN-A

Strain[a]	Sensitivity (BU/ml)
L. lactis subsp. *cremoris*	
IMN C18	5
LMG 2130	NI[b]
LMG 2131	1,000
NCDO 607	1.3
NCDO 924	1,000
NCDO 1198	0.4
BC 101	50
BC 101(pON2)	5,000
BC 101(pON7)	5,000
L. lactis subsp. *lactis*	
NCDO 604	30
IL 1403	0.4
IL 1403(pON2)	1,500
IL 1403(pON7)	1,500
NCDO 176 (biovar diacetylactis)	20
NIZO 4-25 (biovar diacetylactis)	NI
L. raffinolactis NCDO 617	NI
L. garvieae NCDO 2155	5,000

[a] The most and the least sensitive strains tested are included. NCDO strains were from the National Collection of Food Organisms, Reading, United Kingdom.

[b] NI, no inhibition was observed at 10,000 BU/ml.

Nucleotide sequence of *lcnA*. The *Hin*dIII-*Rsa*I fragment was sequenced. The nucleotide sequence of the two consecutive *Dra*I fragments of 625 and 292 nucleotides (Fig. 4) is presented in Fig. 5. The entire *lcnA* gene was contained within the 0.6-kb *Dra*I fragment. Computer analysis of the six possible open reading frames (ORFs) revealed long ORFs only on one of the DNA strands (Fig. 4). Mature LCN-A of 54 amino acid residues is encoded by the DNA segment from nucleotide positions 316 to 477 (Fig. 5). The only possible initiation codon was found at nucleotide position 253, implying that LCN-A is synthesized as a 75-amino-acid precursor containing a 21-amino-acid N-terminal extension. The initiation codon is preceded by the possible Shine-Dalgarno sequence 3′ AGGAGA 5′ (40). Three putative promoter elements, all showing considerable similarity to the *E. coli* σ^{70} consensus and streptococcal promoters, were found just upstream of this ribosome binding site (RBS) (Fig. 5) (27, 35).

Downstream of *lcnA* a second ORF, ORF2, was found. Assuming that there is a translation start site at the ATG at nucleotide position 495, this ORF encodes a 98-amino-acid polypeptide. A possible RBS sequence, 5′ GAGGATTGA 3′, occurs 7 nucleotides from the fMet codon. Downstream of ORF2, extending from nucleotide positions 803 and 896, are two regions of dyad symmetry which could form stem-loop structures with ΔG values of −34.6 and −24.4 kcal/mol (−144.8 and −102.1 kJ/mol), respectively (45). The uridine content in their distal stems suggests that these structures constitute Rho-independent terminators of the *lcnA* transcript. No putative terminator or promoter sequences were found between *lcnA* and ORF2, indicating that *lcnA* and ORF2 may constitute an operon.

No DNA sequence in the EMBL data base showed a high degree of DNA homology to the DNA sequence presented here. The best score found was a 57.4% identity to a 122-bp sequence in the data base.

Cloning in *L. lactis*. When cloned in *E. coli*, the *lcnA* gene did not facilitate the secretion of an active bacteriocin. The *lcnA* gene was therefore also cloned in *L. lactis*. The pIL253::pUC18 constructions carrying the 4-kb *Hin*dIII fragment and the 1.2-kb *Hin*dIII-*Rsa*I fragment were named pON2 and pON7, respectively. Neither of these two plasmids caused detectable bacteriocin production in *L. lactis* subsp. *cremoris* BC 101. However, when present in BC 101, both pON2 and pON7 conferred resistance to LCN-A. With either plasmid, the LCN-A concentration causing 50% growth inhibition increased from 50 to 5,000 BU/ml (Table 3). This result was not seen with transformants containing the cloning vector alone. Similar results were observed with

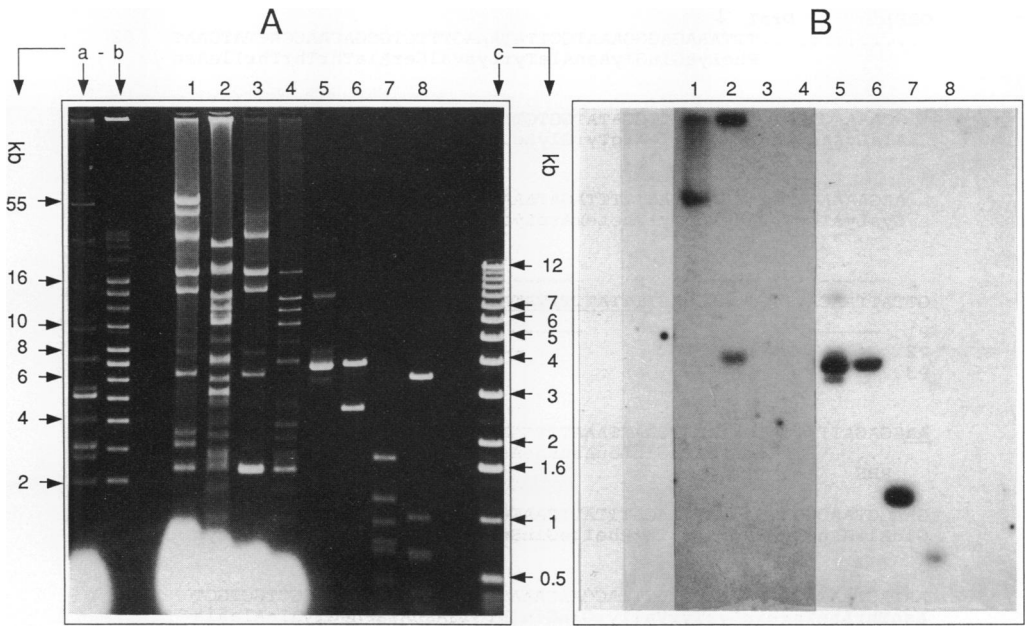

FIG. 3. Analysis of plasmid DNAs of LMG 2130, LMG 2131, and *E. coli*(pON1). (A) Ethidium bromide-stained agarose gel. (B) Gel from panel A subjected to Southern blotting with the *lcnA*-specific oligodeoxynucleotide probe. Plasmid DNA from *E. coli* V517 (24) (lane a) and a supercoiled 1-kb DNA ladder (lane b) were used as size markers for comparison with undigested plasmid DNA. A linear 1-kb DNA ladder (lane c) was used as a size marker for comparison with digested plasmid DNA. Each lane in the Southern blot is numbered to match the corresponding lane in the ethidium bromide-stained agarose gel. Lanes: 1, undigested LMG 2130 plasmid DNA; 2, *Hin*dIII-digested LMG 2130 plasmid DNA; 3, undigested LMG 2131 plasmid DNA; 4, *Hin*dIII-digested LMG 2131 plasmid DNA; 5, undigested pON1; 6, pON1 digested with *Hin*dIII; 7, pON1 digested with *Rsa*I and *Hin*dIII; 8, pON1 digested with *Dra*I. To amplify signals from lanes 1, 2, 3, and 4, we subjected the film to both 20 and 75 h of exposure. Before photography, the 75-h-exposed autoradiogram was cut and placed on top of the 20-h-exposed one, so that the corresponding signals of the two autoradiograms were superimposed.

other strains of *L. lactis* (data not shown). The only strain tested that showed bacteriocin production after transformation with the *lcnA* gene was *L. lactis* subsp. *lactis* IL 1403. When carrying pON2 or pON7, *L. lactis* subsp. *lactis* IL 1403 produced about 60 BU/ml. By comparison, the LCN-A-producing strain, LMG 2130, produces about 1,500 BU/ml.

DISCUSSION

LCN-A, a new lactococcal bacteriocin, has been purified and sequenced. The sequence revealed a protein of 54 amino acids. In terms of primary structure, LCN-A is clearly different from the two lactococcal bacteriocins isolated so far, nisin (16) and diplococcin (10).

Sensitivity to LCN-A appears to be general among strains of *L. lactis*. Since this bacteriocin also is highly specific, it may be used for the identification of *L. lactis* strains. LCN-A is a hydrophobic protein. Its hydrophobic character was demonstrated by its high affinity for phenyl-Superose. This matrix is intended for use in hydrophobic interaction chromatography, and most proteins bind to it only at high salt concentrations. LCN-A bound to the column in the absence of salt and could only be eluted as an active bacteriocin by solvents less polar than water.

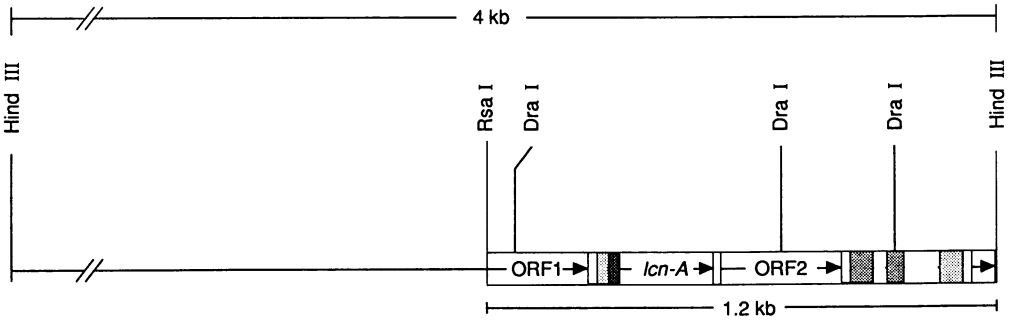

FIG. 4. Map of the cloned 4-kb, *lcnA*-carrying *Hin*dIII fragment. The LCN-A coding region as well as ORF1 and ORF2 are shown within the enlarged 1.2-kb *Rsa*I-*Hin*dIII subfragment. Arrows indicate the orientations of the genes. Possible transcriptional signal sequences are indicated by dark (promoters) and light (terminators) stippled boxes.

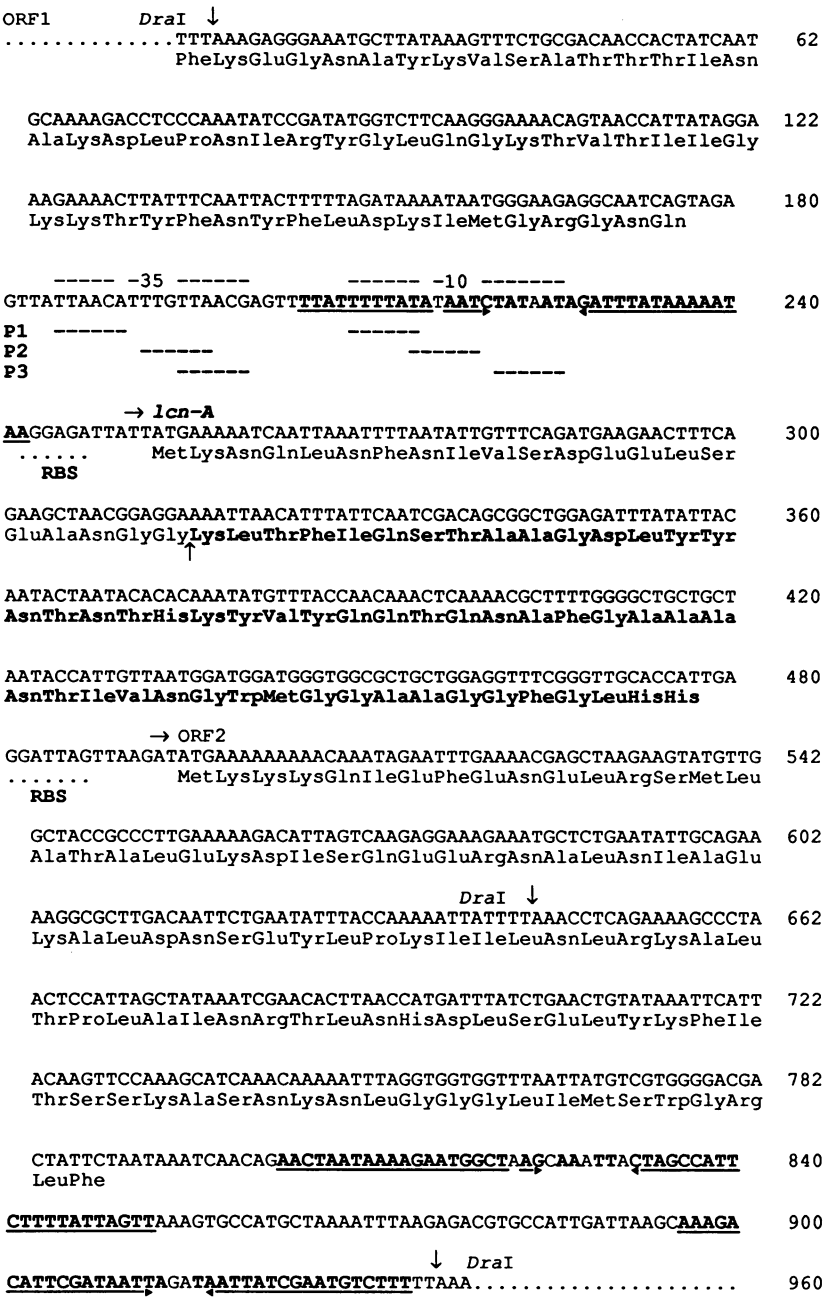

FIG. 5. Nucleotide sequence of the LCN-A operon of *L. lactis* subsp. *cremoris* LMG 2130. The derived amino acid sequences of ORF1, *lcnA*, and ORF2 are shown below the nucleotide sequence. The start sites of *lcnA* and ORF2 translation are shown by horizontal arrows above the nucleotide sequence. The amino acid sequence of mature LCN-A is shown in boldface type, and the site of cleavage is indicated by a vertical arrow pointing up. Also shown are the putative −10 and −35 promoter regions of *lcnA*, promoter candidates (denoted P1, P2, and P3), and putative RBSs. *DraI* restriction sites are indicated by vertical arrows pointing down. Three nucleotide sequence regions with dyad symmetry are indicated by boldface type. Stem-loop structures with the most negative free energy are indicated by facing horizontal arrows below the nucleotide sequence.

The toxic effects of nisin have been ascribed to its ability to form pores in cytoplasmic membranes (36). The hydrophobic character of LCN-A suggests that the cytoplasmic membrane may also be the target for this bacteriocin. Calculations made as described by Rao and Argos (34) predicted that the stretch from amino acids 30 to 52 in LCN-A can form a membrane-spanning helix (data not shown). The idea that LCN-A acts on the membrane is further supported by the finding that this bacteriocin causes leakage of intracellular components even in hypertonic sucrose-containing media (unpublished data).

A DNA probe made on the basis of the amino acid sequence of LCN-A hybridized to a 55-kb plasmid from the producing strain. A derivative of the parental producing

strain that had lost this 55-kb plasmid did not produce LCN-A and became sensitive. Moreover, plasmid DNA from this non-LCN-A-producing derivative did not hybridize to the *lcnA*-specific probe. Taken together, our results demonstrate that LCN-A is encoded by a 55-kb plasmid.

Secreted proteins are usually synthesized as precursors with a short N-terminal extension called the signal peptide, which promotes secretion and which is removed by specific enzymes (1, 43, 49, 50, 52). Comparison of the gene-derived sequence for mature LCN-A with the direct amino acid sequencing data shows that LCN-A is synthesized as a 75-amino-acid precursor. The LCN-A leader peptide of 21 amino acids has a positively charged N terminus followed by a hydrophobic stretch typical of signal peptides of gram-positive bacteria (1). Mature LCN-A has a lysine as its N-terminal amino acid. The sequence Ala-Asn-Gly-Gly precedes this lysine in the LCN-A precursor. According to the "−3, −1" rule of Von Heijne (49, 50), a signal peptidase could cleave the precursor between the two glycines (−2, −1) but not between the glycine and the lysine (−1, +1). This theory may suggest a stepwise processing of the LCN-A precursor in which a 20-amino-acid peptide and then a glycine are removed from the N terminus to yield mature LCN-A of 54 amino acids. The signal peptides of LCN-A and of lactacin F of *L. acidophilus* (28) have in common two glycines preceding the N-terminal amino acid of the mature bacteriocin. However, in the case of lactacin F, the processing has been suggested to follow the rule of Von Heijne (28).

Three putative promoter elements were found upstream of the *lcnA* gene (Fig. 5). Conceivably, transcription initiation could occur 5 to 9 nucleotides downstream of any of the putative Pribnow boxes, yielding leaders of 17 to 33 nucleotides. Overlapping the −10 regions of the putative promoter elements is an inverted repeat sequence that could form a stem-loop structure (Fig. 5). This structure, with a calculated ΔG value of −9.6 kcal/mol (−40.2 kJ/mol) (45), could represent a Rho-dependent terminator of ORF1. Overlapping terminator and promoter elements have previously been reported by Rosenberg and Court (35). It should, however, be noted that the stem-loop structure described above has a relatively low free energy and may therefore represent only a weak terminator. Our data do not exclude the possibility of transcriptional readthrough from ORF1. Alternatively, this palindromic structure, consisting of a 19-nucleotide inverted repeat, could represent a binding site for regulatory proteins affecting *lcnA* transcription. Palindromic structures have been found close to or overlapping the Pribnow boxes of the *E. coli* bacteriocin genes ColE1, ColE3, ColE6, and CloDF13 (2, 13). These inverted repeats have been shown to be SOS boxes and binding sites for the *E. coli* RecA-sensitive LexA repressor (12, 13, 48). However, we have no data suggesting an equivalent regulatory mechanism of *lcnA*.

Strain LMG 2131, which had lost the *lcnA* gene, was sensitive to LCN-A. This result suggests that the producing organism harbors a gene(s) encoding immunity to the bacteriocin. Strain IL 1403 carrying recombinant plasmid pON7 produced LCN-A and was (by necessity) resistant to the bacteriocin. Thus, the 1.2-kb *Rsa*I-*Hin*dIII fragment appears to carry not only the gene encoding LCN-A but also a genetic determinant for resistance. The DNA sequence of this fragment shows only one complete ORF in addition to the *lcnA* gene. This is ORF2, located downstream of and in the same operon as *lcnA*. Hence, the apparently cotranscribed ORF2 is the likely candidate to encode an LCN-A immunity function. A very similar organization of bacteriocin genes and their corresponding immunity genes has been shown for several *E. coli* bacteriocins (2, 26). ORF2 with fMet at nucleotide position 495, preceded by the possible RBS sequence 5′ GGATTAG 3′, encodes a hypothetical polypeptide of 98 amino acids. Alternatively, the ORF2 initiation codon could be TTG at nucleotide position 540, preceded by the possible RBS sequence 5′ GGATTAG 3′. This would yield an ORF2 gene product of 83 amino acids. However, codon usage in the 15 N-terminal amino acids of the 98-amino-acid polypeptide correlates well with the compiled codon usage pattern of the rest of the ORF2 polypeptide and of LCN-A, indicating that ORF2 encodes a 98-amino-acid polypeptide. Its six N-terminal residues (Met-Lys-Lys-Lys-Gln-Ile) show great similarity to signal peptides of gram-positive bacteria (1). Despite the presence of Glu in positions 7, 9, and 11, the putative signal sequence retains a hydrophobic character extending from amino acid positions 5 to 20 (Fig. 5). According to the −3, −1 rule of Von Heijne, there is a possible signal peptidase cleavage site after Ala-Thr-Ala at amino acid position 20. Of the 14 gram-positive signal sequences compiled by Abrahamsén et al. (1), 7 contained Ala-X-Ala at their cleavage sites. Moreover, Ala-Thr-Ala was found to be the −3, −1 amino acid sequence of the signal peptidase cleavage site of *Bacillus subtilis* β-glucanase (29), possibly suggesting a mature ORF2 protein of 79 amino acids. It remains to be shown whether the ORF2-encoded polypeptide is secreted or anchored within the membrane.

The *lcnA* gene was cloned on a plasmid with a high copy number (41). Only one of the strains tested, IL 1403, showed detectable bacteriocin production when carrying *lcnA*. The amount produced was also very low, 60 BU/ml, less than 5% of that produced by strain LMG 2130. However, all of the lactococcal strains tested acquired LCN-A resistance after transformation with the gene, indicating that the *lcnA* operon is transcribed and implying that additional factors are required to make a functional bacteriocin. Since *lcnA* encodes a preprotein, it is likely that processing is required to make an active bacteriocin. The finding that the processing of LCN-A does not appear to follow the general rule of Von Heijne (49, 50) may suggest that a gene(s) encoding a special enzyme(s) for proper processing is required for the production of active LCN-A.

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ADDENDUM

After this paper was submitted for publication, the DNA sequences of two bacteriocin operons from another strain of *L. lactis* subsp. *cremoris* were published (47). The DNA sequence of the stretch carrying one of these operons appeared to be identical to the DNA sequence presented in this work. The bacteriocin genes described by van Belkum et al. (47) are located on a plasmid similar in size to the one described here carrying *lcnA*. However, the two bacteriocin-producing strains have different plasmid profiles (30).

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