APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1993, p. 3906–3915
0099-2240/93/113906-10$02.00/0

Vol. 59, No. 11

Molecular Analysis of the Lactacin F Operon†

C. FREMAUX,[1] C. AHN,[1]‡ AND T. R. KLAENHAMMER[1,2]*

Departments of Food Science[1] and Microbiology,[2] Southeast Dairy Foods Research Center, North Carolina State University, Raleigh, North Carolina 27695-7624

Received 5 May 1993/Accepted 7 September 1993

Lactacin F is a nonlantibiotic, heat-stable, peptide bacteriocin produced by *Lactobacillus johnsonii* VPI11088. Molecular analysis of the lactacin F DNA region characterized a small operon that codes for three open reading frames, designated *lafA*, *lafX*, and ORFZ. The peptide encoded by *lafA*, the lactacin F structural gene, was compared with various peptide bacteriocins from lactic acid bacteria, and similarities were identified in the amino and carboxy termini of the propeptides. Site-directed mutagenesis of the LafA precursor at the two glycine residues in positions −1 and −2 defined an essential motif for processing of mature lactacin F. The involvement of the peptides encoded by *lafX* and ORFZ in bacteriocin expression was investigated by subcloning various fragments from the lactacin F region into the shuttle vector pGKV210. In addition to *lafA*, expression of *lafX* is essential to lactacin F activity. The lactacin F operon resembles the genetic organization of lactococcin M. Although no function has been assigned to ORFZ by genetic analysis, both peptide Z and the lactococcin M immunity protein are predicted to be integral membrane proteins with four putative transmembrane segments. Lactacin F activity, defined by bactericidal action on *Lactobacillus delbrueckii*, is dependent on the expression of two genes, *lafA* and *lafX*.

Bacteriocins are proteinaceous compounds displaying bactericidal activity against closely related species (44). While true for the majority of bacteriocins, it is now well established that these proteins may exist in many forms and exert bactericidal activity beyond that of species that are closely related or confined within the same ecological niche (20). Bacteriocins characterized from lactococcal species were initially organized into eight different types differentiated largely on the basis of host range (11). Biochemical and genetic studies of bacteriocins produced by the lactic acid bacteria (LAB) have now defined four major classes (20). Class I bacteriocins are lantibiotics, of which nisin is the most well-documented (19). Class II bacteriocins are small, mostly hydrophobic, heat-stable peptides that are active on the cell membrane. These peptides are synthesized as a precursor with an N-terminal extension of 18 to 24 residues and a conserved Gly-Gly motif positioned at the −1 and −2 positions before the cleavage site. The peptides do not contain lanthionine residues and appear to be the most prevalent type of bacteriocin produced by the LAB (20). Class III bacteriocins contain large heat-labile peptides (>30 kDa), exemplified in the LAB by helveticin J, which remains to date the only member characterized genetically (6, 17). Finally, complex bacteriocins, formed by the association of bactericidal proteins with one or more other essential chemical moieties, such as lipid and carbohydrate moieties, constitute class IV.

Lactacin F was the first class II bacteriocin from LAB for which both protein and genetic information was available. Lactacin F is produced by *Lactobacillus johnsonii* VPI11088 (classified previously in the *Lactobacillus acidophilus* B2 homology group [8, 18]) and exerts an inhibitory spectrum that includes *Lactobacillus delbrueckii*, *Lactobacillus helveticus*, *Lactobacillus fermentum*, *L. acidophilus*, and *Enterococcus faecalis* (2, 34). The mature bacteriocin was purified, and its N-terminal amino acid sequence was partially determined. By using a degenerate 63-mer oligonucleotide probe deduced from the peptide sequence, a 2.3-kb *Eco*RI fragment was cloned from a conjugative element that encoded genetic determinants for lactacin F production (Laf^+) and immunity (Laf^r) (35). The bacteriocin structural gene (*lafA*) was localized, and its nucleotide sequence was determined. An N-terminal extension for the LafA precursor was deduced from the nucleotide sequence. It contains a Gly-Gly motif in positions −1 and −2, which is commonly found in class II native bacteriocins (13, 20, 21, 26, 30, 45). Two small overlapping open reading frames (ORFs), ORFX and ORFY, were defined downstream of *lafA*, but their involvement in bactericidal production or activity was unknown (35).

In this study, the nucleotide sequences of regions flanking *lafA* were determined, and the essential components of the lactacin F operon were defined. A genetic organization typical among class II LAB bacteriocin determinants was determined. An operon composed of the bacteriocin structural gene and a putative immunity gene is described. In addition, the operon contained an unusual third gene encoding a bacteriocin-like peptide which is required for lactacin F activity. Therefore, the lactacin F system is similar to two other bacteriocin encoded by *Lactococcus lactis*: lactococcin M (45) and lactococcin G (36). The three bacteriocins are proposed to define an important subclass of class II bacteriocins found in LAB.

MATERIALS AND METHODS

Bacterial cultures, phages and plasmids. The bacterial strains, phages, and plasmids used or designed in this study are described in Tables 1 and 2. *Lactobacillus* strains were propagated in MRS broth (Difco Laboratories, Detroit, Mich.) at 37°C, and frozen stocks were maintained at −20°C in MRS broth containing 20% glycerol. MRS agar contained 1.2% granulated agar (BBL Microbiology Systems, Cock-

* Corresponding author.

† Paper no. FS93-15 of the Journal Series of the Department of Food Science, Raleigh, NC 27695-7624.

‡ Present address: Department of Applied Biology and Technology, Kangwon National University, Chuncheon, Kangwondo, Korea 200-701.

TABLE 1. Bacterial strains and plasmids

Strain, plasmid, or phage	Relevant characteristic(s)	Reference or origin
E. coli		
DH5α	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1* Δ*lac*U169 (ϕ80d *lacZ*ΔM15) *deoR* $F^-\lambda^-$	Bethesda Research Laboratories
SDM	*recA1 hsdR17 supE44 mcrAB Tet*^{r} Δ(*lac-proAB*) F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	U.S. Biochemical Corp.
SURE	e14$^-$(*mcrA*) Δ(*mcrCB-hsdSMR-mrr*)*171 endA1 supE44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuC*::Tn*5* (*kan*^{r}) *uvrC* F′ [*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*10*(*Tet*^{r})]	Stratagene
NCK360	DH5α(pTRK160)	35
NCK451	DH5α(pTRK201)	This study
NCK453	DH5α(pTRK203)	This study
NCK454	DH5α(pTRK204)	This study
NCK456	DH5α(pTRK206)	This study
NCK561	SURE(pTRK331)	This study
L. johnsonii[a]		
VPI11088	NCK88, Laf$^+$ Laf^r parent	Virginia Polytechnic Institute, 34, 35
NCK64	NCK88 derivative 88-C, *lafA 729*, Laf$^-$ Laf^r	1, 34, 35
NCK65	NCK88 derivative 88-4, Δ*laf* Laf$^-$ Laf^s	1, 34, 35
NCK89	NCK65 derivative 89, *str-6 rif-7* Δ*laf* Laf$^-$ Laf^s	34, 35
L. delbrueckii subsp. *lactis* NCK235	ATCC 4797, Laf^s, lactacin F indicator	American Type Culture Collection, 34, 35
Plasmids		
pGKV210	Em^r, promoterless *cat-86*, 4.4 kb	47
pTRKH2	*lacZ* Em^r, 6.9 kb	37
pTRK160	pBluescript KS+::2.3-kb *Eco*RI, 5.3 kb, *lafA lafX* ORFZ	This study
pTRK201	pGKV210::0.9-kb *Eco*RI-*Pvu*II from pTRK160, 5.3 kb, *lafA886*	This study
pTRK203	pGKV210::1.05-kb *Eco*RI-*Hae*III from pTRK160, 5.55 kb, *lafAX1047*	This study
pTRK204	pGKV210::2.3-kb *Eco*RI from pTRK160, 6.7 kb, *lafA lafX* ORFZ	This study
pTRK206	pGKV210::0.7-kb *Dra*III from pTRK160, 5.05 kb, *lafA lafX*	This study
pTRK331[b]	pTRKH2::0.7-kb *Eco*RI-*Bam*HI from M13-206, 7.6 kb, *lafA lafX*	This study
Bacteriophages		
M13mp18	Cloning vector, *lacZ*	51
M13-206	M13mp18::0.7-kb *Eco*RI-*Bam*HI from pTRK206, 7.9 kb	This study

[a] *L. johnsonii* is the new species name for the former *L. acidophilus* group B2 (8).
[b] pTRK331 derivatives are listed in Table 2.

eysville, Md.). When appropriate, erythromycin was added to a concentration of 3 μg/ml. *Escherichia coli* strains were propagated at 37°C with shaking in LB broth or agar (1.5%) (41). SURE strains (Stratagene, La Jolla, Calif.) were grown in the presence of 10 μg of tetracycline per ml. Ampicillin, erythromycin, IPTG (isopropyl-β-D-thiogalactopyranoside), and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were used at concentrations of 50, 100, 50, and 50 μg/ml, respectively. Erythromycin-resistant (Em^r) transformants of *E. coli* were selected on brain heart infusion agar plates (37) (BBL Microbiology Systems). Phages M13mp18 and M13-206 and their derivatives were propagated in LB broth, and plaques were obtained on H media (41).

Bacteriocin assays. The critical-dilution assay (31) was used for bacteriocin titration. Lactacin F activity was assayed in MRS broth cultures that were propagated overnight at 37°C. Cell-free supernatants were obtained after centrifugation two times in an Eppendorf microcentrifuge at 14,000 rpm. Ten microliters of serial twofold dilutions of the spent supernatants was spotted on a predried MRS plate. After adsorption, the plate was overlaid with 4 ml of MRS agar inoculated with the lactacin F indicator strain, *L. delbrueckii* subsp. *lactis* ATCC 4797. After incubation for 18 to 24 h at 37°C, bacteriocin titers were determined and expressed in activity units (AU) per milliliter (2). The same protocol was used to determine the level of immunity to lactacin F by titrating a known concentration of lactacin F against the strain to be tested. The MIC was determined and expressed in activity units per milliliter.

DNA manipulation, cloning, and transformation. Plasmid DNA and the replicative and single-stranded forms of phage M13 were purified as described previously (41). Plasmid DNA was digested with restriction enzymes from various manufacturers according to their recommendations. Analytical and preparative agarose gel electrophoresis in Tris-borate-EDTA (pH 8.3) was done according to the method of Sambrook et al. (41). DNA restriction fragments were purified from agarose gels by using the Prep-A-Gene kit (Bio-Rad Laboratories, Richmond, Calif.). Recombinant DNA was obtained with T4 DNA ligase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) following the supplier's recommendations. *E. coli* and *L. johnsonii* strains were transformed or transfected by electroporation in 0.2-cm cuvettes in a Gene Pulser apparatus (Bio-Rad), with settings of 25 μF, 2.1 kV, and 200 ohms. Cells for electroporation were prepared following the protocols described by Dower et al. (4) for *E. coli* and by Raya et al. (39) for *Lactobacillus* species.

Site-directed mutagenesis. A recombinant phage (M13-206) that contained the minimal restriction fragment that could encode lactacin F activity in *L. johnsonii* NCK65 was

TABLE 2. Levels of lactacin F production and immunity exhibited by genetic derivatives of *L. johnsonii* NCK64 and NCK65

Strain	Construction	Relevant plasmid characteristic(s)	Lactacin F activity (AU/ml)	Immunity (MIC [AU/ml])
VPI11088	Parent, NCK88, Laf$^+$ Laf^r		800–12,800[a]	6,400
NCK64	NCK88 variant, Laf$^-$ Laf^r		<100	6,400
NCK65	NCK88 variant, Laf$^-$ Laf^s		<100	800
NCK458	NCK89(pGKV210)		<100	ND[b]
NCK638	NCK65(pTRK201)	*lafA886*	<100	800
NCK639	NCK65(pTRK203)	*lafA lafX1047*	<100	800
NCK640	NCK65(pTRK204)	*lafA lafX* ORFZ	800	1,600
NCK642	NCK65(pTRK206)	*lafA lafX*	800	1,600
NCK465	NCK64(pTRK201)	*lafA886*	<100	ND
NCK466	NCK64(pTRK203)	*lafA lafX1047*	3,200	ND
NCK467	NCK64(pTRK204)	*lafA lafX* ORFZ	12,800	ND
NCK469	NCK64(pTRK206)	*lafA lafX*	12,800	ND
NCK551	NCK65(pTRK331)	*lafA lafX* ORFY	800	ND
NCK552	NCK65(pTRK331.2)	*lafA lafX947 ORFY*	<100	ND
NCK553	NCK65(pTRK331.3)	*lafA lafX* ORFY 964/967	800	ND
NCK573	NCK64(pTRK331)	*lafA lafX*	12,800	ND
NCK555	NCK64(pTRK331.5)	*lafA/G-1V* (Gly [−1] → Val) *lafX*	<100	ND
NCK556	NCK64(pTRK331.6)	*lafA/G-2R* (Gly [−2] → Arg) *lafX*	<100	ND
NCK557	NCK64(pTRK331.7)	*lafA/G-2S* (Gly [−2] → Ser) *lafX*	<100	ND
NCK558	NCK64(pTRK331.8)	*lafA/R1K* (Arg [+1] → Lys) *lafX*	12,800	ND
NCK559	NCK64(pTRK331.9)	*lafA/V-3D* (Val [−3] → Asp) *lafX*	12,800	ND
NCK560	NCK64(pTRK331.10)	*lafA/C30Y* (Cys [+30] → Tyr) *lafX*	<100	ND
NCK574	NCK64(pTRK331.11)	*lafA/R1T* (Arg [+1] → Thr) *lafX*	6,400	ND

[a] Lactacin F production by the parent strain, VPI11088, was variable in batch cultures.
[b] ND, not determined.

constructed. M13-206 was constructed by cloning the 0.7-kb *Bam*HI-*Eco*RI insert from pTRK206, which encoded *lafA*, *lafX*, and their promoter region (Fig. 1), into the multiple-cloning site of the phage M13mp18 (51). Following the manufacturer's protocols, site-directed mutagenesis was performed on phage M13-206 single-stranded DNA by using synthetic oligonucleotides and the T7-GEN in vitro mutagenesis kit (U.S. Biochemical Corp., Cleveland, Ohio). The mutated M13-206 *Bam*HI-*Sac*I insert was subcloned in the multiple-cloning site (*Bgl*II-*Sac*I) of the *E. coli*-LAB shuttle vector pTRKH2 (37). Plasmid pTRK331 contained the unmutated fragment inserted in pTRKH2 and was used as a positive control for production of lactacin F in *L. johnsonii* NCK65.

DNA sequencing. Sequencing-grade plasmid DNA was obtained by proteinase K treatment followed by polyethylene glycol 8000 precipitation of plasmid extracts (41). Phage sequences were determined on single-stranded DNA. Sequencing reactions based on the method of Sanger et al. (42) were performed by using the Sequenase version 2.0 DNA sequencing kit (U.S. Biochemical Corp.) and specific synthetic primers. Sequence analyses were performed with the PC/GENE program (Intelligenetics, Inc., Mountain View, Calif.). Protein sequences deduced from the lactacin F DNA

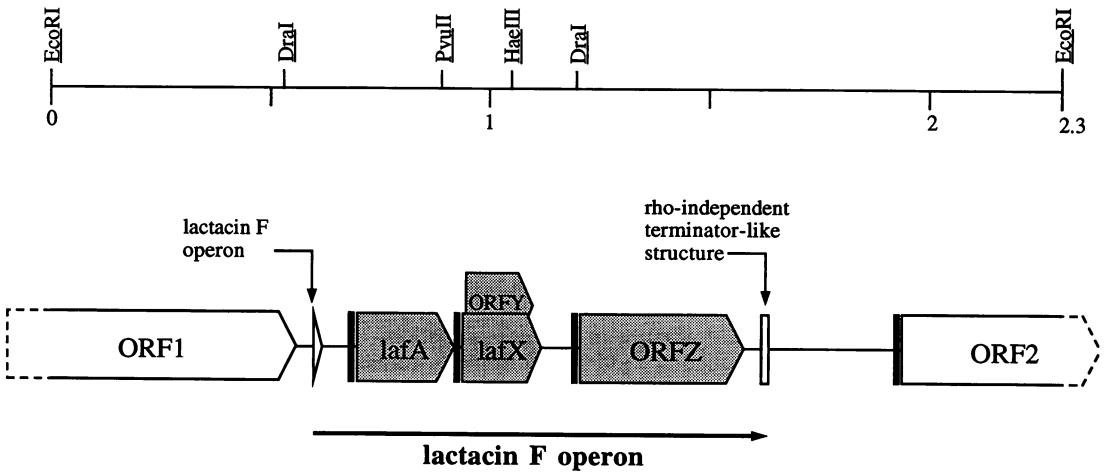

FIG. 1. Genetic organization of the 2.3-kb *Eco*RI fragment encoding the lactacin F operon. The diagram displays a partial restriction map and the positions of ORFs within the lactacin F operon (shaded boxes) and surrounding the operon (open boxes). The promoter ( ), the terminator ( ), and putative ribosome binding sites ( ) are indicated. The black arrow indicates the extent of the lactacin F operon.

region (GenBank sequence M57961) were compared with the protein sequences stored within the Swiss-Prot data bank (release 23).

RNA isolation and analysis. Total RNAs were isolated from *L. johnsonii* clones according to the procedure of van der Vossen et al. (47) modified as described by Fremaux and Klaenhammer (7). RNAs were denatured at 65°C for 15 min in 50% formamide–6% formaldehyde–1×SSC (150 mM NaCl, 15 mM sodium citrate) and then chilled on ice, and 1 volume of cold 20× SSC was added. By using the Bio-dot SF apparatus (Bio-Rad), the denatured samples were blotted on a Magnagraph nylon transfer membrane (Micron Separation, Inc., Honeoye Falls, N.Y.) as specified by the manufacturer and anchored to the membrane with the UV-Stratalinker (Stratagene). Prehybridization and hybridization reactions were performed at 42°C for 2 and 16 h, respectively, in 50% formamide–6× SSC–1× Denhardt's reagent (3)–1% sodium dodecyl sulfate (SDS)–0.1 mg of denatured salmon sperm DNA per ml. The probe, consisting of a gel-purified internal *cat-86* fragment (0.4-kb *Bcl*I-*Hin*dIII restriction fragment from pGKV210) was labeled with [α-^{32}P]dCTP by using the multiprime DNA labeling system (Amersham Corp., Arlington Heights, Ill.). Nonspecific hybridizations were washed at 42°C in 1× SSC–0.1% SDS, after which the membrane was exposed to an autoradiography film.

Primer extensions were performed to map the transcription initiation site of the lactacin F operon. The primer extension oligonucleotide 5′-CCACCATCAACGACTGC was labeled at the 5′ end with [γ-^{32}P]dATP with 16 U of T4 polynucleotide kinase (Promega Corp., Madison, Wis.) in 40 mM Tris-HCl (pH 7.5)–10 mM $MgCl_2$–5 mM dithiothreitol (DTT) for 60 min at 37°C and then purified through a Nuctrap push column (Stratagene). The primer-template hybrid was obtained as described previously (7), and the primer was extended by using the SuperScript RNase H$^-$ reverse transcriptase (GIBCO-BRL, Life Technologies, Inc., Grand Island, N.Y.). Samples were treated with 100 μg of RNase A per ml at 37°C for 60 min, extracted with 1 volume of phenol-chloroform (6:3), and ethanol precipitated. The reverse transcripts were analyzed by electrophoresis through a 5% polyacrylamide sequencing gel; a sequencing reaction of pTRK206 performed with the primer-extension oligonucleotide was used to provide the size markers.

Nucleotide sequence accession number. The DNA sequence information of the lactacin F region has been deposited in GenBank under the accession no. M57961.

RESULTS

DNA sequence determination of the lactacin F region. A 2.3-kb *Eco*RI fragment, isolated from *L. johnsonii* VPI11088 (NCK88), that encodes some of the lactacin F genetic determinants was cloned originally in pTRK160 (35). With synthetic oligonucleotides, the nucleotide sequence of the pTRK160 insert was completed, and its 2,312 bp sequence was deposited in GenBank under accession no. M57961. Computer analysis of the 2.3-kb *Eco*RI fragment identified four complete ORFs, designated *lafA*, *lafX* (previously called ORFX), ORFY, and ORFZ, and two incomplete ORFs (ORF1 and ORF2) located on either end of the fragment (Fig. 1). Four corrections in the previously reported sequence are to be noted (35), two of which affect the genetic organization of the lactacin F region. First, an additional G nucleotide identified in position 1011 of the corrected sequence created a frameshift within both ORFX and ORFY. This change alters the deduced ORFX and ORFY proteins from 32 to 62 amino acids and from 59 to 44 amino acids, respectively. Secondly, an additional T residue at position 1244 was reported in the first sequence determination; this masked the presence of the 375-bp ORFZ.

Computerized screening of the GenBank data bases did not reveal significant homologies of the lactacin F region to any listed DNA sequences. Each ORF, except ORFY, is preceded by a putative ribosome binding site. Comparison of the amino acid sequences deduced from the six ORFs with sequences stored in the Swiss-Prot data base did not reveal significant similarities. Interestingly, proteins deduced from *lafA* (LafA), *lafX* (LafX), and ORFZ (peptide Z) contain a high proportion of hydrophobic residues (56, 42, and 52%, respectively). Their hydropathy profiles (27), therefore, displayed mostly hydrophobic peptides (Fig. 2). In addition, peptide Z was predicted by the methods of Klein et al. (23) to be an integrated protein containing four membrane-spanning segments (Fig. 2C).

The close proximity of *lafA*, *lafX*, ORFY, and ORFZ defines an apparent polycistronic operon. Muriana and Klaenhammer (35) previously described a putative promoter upstream of *lafA* based on consensus sequences described for promoters (12, 14, 24, 32, 40, 47) (Fig. 3A). Expanded sequencing revealed a putative transcription terminator located downstream of ORFZ (Fig. 3B). Two inverted repeats defined an imperfect stem-loop structure with features common to rho-independent terminators (32). The calculated free energy of formation was −21.0 kcal (−87.9 kJ)/mol. Downstream of this terminator-like sequence was a noncoding region with a size of 350 bp followed by an incomplete reading frame, ORF2. On the basis of consensus sequences, no putative promoter that might control the expression of ORF2 was identified. However, the high percentage of A-T nucleotides within this area (77% compared with 65% for the lactacin F region) could mask a promoter or regulatory sequences.

Lactacin F determinant analysis. Selected restriction fragments from the 2.3-kb lactacin F region (Fig. 4A) were subcloned upstream of the promoterless *cat-86* gene in pGKV210 (47). To determine the involvement of the various ORFs encoded within the lactacin F region in bacteriocin production and immunity, various plasmids (pTRK201, pTRK203, pTRK204, and pTRK206) were electroporated into *L. johnsonii* NCK65. The production of lactacin F by each of the transformants was titrated, and the results are displayed in Table 2. The Laf$^-$ Laf^s phenotype of NCK65 has been described previously and appears to be related to a large deletion (ca. 10 kb) that encompasses the entire 2.3-kb *Eco*RI region (1, 35). Transformation of NCK65 with pTRK204 (containing the 2.3-kb *Eco*RI fragment) restores lactacin F production. A similar amount of bacteriocin is produced by NCK65 transformed with pTRK206, which encodes only *lafA*, *lafX*, and ORFY. Plasmid pTRK203, containing only *lafA*, failed to restore lactacin F production (Table 2). These data demonstrated that LafA and LafX and/or ORFY was required for bactericidal activity. The presence or absence of ORFZ or the truncated ORF1 and ORF2 had no detectable effects on bacteriocin production. Therefore, the minimal region essential for lactacin F expression was defined as *lafA*, *lafX*, and/or ORFY with the upstream sequence.

Site-directed mutagenesis was used to construct plasmids in which either *lafX* or ORFY was disrupted. For this purpose, the recombinant bacteriophage M13-206 was constructed to contain the minimal lactacin F region, as defined above. Because these two ORFs overlap, mutagenic oligo-

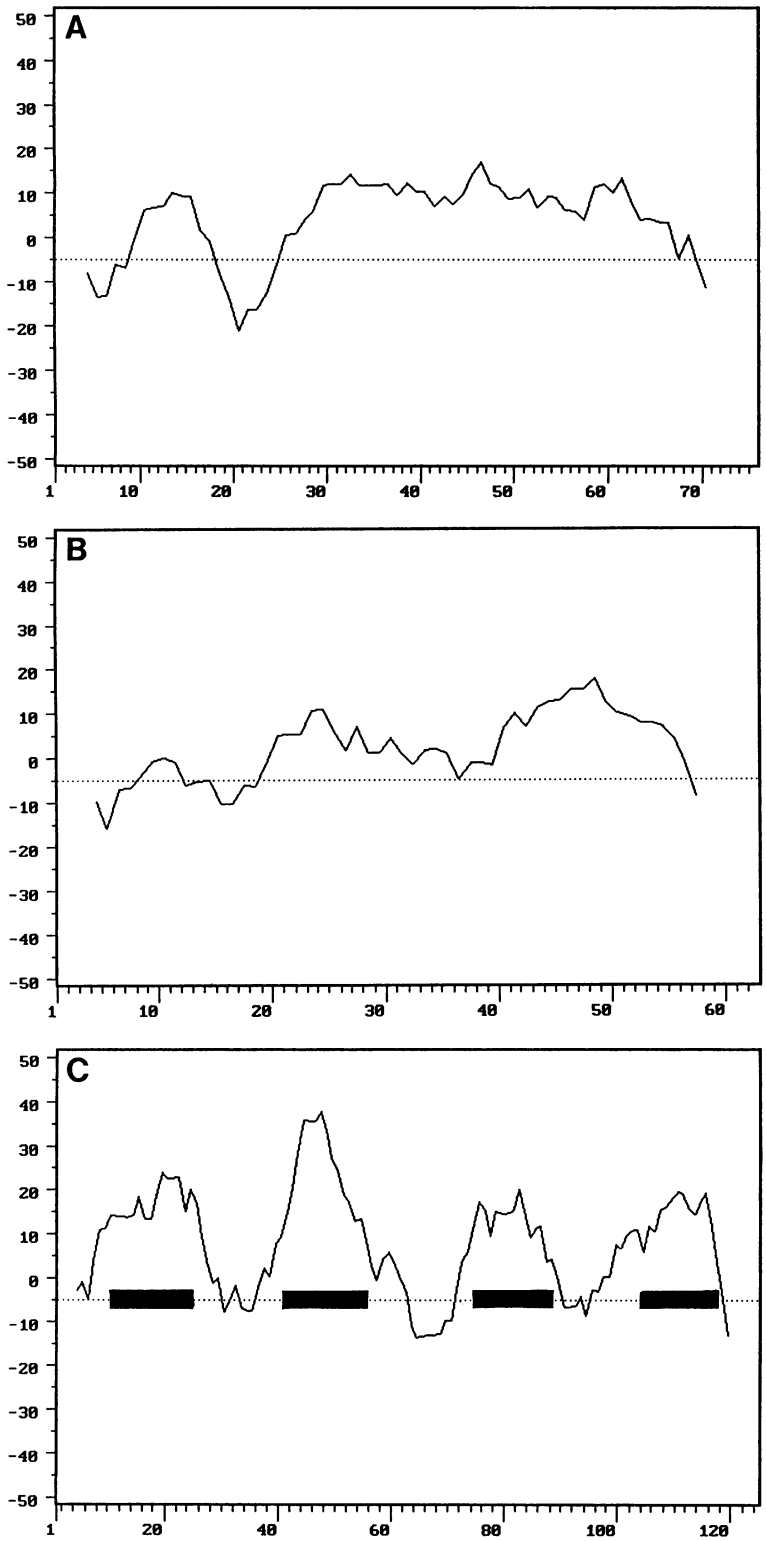

FIG. 2. Hydropathicity plots generated by the method of Kyte and Doolittle (27) by using the PC/GENE program SOAP for the proteins encoded within the lactacin F operon. (A) LafA; (B) LafX; (C) ORFZ deduced peptide. The *x* axis indicates the amino acid residue number, and the horizontal line at the −5 value (*y* axis) divides hydrophobic (top) and hydrophilic (bottom) regions. The closed boxes in panel C indicate the predicted membrane-spanning segments, by using the method of Klein et al. (23).

A

```
GTAATTTTAGACACAAATAGAACAATATTGGTCAATTTTATATCTTAAGATGATAACTTT

          ↓↓↓          RBS          ►lafA
AGTAAGCTATGCATATAAATAAAATTTTAGGAGGTTTCTATCATGAAACAATTTAATTAT
                                     M  K  Q  F  N  Y
```

B

```
          end ORFZ◄
ATTTTGAAAAACAAAAAATAAAGTCTAAATAATTTATCTATTTCAATCGTATATAAATAA
 I  L  K  N  K  K  -

          ──────► ─►      ◄─ ◄──────
AAATTAGAATACCAAATTGGTCTGTAAAACGACTAATTTGGTATTTTTTATCTTATGAAA
```

FIG. 3. Nucleotide sequences of the lactacin F operon promoter (A) and terminator (B). The sequence (M57961) is deposited in GenBank. (A) Nucleotide sequence of the lactacin F operon upstream region. The 5′ end of *lafA* is denoted with the LafA amino acid sequence given in single-letter code. The three concomitant transcription starts are indicated by vertical arrows. (B) Nucleotide sequence of the lactacin F downstream region. The 3′ end of ORFZ is indicated, and its deduced product sequence is given. The facing arrows represent the possible rho-independent terminator.

nucleotides were designed to introduce a stop codon within one amino acid sequence without affecting the other peptide. First, the oligonucleotide used for *lafX* disruption (5′-CT TAGTCATTTAATTTCATTGTC [the mismatched residue is underlined]) generates a stop codon in place of the Lys (+6 position). This transition occurs at the wobble position in the second codon of ORFY (ACA [Thr] to ACT [Thr]) and, thus, does not alter the deduced ORFY protein sequence. Similarly, by using the oligonucleotide 5′-CGATT TCCACCTACTATCTTTG, two stop codons which were introduced at positions +8 and +9 of ORFY did not alter the deduced LafX peptide sequence. The native and the mutated DNA fragments were then inserted into the expression vector pTRKH2. The resulting plasmids (pTRK331, *lafA lafX*$^+$ ORFY; pTRK331.2, *lafA lafX947* ORFY; and pTRK331.3, *lafA lafX* ORFY 964/967) were introduced within NCK65 and analyzed for their ability to produce lactacin F (Table 2). Disruption of ORFY did not affect the production of lactacin F, while disruption of *lafX* led to a Laf$^-$ phenotype. This demonstrated that *lafX* was required for lactacin F production, whereas ORFY was not.

Analyses of lactacin F production were performed in NCK64 (Laf$^-$ Laf^r) transformed with pTRK201, pTRK203, pTRK204, and pTRK206 (Table 2). In this expression host, every recombinant plasmid containing an intact *lafA* resulted in lactacin F production by NCK64. This was anticipated since NCK64 contains a frameshift mutation in *lafA* which does not alter expression of *lafX* (1). Thus, introduction of *lafA* alone is sufficient to complement the mutation and results in lactacin F production. However, the level of bacteriocin produced was four times greater when multiple copies of *lafA* and *lafX* were present in the clones (NCK467 and NCK469) than when *lafA* was multicopy and *lafX* was chromosomally encoded (NCK466). This suggested that a limiting amount of LafX is produced by NCK466, presumably because of fewer *lafX* copies compared with *lafA*. In addition, higher levels of lactacin F were produced when pTRK204 was cloned in NCK64 (NCK469) than in NCK65 (NCK640). This suggests that some important determinants, required for optimum production of lactacin F, are not encoded within the 2.3-kb *Eco*RI fragment, nor are they expressed in NCK65.

The immunity levels of VPI11088, NCK64, and NCK65 and its derivatives were determined by using a concentrated lactacin F extract of known activity. The MICs obtained for

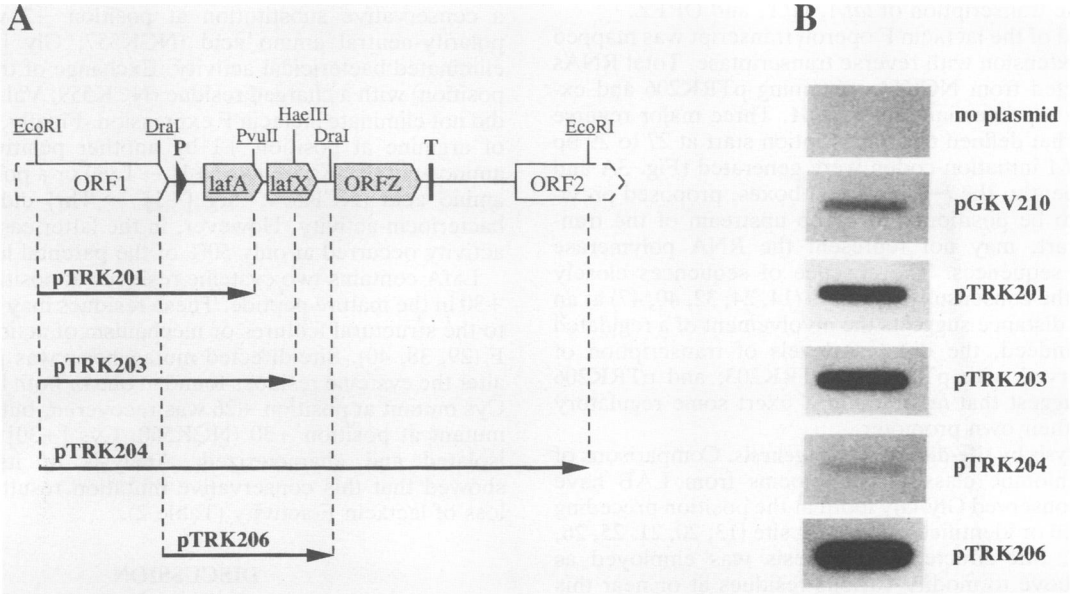

FIG. 4. Subcloning and expression of ORFs in the lactacin F operon. (A) Restriction fragments isolated from the lactacin F region (represented in the upper part of the diagram) and cloned within the promoter probe vector pGKV210 are shown. The enzymes used for cloning are indicated and the orientations of the fragments with respect to the *cat-86* gene of pGKV210 are indicated by arrows. (B) Dot blot analyses of 10 μg of total RNA per ml isolated from NCK64 derivatives containing either no additional plasmid or the plasmid indicated on the right and hybridized with the *cat-86* specific probe.

the various genetic constructions are displayed in Table 2. NCK65 is eight times more sensitive to lactacin F than the parental strain VPI11088, indicating that the immunity determinants were contained within the chromosomal region deleted in NCK65. For NCK65 transformed with pTRK204 (NCK640), a parental level of immunity was not restored. Thus, the 2.3-kb *Eco*RI region fails to encode or fully express immunity determinants.

Characterization of the lactacin F operon. The constructions described in Fig. 4A were cloned in NCK64 in order to evaluate promoter and terminator activities within the lactacin F region. Efforts to assess these functions by enzymatic assay of *cat-86* were inconsistent because of high background levels of chloramphenicol acetyltransferase expression (data not shown). Therefore, expression of *cat-86* in the various constructions was quantitated by analysis of specific mRNAs. Total RNAs extracted from transformants of *L. johnsonii* NCK64 containing either pTRK201, pTRK203, pTRK204, or pTRK206 (Table 2) were blotted and hybridized with a *cat-86* specific probe (Fig. 4B). Basal levels of *cat-86* expression were observed in NCK89 containing pGKV210 (NCK458). This background was related to sequences located within the erythromycin resistance gene terminator that exhibited promoter activity in *L. johnsonii* (data not shown). The sequence upstream of *lafA* cloned within pTRK201 (*Eco*RI-*Pvu*II fragment) drove transcription of *cat-86*. The *Dra*I fragment cloned in pTRK206, which contains both *lafA* and *lafX* and 163-bp upstream sequences, also expresses *cat-86*. This demonstrated that promoter activity occurred between nucleotides 530 (*Dra*III site) and 886 (*Pvu*II). The clone harboring pTRK204 displayed a very low level of transcription, lower than the basal level obtained with pGKV210. This demonstrated the presence of a functional terminator located downstream of ORFZ and indicated the absence of any additional downstream promoter activity. These data indicate that the stem-loop structure downstream of ORFZ acts as a rho-independent terminator. Collectively, the results demonstrated the polycistronic transcription of *lafA*, *lafX*, and ORFZ.

The 5′ end of the lactacin F operon transcript was mapped by primer extension with reverse transcriptase. Total RNAs were extracted from NCK64 containing pTRK206 and extended with a primer internal to *lafA*. Three major reverse transcripts that defined the transcription start at 27 to 29 bp from the *lafA* initiation codon were generated (Fig. 3A and 5). Consequently, the −10 and −35 boxes, proposed previously (35) to be positioned at 40 bp upstream of the transcription start, may not represent the RNA polymerase recognition sequences. The absence of sequences closely resembling the consensus promoters (14, 24, 32, 40, 47) at an appropriate distance suggests the involvement of a regulated promoter. Indeed, the different levels of transcription of *cat-86* observed with pTRK201, pTRK203, and pTRK206 (Fig. 4B) suggest that *lafA* and *lafX* exert some regulatory activity on their own promoter.

LafA analysis by site-directed mutagenesis. Comparisons of the nonlanthionine class II bacteriocins from LAB have revealed a conserved Gly-Gly motif at the position preceding the suspected or identified processing site (13, 20, 21, 25, 26, 30, 35, 46). Site-directed mutagenesis was employed as described above to modify various residues at or near this position within the LafA precursor, specifically, the glycine residues at positions −1 and −2 and the valine (−3 position) and arginine (+1 position) residues. The mutated DNA fragments were cloned into pTRKH2, and the resulting constructions were introduced into NCK64 to evaluate expression of lactacin F (Table 2). Replacement of glycine with valine at position −1 (NCK555, Gly [−1] → Val) or glycine with arginine at position −2 (NCK556, Gly [−2] → Arg) resulted in the complete loss of lactacin F expression. Even a conservative substitution at position −2 with another polarity-neutral amino acid (NCK557, Gly [−2] → Ser) eliminated bactericidal activity. Exchange of the valine (−3 position) with a charged residue (NCK559, Val [−3] → Asp) did not eliminate lactacin F expression. Finally, replacement of arginine at position +1 by another positively charged amino acid (NCK558, Arg [+1] → Lys) or a polarity-neutral amino acid (NCK574, Arg [+1] → Thr) did not disrupt bacteriocin activity. However, in the latter case, lactacin F activity occurred at only 50% of the parental level.

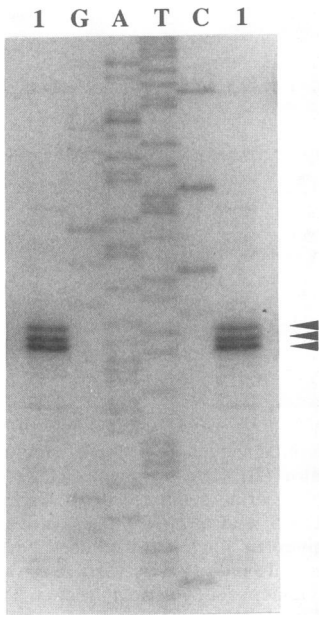

FIG. 5. Identification of the 5′ end of mRNA specified by the promoter of the lactacin F operon. Primer extension was done by using total RNA extracted from NCK642 and a primer complementary to the coding strand in positions 746 to 730 according to the GenBank sequence M57961. The reaction was run in a 5% polyacrylamide gel (lanes 1), and the three major reverse transcripts are indicated (◀). A sequencing reaction generated on pTRK206 with the primer extension oligonucleotide was used as a molecular weight marker (lanes G, A, T, and C).

LafA contains two cysteine residues at positions +26 and +30 in the mature peptide. These residues may be important to the structural features or mechanism of action of lactacin F (29, 38, 40). Site-directed mutagenesis was performed to alter the cysteine residues found in one or both locations. No Cys mutant at position +26 was recovered, but a single Cys mutant at position +30 (NCK560, Cys [+30] → Tyr) was isolated and characterized. Analysis of its phenotype showed that this conservative mutation resulted in a total loss of lactacin F activity (Table 2).

DISCUSSION

Four of the nonlanthionine heat-stable bacteriocins (class II LAB bacteriocins [20]) are encoded within small operons that contain the immunity gene in addition to the bacteriocin structural gene (13, 16, 25, 43, 45, 46). Our results indicate that the lactacin F structural gene (*lafA*) is part of a polycis-

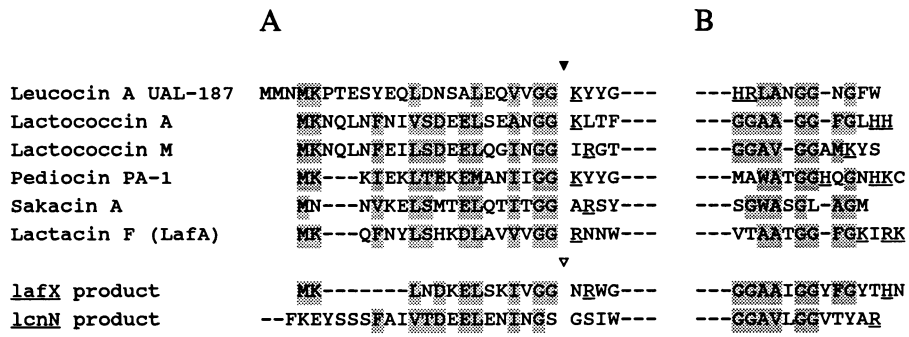

FIG. 6. Amino acid comparisons within the amino (A) and carboxy (B) termini of the class II LAB bacteriocins: lactococcins A (16, 45) and M (45), leucocin A UAL-187 (13), pediocin PA1 (30), sakacin A (15), and lactacin F (35). Also shown are the proteins deduced from *lcnN* (45) and *lafX*. The comparisons were done according to amino acid polarity and charge, and the amino acids were shaded when a majority of residues were similar at a particular position. Positively charged residues at the N or C terminus of the mature peptide are underlined. The bacteriocin processing sites are indicated by a closed arrowhead, and a possible site for the maturation of LcnN and LafX is proposed (▽).

tronic operon composed of two additional ORFs. Downstream of *lafA* (previously denoted *laf*) is a 185-nucleotide ORF (*lafX*, previously noted ORFX) followed by the 375-nucleotide ORFZ (Fig. 1). This peculiar organization is similar to another operon encoding a class II LAB bacteriocin, lactococcin M. As for lactacin F, two genes are required for lactococcin M activity (45).

The complete nucleotide sequence of a 2.3-kb *Eco*RI chromosomal fragment from *L. johnsonii* VPI11088 that encodes lactacin F production (35) has been determined. By using *cat-86* as a reporter gene, a gene cluster containing *lafA*, *lafX*, and ORFZ was found to be transcribed as an operon. Its promoter, characterized by primer extension, does not exhibit features typical of vegetative promoters from *E. coli* and *Bacillus subtilis* (14, 32, 40), suggesting that the operon may be regulated. Interestingly, no terminator was detected between the 3′ end of the truncated upstream ORF (ORF1) and the 5′ end of the lactacin F operon. This implies that the lactacin F operon may be part of a larger operon, thus resembling that observed for the lactococcin A operon (43). Such an organization possibly could enhance bacteriocin production by increasing the number of transcripts over the bacteriocin structural gene. This phenomenon has been suggested for some bacteriocins from LAB (e.g., nisin A [5] and lacticin 481 [38]) and other ribosomally encoded antibiotics (e.g., subtilin, epidermin, and microcin B17 [reviewed in reference 26]). Molecular analyses of various bacteriocin operons in LAB (5, 25, 30, 43, 45) have recently uncovered an array of proteins involved in the processing, maturation, and export of bacteriocins. Therefore, it seems likely that the upstream and/or downstream region of the lactacin F operon encodes some important genes that affect processing, export, or immunity functions. Deletion of this region in strain NCK65 (1), and its absence in the cloned lactacin F fragment, may explain the deficiencies encountered with lactacin F production and immunity in this expression host.

Although computer searches did not reveal significant homologies between LafA and protein sequences stored in the data bank, some important genetic similarities and biochemical properties, such as size, heat stability, and mode of action (9), are shared with other class II LAB bacteriocins (13, 15, 16, 30, 34, 45, 46). First, the hydropathy profile (27) of LafA predicts a very hydrophobic peptide (Fig. 2A), which is typical among class II LAB bacteriocins (20, 21). However, LafA remains the most hydrophobic bactericidal peptide of this class defined thus far. Secondary structure predictions (10) of the N-terminal extensions of these bacteriocins identify a mostly helical sequence followed by a β-turn at the processing site (26). However, as noted by Holo et al. (16), the class II LAB bacteriocins are not likely to be processed by a classic signal peptidase since the propeptide sequences do not fully conform to the rules proposed by von Heijne for signal peptides (49, 50); in particular, the −3 position of LafA can be occupied either by a valine (native bacteriocin) or an aspartate (mutation *lafA/V-3D*) without affecting bactericidal activity. Sequence alignments display a more conserved pattern than was initially recognized, including (i) two conserved glycines (in positions −1 and −2), (ii) hydrophobic residues (in positions −4, −7, −12, and −15), (iii) a core of charged amino acids (in positions −8 to −10), and (iv) a serine (in position −11) (Fig. 6A). The crucial importance of the two glycines in positions −1 and −2 for lactacin F production has been demonstrated in this study. The Gly-Gly motif at the processing site, which is common to all the class II bacteriocin precursors of LAB described to date (20), suggests that a common specific peptidase may be involved in maturation of bacteriocins in this class. This motif has been proposed to provoke a β-turn in the protein that exposes the processing region to the action of this specific protease (26). However, the β-turn predictions for the mutants Gly[−2]→Ser, Gly[−2]→Arg, and Arg[+1]→Thr failed to corroborate a strictly conformational role. These limited data do suggest that processing specificity relies heavily upon sequence recognition of the Gly-Gly motif at the cleavage site.

Another characteristic of the class II LAB bacteriocins concerns their carboxy terminus, including a tail of positively charged residues, also found in lantibiotics isolated from LAB (e.g., nisin [19], lactocin S [33], and lacticin 481 [38]), and a conserved motif formed by an alternating sequence of glycines and hydrophobic residues (G-G-Hy-Hy-X-G-G-X-Hy-G-, where G and Hy indicate glycine and hydrophobic residues, respectively, and X may be either a blank or any residue) that precedes a positively charged terminus (Fig. 6B). Evidence to support the importance of the carboxy terminus for lactacin F is provided by the loss of bactericidal activity occurring in NCK465, in which pTRK201 encodes a truncated LafA where the last 10 C-terminal amino acids are deleted. The conserved C terminus suggests that this part of the molecule may serve a common function of the class II LAB bacteriocins.

Finally, the cysteine located in position 30 of mature LafA seems to play a key role in bactericidal activity. This residue may be involved in intra- or intermolecular bonding since it is located within one of the β-sheets predicted in the mature bacteriocin and it is also near the second cysteine (position 26). Possibly, this reflects some similarities with lactococcin B, whose bactericidal activity depends on the reduced state of its single cysteine (position 24) (48). However, it is more likely that replacement of the cysteine with a tyrosine in LafA modified a critical component essential for tertiary structure in the active bacteriocin.

In light of the above observations, the peptide deduced from *lafX* (LafX) possesses most of the molecular features characterizing the class II LAB bacteriocins, including a hydrophobic profile (Fig. 2B), a consensus processing site, a leader peptide, and a C terminus harboring conserved sequences (Fig. 6). These features are also found in the peptide deduced from *lcnN* encoded within the lactococcin M operon, except for a degenerative processing site (Fig. 6). In both the lactacin F and lactococcin M operons (45), the expression of *lafX* and *lcnN*, in addition to the structural bacteriocin genes *lafA* and *lcnM*, respectively, is required for some bactericidal activity. Lactacin F and lactococcin M may, therefore, represent a subclass of nonlanthionine heat-stable bacteriocin operons for which the complementary action of two bacteriocin-like peptides results in the killing of sensitive cells. This resembles the lactococcin G system in which bactericidal activity occurs through the interaction of two purified peptides, α1 and β, produced by the same strain (36). The two peptides, however, do not display the C-terminal conserved motif and its leader N-terminal sequence has yet to be determined.

Because of its 3′ location within the lactacin F operon, ORFZ was suspected to participate in immunity. Genetic analysis of the operon did not confirm this hypothesis. Nevertheless, the ORFZ deduced peptide (peptide Z) has been compared with other proteins involved in immunity against class II LAB bacteriocins, including LciA and LciM (45), LciB (46), and the pediocin PA-1 and leucocin A UAL-187 immunity proteins (13, 30). Amino acid comparisons did not reveal any significant conserved sequences between these peptides. However, computer analysis of the hydropathy profile and searches for transmembrane segments revealed some interesting structural homologies between the peptide Z and LciM. Similar to peptide Z (Fig. 2C), LciM displayed four consecutive hydrophobic regions, each corresponding to a putative transmembrane segment (data not shown). This suggests that ORFZ encodes an integral membrane peptide that may act in the immunity mechanism against lactacin F. However, contrary to the lactococcin M immunity system, peptide Z probably requires an additional factor encoded within the region that has been deleted in NCK65.

ACKNOWLEDGMENTS

This work was supported in part by the National Dairy Promotion and Research Board and the North Carolina Dairy Foundation.

We thank Gwen Allison for providing unpublished data for consideration in the conduct of this study, Kathy Milton for reviewing the manuscript, and Jeffrey Greene for providing partially purified lactacin F for use in this study.

REFERENCES

1. **Allison, G., C. Fremaux, C. Ahn, and T. R. Klaenhammer.** 1993. Expansion of the bactericidal activity of lactacin F by complementation with a second peptide encoded within the *laf* operon. FEMS Microbiol. Rev. **12**:P122.
2. **Barefoot, S. F., and T. R. Klaenhammer.** 1983. Detection and activity of lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*. Appl. Environ. Microbiol. **45**:1808–1815.
3. **Denhardt, D. T.** 1966. A membrane-filter technique for the detection of complementary DNA. Biochem. Biophys. Res. Commun. **23**:641–646.
4. **Dower, W. J., J. F. Miller, and C. W. Ragsdale.** 1988. High efficiency transformation of *Escherichia coli* by high voltage electroporation. Nucleic Acids Res. **16**:6127–6145.
5. **Engelke, G., Z. Gutowski-Eckel, M. Hammelmann, and K.-D. Entian.** 1992. Biosynthesis of the lantibiotic nisin: genomic organization and membrane localization of the NisB protein. Appl. Environ. Microbiol. **58**:3730–3743.
6. **Fremaux, C., and T. R. Klaenhammer.** Helveticin J, a large heat-labile bacteriocin from *Lactobacillus helveticus*. *In* L. De Vuyst (ed.), Bacteriocins of lactic acid bacteria: microbiology, genetics, and applications, in press. Elsevier Applied Science, London.
7. **Fremaux, C., and T. R. Klaenhammer.** Characterization of a mitomycin C-inducible promoter for a *Lactobacillus* bacteriocin operon. Submitted for publication.
8. **Fujisawa, T., Y. Benno, T. Yaeshima, and T. Mitsuoka.** 1992. Taxonomic study of the *Lactobacillus acidophilus* group, with recognition of *Lactobacillus gallinarum* sp. nov. and *Lactobacillus johnsonii* sp. nov. and synonymy of *Lactobacillus acidophilus* group A3 (Johnson et al. 1980) with the type strain of *Lactobacillus amylovorus* (Nakamura 1981). Int. J. Syst. Bacteriol. **42**:487–491.
9. **Gao, F. H., T. Abee, and W. N. Konings.** 1991. Mechanism of action of the peptide antibiotic nisin in liposomes and cytochrome *c* oxidase-containing proteoliposomes. Appl. Environ. Microbiol. **57**:2164–2170.
10. **Garnier, J., D. J. Osguthorpe, and B. Robson.** 1978. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. J. Mol. Biol. **120**:97–120.
11. **Geis, A., J. Singh, and M. Teuber.** 1983. Potential of lactic streptococci to produce bacteriocin. Appl. Environ. Microbiol. **45**:205–211.
12. **Graves, M. C., and J. C. Rabinowitz.** 1986. *In vivo* and *in vitro* transcription of the *Clostridium pasteurianum* ferredoxin gene. Evidence for "extended" promoter elements in gram-positive organisms. J. Biol. Chem. **261**:11409–11415.
13. **Hastings, J. W., M. Sailer, K. Johnson, K. L. Roy, J. C. Vederas, and M. E. Stiles.** 1991. Characterization of leucocin A UAL 187 and cloning of the bacteriocin gene from *Leuconostoc gelidum*. J. Bacteriol. **173**:7491–7500.
14. **Hawley, D. K., and W. R. McClure.** 1983. Compilation and analysis of *Escherichia coli* promoter DNA sequences. Nucleic Acids Res. **11**:2237–2255.
15. **Holck, A., L. Axelsson, S.-E. Birkeland, T. Aukrust, and H. Blom.** 1992. Purification and amino acid sequence of sakacin A, a bacteriocin from *Lactobacillus sake* Lb706. J. Gen. Microbiol. **138**:2715–2720.
16. **Holo, H., O. Nilssen, and I. F. Nes.** 1991. Lactococcin A, a new bacteriocin from *Lactococcus lactis* subsp. *cremoris*: isolation and characterization of the protein and its gene. J. Bacteriol. **173**:3879–3887.
17. **Joerger, M. C., and T. R. Klaenhammer.** 1990. Cloning, expression, and nucleotide sequence of the *Lactobacillus helveticus* 481 gene encoding the bacteriocin helveticin J. J. Bacteriol. **172**:6339–6347.
18. **Johnson, J. L., C. F. Phelps, C. S. Cummins, J. London, and F. Gasser.** 1980. Taxonomy of the *Lactobacillus acidophilus* group. Int. J. Syst. Bacteriol. **30**:53–68.
19. **Jung, G., and H.-G. Sahl.** 1991. Nisin and novel lantibiotics. ESCOM, Leiden, The Netherlands.
20. **Klaenhammer, T. R.** 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. **12**:39–86.
21. **Klaenhammer, T. R., C. Ahn, C. Fremaux, and K. Milton.** 1992. Molecular properties of *Lactobacillus* bacteriocins, p. 37–58. *In* R. James, C. Lazdunski, and F. Pattus (ed.), Bacteriocins, microcins and lantibiotics. Springer-Verlag, Berlin.

22. **Klaenhammer, T. R., C. Fremaux, C. Ahn, and K. Milton.** 1993. Molecular biology of bacteriocins produced by *Lactobacillus*, p. 151–180. *In* D. Hoover and L. Steenson (ed.), Bacteriocins of lactic acid bacteria. Academic Press, Inc., San Diego, Calif.
23. **Klein, P., M. Kanehisa, and C. DeLisi.** 1985. The detection and classification of membrane spanning proteins. Biochim. Biophys. Acta **815**:468–476.
24. **Koivula, T., M. Sibakov, and I. Palva.** 1991. Isolation and characterization of *Lactococcus lactis* subsp. *lactis* promoters. Appl. Environ. Microbiol. **57**:333–340.
25. **Kok, J., H. Holo, M. J. van Belkum, A. J. Haandrikman, and I. F. Nes.** 1993. Non-nisin bacteriocins in lactococci: biochemistry, genetics, and mode of action, p. 121–150. *In* D. Hoover and L. Steenson (ed.), Bacteriocins of lactic acid bacteria. Academic Press, Inc., San Diego, Calif.
26. **Kolter, R., and P. Moreno.** 1992. Genetics of ribosomally synthesized peptide antibiotics. Annu. Rev. Microbiol. **46**:141–163.
27. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157**:105–132.
28. **Lewus, C. B., A. Kaiser, and T. J. Montville.** 1991. Inhibition of food-borne bacterial pathogens by bacteriocins from lactic acid bacteria isolated from meat. Appl. Environ. Microbiol. **57**:1683–1688.
29. **Liu, W., and J. N. Hansen.** 1990. Some chemical and physical properties of nisin, a small-protein antibiotic produced by *Lactococcus lactis*. Appl. Environ. Microbiol. **56**:2551–2558.
30. **Marugg, J. D., C. F. Gonzalez, B. S. Kunka, A. M. Ledeboer, M. J. Pucci, M. Y. Toonen, S. A. Walker, L. C. M. Zoetmulder, and P. A. Vandenberg.** 1992. Cloning, expression, and nucleotide sequence of genes involved in production of pediocin PA-1, a bacteriocin from *Pediococcus acidilactici* PAC1.0. Appl. Environ. Microbiol. **58**:2360–2367.
31. **Mayr-Harting, A., A. J. Hedges, and R. C. W. Berkeley.** 1972. Methods for studying bacteriocins. Methods Microbiol. **7A**:315–422.
32. **Moran, C. P., N. Lang, S. F. G. Le Grice, G. Lee, M. Stephens, A. L. Sonenshein, J. Pero, and R. Losick.** 1982. Nucleotide sequences that signal the initiation of transcription and translation in *Bacillus subtilis*. Mol. Gen. Genet. **186**:339–346.
33. **Mortvedt, C. I., J. Nissen-Meyer, K. Sletten, and I. F. Nes.** 1991. Purification and amino acid sequence of lactocin S, a bacteriocin produced by *Lactobacillus sake* L45. Appl. Environ. Microbiol. **57**:1829–1834.
34. **Muriana, P. M., and T. R. Klaenhammer.** 1987. Conjugal transfer of plasmid-encoded determinants for bacteriocin production and immunity in *Lactobacillus acidophilus* 88. Appl. Environ. Microbiol. **53**:553–560.
35. **Muriana, P. M., and T. R. Klaenhammer.** 1991. Cloning, phenotypic expression, and DNA sequence of the gene for lactacin F, an antimicrobial peptide produced by *Lactobacillus* spp. J. Bacteriol. **173**:1779–1788.
36. **Nissen-Meyer, J., H. Holo, L. S. Harvarstein, K. Sletten, and I. F. Nes.** 1992. A novel lactococcal bacteriocin whose activity depends on the complementary action of two peptides. J. Bacteriol. **174**:5686–5692.
37. **O'Sullivan, D. J., and T. R. Klaenhammer.** High- and low-copy-number *Lactococcus* shuttle cloning vectors with features for clone screening. Gene, in press.
38. **Piard, J.-C., O. P. Kuipers, H. S. Rollema, M. J. Desmazeaud, and W. M. de Vos.** 1993. Structure, organization, and expression of the *lct* gene for lacticin 481, a novel lantibiotic produced by *Lactococcus lactis*, p. 85–116. *In* J.-C. Piard. La lacticine 481, une nouvelle bacteriocine de type lantibiotique proderite par *Lactococcus lactis*: caracterisation biochemique et genetique. Ph.D. thesis. Université de Caen, Caen, France.
39. **Raya, R. R., C. Fremaux, G. L. De Antoni, and T. R. Klaenhammer.** 1992. Site-specific integration of the temperate bacteriophage φadh into the *Lactobacillus gasseri* chromosome and molecular characterization of the phage (*attP*) and bacterial (*attB*) attachment sites. J. Bacteriol. **174**:5584–5592.
40. **Rosenberg, M., and D. Court.** 1979. Regulatory sequences involved in the promotion and termination of RNA transcription. Annu. Rev. Genet. **13**:319–353.
41. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
42. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
43. **Stoddart, G. W., J. P., Petzel, M. J. van Belkum, J. Kok, and L. L. McKay.** 1992. Molecular analyses of the lactococcin A gene cluster from *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* WM4. Appl. Environ. Microbiol. **58**:1952–1961.
44. **Tagg, J. R., A. S. Dajani, and L. W. Wannamaker.** 1976. Bacteriocins of gram-positive bacteria. Bacteriol. Rev. **40**:722–756.
45. **van Belkum, M. J., B. J. Hayema, R. E. Jeeninga, J. Kok, and G. Venema.** 1991. Organization and nucleotide sequences of two lactococcal bacteriocin operons. Appl. Environ. Microbiol. **57**:492–498.
46. **van Belkum, M. J., J. Kok, and G. Venema.** 1992. Cloning, sequencing, and expression in *Escherichia coli* of *lcnB*, a third bacteriocin determinant from the lactococcal bacteriocin plasmid p9B4-6. Appl. Environ. Microbiol. **58**:572–577.
47. **van der Vossen, J. M. B. M., D. van der Lelie, and G. Venema.** 1987. Isolation and characterization of *Streptococcus cremoris* Wg2-specific promoters. Appl. Environ. Microbiol. **53**:2452–2457.
48. **Venema, K., T. Abee, A. J. Haandrikman, K. J. Leenhouts, J. Kok, W. N. Konings, and G. Venema.** 1993. Mode of action of lactococcin B, a thiol-activated bacteriocin from *Lactococcus lactis*. Appl. Environ. Microbiol. **59**:1041–1048.
49. **von Heijne, G.** 1983. Patterns of amino acids near signal sequence cleavage sites. Eur. J. Biochem. **133**:17–21.
50. **von Heijne, G.** 1986. A new method for predicting signal sequence cleavage sites. Nucleic Acids Res. **14**:4683–4690.
51. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.