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Molecular Analyses of the Lactococcin A Gene Cluster from *Lactococcus lactis* subsp. *lactis* Biovar Diacetylactis WM4†

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The genes responsible for bacteriocin production and immunity in *Lactococcus lactis* subsp. *lactis* biovar diacetylactis WM4 were localized and characterized by DNA restriction fragment deletion, subcloning, and nucleotide sequence analysis. The nucleotide sequence of a 5.6-kb *Ava*II restriction fragment revealed a cluster with five complete open reading frames (ORFs) in the same orientation. DNA and protein homology analyses, combined with deletion and Tn5 insertion mutagenesis, implicated four of the ORFs in the production of and immunity to lactococcin A. The last two ORFs in the cluster were the lactococcin A structural and immunity genes, *lcnA* and *lciA*. The two ORFs immediately upstream of *lcnA* and *lciA* were designated *lcnC* and *lcnD*, and the proteins that they encoded showed similarities to proteins of signal sequence-independent secretion systems. *lcnC* encodes a protein of 716 amino acids that could belong to the HlyB family of ATP-dependent membrane translocators. *LcnC* contains an ATP binding domain in a conserved C-terminal stretch of approximately 200 amino acids and three putative hydrophobic segments in the N terminus. The *lcnD* product, *LcnD*, of 474 amino acids, is essential for lactococcin A expression and shows structural similarities to HlyD and its homologs. On the basis of these results, a secretion apparatus that is essential for the full expression of active lactococcin A is postulated.

The role of lactococci and other lactic acid bacteria in the production of fermented foods has created interest in their inhibitory properties (18, 28). The lactococci have long been recognized as producers of various bacteriocins (45, 54). These naturally produced, food-grade, inhibitory proteins provide potential for improved food safety (12, 25) in addition to that imparted by lactic acid production. Nisin, produced by some strains of *Lactococcus lactis* subsp. *lactis*, has been the predominant lactococcal bacteriocin reported in the literature (for reviews, see references 24 and 28). DNA sequence analysis of the nisin production (4, 15, 26) and resistance (17) genes provided essential information for the further study of nisin for increased use in fermented foods. Diplococcin (9), lactostrepcins (56), and other lactococcal bacteriocins (23, 50) have received less attention than nisin but have become the subject of increased research.

Recently, two bacteriocin and immunity gene systems from *Lactococcus lactis* subsp. *cremoris* 9B4 were cloned and sequenced by van Belkum et al. (50, 51). The nucleotide sequence analysis of the two DNA regions specifying these activities revealed several open reading frames (ORFs) involved in bacteriocin production and immunity. One region, specifying low antagonistic activity, possessed three ORFs. The first two were involved in bacteriocin production, whereas the third appeared to be responsible for bacteriocin

immunity. Sequence and mutation analyses of the second region, specifying higher antagonistic activity, revealed that one ORF (ORF-B1) was responsible for bacteriocin activity and that another (ORF-B2) was responsible for bacteriocin immunity. More recently, Holo et al. (23) described the isolation and characterization of a bacteriocin that they termed lactococcin A, produced by *L. lactis* subsp. *cremoris* LMG2130. The gene encoding lactococcin A (*lcnA*) was sequenced and found to be identical to the DNA sequence for the ORF-B1-produced bacteriocin from *L. lactis* subsp. *cremoris* 9B4 (51). The two reported lactococcin A-producing strains had different plasmid profiles (23). Holo et al. (23) transformed several lactococcal strains with *lcnA* and found that only the *L. lactis* subsp. *lactis* IL1403 transformants showed detectable bacteriocin production. The amount produced, however, was less than 5% that produced by the parental strain, LMG2130, even though all of the lactococcal strains tested acquired lactococcin A immunity after transformation with the *lcnA* operon. As suggested by Holo et al. (23), these results indicated that the *lcnA* operon was transcribed and implied that additional factors were required for the expression of functional lactococcin A. Posttranslational processing was also suggested by van Belkum et al. (51) for the bacteriocins produced by *L. lactis* subsp. *cremoris* 9B4.

The production of a bacteriocin by *Lactococcus lactis* subsp. *lactis* biovar diacetylactis WM4 was linked to a 131-kb plasmid, pNP2. The gene(s) for the WM4 bacteriocin was isolated on a recombinant plasmid that contained three *Bcl*I fragments of pNP2 subcloned into pGB301 (21). The resultant plasmid, pKSH1, conferred the bacteriocin production (Bac⁺) phenotype when transformed into the Bac⁻ *L. lactis* subsp. *lactis* LM0230 host. Clones that independently contained the 9.5-, 13.4-, and 12.4-kb *Bcl*I restriction

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TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant phenotypes, properties, and resident plasmids ^a	Source or reference
Strains		
<i>E. coli</i>		
XL1-Blue	Ap ^s <i>recA1 lac supE44</i> F' [<i>lacI^q lacZΔM15, Tn10</i> (Tc ^r)]	5
JM109	Tc ^s Cm ^s <i>recA1 Δlac supE44</i>	40
MC1061	Tc ^s Cm ^s <i>ΔlacX74</i>	40
LLM16-1	JM109 transformed with pLLM16-1, encoding Cm ^r Tc ^r	This study
LLM17	XL1-Blue transformed with pLLM17, encoding Ap ^r	This study
LLM20	JM109 transformed with pLLM20, encoding Cm ^r Tc ^s	This study
LLM23	MC1061 transformed with pLLM23, encoding Cm ^r Tc ^s	This study
<i>L. lactis</i> subsp. <i>lactis</i>		
LM0230	Lac ⁻ LcnA ⁻ LciA ⁻ plasmid-free derivative of <i>L. lactis</i> subsp. <i>lactis</i> C2	36
IL1403	Lac ⁻ LcnA ⁻ LciA ⁻ plasmid-free derivative of <i>L. lactis</i> subsp. <i>lactis</i> IL594	23, 49
LLM24-1	<i>L. lactis</i> subsp. <i>lactis</i> IL1403 transformed with pLLM24, encoding LcnA ⁺ LciA ⁺ Em ^r	This study
<i>L. lactis</i> subsp. <i>lactis</i> LM0230 trans-formants		
JK301	pGB301, encoding Em ^r	31
KSH1	pKSH1, encoding LcnA ⁺ LciA ⁺ Em ^r	21
LLM12	pLLM12, encoding LcnA ⁻ LciA ⁺ Em ^r	This study
LLM13	pLLM13, encoding LcnA ⁻ LciA ⁻ Em ^r	This study
LLM14	pLLM14, encoding LcnA ⁻ LciA ⁻ Em ^r	This study
LLM15	pLLM15, encoding LcnA ⁻ LciA ⁺ Em ^r	This study
LLM16	pLLM16, encoding LcnA ⁻ LciA ⁺ Em ^r	This study
LLM21	pLLM21, encoding LcnA ⁺ LciA ⁺ Em ^r	This study
LLM22	pLLM22, encoding LcnA ⁺ LciA ⁺ Em ^r	This study
LLM24	pLLM24, encoding LcnA ⁻ LciA ⁺ Em ^r	This study
Plasmids		
pBluescript II	Ap ^r <i>lacZα</i> ⁺	Stratagene
pACYC184	Cm ^r Tc ^r	6
pGB301	Em ^r Cm ^r	2
pSA3	Em ^r Cm ^r Tc ^r shuttle vector	8
pIL253	Em ^r	42
pKSH1	pGB301 carrying 9.5-, 13.4-, and 12.1-kb <i>BclI</i> pNP2 fragments	21
pLLM12	pGB301 carrying 9.5- and 3.3-kb <i>BclI</i> fragments of pNP2	This study
pLLM13	pGB301 carrying the 13.4-kb <i>BclI</i> fragment of pNP2	This study
pLLM14	pGB301 carrying the 12.1-kb <i>BclI</i> fragment of pNP2	This study
pLLM15	<i>EcoRI</i> deletion derivative of pLLM12	This study
pLLM16	pSA3 carrying the 5.5-kb <i>BclI-EcoRI</i> fragment of pLLM12	This study
pLLM16-1	<i>AvaI</i> deletion derivative of pLLM16 that eliminated the pGB305 moiety of pSA3	This study
pLLM17	pBluescript II carrying the 0.75-kb <i>BclI-EcoRV</i> fragment of pLLM15	This study
pLLM20	pACYC184 carrying the 1.9-kb <i>EcoRV</i> fragment of pKSH1	This study
pLLM21	<i>EcoRV</i> partial deletion derivative of pKSH1	This study
pLLM22	pIL253 carrying the 5.6-kb <i>AvaII</i> fragment of pLLM21	This study
pLLM23	pACYC184 carrying pLLM22	This study
pLLM24	<i>EcoRV</i> deletion derivative of pKSH1	This study

^a Ap^r, Em^r, and Tc^r, resistant to ampicillin, erythromycin, and tetracycline, respectively; Ap^s and Tc^s, sensitive to ampicillin and tetracycline, respectively.

fragments of pKSH1 (designated in this study pLLM12, pLLM13, and pLLM14, respectively) were each shown to be Bac⁻ by Harmon and McKay (21). The present research reports the further isolation, nucleotide sequence analysis, and molecular characterization of the WM4 bacteriocin production and immunity system. The results indicate the presence of a gene cluster comprising four ORFs involved in bacteriocin production and immunity. The last two ORFs in the cluster were found to be identical to the lactococcin A production and immunity genes (23, 51). By protein homology studies, the products of the other two ORFs were implicated in lactococcin A secretion and, probably, processing.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are described in Table 1. Parental strains were obtained from our frozen stock culture collection. *L. lactis* subsp. *lactis* strains were propagated in M17 medium (46) supplemented with 0.5% glucose (M17-G) and incubated at 32°C. *Escherichia coli* cultures were propagated in Luria-Bertani medium (40) and incubated at 37°C. Agar plates and soft agar overlays were made by adding 1.5 and 0.7% agar (Difco, Detroit, Mich.), respectively, to broth media. Antibiotics were added to media for the selection of appropriate plasmids and vectors as follows:

erythromycin, 15 µg/ml in broth and 5 µg/ml in plates; chloramphenicol, 35 µg/ml; tetracycline, 12.5 µg/ml; kanamycin, 20 µg/ml; and ampicillin, 50 µg/ml. Isopropyl-β-D-thiogalactopyranoside and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Life Technologies, Inc., Grand Island, N.Y.) were added to obtain final levels in Luria-Bertani plates of 0.1 mM and 40 µg/ml, respectively, for blue or white colony selection of pBluescript II transformants of *E. coli* XL1-Blue.

Lactococcin A production and immunity screening. Screening for the lactococcin A production (LcnA⁺) phenotype was conducted as described by Kekesy and Piquet (27) by use of seeded soft agar overlays and culture smears without soft agar. Screening for the lactococcin A immunity (LciA⁺) phenotype was conducted by use of a modification of the method of Davies and Reeves (10). *L. lactis* subsp. *lactis* KSH1 was streaked across the surface of M17-G agar in a standard petri plate or in a Lutri plate (Lutri Plate, Inc., Starkville, Miss.). Plates were incubated at 32°C for 16 to 20 h. With a sterile spatula, the agar from the standard petri plate was flipped into the lid to expose the bottom of the agar disk. The bottom of the agar disk was exposed in the Lutri plate by removing the pouring platform. Strains to be tested for the LciA⁺ phenotype were cross-streaked on the newly exposed surface and incubated at 32°C for 20 to 24 h.

Plasmid DNA isolation, analysis, and molecular manipulation. *L. lactis* subsp. *lactis* plasmids were isolated by the lysis method of Anderson and McKay (1) and purified by CsCl-ethidium bromide buoyant density gradient centrifugation (40). *E. coli* plasmids were isolated for restriction enzyme analysis by the boiling lysis method (22, 40). Plasmid DNA for sequencing was isolated from *E. coli* by an alkaline lysis procedure (39) and further purified by CsCl-ethidium bromide buoyant density gradient centrifugation (40). Restriction enzyme digestions were performed in accordance with the supplier's instructions (Life Technologies). Agarose gel electrophoresis was conducted with Tris-acetate-EDTA buffer (pH 8.0) at 4 V/cm (40), and gels were stained with ethidium bromide (0.5 µg/ml). Ligations were conducted with T4 DNA ligase (Life Technologies) at 13°C. Isolated restriction fragments for subcloning were obtained from agarose gels with GeneClean (Bio 101, Inc., La Jolla, Calif.). Transformation of *L. lactis* subsp. *lactis* LM0230 was performed by protoplast transformation (31) and electroporation (35). Electroporation was modified such that frozen cell pellets of *L. lactis* subsp. *lactis* LM0230, obtained from 400 µl of harvested, washed, and concentrated cells, were resuspended in 90 µl of distilled deionized H₂O, and 50 µl of the resuspended cells was added to 1 µg of plasmid DNA (resuspended in ≤5 µl of distilled deionized H₂O) and mixed by drawing the mixture up and down with the pipettor. The mixture was added to the electroporation cuvette, and the pulse was applied (25-µF capacitance, 200-Ω parallel resistance, and a field strength of 16 kV/cm [0.1-cm cuvette] or 12.5 kV/cm [0.2-cm cuvette]) with a Gene Pulser Apparatus (Bio-Rad Laboratories, Richmond, Calif.). The pulsed mixture was immediately diluted with 750 µl of M17-G broth, transferred to a 1.5-ml microcentrifuge tube, incubated at 32°C for 2 h, and then plated onto M17-G selective medium. Electroporation procedure modifications improved transformation efficiency from 10⁴, as reported by McIntyre and Harlander (35), to 10⁵ transformants per µg of DNA. Transformation and electroporation of *E. coli* were performed as described by Hanahan (20) and Dower (16), respectively.

DNA sequencing and sequence analysis. DNA sequencing was performed with three plasmids (pLLM16-1 [same frag-

ment as in pLLM16], pLLM17, pLLM20, and pLLM23; Fig. 1B) whose cloned restriction fragments encompassed the regions of pLLM12 and pLLM13 involved in the LcnA⁺ and LciA⁺ phenotypes (Fig. 1A). Fragments were sequenced by the dideoxy chain termination method (41) with the Sequenase version 2.0 DNA sequencing kit (United States Biochemical, Cleveland, Ohio) as recommended by the manufacturer. DNA was labeled with [³⁵S]dATP (New England Nuclear Corp., Boston, Mass.). Plasmid DNA denaturation and primer annealing were performed as described for double-stranded DNA by Toneguzzo et al. (48) with a final EDTA concentration of 0.22 mM (55) and a primer/template molar ratio of 4:1. For sequencing, 3 to 4 µg of DNA per reaction was used for the pBluescript II clone (pLLM17) and 20 to 25 µg of DNA per reaction was used for pACYC184 clones (pLLM16-1, pLLM20, and pLLM23). Plasmid DNA was denatured at 85°C by incubation for 5 min for pLLM17 and 10 min for pLLM16-1, pLLM20, and pLLM23. Electrophoresis was conducted through 6% polyacrylamide-7 M urea gels in Tris-borate-EDTA buffer (pH 8.3) on a SequiGen nucleic acid sequencing system (Bio-Rad Laboratories). Sequencing gels were dried, and autoradiograms were developed on Kodak XAR-5 film exposed at 25 to 27°C. DNA sequencing was initiated on pLLM17 with the M13 “-20” and “Reverse” primers of the pBluescript II vector. Subsequent DNA sequences of both strands were determined with 15- to 18-mer oligonucleotide primers synthesized on a model 391 PCR-Mate DNA synthesizer (Applied Biosystems, Inc., Foster City, Calif.) in a primer-walk sequencing scheme. Sequence data were analyzed on a SUN minicomputer at the University of Minnesota Molecular Biology Computing Center facility by use of the IntelliGenetics suite of programs (release 6.01) and the PC/GENE program (release 5.35) (IntelliGenetics, Inc., Mountain View, Calif.). The SUN computer was used to search GenBank (release 66) and EMBL (release 25-66) DNA data bases and the Swiss-Prot (release 15) and NBRF-PIR (release 26) protein data bases for homologous DNA and protein sequences, respectively.

Tn5 mutagenesis. Transposon (Tn5) mutagenesis (11) was conducted to identify the genes responsible for the LcnA⁺ and LciA⁺ phenotypes. *E. coli* LLM23 was infected with λ::Tn5 (λ b221 rex::Tn5 c1857 Oam29 Pam80) at a 10:1 (phage particles to bacterial cells) multiplicity of infection. Chloramphenicol-resistant (Cm^r), kanamycin-resistant (Km^r) colonies were washed from the plates and lysed (39). The plasmid pool was electroporated into *E. coli* JM109 to select for Tn5 insertions into pLLM23. Plasmid DNA from Cm^r Km^r transformants was digested (*Bam*HI and *Bam*HI-*Kpn*I) and screened for Tn5 insertion into the *lcn* genes on the *Ava*II restriction fragment of pLLM23. Plasmids containing single Tn5 insertions into independent *lcn* genes were electroporated into *L. lactis* subsp. *lactis* LM0230, and transformants were screened for the LcnA⁺ and LciA⁺ phenotypes.

Nucleotide sequence accession number. The GenBank accession number for the DNA sequence of the lactococcin A production, immunity, and expression gene cluster is M90969.

RESULTS

Isolation of the bacteriocin production and immunity genetic loci of *L. lactis* subsp. *lactis* biovar diacetylactis WM4. *L. lactis* subsp. *lactis* LLM12, LLM13, and LLM14 were screened to determine whether the fragments cloned in pLLM12, pLLM13, and pLLM14, respectively (Fig. 1A), conferred an immunity (Imm⁺) phenotype to the bacteriocin produced by *L. lactis* subsp. *lactis* biovar diacetylactis WM4

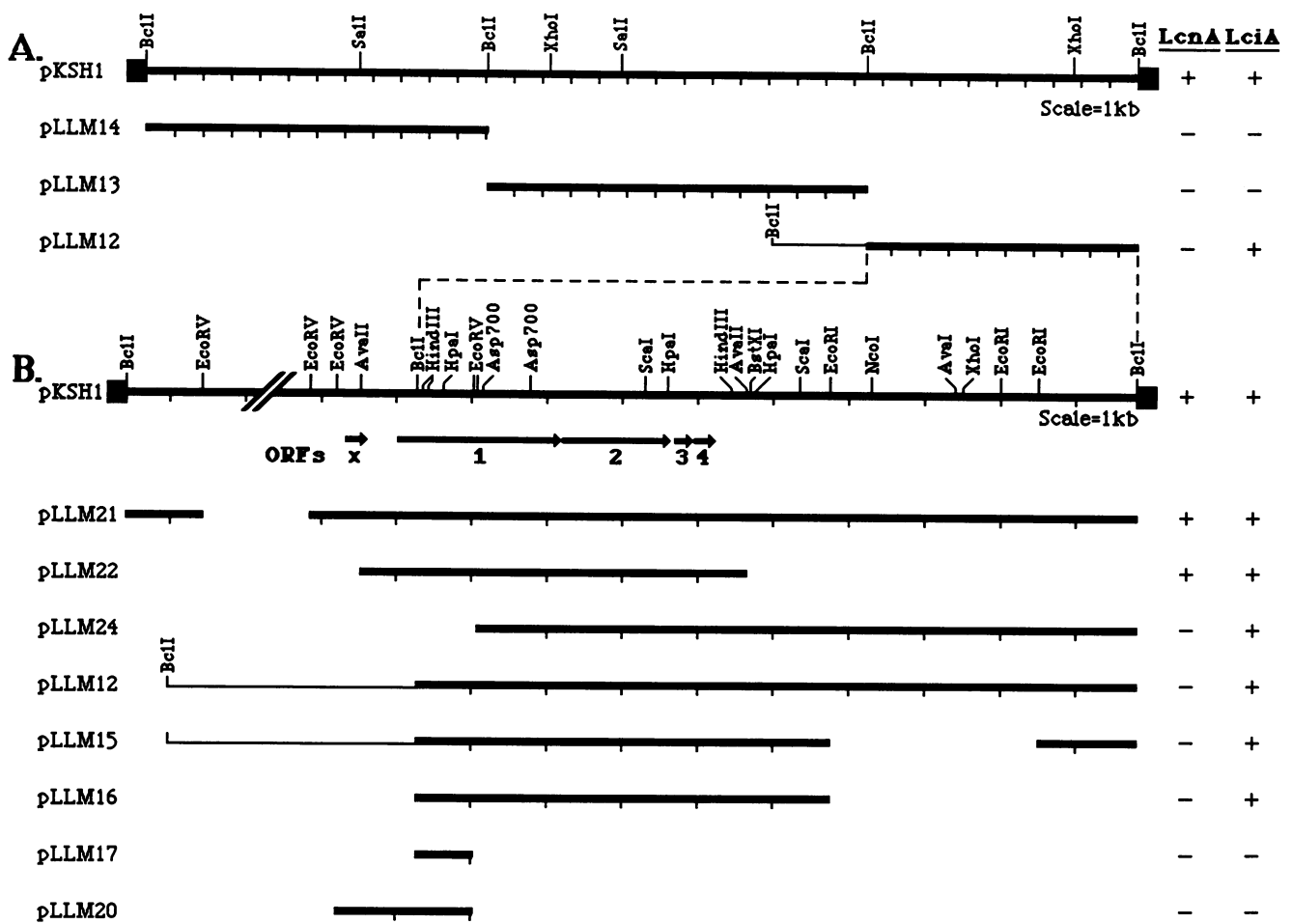


FIG. 1. Linear restriction enzyme maps of pKSH1 (adapted from reference 21), deletion derivatives, and subclones. (A) pKSH1 (LcnA⁺ LciA⁺), pLLM14 (LcnA⁻ LciA⁻), pLLM13 (LcnA⁻ LciA⁻), and pLLM12 (LcnA⁻ LciA⁻) in *L. lactis* subsp. *lactis* LM0230. (B) Expanded restriction map of pKSH1 (the gap between the double slash represents 21.7 kb), with the locations of ORFx and ORF1 through ORF4 shown for reference (see Fig. 2 and 5). The deleted or subcloned pKSH1 restriction fragments in pLLM21, pLLM22, and pLLM24 were used to localize the LcnA⁺ and LciA⁺ phenotypes as expressed in *L. lactis* subsp. *lactis* LM0230. Deleted or subcloned restriction fragments in pLLM12, pLLM15, and pLLM16 were used to isolate the LciA⁺ phenotype. Subcloned restriction fragments in pLLM17 and pLLM20 (LcnA⁻ LciA⁻) were used for dideoxy DNA sequencing. Dark boxes on the pKSH1 map represent vector DNA.

even though all three strains were reported to be Bac⁻ by Harmon and McKay (21). *L. lactis* subsp. *lactis* LLM12 demonstrated an Imm⁺ phenotype, while *L. lactis* subsp. *lactis* LLM13 and LLM14 were Imm⁻. Thus, the 9.5-kb *BclI* cloned restriction fragment of pLLM12 carried a functional Imm⁺ locus (loci), while bacteriocin activity was not detected. Further restriction endonuclease analyses of pKSH1 and pLLM12 were performed to develop a more complete restriction map of regions involved with bacteriocin production and immunity. The 3.3-kb *BclI* restriction fragment of pLLM12 (Fig. 1A and B, thin line) was an extraneous pNP2 restriction fragment that was coligated into pGB301 with the 9.5-kb *BclI* restriction fragment during subcloning. Localization of bacteriocin production and immunity genetic loci was achieved through restriction fragment deletions and subclones. The genes encoding the Bac⁺ and Imm⁺ phenotypes were isolated on 11.2-kb *EcoRV-BclI* (pLLM21) and 5.6-kb *AvaII* (pLLM22) restriction fragments of pKSH1 (Fig. 1B). Efforts to further localize the bacteriocin production and immunity genes together on the 3.8-kb *AvaII-ScaI* restriction fragment from pLLM22 were unsuccessful (data not

shown). The gene(s) encoding the Imm⁺ phenotype was localized on the 5.5-kb *BclI-EcoRI* (pLLM15 and pLLM16) restriction fragment of pLLM12 (Fig. 1B).

DNA sequence analysis. The complete nucleotide sequence of the inserts in pLLM17 and pLLM20 was determined (Fig. 1B), in addition to 3,324 bp downstream of the *EcoRV* site in the pLLM16-1 and pLLM23 inserts. A total of 5,186 bp was sequenced (Fig. 2). Both strands of the DNA sequence were analyzed for ORFs; five ORFs (≥150 bp) were found on one strand. These ORFs were designated ORFx and ORF1 through ORF4 (Fig. 1B and 2). Potential promoters (-10 and -35 sequences) were located upstream of ORFx, ORF1, and ORF3. Putative ribosome binding sites were located upstream of all five ORFs. ORFx was not involved in bacteriocin production or immunity, as the subcloned 5.6-kb *AvaII* restriction fragment of pLLM22 (Bac⁺ Imm⁺) deleted the 5' end of this ORF. ORF1 and ORF2 were probably involved in bacteriocin activity, as the junction *BclI* restriction site of the contiguous 9.5- and 13.4-kb *BclI* restriction fragments of pKSH1 was located within ORF1 and deletion of DNA upstream of the junction *BclI* restriction site (as in pLLM12,

<p>1 <u>EcoRV</u> GATATTCCTATAAATCTCACTTTTCAAATATTATTATATAACGTGATATGTAGAAGGTCAGAT 60</p> <p>61 ATGTTGAGCAATTAAGAAAGAAATCATTTGAATTAATACTAGAGCTAGAACAATGGCATA 120 -35</p> <p>121 GAAATATTATATATTAGAGAGGTAATAAAGAGGAAATAACAGGAAGCTCATTACAAAGC 180 -10 RBS METArgGlyIleThrGlySerSerLeuGlnSer ORF x ==></p> <p>181 CGAAGCTGTATACAAAAAGCCAAAAAGAAAGATTGTGAAGATTGTGACTCTGAATTT 240 ArgThrValTyrThrLysLysProLysLysLysIleCysLysAsnCysAspSerGluPhe</p> <p>241 TGGGCTAAGCAAAATACACTGAAGGCTACCAAGCACTATTGTGGTTTTGTCGGATGGTG 300 TrpAlaLysAlaLysTyrThrGluGlyTyrGlnAspTyrCysGlyPheCysArgMETVal</p> <p>301 TATCGTCAGATAAAAAGAAGATTATAAAAAAAGTAAAGTTAAAGGACCACTCTATAT 360 TyrArgGlnAspLysArgArgIleIleLysLysSerLysValLysGlyProThrLeuTyr</p> <p>361 AATCCAAAGTATTTCTAAGAGAAAGCCCAAAATAGTAAAATAAGATATTTTTCATAAG 420 AsnProLysValPheSerLysArgLysProLys *</p> <p>421 TTTCCGAAAATTAGCTTTTCAATAAATCTAGTTTAACTTTTCTTTGGATAAAAAA 480 -> <-></p> <p>481 GCGATAATGAGTCAGCTATTGTCGCTTTTACTATTTTAAATCTTATGGTGAAGTGT 540</p> <p>541 CTAATTTAATCTCGATTATAGGCTGTGATATAAATAACCAAGGCTTTTTGGCAT 600</p> <p>601 CTATACTACTAATTTTAAATAAAGAGGCAAGTAAGTATATTATTTTCACTTATCTAG 660</p> <p>661 GTTAAACTACTAAGCTTATTTTCTCACTATCTTTTCTCGATTTTAAACTAAAAT 720</p> <p>721 AGATTTTATGAAAATCTCTGTAACGCAACCGCAAAATATTTATGCTCCAGTATTTA 780 -35 -10</p> <p>781 CTAATAATTTAACTCAGAAAGTAAAGAAAGATGAAATTTAAAAGAAAATTTATACCTCA 841 RBS METLysPheLysLysLysAsnTyrThrSer ORF 1 (lcnC) ==></p> <p>842 CAAGTAGATGAAATGGAGCTGGCTGTGCTGCCTATCAATGATTTTAAAGTCTTATGGC 901 GlnValAspGluMETAspCysGlyCysAlaAlaLeuSerMETIleLeuLysSerTyrGly</p> <p>902 ACAGAAAATCTCGCTTCTATTGCGCTTACTTGCAGGTACAAACAATCGAAGGAACCTCC 961 ThrGluLysSerLeuAlaSerLeuArgLeuLeuAlaGlyThrThrIleGluGlyThrSer</p> <p>962 GCTTTAGGGATAAAAAGGCGACCTGAAATATTAGAGTTTTCAGTTCAGGCGCTAAGAAC 1021 AlaLeuGlyIleLysLysAlaAlaGluIleLeuGluPheSerValGlnAlaLeuArgThr</p> <p>1022 GATGCAAGCCTTTTGAATGAAAACGCTCCTTACCATTATTTGCTCATGTCTATAA 1081 AspAlaSerLeuPheGluMETLysAsnAlaProTyrProPheIleAlaHisValIleLys</p> <p>1082 GACCAAAAATACCCATTTATGTGATCACTGGCCGCAATAAAAAATTCGGTATTCATT 1141 AspGlnLysTyrProHisTyrValIleThrGlyAlaAsnLysAsnSerValPheIle</p> <p>1142 GCTGATCCTGACCAACCAATTAATAAGCAAAATATCAAAAGAGCTTTTATCGGAA 1201 AlaAspProAspProThrIleLysMETThrLysLysSerLysGluAlaPheLeuSerGlu</p> <p>1202 TGGACTGGGATTAGTTTATTTCTTTCGACTACACACTTATCATCCCACTAAAGAAAA 1261 TepThrGlyIleSerLeuPheLeuSerThrThrProSerTyrHisProThrLysLysGluLys</p> <p>1262 GCTTCCTCATTTATCTTTTATCCCAATATCAACCGTCAAAGAAGTCACTTCTCAAT 1321 AlaSerSerLeuLeuSerPheIleProIleIleThrArgGlnLysLysValIleLeuAsn</p> <p>1322 ATTGTTATGGCTCATTCATTGTAACACTGATTAATATCTAGGCTCTTACTACCTCCAG 1381 IleValIleAlaSerPheIleValThrLeuIleAsnIleLeuGlySerTyrLeuGln</p>	<p>1382 AGCATGATTGACAGCTATATTCCAAATGCCTTAATGGAACCTTTAGGGATTATCTCAGTA 1441 SerMETIleAspSerTyrIleProAsnAlaLeuMETGlyThrLeuGlyIleIleSerVal</p> <p>1442 GGGCTATGTTAACCTATATTTCAACAGGCTCTTAGAATTTGCTAAGGCTTCTTATTTG 1501 GlyLeuLeuLeuThrTyrIleIleGlnGlnValLeuGluPheAlaLysAlaPheLeuLeu</p> <p>1502 AACGTCTTCTCAAAGATTAGCTATTGATGTCATTCTTCTTATATAGACACATTTTC 1561 AsnValLeuSerGlnArgLeuAlaIleAspValIleLeuSerTyrIleArgHisIlePhe</p> <p>1562 CAACTCCCATGCTCTTTCTTTCCAGCCGAGAACAGGAGAATACCAGTCGGTTTTCC 1621 GlnLeuProMETSerPhePheSerThrArgArgThrGlyGluIleThrSerArgPheSer</p> <p>1622 GATCGAGTCTATTTTAGATGCTATTGCTCAACGATCTTTCGCTCTTTTAGATTG 1681 AspAlaSerSerIleLeuAspAlaIleAlaSerThrIleLeuSerLeuPheLeuAspLeu</p> <p>1682 ACGATTGTCGTCATGACAGGACTAATTTAGGCCCTCAAATATGCACTCTTCTTCTCT 1741 ThrIleValValMETThrGlyLeuIleLeuGlnAsnMETGlnLeuPheLeuLeu</p> <p>1742 GTTCTTTGGCAATCCCACTCTATATTTGTTTATTTATTTTACGCCCTTTTGGAA 1801 ValLeuLeuAlaIleProLeuTyrIleValValIleIleIlePheThrProLeuPheGlu</p> <p>1802 AAACAAAACCATGAAGTCATGCAGCAACGCTTAAACTCCTCAATTTATGAGAT 1861 LysGlnAsnHisGluValMETGlnThrAsnAlaValLeuAsnSerSerIleIleGluAsp</p> <p>1862 ATCAATGGGATGAAACTATAAAGCACTTGGCAGTGAACAAGAAGATATCAAAAAAT 1921 IleAsnGlyIleGluThrIleLysAlaLeuAlaSerGluGlnGluArgTyrGlnLysIle</p> <p>1922 GACTACGAATTTGCAAGTTATCTAAAAGGCTTTCACCTTACAAAAATCAGAGACTT 1981 AspTyrGluPheAlaSerTyrLeuLysLysAlaPheThrLeuGlnLysSerGluAlaIle</p> <p>1982 CAAGGCTTAATTAAGCAATTACAACCTAACATTGAGTGCACCACTTTTATGGTTGGT 2041 GlnGlyLeuIleLysAlaIleIleGlnLeuThrLeuSerValThrIleThrTrpPheGly</p> <p>2042 GCCACATAGTAATAAGTCAAAAAATTCAGCTCGGACAATGATTTACTTTAATGCCCTG 2101 AlaThrLeuValIleSerGlnLysIleThrLeuGlyGlnLeuIleThrPheAsnAlaLeu</p> <p>2102 CTTCTTACTTTACAATCCAATTACCAATATCATTAACTTCAACAAAACACTACAAAG 2161 LeuSerTyrPheThrAsnProIleThrAsnIleIleAsnLeuGlnThrLysGlnLys</p> <p>2162 GCAAGGTAGCCAATGAACGATTAATGAGGCTCTATCTGTACCAGTGAATTTGAGGAA 2221 AlaArgValAlaAsnGluArgLeuAsnGluValTyrLeuValProSerGluPheGluGlu</p> <p>2222 AAGAAAACAGAACTGCCCTCACATTTTAACTTAACATGCCGATTTTCAATACAA 2281 LysLysThrGluLeuSerLeuSerHisPheAsnLeuAsnMETSerAspIleSerTyrGln</p> <p>2282 TATGGTTTGGCAGAAAAGCTTATCTGAGATAGAATCTCTTATAAAGAAAATGAAAA 2341 TyrGlyPheGlyArgLysValLeuSerGluIleGluLeuSerIleLysGluAsnGluLys</p> <p>2342 TTGACTTGTGGGCATGAGTGGTTCAGGAAGATCACTCTGTTAAATTTTGGTCAAC 2401 LeuThrIleValGlyMETSerGlySerGlyLysSerThrLeuValLysLeuAlaAsn</p> <p>2402 TCTTCTCAACCCACTTCTGGCACCATCCTTAGTGGAAATGACCTGCACAGTTTGGAT 2461 PhePheGlnProThrSerGlyThrIleThrLeuGlyGlyIleAspLeuGlnGlnPheAsp</p> <p>2462 AAACCCAACCTCGAAGATTAATCAACTATCTCCCAACACCTTATTTTTACTGGT 2521 LysHisGlnLeuArgArgLeuIleAsnTyrLeuProGlnGlnProTyrIlePheThrGly</p> <p>2522 TCAATTTAGATAACTACTTCTAGGAGCTAATGAGAATGCATCACAAGAAGAGATCTT 2581 SerIleLeuAspAsnLeuLeuLeuGlyAlaAsnGluAsnAlaSerGlnGluGluIleLeu</p> <p>2582 AAAGCGTGGAAATGGCAGAAATCCGTGCAGATATTGAACAAATGCAGTTGGGCTATCAG 2641 LysAlaValGluLeuAlaGluIleArgAlaAspIleGluGlnMETGlnLeuGlyTyrGln</p>
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FIG. 2. Nucleotide sequence encompassing the lactococci A gene cluster and deduced amino acid sequences of five ORFs. The gene names (*lcnC*, *lcnD*, *lcnA*, and *lciA* [22; this study]) are given within parentheses. Predicted -10 and -35 promoter sites and potential ribosome binding sites (RBS) are indicated. The lactococci A N-terminal processing site (23) is indicated by the single vertical arrow under the primary protein sequence. The transcription start site for the *lcnA* and *lciA* mRNAs (51) is shown by the double vertical arrow below the DNA sequence upstream of the *lcnA* start codon. Translation termination codons are labeled by asterisks. Horizontal solid arrows indicate regions of dyad symmetry.

pLLM15, and pLLM16) resulted in a Bac⁻ phenotype (21). The putative promoter for ORF1 and ORF2 would be removed in these cloned derivatives.

Computerized data base searches comparing the sequences of ORF1 through ORF4 with all sequences in the GenBank (release 66) and EMBL (release 23-66) data bases did not reveal significant homologies to any data base-listed DNA sequences. However, 100% homology of ORF3 and ORF4 to the lactococci A production and immunity genes (*lcnA* and *lciA*), respectively, was noted by comparison with their published nucleotide sequences reported by van Belkum et al. (51) and Holo et al. (23). Furthermore, the partial sequence of the ORF1 reported by Holo et al. (23) and van Belkum et al. (51) was homologous to the 3' portion of our ORF2 sequence (Fig. 1B and 2). ORF2 contained 474 codons and could encode a protein of 52.5 kDa. Another ORF (ORF1) that contained 716 codons and had the capacity to encode a protein of 79.9 kDa was located immediately upstream of the ORF2 (Fig. 1B and 2).

Two regions of dyad symmetry located immediately downstream of the *lciA* stop codon (indicated by horizontal solid arrows in Fig. 2) have calculated free energies of -24.9 and -10.2 kcal/mol (ca. -104.2 and -42.7 kJ/mol, respec-

tively) (47). Another region of dyad symmetry (calculated free energy, -13.5 kcal/mol [ca. -56.5 kJ/mol]) was located 70 bp downstream (nucleotides 467 to 519) from the stop codon of ORF2 (Fig. 2). These palindrome types are characteristic of rho-independent transcription termination structures, and the ones identified in this study are therefore putative transcription terminators.

Similarity of the ORF-1-encoded protein to ATP-dependent translocators. A protein homology search revealed that the deduced proteins encoded by ORF1 and ORF2 share significant similarity with proteins that form secretion systems in various gram-negative bacteria for extracellular proteins whose secretion does not depend on the general signal peptide-dependent export pathway (37, 38). Such examples include the hemolysin and colicin V proteins of *E. coli*, cyclolysin of *Bordetella pertussis*, leucotoxin of *Pasteurella haemolytica*, and metalloproteases B and C of *Erwinia chrysanthemi* (13, 19, 33, 44, 53). These proteins lack a typical N-terminal signal peptide and are dependent on the products of at least two other genes for their externalization. Most or all of these secretion genes are closely linked genetically to the corresponding structural genes of the secreted proteins, and their products form a dedicated

LcnC-	MKF-KKKNYTSQVDE--MDCGCAALSM-----ILKSYGTEKSLASLRLLAGTITTEGTSAL	- 52
HlyB-	MDSCHKIDYGLYALEILAQYHNVSVPNEEIKHRFDTDGTLGLTSW-LLAAKSLELK-VK	- 58
LcnC-	GIKKAAEILEFSVQALRTDASLFEMKNAPYFFIAHVIKDKQYPHYVYITGANKNSVFIAD	-112
HlyB-	QVKKTIDRLNFIFL-----PALVWREDGRHFILTKISKEVN--RYLIFDLEQRNPRVLEQ	-111
LcnC-	PDPTIKMTKLSKEAFLSEWTLGSLFSLSTTPSYHPTKEKASSLLSFPIITRQKVVILNIV	-172
HlyB-	SEF-----EALYQG-----HIILITSRSSVTGKLAQKDFTFWIFALIIKYRRIFIETL	-158
LcnC-	IASFIVTLINILGSYYLQSMIDSYIPNALMGTGLGII SVGLLLTYITQVLEFAKAFLLNV	-232
HlyB-	VVSVFLQLFALITPLFQVVMVKLVHRGFTLNIVITVALSVVVFEIILSGLRTYIFAH	-218
LcnC-	LSQRLAIDVILSYIRHFQLPMSFFSTRRTGETISRFSDASSILDAAISTILSLFLDLTI	-292
HlyB-	STSRIDVELGAKLFRHLLALPISYFESRRVGDVTVARVRELDQIRNFLTQALTSVLDL--	-276
LcnC-	VVMTGLLILGLQ---NMQLFLLVLLAIPLYIVVIIIFTFPEKONHEVMQTNAVINSIIIE	-349
HlyB-	-LFLSIFFAVMWYSPKLTLLVILFSLPCYAAWSVFI SPILRRRLDDKFRNADNQSFIVE	-335
LcnC-	DINGIETIKALASEQERYQKIDYEFASYLKAFTLQKSEAI--QGLIKAIQLTSLVITL	-407
HlyB-	SVTAINTIKAMAVSPQMTNIDWKLAGYVAAGFKVTVLATIGQQGI--QLIQTKVMIINL	-393
LcnC-	WFGATLVISQKITLGLQITFNALLSYFTNPITNIINLQTKLQKARVANERLNEVYLVPE	-467
HlyB-	WLGALHVISGDLISGQLIAFNMLAGQIVAVPVRILAQIWNQDFQVGVSVTRIGDVLNSPTE	-453
LcnC-	FEEKTELSSLHFNINMSDISYQYGF-GRKVLSEIELSIKENEKLTIVGMSGSGKSTLVK	-526
HlyB-	SYHGKLTPEINGDITFRNIRFRYKPDSPVILDNINLSIKQGEVIGIVGRSGSGKSTLTK	-513
LcnC-	LLVNFQOPTSGTITLGGIDLQFQDKHQLRRLINYLDPQQYIFTGSIIDNLLGANENAS	-586
HlyB-	LIQRFYIPENGQVLIDGHDLADLPNWRQVGVVL-QDNVLLNRSIIDNISL-ANPQMS	-571
LcnC-	QBEILKAVELAEIRADIEQMLGYQTESSDASSLSFLQKQRIARALLSPAKILILDE	-646
HlyB-	VERVYAAKLAGAHDPISELREGYNTIVGEGAGLGGQRQRIARALVNNPKILIFDE	-631
LcnC-	ATSNLDMITEKKILKNLLPL--DKTIIFIAHRLSVAEMSHRIIVVDQGVIESGSHVDLL	-704
HlyB-	ATSALDYSESHVIMRNMHKICKGRVTVIIAHLRSLTVKNADRIIVMEKGRIVEQKHKELL	-691
LcnC-	AQ-NGFYEQLYH---N	-716
HlyB-	SEPELSYSYLYQLQSD	-707

FIG. 3. Homology between the deduced protein sequences of LcnC in the lactococcin A gene cluster and HlyB in the hemolysin operon. Numbers refer to amino acid residues in the respective proteins. The homology comparison demonstrated 28.7% identity (indicated by vertical lines) and 18.7% similarity (indicated by dots) between LcnC and HlyB. Homologies between LcnC and HlyB include both the N-terminal membrane-associated domain and the C-terminal cytoplasmic ATP binding domain of HlyB. The HlyB amino acid sequence was obtained from the Swiss-Prot data base.

for parental levels of expression of lactococcin A in *L. lactis* subsp. *lactis* LM0230. Analysis of the DNA sequence that encompassed the regions essential for lactococcin A production and immunity revealed the presence of five complete ORFs in the same orientation and preceded by ribosome binding sites. No significant ORFs with proper regulatory regions were found on the opposite DNA strand. The last two ORFs in the cluster were *lcnA* and *lciA*, respectively. ORF_x had no function in lactococcin A production or immunity, and the potential rho-independent transcription termination stem-loop downstream of ORF_x provided a 5' limit to regions responsible for transcription of the lactococcin A gene cluster. Upstream of the first gene in the cluster, *lcnC*, a putative promoter that could function in the expression of *lcnC* and *lcnD* was present. van Belkum et al. (51) showed that the *lcnA* and *lciA* genes can be transcribed independently of *lcnC* and *lcnD* from the promoter immediately upstream of *lcnA*. The start site of transcription was determined to be at an A residue 20 bases upstream of the

ATG start codon of *lcnA* (Fig. 2). As there is no obvious transcription termination structure between *lcnC* and *lcnD* and *lcnA* and *lciA*, the probability exists that another mRNA that encompasses all four *lcn* genes can be produced. Deletion of the promoter upstream of *lcnC*, as in pLLM12, produces an LcnA⁻ phenotype for reasons to be discussed below but does not lead to reduced levels of immunity. Apparently, the *lcnA* and *lciA* mRNAs are still produced to yield wild-type levels of immunity. The *BclI* restriction site at the junction of the contiguous 9.5- and 13.4-kb restriction fragments of pKSH1 is within *lcnC*. The region around this site has been implicated in bacteriocin production in *L. lactis* subsp. *lactis* biovar diacetylactis WM4 (21). Harmon and McKay (21) showed that these two *BclI* restriction fragments, when cloned separately, did not lead to detectable bacteriocin production. The current study reports that the clone containing the 9.5-kb *BclI* restriction fragment (*L. lactis* subsp. *lactis* LLM12) expresses lactococcin A immunity without any detectable lactococcin A activity, yet it still carries the intact lactococcin A structural gene with a functional promoter. These results can now be explained as follows. Tn5 insertions in *lcnD* revealed that this gene is essential for the LcnA⁺ phenotype. These *lcnD* Tn5 insertions resulted in an LcnA⁻ phenotype without the loss of immunity (the LciA⁺ phenotype). This observation suggests that there is no polar effect of these Tn5 insertions on the transcription of *lcnA* and *lciA* from the promoter immediately upstream of *lcnA*. The putative promoter for *lcnC* and *lcnD* is lacking on the 9.5-kb *BclI* restriction fragment in pLLM12, and cells carrying this plasmid have the same phenotype as those carrying *lcnD*::Tn5. Tn5 insertions in *lcnC* also resulted in an LcnA⁻ LciA⁺ phenotype. The current study cannot exclude the possibility that a polar effect of the *lcnC*::Tn5 insertions on *lcnD* is responsible for the LcnA⁻ phenotype. However, if we assume that there is no polar effect, the results suggest that *lcnC* is also essential for the production of active secreted lactococcin A.

A protein homology comparison of LcnC and LcnD revealed similarities to proteins implicated in the signal sequence-independent secretion of certain proteins produced by gram-negative bacteria. This similarity is most pronounced with LcnC. This protein is apparently a member of the HlyB-like family of ATP-dependent membrane translocators (3). As for all members of this family, the C terminus of LcnC contains the highly conserved ATP binding domain, and the general structure of the N terminus (three hydrophobic regions) is also conserved. The similarity of LcnD to other inner membrane proteins in these transport systems (HlyD and PrtE in the secretion apparatus of *E. coli* hemolysin A and *E. chrysanthemi* protease B, respectively [33, 53]) is less obvious (less than 20% identity for both comparisons and 13 and 19% similarities for HlyD and PrtE, respectively). However, the structures of the N termini of these proteins appear to be conserved, and while all three proteins are hydrophilic, their N termini are strongly hydrophobic. PrtE is attached to the inner membrane by its hydrophobic N terminus (14).

The outcome of these protein homology studies allows the following interpretation of the mutation and transposon insertion data. The loss of LcnD and, probably, LcnC results in blocking of the N-terminal processing and the secretion of lactococcin A into the external medium and an LcnA⁻ phenotype. Lactococcin A is probably still produced, as cells lacking LcnC and/or LcnD produce the immunity protein (the LciA⁺ phenotype). This probability is currently under further investigation. These studies will also reveal

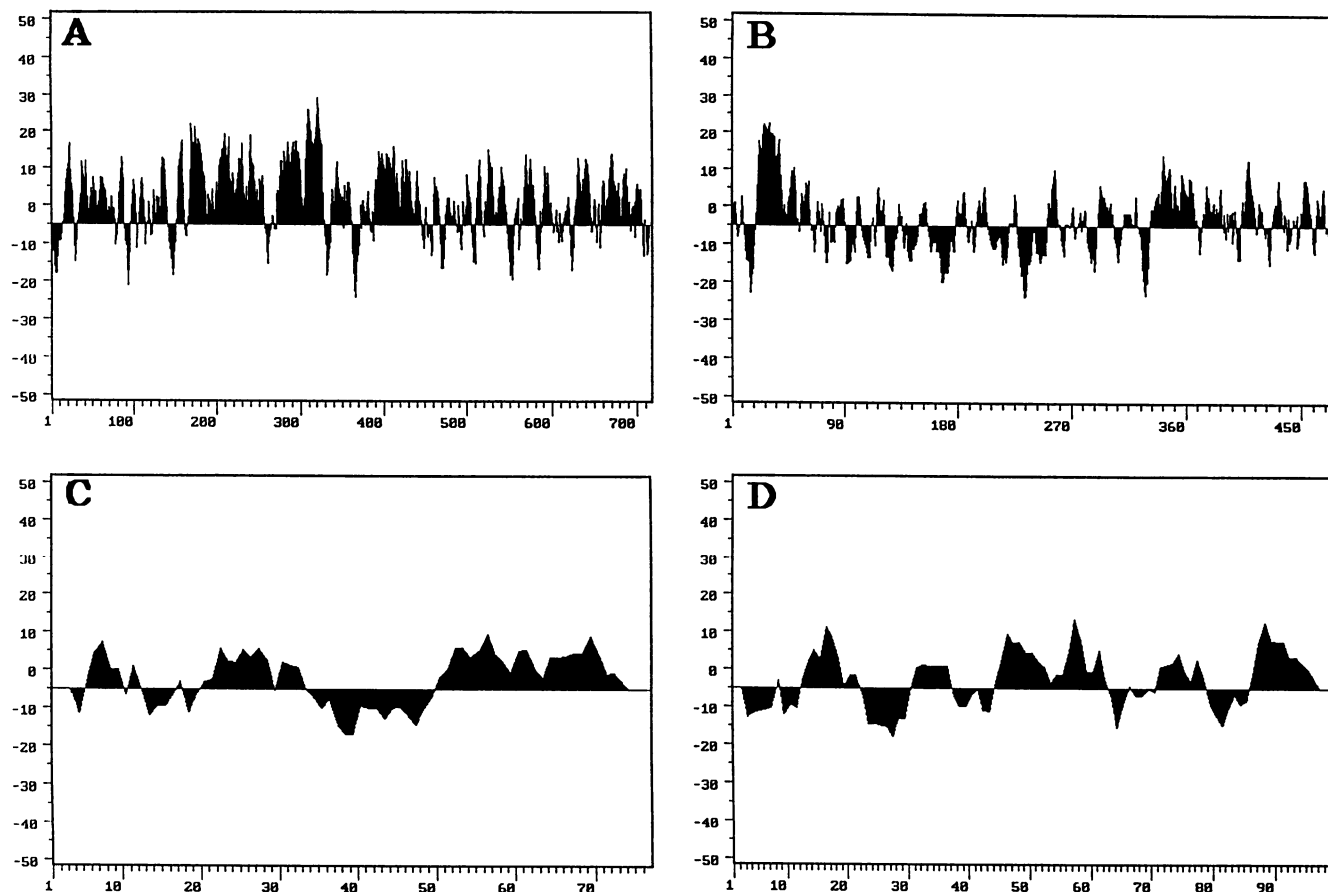


FIG. 4. Hydropathy plots of LcnC (A), LcnD (B), LcnA (C), and LciA (D). Hydropathy plots were calculated by the method of Kyte and Doolittle (32), with a calculation interval of 7 amino acid residues. *x* axis, amino acid residues; *y* axis, hydropathy index showing hydrophobicity above the baseline and hydrophilicity below the baseline. These deduced proteins of the lactococcin A gene cluster are involved in the expression of the LcnA⁺ and LciA⁺ phenotypes. (See the text for a description of hydropathy plot characteristics.)

whether the removal of the initial 21 amino acids from the N terminus of the lactococcin A precursor (23) is part of the secretion process. The possibility that lactococcin A translocation across a single membrane requires, apart from the ATP translocator, only one additional cytoplasmic membrane protein (LcnD) is an interesting aspect of the putative lactococcin A secretion machinery. In the examples of signal sequence-independent secretion in gram-negative bacteria, the proteins must cross two membranes. The two inner membrane proteins, HlyB and HlyD, and the minor outer membrane protein, TolC, are required for the secretion of hemolysin A by *E. coli* (53), while PrtD and PrtE are the inner membrane components and PrtF is the outer membrane component of the protease B secretion apparatus of *E. chrysanthemi* (33).

On the basis of the above discussion, it is clear that for wild-type expression (production, processing, and secretion) of lactococcin A, plasmid-encoded secretion and processing functions are essential. It is surprising that both Holo et al. (23) and van Belkum et al. (50–52) detected lactococcin A expression with strains carrying only the *lcnA* and *lciA* genes of the lactococcin A gene cluster. Both groups observed a slight to marked decrease in the halo diameters produced by their clones, compared with the halo produced by the wild-type strain. van Belkum et al. (51, 52) excluded the possibility that this result was caused by copy number

differences and/or the need for additional plasmid-linked bacteriocin genes. It now appears that the lactococcin A secretion and processing observed in the clones carrying only the lactococcin A structural and immunity genes were caused by the choice of the host strain (*L. lactis* subsp. *lactis* IL1403) used in their cloning experiments. This finding was confirmed when transformants of *L. lactis* subsp. *lactis* IL1403 containing pLLM24 (Fig. 1B) were found to have an LcnA⁺ phenotype (43). *L. lactis* subsp. *lactis* IL1403 was shown to carry a functional set of secretion genes on its chromosome by Southern hybridization with a DNA fragment carrying the entire lactococcin A gene cluster (30). The reduced halo diameter observed with this strain could be ascribed to either a lower copy number of the chromosomal *lcnC* and *lcnD* genes or by assuming that lactococcin A is not the natural substrate of the secretion system encoded on the *L. lactis* subsp. *lactis* IL1403 chromosome. Partial complementation of the *lcnC* and *lcnD* genes by similar genes of a heterologous secretion system could result in a reduced secretion and processing efficiency for lactococcin A. Copy number effects and heterologous complementation have been observed with some of the gram-negative bacterial examples of this type of secretory apparatus (13, 34, 44). *L. lactis* subsp. *lactis* IL1403 secretes the three lactococcins (A, B, and M) described by van Belkum et al. (51, 52). The putative *L. lactis* subsp. *lactis* IL1403 secretion machinery is

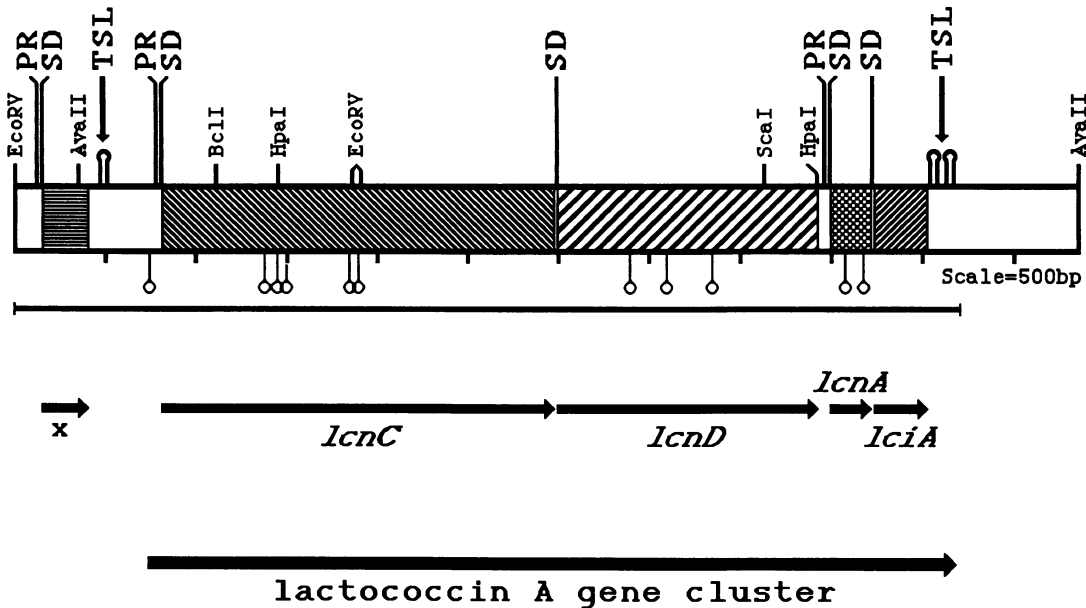


FIG. 5. Linear restriction map showing the sequenced regions (solid blunt-end line) of pLLM16-1, pLLM17, and pLLM20. The orientation and organization of the *lcnC*, *lcnD*, *lcnA*, and *lciA* genes; promoter regions (PR) and Shine-Dalgarno sequences (SD); rho-independent transcription termination stem-loop structures (TSL); the deduced location of the Tn5 insertion sites (depicted by circles); and the lactococcin A gene cluster are illustrated.

apparently capable of handling molecules that are quite different in their amino acid sequences. The only similarity among the three lactococcins is the N-terminal extension of the pre-lactococcins (52). The exciting possibility that these N termini impart functional recognition by the secretion apparatus is under investigation. Preliminary sequence data indicate that upstream of the lactococcin M structural gene, *lcnM*, an exact copy of *lcnD* is present, and the possible presence of a copy of *lcnC* further upstream of *lcnM* is being investigated (49, 51).

The data from this study suggest the existence of a signal sequence-independent secretory pathway in the lactococci. The presence of similar components of a secretion and processing system for *Bacillus subtilis* has been reported recently (7, 29) and further suggests the existence of signal sequence-independent secretory pathways in gram-positive bacteria. The results reported here will facilitate studies of the transport of naturally occurring and cloned proteins in lactococci and of the development of a food-grade suicide cloning vector system. The sequence data provide information for the deliberate and directed alteration of lactococcins for the enhanced inhibition of undesirable bacterial species in food products and for the improved secretion of industrially useful proteins in food-grade bacterial species.

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