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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 Tn*5* 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 | | |
| *E. coli* | | |
| XL1-Blue | Ap$^s$ *recA1 lac supE44* F′ [*lacI*$^q$ *lacZ*ΔM15, Tn*10* (Tc$^r$)] | 5 |
| JM109 | Tc$^s$ Cm$^s$ *recA1* Δ*lac supE44* | 40 |
| MC1061 | Tc$^s$ Cm$^s$ Δ*lacX74* | 40 |
| LLM16-1 | JM109 transformed with pLLM16-1, encoding Cm$^s$ 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 |
| *L. lactis* subsp. *lactis* | | |
| LM0230 | Lac$^-$ LcnA$^-$ LciA$^-$ plasmid-free derivative of *L. lactis* subsp. *lactis* C2 | 36 |
| IL1403 | Lac$^-$ LcnA$^-$ LciA$^-$ plasmid-free derivative of *L. lactis* subsp. *lactis* IL594 | 23, 49 |
| LLM24-1 | *L. lactis* subsp. *lactis* IL1403 transformed with pLLM24, encoding LcnA$^+$ LciA$^+$ Em$^r$ | This study |
| *L. lactis* subsp. *lactis* LM0230 transformants | | |
| 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$^r$ *lacZ*α$^+$ | 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 *Bcl*I pNP2 fragments | 21 |
| pLLM12 | pGB301 carrying 9.5- and 3.3-kb *Bcl*I fragments of pNP2 | This study |
| pLLM13 | pGB301 carrying the 13.4-kb *Bcl*I fragment of pNP2 | This study |
| pLLM14 | pGB301 carrying the 12.1-kb *Bcl*I fragment of pNP2 | This study |
| pLLM15 | *Eco*RI deletion derivative of pLLM12 | This study |
| pLLM16 | pSA3 carrying the 5.5-kb *Bcl*I-*Eco*RI fragment of pLLM12 | This study |
| pLLM16-1 | *Ava*I deletion derivative of pLLM16 that eliminated the pGB305 moiety of pSA3 | This study |
| pLLM17 | pBluescript II carrying the 0.75-kb *Bcl*I-*Eco*RV fragment of pLLM15 | This study |
| pLLM20 | pACYC184 carrying the 1.9-kb *Eco*RV fragment of pKSH1 | This study |
| pLLM21 | *Eco*RV partial deletion derivative of pKSH1 | This study |
| pLLM22 | pIL253 carrying the 5.6-kb *Ava*II fragment of pLLM21 | This study |
| pLLM23 | pACYC184 carrying pLLM22 | This study |
| pLLM24 | *Eco*RV 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 Kekessy 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 $H_2O$, and 50 μl of the resuspended cells was added to 1 μg of plasmid DNA (resuspended in ≤5 μl of distilled deionized $H_2O$) 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 $10^4$, as reported by McIntyre and Harlander (35), to $10^5$ 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 fragment 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 [$^{35}$S]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.

**Tn*5* mutagenesis.** Transposon (Tn*5*) mutagenesis (11) was conducted to identify the genes responsible for the $LcnA^+$ and $LciA^+$ phenotypes. *E. coli* LLM23 was infected with λ::Tn*5* (λ *b*221 *rex*::Tn*5* *c*I857 *O*am29 *P*am80) 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 Tn*5* insertions into pLLM23. Plasmid DNA from $Cm^r$ $Km^r$ transformants was digested (*Bam*HI and *Bam*HI-*Kpn*I) and screened for Tn*5* insertion into the *lcn* genes on the *Ava*II restriction fragment of pLLM23. Plasmids containing single Tn*5* 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

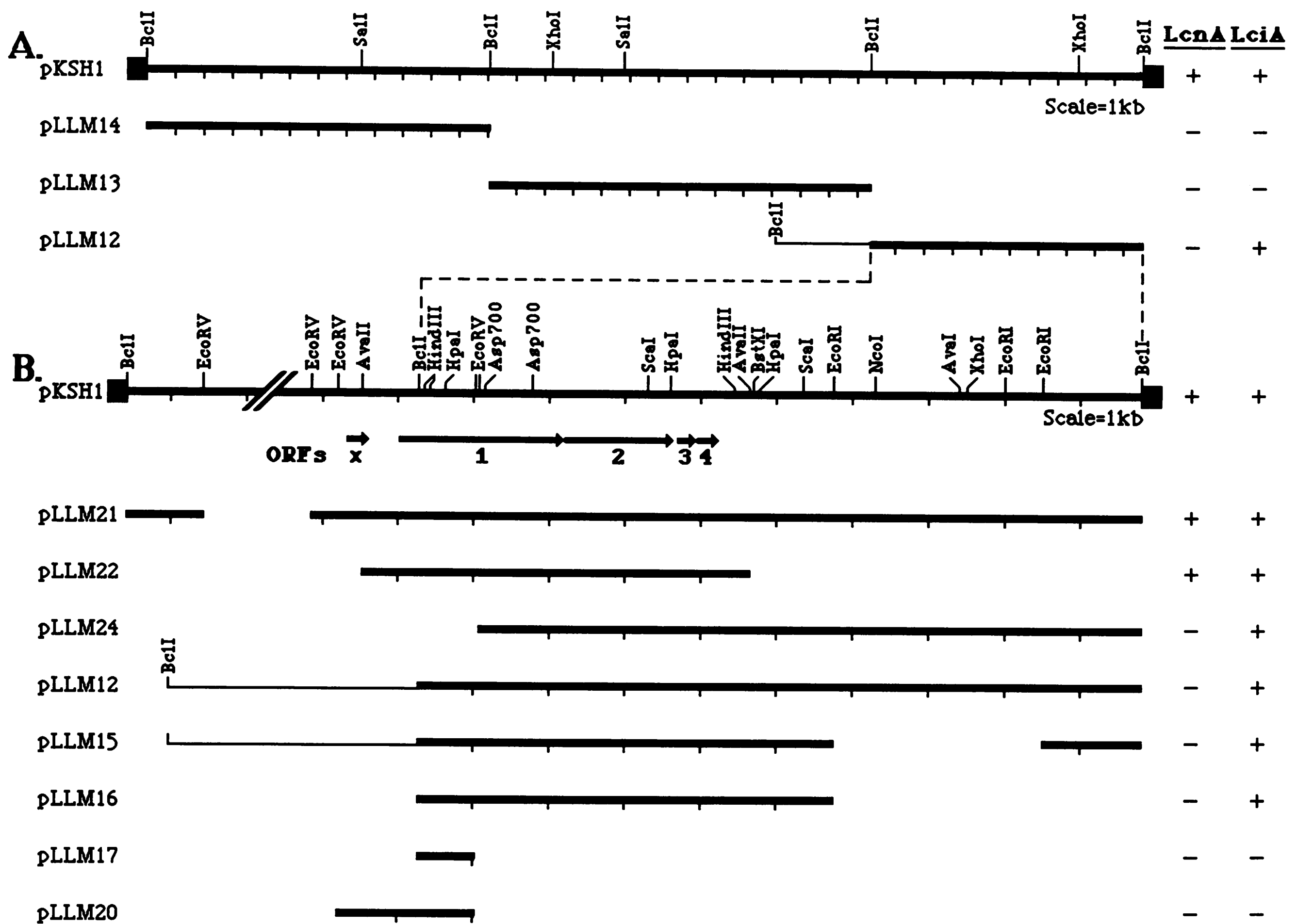


FIG. 1. Linear restriction enzyme maps of pKSH1 (adapted from reference 21), deletion derivatives, and subclones. (A) pKSH1 (LcnA$^+$ LciA$^+$), pLLM14 (LcnA$^-$ LciA$^-$), pLLM13 (LcnA$^-$ LciA$^-$), and pLLM12 (LcnA$^-$ LciA$^+$) in *L. lactis* subsp. *lactis* LM0230. (B) Expanded restriction map of 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 *Bcl*I 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 *Bcl*I 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 *Bcl*I 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 *Eco*RV-*Bcl*I (pLLM21) and 5.6-kb *Ava*II (pLLM22) restriction fragments of pKSH1 (Fig. 1B). Efforts to further localize the bacteriocin production and immunity genes together on the 3.8-kb *Ava*II-*Sca*I restriction fragment from pLLM22 were unsuccessful (data not shown). The gene(s) encoding the Imm$^+$ phenotype was localized on the 5.5-kb *Bcl*I-*Eco*RI (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 *Eco*RV 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 *Ava*II 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 *Bcl*I restriction site of the contiguous 9.5- and 13.4-kb *Bcl*I restriction fragments of pKSH1 was located within ORF1 and deletion of DNA upstream of the junction *Bcl*I restriction site (as in pLLM12,

```
      EcoRV
   1  GATATCCTATAAATCTCACTTTTCAAATTATTATATAACGTGATATGTAGAAGGTCAGAT    60

  61  ATGTTGAGCAATTATGAAAGAAATCATTGAATAATTAACTAGAGCTAGAACTATGGCATA   120
                                -35

 121  GAAATATTATATATTAGAGAGGTAAAAATGAGAGGAATAACAGGAAGCTCATTACAAAGC   180
          -10        RBS          METArgGlyIleThrGlySerSerLeuGlnSer
                                  ORFx ===>

 181  CGAACTGTCTATACAAAAAAGCCCAAAAAGAAGATTTGTAAGAATTGTGACTCTGAATTT   240
      ArgThrValTyrThrLysLysProLysLysLysIleCysLysAsnCysAspSerGluPhe

 241  TGGGCTAAAGCAAAATACACTGAAGGCTACCAAGACTATTGTGGTTTTTGTCGGATGGTG   300
      TrpAlaLysAlaLysTyrThrGluGlyTyrGlnAspTyrCysGlyPheCysArgMETVal

                                                      AvaII
 301  TATCGTCAGGATAAAAGAAGAATTATAAAAAAAAGTAAAGTTAAAGGACCAACTCTATAT   360
      TyrArgGlnAspLysArgArgIleIleLysLysSerLysValLysGlyProThrLeuTyr

 361  AATCCCAAAGTATTTTCTAAGAGAAAGCCCAAATAGTAAAATAAGATATTTTTTCATAAG   420
      AsnProLysValPheSerLysArgLysProLys *

                                                    _______________
 421  TTTCGGAAAAATTAGTCTTTTCAATAAACTTAGTTTTAACTTTTTCTTTTGATAAAAAAA   480

      ---------->    <--------------------------
 481  GCGATAATGAGTCAGTCATTGTCGCTTTTACTATTTTAATATCTTATGGTGTAAGTGTTT   540

 541  CTAATTATAATCTCGATTATAAGGCTTGGTATATAATTATAACAAGTGCTTTTTTGGCAT   600

 601  CTATACTACTTAATTTTAATAAAAAAGAGGCAGTAAGTAATATTATTTTCATTTATCTAG   660

 661  GTTTAAACTACTAAGTCCTTATTATTTTCACTATCTTTTTCTTCGATTTTAAACTAAAAT   720

 721  AGATTTATTGAAATTCTCTGTAACTGAACCGCAAACAATATTATTGGTTCCAGTGATTTA   780
              -35                           -10

 781  CTAAAATTTTAACTCAGAAAGTTAAGAAAAGATGAAATTTAAAAAGAAAAATTATACCTCA  841
                    RBS               METLysPheLysLysLysAsnTyrThrSer
                                      ORF1(lcnC)===>

 842  CAAGTAGATGAAATGGACTGTGGCTGTGCTGCCTTATCAATGATTTTAAAGTCTTATGGC   901
      GlnValAspGluMETAspCysGlyCysAlaAlaLeuSerMETIleLeuLysSerTyrGly

 902  ACAGAAAAATCTCTCGCTTCATTGCGCTTACTTGCAGGTACAACAATCGAAGGAACCTCC   961
      ThrGluLysSerLeuAlaSerLeuArgLeuLeuAlaGlyThrThrIleGluGlyThrSer

 962  GCTTTAGGGATAAAAAAGGCAGCTGAAATATTAGAGTTTTCAGTTCAGGCCCTAAGAACA  1021
      AlaLeuGlyIleLysLysAlaAlaGluIleLeuGluPheSerValGlnAlaLeuArgThr

1022  GATGCAAGCCTTTTTGAAATGAAAAACGCTCCTTACCCATTTATTGCTCATGTCATTAAA  1081
      AspAlaSerLeuPheGluMETLysAsnAlaProTyrProPheIleAlaHisValIleLys

                                 BclI
1082  GACCAAAAATACCCACATTATTATGTGATCACTGGCGCAAATAAAAATTCGGTATTCATT  1141
      AspGlnLysTyrProHisTyrTyrValIleThrGlyAlaAsnLysAsnSerValPheIle

1142  GCTGATCCTGACCCAACCATTAAAATGACAAAATTATCAAAAGAAGCTTTTTTATCGGAA  1201
      AlaAspProAspProThrIleLysMETThrLysLeuSerLysGluAlaPheLeuSerGlu

1202  TGGACTGGGATTAGTTTATTTCTTTCGACTACACCATCTTATCATCCCACTAAAGAAAAA  1261
      TrpThrGlyIleSerLeuPheLeuSerThrThrProSerTyrHisProThrLysGluLys

1262  GCTTCCTCATTATTATCTTTTATCCCAATTATCACCCGTCAAAAGAAAGTCATTCTCAAT  1321
      AlaSerSerLeuLeuSerPheIleProIleIleThrArgGlnLysLysValIleLeuAsn

1322  ATTGTTATTGCCTCATTCATTGTAACACTGATTAATATTCTAGGCTCTTACTACCTCCAG  1381
      IleValIleAlaSerPheIleValThrLeuIleAsnIleLeuGlySerTyrTyrLeuGln

1382  AGCATGATTGACAGCTATATTCCAAATGCCTTAATGGGAACTTTAGGGATTATCTCAGTA  1441
      SerMETIleAspSerTyrIleProAsnAlaLeuMETGlyThrLeuGlyIleIleSerVal

           HpaI
1442  GGGCTATTGTTAACCTATATTATCCAACAGGTCTTAGAATTTGCTAAGGCCTTCTTATTG  1501
      GlyLeuLeuLeuThrTyrIleIleGlnGlnValLeuGluPheAlaLysAlaPheLeuLeu

1502  AACGTTCTTTCTCAAAGATTAGCTATTGATGTCATTCTTTCTTATATTAGACACATTTTC  1561
      AsnValLeuSerGlnArgLeuAlaIleAspValIleLeuSerTyrIleArgHisIlePhe

1562  CAACTTCCCATGTCTTTCTTTTCGACCCGAAGAACAGGAGAAATTACCAGTCGGTTTTCC  1621
      GlnLeuProMETSerPhePheSerThrArgArgThrGlyGluIleThrSerArgPheSer

1622  GATGCGAGTTCTATTTTAGATGCTATTGCCTCAACGATTCTTTCGCTCTTTTTAGATTTG  1681
      AspAlaSerSerIleLeuAspAlaIleAlaSerThrIleLeuSerLeuPheLeuAspLeu

1682  ACGATTGTCGTCATGACAGGACTAATTTTAGGCCTTCAAAATATGCAACTCTTTCTTCTC  1741
      ThrIleValValMETThrGlyLeuIleLeuGlyLeuGlnAsnMETGlnLeuPheLeuLeu

1742  GTTCTTTTGGCAATCCCACTCTATATTGTTGTTATTATTATTTTTACGCCCCTTTTTGAA  1801
      ValLeuLeuAlaIleProLeuTyrIleValValIleIleIlePheThrProLeuPheGlu

                                                                ___
1802  AAACAAAACCATGAAGTCATGCAGACCAATGCCGTCTTAAACTCCTCAATTATTGAAGAT  1861
      LysGlnAsnHisGluValMETGlnThrAsnAlaValLeuAsnSerSerIleIleGluAsp

      EcoRV                                           EcoRV
1862  ATCAATGGGATTGAAACTATAAAGGCACTTGCCAGTGAACAAGAAAGATATCAAAAAATT  1921
      IleAsnGlyIleGluThrIleLysAlaLeuAlaSerGluGlnGluArgTyrGlnLysIle

1922  GACTACGAATTTGCAAGTTATCTAAAAAAGGCTTTCACTTTACAAAAATCAGAAGCTATT  1981
      AspTyrGluPheAlaSerTyrLeuLysLysAlaPheThrLeuGlnLysSerGluAlaIle

1982  CAAGGCTTAATTAAAGCAATTATACAACTAACATTGAGTGTCACCATTTTATGGTTTGGT  2041
      GlnGlyLeuIleLysAlaIleIleGlnLeuThrLeuSerValThrIleLeuTrpPheGly

2042  GCCACATTAGTAATAAGTCAAAAAATTACGCTCGGACAATTGATTACTTTTAATGCCCTG  2101
      AlaThrLeuValIleSerGlnLysIleThrLeuGlyGlnLeuIleThrPheAsnAlaLeu

2102  CTTTCTTACTTTACAAATCCAATTACCAATATCATTAACCTTCAAACAAAACTACAAAAG  2161
      LeuSerTyrPheThrAsnProIleThrAsnIleIleAsnLeuGlnThrLysLeuGlnLys

2162  GCAAGGGTAGCCAATGAACGATTAAATGAGGTCTATCTTGTACCCAGTGAATTTGAGGAA  2221
      AlaArgValAlaAsnGluArgLeuAsnGluValTyrLeuValProSerGluPheGluGlu

2222  AAGAAAACAGAACTGTCCCTCTCACATTTTAACTTAAACATGTCCGATATTTCATATCAA  2281
      LysLysThrGluLeuSerLeuSerHisPheAsnLeuAsnMETSerAspIleSerTyrGln

2282  TATGGTTTTGGCAGAAAAGTCTTATCTGAGATAGAACTCTCTATTAAAGAAAATGAAAAA  2341
      TyrGlyPheGlyArgLysValLeuSerGluIleGluLeuSerIleLysGluAsnGluLys

2342  TTGACTATTGTGGGCATGAGTGGTTCAGGAAAGAGTACCCTTGTTAAATTATTGGTCAAC  2401
      LeuThrIleValGlyMETSerGlySerGlyLysSerThrLeuValLysLeuLeuValAsn

2402  TTCTTTCAACCCACTTCTGGCACCATCACTTTAGGTGGAATTGACCTGCAACAGTTTGAT  2461
      PhePheGlnProThrSerGlyThrIleThrLeuGlyGlyIleAspLeuGlnGlnPheAsp

2462  AAACACCAACTCCGAAGATTAATCAACTATCTTCCCCAACAACCTTATATTTTTACTGGT  2521
      LysHisGlnLeuArgArgLeuIleAsnTyrLeuProGlnGlnProTyrIlePheThrGly

         ----------------------->      <---------------------
2522  TCAATTTTAGATAATCTACTTCTAGGAGCTAATGAGAATGCATCACAAGAAGAGATTCTT  2581
      SerIleLeuAspAsnLeuLeuLeuGlyAlaAsnGluAsnAlaSerGlnGluGluIleLeu

      ___
2582  AAAGCGGTGGAATTGGCAGAAATCCGTGCAGATATTGAACAAATGCAGTTGGGCTATCAG  2641
      LysAlaValGluLeuAlaGluIleArgAlaAspIleGluGlnMETGlnLeuGlyTyrGln
```

FIG. 2. Nucleotide sequence encompassing the lactococcin 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 lactococcin 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 lactococcin 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, respectively) (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 ORFx (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

```
2642  ACCGAACTTTCAAGTGATGCAAGTAGTCTATCGGGGGACAAAAACAACGCATTGCTTTA  2701
      ThrGluLeuSerSerAspAlaSerSerLeuSerGlyGlyGlnLysGlnArgIleAlaLeu

2702  GCTCGTGCGCTCCTTTCCCCTGCCAAAATCCTCATTTTAGATGAAGCAACCAGTAACCTT  2761
      AlaArgAlaLeuLeuSerProAlaLysIleLeuIleLeuAspGluAlaThrSerAsnLeu

2762  GATATGATTACAGAGAAGAAAATATTAAAGAACTTGTTGCCCTTGGATAAAACCATTATT  2821
      AspMETIleThrGluLysLysIleLeuLysAsnLeuLeuProLeuAspLysThrIleIle

2822  TTCATTGCTCACCGCCTCTCTGTGGCGGAAATGAGTCATCGAATTATTGTTGTCGATCAA  2881
      PheIleAlaHisArgLeuSerValAlaGluMETSerHisArgIleIleValValAspGln

2882  GGAAAAGTGATAGAGAGTGGCTCACATGTTGACCTGCTTGCACAAAATGGCTTTTATGAA  2941
      GlyLysValIleGluSerGlySerHisValAspLeuLeuAlaGlnAsnGlyPheTyrGlu

2942  CAACTTTACCATAACTGAATCAGGAGAAAAAGATGTTTGATAAAAAATTACTGGAAAGT  3000
      GlnLeuTyrHisAsn *       RBS      METPheAspLysLysLeuLeuGluSer
                                        ORF2(lcnD)===>

3001  TCAGAGCTTTATGATAAACGCTATCGAAATTTCTCGACCTTAATTATTCTTCCACTTTTT  3060
      SerGluLeuTyrAspLysArgTyrArgAsnPheSerThrLeuIleIleLeuProLeuPhe

3061  ATCCTTCTTGTTGGAGGAGTAATTTTTACTTTCTTTGCCCATAAGGAGTTGACCGTAATA  3120
      IleLeuLeuValGlyGlyValIlePheThrPhePheAlaHisLysGluLeuThrValIle

3121  AGTACAGGAAGTATTGAACCCACAAAAATAGTTGCTAAAATTCAATCAACGAATGCCAAT  3180
      SerThrGlySerIleGluProThrLysIleValAlaLysIleGlnSerThrAsnAlaAsn

3181  CCAATCATTGAGAATAACCTCAAAGAAGGCGAAGCGGTTAAAGAAAATAGCTTACTTCTC  3240
      ProIleIleGluAsnAsnLeuLysGluGlyGluAlaValLysGluAsnSerLeuLeuLeu

3241  AAATATAACGGGACACCAGAACAGACCCAACTCAGTGAGCTACTGACTCAAAAGAAACAA  3300
      LysTyrAsnGlyThrProGluGlnThrGlnLeuSerGluLeuLeuThrGlnLysLysGln

3301  GCGTTAGACAAAAAAGTACAACTCGACCTTCTCCAAAGAAGTTTGACCAACGAAAAAAAT  3360
      AlaLeuAspLysLysValGlnLeuAspLeuLeuGlnArgSerLeuThrAsnGluLysAsn

3361  GAATTTCCCACTGCCGATAGTTTTGGCTATGAGAAAAGTTTTGAAAACTATGAGGCACAA  3420
      GluPheProThrAlaAspSerPheGlyTyrGluLysSerPheGluAsnTyrGluAlaGln

3421  GTAAAGAGTCTTGAAGCAACCATACAAAAGTCAAATCAAGCCGTAGAAGATCAAAATAAA  3480
      ValLysSerLeuGluAlaThrIleGlnLysSerAsnGlnAlaValGluAspGlnAsnLys

3481  AGTACCGAGAGTCAAAAGCAAGCCATTCAAAATCAGGTGGCAACACTCCAGCAAGCTATT  3540
      SerThrGluSerGlnLysGlnAlaIleGlnAsnGlnValAlaThrLeuGlnGlnAlaIle

3541  CAGAATTACTCTGAAATCGAAAATGCGGTATCGAGTGGTGGTGGAGTTTCACAAGATAAT  3600
      GlnAsnTyrSerGluIleGluAsnAlaValSerSerGlyGlyGlyValSerGlnAspAsn

3601  CCCTACCTCTCTCAATATAACAGTTACCAAGCCCAACAAGCGACTTTAGAGGCCGATTTA  3660
      ProTyrLeuSerGlnTyrAsnSerTyrGlnAlaGlnGlnAlaThrLeuGluAlaAspLeu

3661  AAAAATCAAAAAAATCCAGATGAAACTGCTAAGCAAGCGGCTAAAAGTCAAGAGGAGTCT  3720
      LysAsnGlnLysAsnProAspGluThrAlaLysGlnAlaAlaLysSerGlnGluGluSer

3721  TTAAAAAGTCAATTTTTATCAGGTTTAGCTTCGAGTAAAGACAGCTTAAAAAGCCAAATT  3780
      LeuLysSerGlnPheLeuSerGlyLeuAlaSerSerLysAspSerLeuLysSerGlnIle

3781  CAATCCTTTAATGTGCAAGAAAGTAGCTTAACAGGAAGTAATGCTTATGATAATAGCCAA  3840
      GlnSerPheAsnValGlnGluSerSerLeuThrGlySerAsnAlaTyrAspAsnSerGln

3841  TCTAGTCAAATCTTAACTTTAAAAAGTCAAGCACTTTCAGCTTCAAATAAAGAAATGACA  3900
      SerSerGlnIleLeuThrLeuLysSerGlnAlaLeuSerAlaSerAsnLysGluMETThr

3901  GACCTAAATAGTACCCTTACTGATTTAGAAACAAAAATTAGCTTGCAAAAACAAGATGAC  3960
      AspLeuAsnSerThrLeuThrAspLeuGluThrLysIleSerLeuGlnLysGlnAspAsp

3961  CAATACAGTCAAGTTTTTGCGGAACAGGCTGGAGTCCTGCATGTGCTCCCAGATATTTTA  4020
      GlnTyrSerGlnValPheAlaGluGlnAlaGlyValLeuHisValLeuProAspIleLeu

4021  GGAATGAAAAAGATTCCGATTGGAACACCTATCGCAGAAATCTATCCTTTATTAAAGTCA  4080
      GlyMETLysLysIleProIleGlyThrProIleAlaGluIleTyrProLeuLeuLysSer

                                          ScaI
4081  GAAACACAAGTTAATCTGACAAGTTATATCCCAAGTACTCAAATTTCTGGAATGAAAGTC  4140
      GluThrGlnValAsnLeuThrSerTyrIleProSerThrGlnIleSerGlyMETLysVal

4141  GGTCAAAAAGTGAGATTTACAGTACAGCAAAATTTACCTCAACCTGAAATTTTAACTGGA  4200
      GlyGlnLysValArgPheThrValGlnGlnAsnLeuProGlnProGluIleLeuThrGly

4201  ATAATCAACCAAATAGATAGTGCTCCCACAGCTTTTAAAGAGGGAAATGCTTATAAAGTT  4260
      IleIleAsnGlnIleAspSerAlaProThrAlaPheLysGluGlyAsnAlaTyrLysVal

4261  TCTGCGACAACCACTATCAATGCAAAAGACCTCCCAAATATCCGATATGGTCTTCAAGGG  4320
      SerAlaThrThrThrIleAsnAlaLysAspLeuProAsnIleArgTyrGlyLeuGlnGly

4321  AAAACAGTAACCATTATAGGAAAGAAAACTTATTTCAATTACTTTTTAGATAAAATAATG  4380
      LysThrValThrIleIleGlyLysLysThrTyrPheAsnTyrPheLeuAspLysIleMET

                                         HpaI        ______________
4381  GGAAGAGGCAATCAGTAGAGTTATTAACATTTGTTAACGAGTTTTATTTTTATATAATCT  4440
      GlyArgGlyAsnGln *                  -35

      >   <--------------
4441  ATAATAGATTTATAAAAATAAGGAGATTATTATGAAAAATCAATTAAATTTTAATATTGTT  4501
      -10        ⇑            RBS       METLysAsnGlnLeuAsnPheAsnIleVal
                                        ORF3(lcnA)===>

4502  TCAGATGAAGAACTTTCAGAAGCTAACGGAGGAAAATTAACATTTATTCAATCGACAGCG  4561
      SerAspGluGluLeuSerGluAlaAsnGlyGlyLysLeuThrPheIleGlnSerThrAla
                                          ↑

4562  GCTGGAGATTTATATTACAATACTAATACACACAAATATGTTTACCAACAAACTCAAAAC  4621
      AlaGlyAspLeuTyrTyrAsnThrAsnThrHisLysTyrValTyrGlnGlnThrGlnAsn

4622  GCTTTTGGGGCTGCTGCTAATACCATTGTTAATGGATGGATGGGTGGCGCTGCTGGAGGT  4681
      AlaPheGlyAlaAlaAlaAsnThrIleValAsnGlyTrpMETGlyGlyAlaAlaGlyGly

4682  TTCGGGTTGCACCATTGAGGATTAGTTAAGATATGAAAAAAAAACAAATAGAATTTGAA  4740
      PheGlyLeuHisHis *                 METLysLysLysGlnIleGluPheGlu
                          RBS           ORF4(lciA)===>

4741  AACGAGCTAAGAAGTATGTTGGCTACCGCCCTTGAAAAAGACATTAGTCAAGAGGAAAGA  4800
      AsnGluLeuArgSerMETLeuAlaThrAlaLeuGluLysAspIleSerGlnGluGluArg

4801  AATGCTCTGAATATTGCAGAAAAGGCGCTTGACAATTCTGAATATTTACCAAAAATTATT  4860
      AsnAlaLeuAsnIleAlaGluLysAlaLeuAspAsnSerGluTyrLeuProLysIleIle

4861  TTAAACCTCAGAAAAGCCCTAACTCCATTAGCTATAAATCGAACACTTAACCATGATTTA  4920
      LeuAsnLeuArgLysAlaLeuThrProLeuAlaIleAsnArgThrLeuAsnHisAspLeu

4921  TCTGAACTGTATAAATTCATTACAAGTTCCAAAGCATCAAACAAAAATTTAGGTGGTGGT  4980
      SerGluLeuTyrLysPheIleThrSerSerLysAlaSerAsnLysAsnLeuGlyGlyGly

                                  __________________________
4981  TTAATTATGTCGTGGGGACGACTATTCTAATAAATCAACAGAACTAATAAAAGAATGGCT  5040
      LeuIleMETSerTrpGlyArgLeuPhe *

      >      <------------------------------
5041  AAGCAAATTACTAGCCATTCTTTTATTAGTTAAAGTGCCATGCTAAAATTTAAGAGACGT  5100

           ------------------->  <--------------------
5101  GCCATTGATTAAGCAAAGACATTCGATAATTAGATAATTATCGAATGTCTTTTTAAATCT  5160

5161  TCTTTTTTTAATTACTTTTTAGATAA                                    5186
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FIG. 2—*Continued*.

export apparatus. One of the proteins invariably found in the signal peptide-independent export system is an ATP-dependent membrane translocator (3).

The deduced protein encoded by ORF1 in the lactococcin A gene cluster (ORF1, ORF2, *lcnA*, and *lciA*) shares similarity with ATP-dependent translocators of the HlyB family (3). ORF1 and HlyB show 28.7% identity and 18.7% similarity at the amino acid level. The similarity between ORF1 and HlyB is most pronounced in the C-terminal stretch of 200 amino acids that contains the conserved ATP binding domain (Fig. 3). As is the case for HlyB and its homologs, the ORF1 protein has a highly hydrophobic N terminus. Hydrophobicity analysis predicts three hydrophobic domains in the putative ORF1 product (Fig. 4A). Hydrophobicity analysis of the deduced protein encoded by ORF2 indicates that it is largely hydrophilic, with the exception of the 43 N-terminal amino acids (Fig. 4B). This region does not contain a typical signal peptide found in most transported proteins and in this respect resembles CvaA (19), PrtE (33), and HlyD (53). On the basis of this analysis and the fact that both ORFs are critical for lactococcin A activity (see below), ORF1 and ORF2 were renamed *lcnC* and *lcnD*, respectively. The hydropathic character of the deduced LcnA and LciA proteins was determined (Fig. 4C and D), but no similarities to published proteins were found.

**LcnD and, probably, LcnC are essential for lactococcin A activity.** Transposon mutagenesis was performed on LcnA$^+$ LciA$^+$ pLLM23 (Fig. 5). Tn*5* insertion sites and the resulting phenotypes were compared to determine the involvement of *lcnC* and *lcnD* in lactococcin A production and immunity. The single Tn*5* insertion upstream of *lcnC* and several Tn*5* insertions downstream of *lciA* (in the pIL253 cloning vector) allowed the LcnA$^+$ LciA$^+$ phenotype to be retained. Tn*5* insertions into three of the four genes were readily obtained. All insertions into *lcnD* resulted in an LcnA$^-$ LciA$^+$ phenotype. As *lcnA* and *lciA* can be transcribed from their own promoter (51), the loss of lactococcin A activity is not caused by a polar effect of Tn*5* on the lactococcin A structural gene; therefore, LcnD is essential for the LcnA$^+$ phenotype. The same phenotype (LcnA$^-$ LciA$^+$) was observed with the five Tn*5* mutations in *lcnC*. If it is assumed that Tn*5* insertions do not exert a polar effect on *lcnD* transcription, the latter result would indicate that LcnC is also essential for the LcnA$^+$ phenotype. Two Tn*5* mutations were obtained in the lactococcin A structural gene (*lcnA*), and they both resulted in an LcnA$^-$ LciA$^-$ phenotype. The inability to obtain Tn*5* insertions into the immunity gene (*lciA*) suggests that such mutations are lethal.

## DISCUSSION

The present report describes the nucleotide sequence of the lactococcin A gene cluster from *L. lactis* subsp. *lactis* biovar diacetylactis WM4, which encodes the genes required

```
LcnC- MKF-KKKNYTSQVDE--MDCGCAALSM-----ILKSYGTEKSLASLRLLAGTTIEGTSAL - 52
HlyB- MDSCHKIDYGLYALEILAQYHNVSVNPEEIKHRFDTDGTGLGLTSW-LLAAKSLELK-VK - 58

LcnC- GIKKAAEILEFSVQALRTDASLFEMKNAPYPFIAHVIKDQKYPHYYVITGANKNSVFIAD -112
HlyB- QVKKTIDRLNFIFL-----PALVWREDGRHFILTKISKEVN--RYLIFDLEQRNPRVLEQ -111

LcnC- PDPTIKMTKLSKEAFLSEWTGISLFLSTTPSYHPTKEKASSLLSFIPIITRQKKVILNIV -172
HlyB- SEF---------EALYQG----HIILITSRSSVTGKLAKFDFTWFIPAIIKYRRIFIETL -158

LcnC- IASFIVTLINILGSYYLQSMIDSYIPNALMGTLGIISVGLLLTYIIQQVLEFAKAFLLNV -232
HlyB- VVSVFLQLFALITPLFFQVVMDKVLVHRGFSTLNVITVALSVVVVFEIILSGLRTYIFAH -218

LcnC- LSQRLAIDVILSYIRHIFQLPMSFFSTRRTGEITSRFSDASSILDAIASTILSLFLDLTI -292
HlyB- STSRIDVELGAKLFRHLLALPISYFESRRVGDTVARVRELDQIRNFLTGQALTSVLDL-- -276

LcnC- VVMTGLILGLQ---NMQLFLLVLLAIPLYIVVIIIFTPLFEKQNHEVMQTNAVLNSSIIE -349
HlyB- -LFSLIFFAVMWYYSPKLTLVILFSLPCYAAWSVFISPILRRRLDDKFSRNADNQSFLVE -335

LcnC- DINGIETIKALASEQERYQKIDYEFASYLKKAFTLQKSEAI--QGLIKAIIQLTLSVTIL -407
HlyB- SVTAINTIKAMAVSPQMTNIWDKQLAGYVAAGFKVTVLATIGQQGI--QLIQKTVMIINL -393

LcnC- WFGATLVISQKITLGQLITFNALLSYFTNPITNIINLQTKLQKARVANERLNEVYLVPSE -467
HlyB- WLGAHLVISGDLSIGQLIAFNMLAGQIVAPVIRLAQIWQDFQQVGISVTRLGDVLNSPTE -453

LcnC- FEEKKTELSLSHFNLNMSDISYQYGF-GRKVLSEIELSIKENEKLTIVGMSGSGKSTLVK -526
HlyB- SYHGKLTLPEINGDITFRNIRFRYKPDSPVILDNINLSIKQGEVIGIVGRSGSGKSTLTK -513

LcnC- LLVNFFQPTSGTITLGGIDLQQFDKHQLRRLINYDLPQQPYIFTGSILDNLLLGANENAS -586
HlyB- LIQRFYIPENGQVLIDGHDLALADPNWLRRQVGVVL-QDNVLLNRSIIDNISL-ANPGMS -571

LcnC- QEEILKAVELAEIRADIEQMQLGYQTELSSDASSLSFLQKQRIALARALLSPAKILILDE -646
HlyB- VEKVIYAAKLAGAHDFISELREGYNTIVGEQGAGLSGGQRQRIAIARALVNNPKILIFDE -631

LcnC- ATSNLDMITEKKILKNLLPL--DKTIIFIAHRLSVAEMSHRIIVVDQGKVIESGSHVDLL -704
HlyB- ATSALDYESEHVIMRNMHKICKGRTVIIIAHRLSTVKNADRIIVMEKGKIVEQGKHKELL -691

LcnC- AQ-NGFYEQLYH---N                                             -716
HlyB- SEPESLYSYLYQLQSD                                             -707
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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. ORFx had no function in lactococcin A production or immunity, and the potential rho-independent transcription termination stem-loop downstream of ORFx 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 *Bcl*I 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 *Bcl*I restriction fragments, when cloned separately, did not lead to detectable bacteriocin production. The current study reports that the clone containing the 9.5-kb *Bcl*I 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. Tn*5* insertions in *lcnD* revealed that this gene is essential for the LcnA$^+$ phenotype. These *lcnD* Tn*5* 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 Tn*5* 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 *Bcl*I restriction fragment in pLLM12, and cells carrying this plasmid have the same phenotype as those carrying *lcnD*::Tn*5*. Tn*5* insertions in *lcnC* also resulted in an LcnA$^-$ LciA$^+$ phenotype. The current study cannot exclude the possibility that a polar effect of the *lcnC*::Tn*5* 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

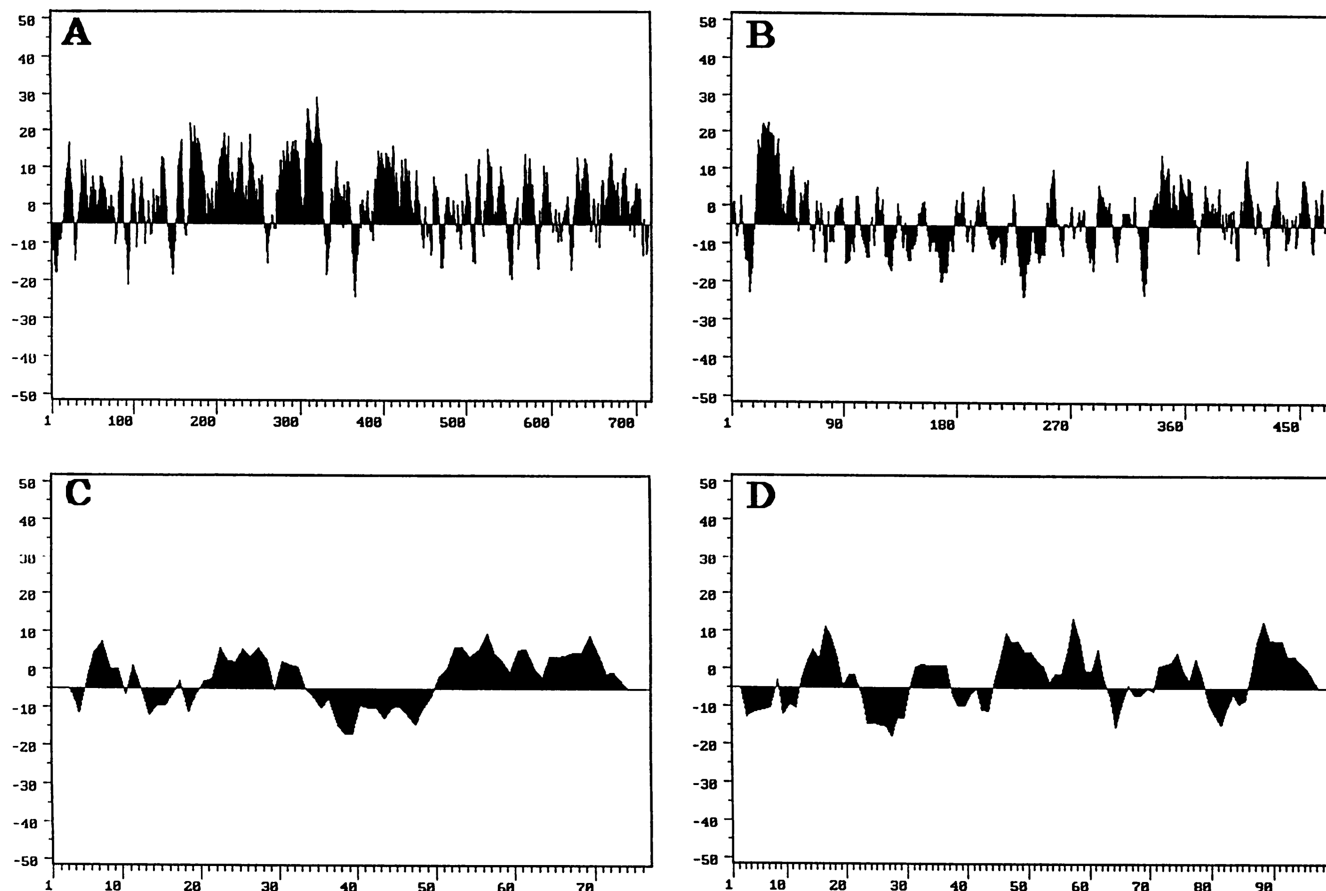


FIG. 4. Hydropathy plots of LcnC (A), LcnD (B), LcnA (C), and LciA (D). Hydropathy plots were calculated by the method of Kyte and Doolittle (32), with a calculation interval of 7 amino acid residues. *x* axis, amino acid residues; *y* axis, hydropathy index showing hydrophobicity above the baseline 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

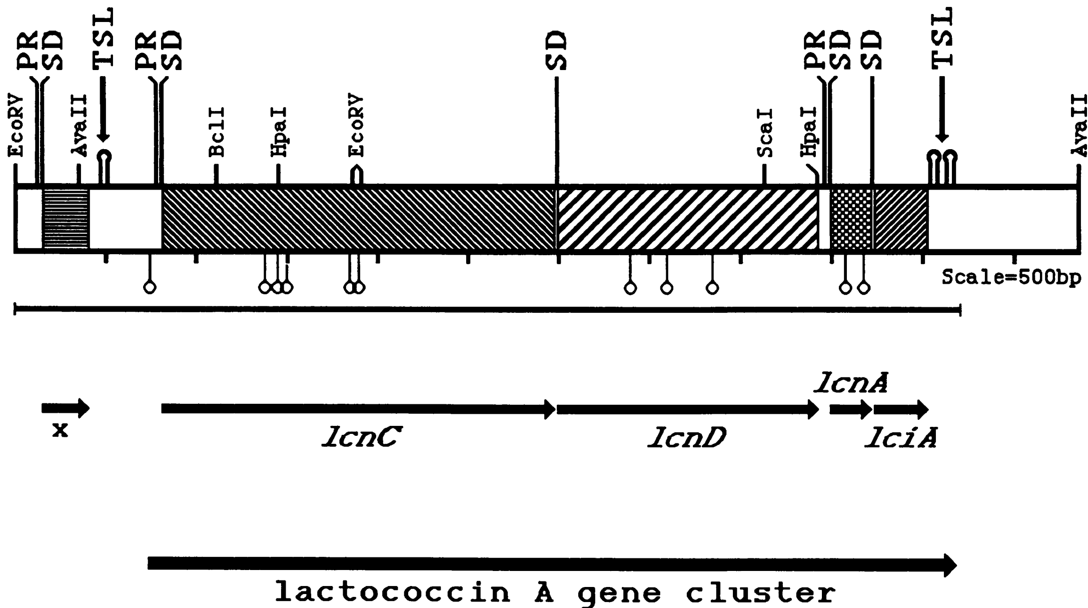


FIG. 5. Linear restriction map showing the sequenced regions (solid blunt-end line) of pLLM16-1, pLLM17, and pLLM20. The orientation and organization of the *lcnC*, *lcnD*, *lcnA*, and *lciA* genes; promoter regions (PR) and Shine-Dalgarno sequences (SD); rho-independent transcription termination stem-loop structures (TSL); the deduced location of the Tn*5* 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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