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Molecular Characterization of Genes Involved in the Production of the Bacteriocin Leucocin A from *Leuconostoc gelidum*

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Leucocin A is a small heat-stable bacteriocin produced by *Leuconostoc gelidum* UAL187. A 2.9-kb fragment of plasmid DNA that contains the leucocin structural gene and a second open reading frame (ORF) in an operon was previously cloned (J. W. Hastings, M. Sailer, K. Johnson, K. L. Roy, J. C. Vederas, and M. E. Stiles, J. Bacteriol. 173:7491–7500, 1991). When a 1-kb *Dra*I-*Hpa*I fragment containing this operon was introduced into a bacteriocin-negative variant (UAL187-13), immunity but no leucocin production was detected. Leucocin production was observed when an 8-kb *Sac*I-*Hin*dIII fragment of the leucocin plasmid was introduced into *L. gelidum* UAL187-13 and *Lactococcus lactis* IL1403. Nucleotide sequence analysis of this 8-kb fragment revealed the presence of three ORFs in an operon upstream of and on the strand opposite from the leucocin structural gene. The first ORF (*lcaE*) encodes a putative protein of 149 amino acids with no apparent function in leucocin A production. The second ORF (*lcaC*) contains 717 codons that encode a protein homologous to members of the HlyB family of ATP-binding cassette transporters. The third ORF (*lcaD*) contains 457 codons that encode a protein with marked similarity to LcnD, a protein essential for the expression of the lactococcal bacteriocin lactococcin A. Deletion mutations in *lcaC* and *lcaD* resulted in loss of leucocin production, indicating that LcaC and LcaD are involved in production and translocation of leucocin A. The secretion apparatus for lactococcin A did not complement mutations in the *lcaCD* genes to express leucocin A in *L. lactis*. However, lactococcin A production was observed when the structural and immunity genes for this bacteriocin were introduced into a leucocin producer of *L. gelidum* UAL187, indicating that lactococcin A could be exported by the leucocin A secretion machinery.

In recent years, numerous reports have been published on antimicrobial peptides or proteins produced by lactic acid bacteria (LAB). The potential for use of these naturally produced inhibitory substances, termed bacteriocins, for food preservation has created interest in their characterization. On the basis of biochemical and genetic studies, bacteriocins from LAB were divided by Klaenhammer (25) into four major classes, of which classes I and II are the best documented. Class I bacteriocins are lantibiotics which contain unusual amino acids, lanthionine and β-methyllanthionine. A prominent member of this class of bacteriocins is nisin, which is produced by *Lactococcus lactis* and is used as an important food preservative in many countries (9). For nisin to be produced, a polycistronic gene cluster of up to 15 kb of DNA that contains all of the genes necessary for its production, posttranslational modification, and secretion and for rendering the host cell immune to its own bacteriocin is required (11, 26, 37, 48). Class III and IV bacteriocins consist of large proteins and protein complexes associated with other chemical moieties, respectively.

Class II bacteriocins are characterized as small, heat-stable, hydrophobic peptides with a high isoelectric point. They are produced as precursors with an N-terminal extension of 18 to 24 amino acids. This extension is cleaved at the C terminus side of two glycine residues to give the mature bacteriocin. Sequence alignment of the N termini revealed a remarkable degree of similarity in their hydropathic profiles (13). The nucleotide sequences of the structural genes for several class II bacteriocins have been published, including pediocin PA-1/AcH (5, 30); sakacins A and P (20, 41); lactacin F (13, 31); leucocin A (16); lactococcins A, B, and M (22, 38, 44, 45); plantaricin A (10); and carnobacteriocins A, BM1, and B2 (33, 52). However, the additional genes necessary for bacteriocin production have been determined only for the lactococcins and pediocin PA-1/AcH, and in the case of the lactococcins, the gene for immunity has also been confirmed. Genetic characterization of the lactococcin and pediocin gene clusters indicates that they have similar features. They both have genes for bacteriocin production in an operon structure, although the structural and immunity genes for the lactococcins can be transcribed independently of the other genes in the operon. Furthermore, one of the genes in each of the lactococcin and pediocin operons encodes a protein which belongs to the HlyB family of ATP-binding cassette (ABC) transporters (19). This protein is thought to be involved in the signal sequence-independent secretion of the bacteriocins. Recently, genes encoding proteins which resemble members of a two-component signal transduction system which are involved in the expression of plantaricin A and sakacin A have been identified (2, 10).

Leucocin A is a bacteriocin produced by *Leuconostoc gelidum* UAL187 isolated from vacuum-packaged meat (17). It inhibits a wide spectrum of LAB as well as some strains of *Listeria monocytogenes* and *Enterococcus faecalis*. Curing experiments with UAL187 showed that the genetic determinant for leucocin A was located on one of the three plasmids found in this organism. The bacteriocin was purified and shown to contain 37 amino acids (16). A degenerate oligonucleotide probe was used for hybridization with plasmid DNA of UAL187-22, which has only two of the three plasmids, pLG7.6 and pLG9.2, and still produces bacteriocin (17). A 2.9-kb *Hpa*II fragment of pLG7.6 showing homology was cloned and sequenced, revealing the structural gene for leucocin A (*lcnA*) and a second open reading frame (ORF). It was postulated that this second ORF encodes an immunity protein (16). Leucocin A was shown to be produced as a precursor with a

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TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Description[a]	Reference or source
Strains		
Escherichia coli		
MH1	MC1061 derivative; *araD139 lacX74 galU galK hsr hsm*$^+$ *strA*	6
DH5α	F$^-$ ϕ80d*lacZ* ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17*(r_k^-, m_k^+) *supE44* λ$^-$ *thi-1 gyrA96 relA1*	BRL Life Technologies Inc.
Leuconostoc gelidum		
UAL187-22	Lca$^+$ Imm$^+$ containing native plasmids pLG9.2 and pLG7.6	17
UAL187-13	Lca$^-$ Imm$^-$ containing native plasmid pLG9.2	17
Carnobacterium piscicola		
LV17C	Plasmid free	1
Lactococcus lactis subsp. *lactis*		
IL1403	Plasmid free	7
Plasmids		
pUC118	*lacZ′*, Amp^r, 3.2 kb	50
pLG7.6	Lca$^+$ Imm$^+$, 18 kb	17
pJH6.1F	pUC118 containing 2.9-kb *Hpa*II fragment from pLG7.6, Amp^r, 6.1 kb	16
pMG36e	Em^r, 3.6 kb	47
pGKV210	Em^r, 4.4 kb	49
pMB553	Em^r, 5.1 kb; specifying lactococcin A	43
pMB500	Km^r, 18.2 kb; specifying lactococcins A and B	43
pMJ1	pGKV210 containing 2.9-kb *Hpa*II fragment from pJH6.1F, Em^r, 6.8 kb	This study
pMJ3	pGKV210 containing 1-kb *Hpa*I-*Dra*I fragment from pJH6.1F, Em^r, 5.4 kb	This study
pMJ4	pUC118 containing 12.3-kb *Hin*dIII fragment from pLG7.6, Amp^r, 15.5 kb	This study
pMJ6	pMG36e containing the 8-kb *Sac*I-*Hin*dIII fragment from pMJ4, Em^r, 11.6 kb	This study
pMJ10	pMG36e containing the 7.9-kb *Hin*dIII-*Nru*I fragment from pMJ4, Em^r, 11.4 kb	This study
pMJ16	*Eco*RV-*Bam*HI deletion derivative of pMJ6, Em^r, 10.6 kb	This study
pMJ17	*Bst*EII-*Stu*I deletion derivative of pMJ6, Em^r, 10.8 kb	This study
pMJ18	*Eco*RV-*Hin*dIII deletion derivative of pMJ6, Em^r, 8.7 kb	This study
pMJ20	Frameshift mutation in *Cla*I site of pMJ3, Em^r, 5.4 kb	This study
pMJ26	Frameshift mutation in *Nsi*I site of pMJ6, Em^r, 11.6 kb	This study

[a] Lca$^+$, producing leucocin A; Lca$^-$, not producing leucocin A; Imm$^-$ and Imm$^+$, sensitivity and immunity to leucocin A, respectively; Amp^r, Em^r, and Km^r, resistance to ampicillin, erythromycin, and kanamycin, respectively.

24-amino-acid N-terminal extension. Transformation of several LAB with constructs containing the 2.9-kb fragment did not show production of leucocin A. UAL187-13, a cured, bacteriocin-negative derivative of the wild-type strain, was refractory to transformation.

To prevent confusion with nomenclature used for the genes involved in the expression of lactococcins, *lcnA* and ORF2 (16) have been renamed *lcaA* and *lcaB*, respectively. In this paper, we report the cloning and nucleotide sequence analysis of a second operon which is located adjacent to, and on the strand opposite from, the *lcaAB* operon. A construct containing the two operons was successfully transferred into *L. gelidum* UAL187-13 and resulted in leucocin production.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown in TY broth (34) at 37°C; *L. lactis* was grown in glucose-M17 broth (40) at 30°C; and *L. gelidum* and *Carnobacterium piscicola* were grown in APT broth (All Purpose Tween; Difco Laboratories Inc., Detroit, Mich.) at 25°C. Broth media were supplemented with 1.2% (wt/vol) agar for solid plating media. Selective concentrations of erythromycin for growth of *E. coli*, *L. lactis*, and *L. gelidum* containing recombinant plasmids were 200, 5, and 5 μg/ml, respectively. When appropriate, ampicillin was used at a final concentration of 150 μg/ml for *E. coli*, and kanamycin was used at a final concentration of 50 μg/ml for *L. lactis.*

Bacteriocin assay. To test for production of leucocin, cells of *L. gelidum* or *L. lactis* were inoculated, unless otherwise stated, onto APT and glucose-M17 agar plates, respectively, and incubated at 25°C for 18 h. Soft APT agar (0.7% [wt/vol]) containing *C. piscicola* LV17C as the indicator strain was then poured onto the surface. After 15 h of incubation, the plates were examined for zones of inhibition. Immunity or resistance of the different strains to leucocin was determined by a spot-on-lawn test of 0.5 μg of the bacteriocin (1). Lactococcin production was tested as described above with *L. lactis* IL1403 as the indicator strain in soft glucose-M17 agar (0.7% [wt/vol]).

Molecular cloning. Plasmids from *E. coli* were isolated by the method described by Birnboim and Doly (3). With some modifications, the same method was used to isolate plasmids from *L. gelidum* and *L. lactis*. Cells were lysed at 37°C in 50 mM Tris-HCl (pH 8)–10 mM EDTA containing 5 mg of lysozyme and 100 μg of mutanolysin (Sigma, St. Louis, Mo.) per ml for 20 min. Restriction endonucleases, the Klenow fragment of the *E. coli* DNA polymerase I, and T4 DNA ligase were obtained from Promega (Madison, Wis.), Bethesda Research Laboratories (Burlington, Ontario, Canada), Boehringer Mannheim (Dorval, Quebec, Canada), or New England Biolabs (Mississauga, Ontario, Canada) and used as recommended by the supplier. Cloning and DNA manipulations were performed as described by Sambrook et al. (35). Competent *E. coli* cells were transformed by the method of Mandel and Higa (29). Transformation of *L. lactis* by electroporation was performed with a Bio-Rad gene pulser (Bio-Rad Laboratories, Richmond, Calif.) by the method of Holo and Nes (21). For transformation of *L. gelidum*, cells were cultivated in APT broth supplemented with 3% (wt/vol) glycine. Exponentially growing cells were harvested, washed once with water and twice with ice-cold electroporation buffer (5 mM potassium phosphate buffer [pH 7], 3 mM $MgCl_2$, in 1 M sucrose), and concentrated 100-fold in the same buffer. Subsequently, 50 μl of the cell suspension was mixed with 2 μl of plasmid DNA and held on ice for 5 min prior to electroporation. Immediately after electroporation, cells were diluted in 1 ml of APT containing 0.5 M sucrose and 20 mM $MgCl_2$ and incubated for 3 h at 25°C. Cells were plated on APT agar containing the appropriate antibiotic, and transformants were visible after 3 to 4 days of incubation at 25°C.

Southern hybridization. For Southern hybridization, DNA was transferred to Hybond N (Amersham Canada Ltd., Oakville, Ontario, Canada), as described by Sambrook et al. (35). Nonradioactive DNA probes were made with a random-primed labeling and detection kit (Boehringer Mannheim). Hybridization and immunological detection were performed as recommended by the supplier.

DNA sequencing. Nucleotide sequence analysis was performed by sequencing the DNA in both orientations by the dideoxy-chain method of Sanger et al. (36). DNA was sequenced by *Taq* DyeDeoxy Cycle sequencing on an Applied Biosystems 373A DNA sequencer (Applied Biosystems, Foster City, Calif.). For sequencing, stepwise deletion derivatives of cloned DNA fragments were made

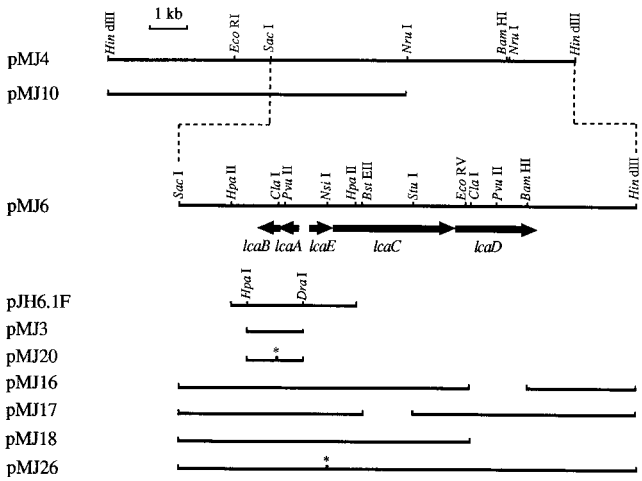

FIG. 1. Schematic representation of the 12.3-kb *Hin*dIII insert of pMJ4 and its subclones. Partial restriction maps of some of the inserts are shown. Not all of the *Hpa*II restriction sites on the insert of pMJ6 are indicated. The positions and direction of transcription of *lcaA*, *lcaB*, *lcaC*, *lcaD*, and *lcaE* on the insert of pMJ6 are shown. The asterisks on pMJ20 and pMJ26 indicate frameshift mutations of *lcaB* and *lcaE*, respectively.

with the Erase-a-Base system from Promega. In addition, a primer-walking strategy was used for nucleotide sequencing. Synthetic oligonucleotides were made with an Applied Biosystems 391 PCR-Mate DNA synthesizer. Analysis of the nucleotide sequence was performed with a software program (DNASTAR, Inc., Madison, Wis.). The search for homology of the predicted amino acid sequences with those of proteins in the Swiss-Prot protein sequence database (release 30) was based on the FASTA algorithm of Pearson and Lipman (32).

Nucleotide sequence accession number. The nucleotide sequence presented in this paper was submitted to GenBank (Los Alamos, N.Mex.) and was given the accession number L40491.

RESULTS

Cloning of the genes involved in production of leucocin A. The 2.9-kb *Hpa*II fragment containing the *lcaAB* operon was previously cloned in pUC118, resulting in pJH6.1F (Fig. 1), and in the shuttle vector pNZ19 to form the plasmid pJH8.6L. Attempts to transform *L. gelidum* UAL187-13 with pJH8.6L were unsuccessful (16). Therefore, a different vector was used to introduce the 2.9-kb fragment into strain UAL187-13. With the multiple cloning site of pUC118, the 2.9-kb insert in plasmid pJH6.1F was excised by digestion with *Eco*RI and *Hin*dIII and cloned into the *Eco*RI-*Hin*dIII sites of pGKV210. The resulting plasmid, pMJ1, was used to transform strain UAL187-13. However, all of the transformants examined showed the presence of spontaneous deletion derivatives of pMJ1. When a 1-kb *Dra*I-*Hpa*I fragment containing *lcaA* and *lcaB* was subcloned from the 2.9-kb fragment into the *Sma*I site of pGKV210, the resulting recombinant plasmid, pMJ3 (Fig. 1), formed a stable transformant in *L. gelidum* UAL187-13. This transformant was immune to leucocin A but did not produce the bacteriocin. Apparently, additional information encoded on pLG7.6 is required for expression of the bacteriocin phenotype. The plasmid pMJ20 (Fig. 1) was constructed by introducing a frameshift mutation in *lcaB*, by filling in the unique *Cla*I site with Klenow DNA polymerase. Immunity was not observed for UAL187-13 carrying this plasmid, indicating that *lcaB* encodes the protein necessary for immunity to leucocin A.

Because additional genetic information is required for leucocin A production, regions adjacent to the *lcaAB* operon (Fig. 1) were cloned. It was previously reported that the producer strain UAL187-22 contains plasmids pLG7.6 and pLG9.2 of 7.6 and 9.2 MDa, respectively (17). Restriction analysis of plasmid DNA from UAL187-22 revealed that the actual sizes of pLG7.6 and pLG9.2 were 18 and 21 kb, respectively (data not shown). To localize the *lcaAB* genes, Southern analysis of plasmid DNA with the 1-kb *Dra*I-*Hpa*I fragment as probe detected a 12.3-kb *Hin*dIII fragment that was cloned into pUC118 to give pMJ4. Subclones of this fragment in a shuttle vector gave rise to plasmids pMJ6 and pMJ10 containing an 8-kb *Sac*I-*Hin*dIII insert and a 7.9-kb *Hin*dIII-*Nru*I insert, respectively (Fig. 1).

Plasmids pMJ6 and pMJ10 were transformed into *L. lactis* IL1403 and screened for leucocin A production. Transformants containing pMJ6 but not pMJ10 inhibited the growth of the indicator strain *C. piscicola* LV17C. However, the zones of inhibition of these transformants were clearly smaller than those formed by *L. gelidum* 187-22 (Fig. 2A). *L. lactis* is naturally resistant to leucocin (data not shown); therefore, the phenotype of immunity to leucocin A could not be detected in *L. lactis*. Transformation of the bacteriocin-negative strain *L. gelidum* UAL187-13 with pMJ6 resulted in several transformants containing deletion derivatives of pMJ6 that did not produce the bacteriocin. A transformant of UAL187-13 which contained a plasmid with the expected size and restriction pattern of pMJ6 produced a zone of inhibition comparable to that formed by UAL187-22 (Fig. 2A). These results indicate that the genes responsible for the production of leucocin A are located on an 8-kb *Sac*I-*Hin*dIII fragment of pLG7.6.

Nucleotide sequence analysis. Restriction analysis of pMJ6 revealed the location and orientation of the *lcaAB* operon on the 8-kb fragment (Fig. 1). The nucleotide sequence of the region upstream of the *lcaAB* operon was determined in both directions by the dideoxy-chain termination method. The nucleotide sequence in Fig. 3 shows a 4.3-kb segment located adjacent to the previously reported nucleotide sequence containing the *lcaAB* operon as well as part of this previously reported nucleotide sequence (16). The start of an ORF was identified 151 bases from, and on the strand opposite to, the start codon of *lcaA*. This ORF, designated *lcaE*, could encode

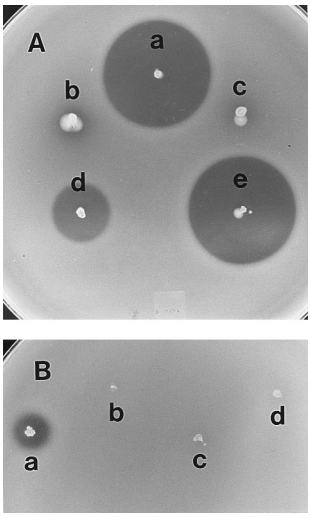

FIG. 2. Deferred inhibition of leucocin A transformants with *C. piscicola* LV17C as the indicator strain (A) and lactococcin A transformants with *L. lactis* IL1403 as the indicator strain (B). (A) a, *L. gelidum* UAL187-22; b, *L. lactis* IL1403; c, *L. gelidum* UAL187-13; d, *L. lactis* IL1403 (pMJ6); e, *L. gelidum* UAL187-13 (pMJ6). APT was used as solid medium. (B) a, *L. gelidum* UAL187-22 (pMB553); b, *L. gelidum* UAL187-13 (pMB553); c, *L. gelidum* UAL187-22; d, *L. gelidum* UAL187-13. Glucose-M17 was used as solid medium.

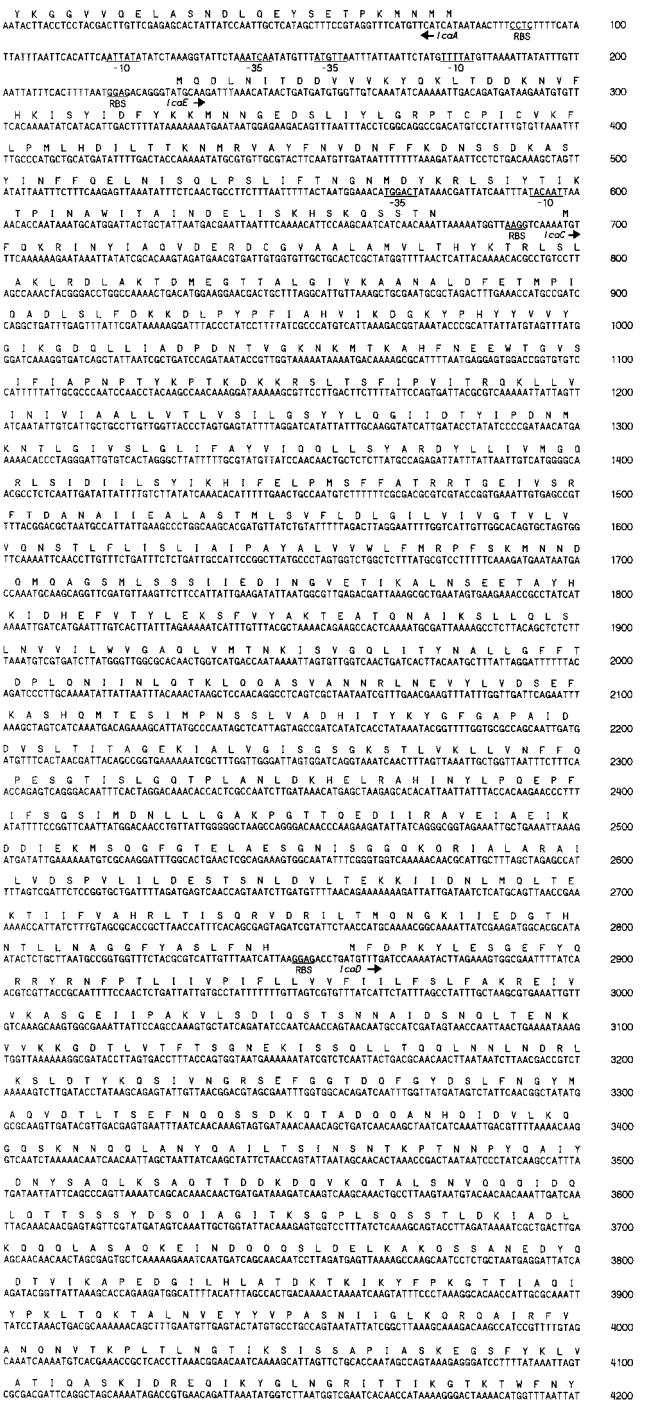

FIG. 3. Nucleotide sequence of a 4.7-kb fragment of plasmid pLG7.6 containing the *lcaECD* gene cluster and the 5′ part of *lcaA*. The first 384 bp of the sequence is part of the *lcaAB* operon sequence previously reported by Hastings et al. (16). The directions of transcription of the different genes are indicated by arrows. The deduced amino acid sequences for *lcaA*, *lcaE*, *lcaC*, and *lcaD* are shown above the nucleotide sequence. Potential −35 and −10 promoter regions and possible ribosome binding sites (RBS) are indicated. The inverted repeat downstream of *lcaD* which might act as a rho-independent terminator is indicated by reversed arrows.

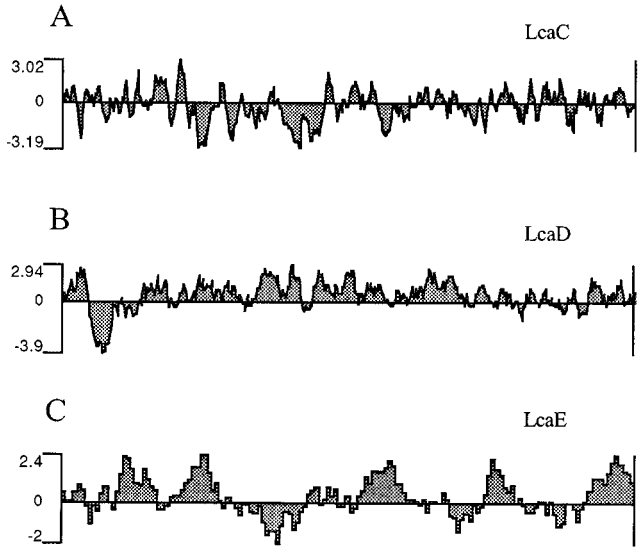

FIG. 4. Hydropathy plots of LcaC (A), LcaD (B), and LcaE (C). Hydropathy plots were calculated by the method of Kyte and Doolittle (27). *x* axis, amino acid residues; *y* axis, hydropathy index showing hydrophilicity above and hydrophobicity below the baseline.

a protein of 149 amino acids and is followed by a TAA stop codon. Immediately downstream of *lcaE*, a second ORF (*lcaC*) that contains 717 codons was found. The TAA stop codon of *lcaC* is followed immediately by an ORF that could encode a protein of 457 amino acids and has a TAG stop codon. All three of the ORFs are preceded by probable ribosomal binding sites. A possible promoter sequence was found upstream of *lcaE* (Fig. 3). A putative promoter sequence was also found within the *lcaE* gene (Fig. 3). The sequence of its −35 (TGGACT) and −10 (TACAAT) regions closely resembles the consensus sequence of constitutive promoters found in other LAB (46). The spacing of 16 and 19 bases between the −35 and −10 regions of these promoter sequences agrees with that of the usual spacing found in LAB promoters. An imperfect inverted repeat was found 6 bases downstream of the stop codon of *lcaD*, which has the characteristics of a possible rho-independent terminator. No other ORFs or palindromic structures were found in either strand in the 4.6-kb region upstream of *lcaA*.

Similarity of LcaC and LcaD to bacterial transport proteins. The hydrophobicity plot of the putative LcaC protein revealed that the N-terminal region contains several hydrophobic domains (Fig. 4A). A homology search with other amino acid sequences in the SwissProt database showed that LcaC belongs to the HlyB-like family of ABC transporters (4, 19). These proteins contain a highly conserved ATP-binding domain in the C-terminal region and several membrane-spanning domains in the N-terminal half of the sequence. Homology comparison of HlyB, which is involved in the secretion of hemolysin A, and LcaC revealed that 58% of the amino acids were similar when conserved residue substitutions were included and 27% were identical. However, LcaC has a much higher degree of homology with several other ABC transporters. ComA, a protein from *Streptococcus pneumoniae* that is required for competence induction for genetic transformation (23), shares 82% similarity and 59% identity with LcaC. Comparison of LcaC with LcnC, a protein that is implicated in the secretion of the lactococcal bacteriocin lactococcin A and possibly in the secretion of lactococcins B and M (38, 42), and

PedD, which is involved in the production of pediocin PA-1 (30), revealed 81% similarity and 57% identity and 73% similarity and 50% identity at the amino acid level, respectively. The data bank search showed further that LcaC was very homologous to SapT (82% similarity, 57% identity) and SppT (81% similarity, 58% identity), proteins that are encoded by DNA sequences linked to sakacins A and P, respectively. The highest score however, was found with MesD, a protein encoded in a DNA sequence linked to mesentericin Y105 (18) that was nearly identical to LcaC, with 99% similarity and 98% identity.

Analysis of the hydropathy profile of LcaD showed a largely hydrophilic protein with the exception of a strong hydrophobic region at the N terminus (Fig. 4B). A homology search in the data bank revealed that LcaD is similar to LcnD, another protein that is essential for lactococcin production in *L. lactis* (38). LcaD showed 54% similarity and 35% identity to LcnD. Additional homologs of LcaD that were found were SapE (65% similarity, 35% identity), SppE (62% similarity, 32% identity), and MesE (96% similarity, 87% identity), whose genes are linked to the genetic determinants for sakacins A and P and mesentericin Y105, respectively. Other proteins with similarity to the LcaD protein were ComB from *S. pneumoniae* (24) with 61% similarity and 29% identity and the ORF1 protein encoded by *Lactobacillus johnsonii* (13). The ORF1 protein has similarity with the N and C termini of LcaD (data not shown). The ORF1 protein is encoded by a 5′-end-truncated ORF of 540 bases located upstream of the bacteriocin operon responsible for lactacin F production (13).

The hydropathy profile of the putative protein LcaE indicated that this is a hydrophilic protein (Fig. 4C). A search of the data bank revealed only homology of LcaE to MesC (85% similarity, 70% identity), a protein encoded by a DNA sequence associated with mesentericin Y105 production.

Functional and complementation analyses of LcaC and LcaD. To establish whether *lcaE*, *lcaC*, and *lcaD* are essential for leucocin production, deletion and mutation derivatives of pMJ6 were constructed in *E. coli* (Fig. 1). Deletion of the *Bst*EII-*Stu*I fragment in *lcaC* resulted in plasmid pMJ17. Cells of *L. gelidum* UAL187-13 containing this construct were immune to leucocin, but bacteriocin was not produced. If it is assumed that the deletion did not have a polar effect on *lcaD*, the result would indicate that *lcaC* is involved in leucocin production. Two deletion constructs in *lcaD* were made, namely, pMJ16 and pMJ18. In plasmid pMJ16, an *Eco*RV-*Bam*HI fragment was deleted, whereas an *Eco*RV-*Hin*dIII fragment was deleted in pMJ18. A frameshift mutation in *lcaE* was made with the *Nsi*I restriction site, giving plasmid pMJ26. Several attempts to introduce pMJ16, pMJ18, and pMJ26 into UAL187-13 were unsuccessful. When pMJ16 and pMJ17 were introduced into *L. lactis* IL1403, bacteriocin production was not detected. However, transformation of *L. lactis* IL1403 with pMJ26 did not affect leucocin production. These results indicate that LcaD, but not LcaE, is essential for leucocin production. Given the high degree of similarity between LcaC and LcaD of *L. gelidum* and LcnC and LcnD of *L. lactis*, it was decided to determine whether the mutations in *lcaC* and *lcaD* could be complemented by the lactococcin A gene cluster in *L. lactis* IL1403 carrying pMB500 (38, 43). Plasmids pMJ3, pMJ16, and pMJ17 were used to transform IL1403(pMB500). Although the different plasmids contain the same replicon as pMB500, transformants can be selected for erythromycin resistance and pMB500 can be selectively retained by its own lactococcin production and resistance to kanamycin. However, leucocin production was not observed in these transformants, indicating that proper complementation by the lactococcin secretion apparatus was not possible. Only transformation of IL1403(pMB500) with pMJ6 resulted in a zone of inhibition (data not shown). In contrast, transformation of *L. gelidum* UAL187-22 with plasmid pMB553, which carries the structural and immunity genes for lactococcin A, showed a small zone of inhibition with *L. lactis* IL1403 as an indicator (Fig. 2B). Lactococcin A is active only against lactococci (22). No such zone of inhibition was observed when UAL187-13 was transformed with pMB553. Apparently, the leucocin secretion system is able to complement the *lcnC* and *lcnD* genes for the secretion of lactococcin A to a limited extent.

DISCUSSION

For expression of leucocin in the bacteriocin-negative variant *L. gelidum* UAL187-13, the presence of the *lcaAB* operon described by Hastings et al. (16) as well as *lcaC* and *lcaD* described in this paper is required. A definite but reduced level of leucocin production was also detected with the transformed heterologous host strain *L. lactis* IL1403. The reason why bacteriocin production in *L. lactis* was not as high as in *L. gelidum* is not clear. Either the expression of the genes involved in leucocin production is low, or the gene products do not function in *L. lactis* as well as they do in *L. gelidum*, or there is a difference in the copy number of the plasmid. The gene clusters involved in the expression of lactococcins and pediocin PA-1/AcH are organized in a single operon-like structure, with the secretion genes preceding the structural and immunity genes for lactococcin A (38) and following the structural gene for pediocin PA-1/AcH (5, 30). Interestingly, for leucocin A the two operons involved in bacteriocin production and secretion are located on opposite strands and they are transcribed divergently. It was postulated by Hastings et al. (16) that the ORF downstream of the structural gene for leucocin, *lcaB*, was the immunity gene. This was confirmed in this study by a frameshift mutation in *lcaB*. Such an arrangement of an immunity gene following the bacteriocin structural gene was also reported for the lactococcin A, B, and M operons (44, 45). Mutation analyses showed that *lcaC* and *lcaD* were essential for leucocin production in *L. lactis*, but not *lcaE*. In *L. gelidum*, LcaC was required for bacteriocin production. It was not possible to obtain transformants of *L. gelidum* containing constructs with a mutation in *lcaD* or *lcaE*. This could mean either that such mutations are lethal for *L. gelidum* or that the unsuccessful transformation of UAL187-13 with those mutation derivatives was due to the poor transformability of *L. gelidum* with plasmids exceeding 8 kb (data not shown).

Homology comparisons of LcaC and LcaD with other proteins showed distinct similarities with secretion proteins that differ from those involved in the general signal sequence-dependent export pathway. These proteins form a dedicated transport apparatus for certain proteins that lack a typical N-terminal signal peptide. A typical representative of this family is the HlyB-HlyD secretion apparatus for hemolysin A in *E. coli* (12, 51). HlyB belongs to a family of ATP-dependent membrane translocator proteins (4). The genes for HlyB and HlyD are closely linked to the structural gene for hemolysin and are both necessary for hemolysin secretion. A genetically linked secretion apparatus similar to that of HlyB- and HlyD-type gene products has also been described for several other secretion systems in gram-negative bacteria, including colicin V of *E. coli* (14), leukotoxin of *Pasteurella haemolytica* (39), cyclolysin of *Bordetella pertussis* (15), and proteases of *Erwinia chrysanthemi* (28). Proteins similar to the HlyB-HlyD family of secretion proteins have also been described for lactococcin A (38). A similar secretion apparatus is encoded on the leucocin

plasmid pLG7.6 for leucocin A. LcaC contains a highly conserved ATP-binding domain in the C-terminal 200 amino acids, and it has several hydrophobic domains toward the N terminus. A high degree of homology of LcaC with several other ABC transporters including ComA, PedD, SapT, SppT, and MesD is also observed. The marked similarity between these proteins is found not only in the ATP-binding domain but also in the hydrophobic N-terminal region (data not shown). Six membrane-spanning segments are postulated for ComA (23). These data indicate that LcaC belongs to the family of ABC transporters.

As stated earlier, LcaD is structurally similar to the HlyD homolog from gram-negative bacteria. The protein is largely hydrophilic with a hydrophobic section at the N terminus which could function as a membrane anchor. The HlyD homolog for the protease B secretion system, PrtE, has been shown to be inserted in the inner membrane by its N terminus (8). However, the amino acid sequences of the HlyD homologs from gram-negative bacteria show only a limited extent of homology. Interestingly, the amino acid sequences of the HlyD homologs LcaD, LcnD, SapE, SppE, and MesE, which are involved in bacteriocin production, and of ComB share significant homology. When LcaD was compared with other protein sequences from the database, additional similarity to LcaD was found for the product of ORF1 of *L. johnsonii*. Only the last 180 codons could be determined after cloning of the fragment containing ORF1 (13, 31). The deduced amino acid sequence of ORF1 showed homology only with the N- and C-terminal parts of LcaD. This indicates either that the ORF1 product is different in size compared with LcaD or that the cloning of the fragment resulted in a deletion in ORF1. ComB from *S. pneumoniae* is a protein encoded by *comB* located immediately downstream of the gene for the ABC transporter ComA (23, 24). It is speculated that ComA and ComB, which are both required for competence induction, might be involved in the secretion of a competence factor which is probably a small protein (23).

LcaE did not appear to be required for bacteriocin production and showed homology only to a protein that is genetically linked to the bacteriocin mesentericin Y105. The role of this protein remains unclear. The fact that LcaC, LcaD, and LcaE are very homologous to similar proteins that are genetically linked to mesentericin Y105 is not surprising. Leucocin A differs from mesentericin Y105 in only two residues within its sequence. However, the overall similarity of the proteins linked to leucocin A and mesentericin Y105 production is remarkable given the different sources of origin of the producer bacteria. *L. gelidum* UAL187 was isolated from vacuum-packaged meat in Canada (17), and mesentericin Y105 is produced by a strain of *Leuconostoc mesenteroides* isolated from goat's milk in France (18).

It is intriguing to note that both LcaC and LcaD share a high degree of homology with several proteins that form a dedicated secretion system consisting of an ABC transporter and a second protein which is most likely attached to the membrane. The precise function of the LcaD-type proteins is not known, but they might be involved in facilitating secretion of the bacteriocin. The similarity between the LcaC-LcaD secretion system for leucocin and that for others, such as lactococcins, indicates that bacteriocin expression with heterologous secretion systems should be feasible. However, *lcaC* and *lcaD* could not be complemented by *lcnC* and *lcnD* to produce leucocin in *L. lactis*, in contrast to the partial complementation observed when lactococcin A was produced in UAL187-22. The reason for this difference in complementation of heterologous secretion proteins is unclear. Class II bacteriocins are all produced as precursors with an N-terminal extension. These N termini are very similar in their amino acid sequences and hydropathic profiles, and they contain a Gly-Gly processing site at positions -1 and -2 of the cleavage site. The role of these N termini in recognition of the prebacteriocin by the secretion apparatus is under investigation. Further study of the factors that determine recognition, secretion, and processing of bacteriocins by the secretion apparatus will facilitate the development of heterologous secretion systems for these compounds.

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