APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1995, p. 1371–1377 Vol. 61, No. 4
0099-2240/95/$04.00+0

Heterologous Expression of the Lactacin F Peptides by *Carnobacterium piscicola* LV17†

G. E. ALLISON,[1] R. W. WOROBO,[2] M. E. STILES,[2] AND T. R. KLAENHAMMER[1,3]*

Departments of Microbiology[1] and Food Science,[3] Southeast Dairy Foods Research Center, North Carolina State University, Raleigh, North Carolina 27695-7624, and Department of Agricultural, Food, and Nutritional Science,[2] University of Alberta, Edmonton, Alberta, Canada T6G 2P5

Received 17 October 1994/Accepted 21 December 1994

The lactacin F complex, composed of LafA and LafX peptides, is produced by *Lactobacillus johnsonii* VPI 11088 and is active against five other *Lactobacillus* species and *Enterococcus faecalis*. The genetic determinants encoding the lactacin F complex are organized in a 1-kb polycistronic operon which comprises three genes, *lafA*, *lafX*, and ORFZ (encoding the putative immunity protein). The *lafA* and *lafX* genes encode the bacteriocin precursors with N-terminal extensions characterized by a Gly-Gly^{-1}*Xaa^{+1} cleavage site (*). The Gly-Gly motif is conserved in several other bacteriocins, including carnobacteriocins A, BM1, and B2. *Carnobacterium piscicola* LV17 produces carnobacteriocins which are active against *Listeria monocytogenes* and other lactic acid bacteria. In this study, the lactacin F operon was introduced into *C. piscicola* LV17. The transformants produced lactacin F concurrently with the carnobacteriocins. When the *lafA* and *lafX* genes were separated and cloned individually into LV17, production of either LafA or LafX by *C. piscicola* LV17 was detected by complementation with *L. johnsonii* clones producing LafX or LafA, respectively. Transformants of *C. piscicola* LV17 which produced lactacin F, LafA, or LafX, in combination with the carnobacteriocins, were assayed for an increased and expanded inhibitory spectrum. The recombinant organisms were only active against lactacin F- and carnobacteriocin-sensitive strains. A plasmidless derivative of LV17 which does not produce the carnobacteriocins failed to produce lactacin F, LafA, or LafX when transformed with the appropriate recombinant plasmids. The ability of *C. piscicola* LV17 to produce lactacin F demonstrates that the machinery for the carnobacteriocins is capable of processing and exporting bacteriocins from both systems.

Growing interest in new and improved preservatives for food and dairy products has fueled investigation of bacteriocins produced by lactic acid bacteria. An explosion of research in this area has provided a wealth of new information focusing on three major areas: identification of new bacteriocins with broad inhibitory spectra; characterization of the processing, excretion, and regulatory mechanisms; and discovery of the mode of action. Bacteriocins are proteinaceous antimicrobial compounds (36). On the basis of their biochemical characteristics, bacteriocins have been categorized into four different classes (16). The class II bacteriocins are produced by a wide variety of genera, yet they share the following biochemical characteristics: heat stability, the presence of small membrane-active peptides predicted to form amphiphilic helices or beta sheets, the absence of dehydro amino acid residues and lanthionine bridges, and a bacteriocin precursor characterized by a Gly-Gly^{-1} proteolytic processing site at the C terminus of the N-terminal extension. Examples of class II bacteriocins include pediocin PA-1; lactococcins A, B, G, and M; sakacins A and P; curvacin A; leucocin A; lactacin F; carnobacteriocins A, BM1, and B2, and others (reviewed in reference 16).

The leader peptides of class II bacteriocins share many features. These N-terminal sequences are usually 18 to 24 amino acids long (reviewed in reference 16) but are not typical of those cleaved by classic signal peptidases (14, 38, 39). The sequence similarity among many of the class II bacteriocin N-terminal extensions is striking and includes the following: hydrophobic residues in positions −4, −7, −12, and −15; a core of charged amino acids in positions −8 to −10; and polar or charged residues at the N terminus (10, 28). Furthermore, the N-terminal extensions of many bacteriocin precursors have specific amino acid residues conserved in certain positions: Gly^{-1}, Gly^{-2}, Ile^{-4}, Leu^{-7}, Glu^{-8}, Ser^{-11}, and Leu^{-12} (13, 28). Of all of the class II bacteriocins, the N-terminal leader sequences of the lactacin F peptides and the carnobacteriocins (A, BM1, and B2) have the greatest similarity, suggesting that systems for proteolytic processing and export may be interchangeable.

Lactacin F, produced by *Lactobacillus johnsonii* VPI 11088 (ATCC 11506), is a membrane-active bacteriocin consisting of two peptides, LafA and LafX (1, 4, 10). The lactacin F operon is composed of three genes. The bacteriocin structural genes, *lafA* and *lafX*, are adjacent to one another and followed by ORFZ, which encodes the putative immunity protein (10, 23). *Carnobacterium piscicola* LV17 (NCFB 2855) was originally isolated as an atypical *Lactobacillus* strain in a study of the bacterial composition of vacuum-packaged meat by Shaw and Harding (34) and later identified as *C. piscicola* by Ahn and Stiles (2). *C. piscicola* LV17 produces three bacteriocins, carnobacteriocins A, BM1, and B2. Carnobacteriocins A and B2 are encoded by plasmids pCP49 and pCP40, respectively, whereas BM1 is encoded from a genomic fragment (2, 28, 41). While different in ecological origins and fermentation characteristics, these two species produce bacteriocins with very similar N-terminal extensions.

The objective of this study was to determine if lactacin F could be expressed in the bacteriocin-producing strain of *C. piscicola*.

* Corresponding author.

† Paper number FSR94-43 of the Journal Series of the Department of Food Science, Southeast Dairy Foods Research Center, North Carolina State University, Raleigh, NC 27695-7624.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Reference or source
Strains		
L. johnsonii		
VPI 11088 (ATCC 11506)	LafAX$^+$ Laf^r Cbn^r, parental strain	VPI[b]; 22
NCK65	88-4; LafAX$^-$ Laf^s Cbn^r, VPI 11088 derivative	22, 23
NCK719	NCK65(pTRK386), LafAX$^+$ Em^r	This study
NCK639	NCK65(pTRK203), LafA$^+$ Em^r	10
NCK659	NCK65(pTRK92), LafX$^+$ Em^r	4
C. piscicola		
LV17 (NCFB 2855)	CbnAB$^+$ Cbn^r Laf^r, parental strain	NCFB[c]; 2
LV17C	CbnAB$^-$ Cbn^s Laf^r, plasmidless mutant	2
NCK493	LV17C(pTRK201), CbnAB$^-$ Cbn^s LafAX$^-$	This study
NCK720	LV17(pTRK386), CbnAB$^+$ Cbn^r LafAX$^+$ Laf^r	This study
NCK721	LV17C(pTRK386), CbnAB$^-$ Cbn^s LafAX$^-$ Laf^r	This study
NCK722	LV17(pTRK92), CbnAB$^+$ Cbn^r LafX$^+$	This study
NCK724	LV17C(pTRK92), CbnAB$^-$ Cbn^s LafX$^-$	This study
NCK723	LV17(pTRK203), CbnAB$^+$ Cbn^r LafA$^+$	This study
NCK725	LV17C(pTRK203), CbnAB$^-$ Cbn^s LafA$^-$	This study
C. divergens LV13 (NCFB 2852)	Cbn^s, carnobacteriocin indicator	NCFB; 2
Lactobacillus bulgaricus 1489	Laf^s, lactacin F indicator	NCDO[d]; 22
L. delbrueckii subsp. *lactis* ATCC 4797	Laf^s, lactacin F indicator	ATCC[e]; 22
Lactobacillus fermentum 1750	Laf^s, lactacin F indicator	NCDO; 22
L. helveticus 87	Laf^s, lactacin F indicator; LafA^s, LafA indicator	NCDO; 4, 22
Lactobacillus lactis 970	Laf^s, lactacin F indicator	NCDO; 22
E. faecalis ATCC 19433	Laf^s Cbn^s	ATCC; 2, 22
L. plantarum C-11	Plantaricin A$^+$	M. A. Daeschel[f]
Lactococcus lactis IL1403	Lactococcin A sensitive	5
L. monocytogenes ATCC 19115	Serotype 4b	ATCC
L. monocytogenes F5069	Serotype 4b, isolated from raw milk	C. Donnelley[g]
L. monocytogenes Scott A	Serotype 4b, isolated from patient	C. Donnelley
P. pentosaceus FBB61/2	Pediocin A$^-$ pediocin A sensitive	6
E. coli SURE	e14$^-$ (*mcrA*) Δ(*mcrCB-hsdSMR-mrr*)*171 sbcC recB recJ umuC*::Tn*5* (Kan^r) *uvrC supE44 lac gyrA96 relA1 thi-1 endA1* [F′ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^r)]	Stratagene
E. coli NCK718	*E. coli* SURE(pTRK386)	This study
Plasmids		
pBluescript KS$^+$	3.0 kb, Am^r	Stratagene
pTRK160	pBluescript KS$^+$::2.3-kb *Eco*RI (*lafA*$^+$ *lafX*$^+$ ORFZ$^+$), 5.3 kb	10, 23
pTRKH2	6.9 kb, *lacZ* Em^r	26
pTRK92	pTRKH2::0.7-kb *Dra*I-64 PCR product (*lafA729 lafX*$^+$), 7.6 kb, Em^r	4
pTRK386	pTRKH2::2.3-kb *Eco*RI (*lafA*$^+$ *lafX*$^+$ ORFZ$^+$), 9.2 kb	This study
pGKV210	4.4 kb, Em^r, promoterless *cat86*	37
pTRK201	pGKV210::0.89-kb *Eco*RI-*Pvu*II pTRK160 (*lafA886*), 5.29 kb, Em^r Cm^r	10
pTRK203	pGKV210::1.05-kb *Eco*RI-*Hae*III pTRK160 (*lafA*$^+$ *lafX1047*), 5.45 kb, Em^r Cm^r	10

[a] LafAX$^+$, LafAX$^-$, LafA$^+$, LafA$^-$, LafX$^+$, LafX$^-$, CbnAB$^+$, and CbnAB$^-$: lactacin F, lactacin A, lactacin X, and carnobacteriocin producers and nonproducers, respectively. Laf^r, Laf^s, Cbn^r, and Cbn^s: lactacin F and carnobacteriocin resistant and sensitive, respectively. Am^r, ampicillin resistant; Em^r, erythromycin resistant; Cm^r, chloramphenicol resistant.

[b] J. L. Johnson, Virginia Polytechnic Institute, Blacksburg.

[c] National Collection of Food Bacteria, Reading, United Kingdom.

[d] National Collection of Dairy Organisms, National Institute for Dairying, Reading, United Kingdom.

[e] American Type Culture Collection, Rockville, Md.

[f] U.S. Department of Agriculture, North Carolina State University, Raleigh.

[g] University of Vermont, Burlington.

MATERIALS AND METHODS

Bacteriology. The bacterial strains and plasmids used in this study are described in Table 1. Cultures were maintained and stored at −20°C in 20% glycerol. The organisms used in this study were propagated as follows: *Lactobacillus* strains, at 37°C in Lactobacilli MRS or APT medium (Difco Laboratories, Detroit, Mich.); *Carnobacterium* strains, at 25°C in APT medium; *Enterococcus faecalis* and *Listeria monocytogenes*, at 37°C in brain heart infusion medium (Difco); and *Lactococcus* and *Pediococcus* strains, at 30°C in M17 (Difco) containing 5 g of glucose per liter (M17G). Agar growth medium was prepared by adding either 0.75 or 1.5% granular agar (BBL Microbiology Systems, Cockeysville, Md.) to broth medium. When grown on agar medium, *Lactobacillus* and *Carnobacterium* strains were propagated under anaerobic gas (85% N_2, 10% CO_2, 5% H_2). *Lactobacillus* strains harboring recombinant plasmids were grown in MRS broth with 3 μg of erythromycin per ml. *Carnobacterium* transformants were selected on APT agar and cultured in broth supplemented with 5 μg of erythromycin per ml. *Escherichia coli* SURE (Stratagene, La Jolla, Calif.) was propagated at 37°C in Luria-Bertani medium (31) with 10 μg of tetracycline per ml. When appropriate for selection of clones, X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside) were used at a concentration of 50 μg/ml (31). Erythromycin-resistant (Em^r) transformants of *E. coli* were selected on brain heart infusion agar with 200 μg of erythromycin per ml (26).

Bacteriocin assay. Prior to bacteriocin assays, frozen stocks of recombinant strains of *Carnobacterium* spp. were streaked onto selective APT agar, incubated for 24 to 36 h, transferred into selective APT broth, and incubated for 18 to 24

h. *C. piscicola* LV17 and LV17C and *Lactobacillus* recombinant strains were inoculated (1% from frozen stocks) into broth, incubated for 18 to 24 h, subcultured, and incubated for 18 to 24 h. Bacteriocin production by *Carnobacterium* strains was assayed by spotting 5 μl from a broth culture onto APT agar (1.5%) and incubating at 25°C for 24 h under anaerobic gas. The plate was then overlaid with 5.5 ml of the appropriate type of agar (0.75%) containing a 1% inoculum of indicator cells prepared under the general conditions described for *Carnobacterium* and *Lactobacillus* strains. Overlaid plates were incubated at optimal temperatures for 12 to 18 h before reading of the results. For assays of bacteriocin complementation between *Lactobacillus* and *Carnobacterium* clones, 5 μl of *Carnobacterium* cultures was spotted onto APT agar and grown under anaerobic conditions for 24 h at 25°C. *Lactobacillus* cultures (5 μl) were then spotted close to the existing *Carnobacterium* colony, and the agar plates were incubated anaerobically at 37°C for an additional 6 to 8 h. Indicators were then overlaid as described above.

Molecular transformation and cloning. *E. coli* and *Lactobacillus* cells were prepared for electrotransformation by following the protocols outlined by Dower et al. (7) and Raya et al. (30), respectively. *Carnobacterium* cells were prepared for electrotransformation by using the latter protocol with the following modifications: electroporation buffer was composed of 0.5 M sucrose and 2.5 mM $CaCl_2$; freshly inoculated medium was incubated at 25°C for 4 to 5 h prior to preparation of electrocompetent cells; after washing once with sterile picopure water (50 ml) and twice with electroporation buffer (25 ml and then 10 ml), the original 100-ml culture was resuspended in 250 μl of electroporation buffer and 50 μl of this cell suspension was used for electroporation. The cells were electroporated in 0.2-cm cuvettes with a Gene Pulser apparatus (Bio-Rad, Richmond, Calif.) set at 25 μF, 200 Ω and 2.45 kV for *E. coli* or 2.1 kV for *Lactobacillus* and *Carnobacterium* strains. After electroporation, the cells were incubated in the appropriate nonselective medium for 1 h (*E. coli*) or for 2 to 4 h (*Lactobacillus* and *Carnobacterium* strains), prior to plating on selective agar media.

Plasmid DNA was isolated from *E. coli* as described by Sambrook et al. (31) and from *Lactobacillus* spp. as described by Walker and Klaenhammer (40). The *Lactobacillus* protocol was modified for *Carnobacterium* strains as follows: lysozyme concentration was increased to 20 mg/ml, the high salt solution was replaced by a mixture of 50 μl of 2 M Tris-HCl (pH 7.0) and 70 μl of 5 M NaCl, phenol extractions were shaken at room temperature for 1 h, and DNA was precipitated with 95% ethanol at −20°C.

The lactacin F operon was cloned into pTRKH2 for the heterologous expression experiments. Restriction endonucleases and T4 ligase were used as recommended by the manufacturer (New England Biolabs, Beverly, Mass.). pTRKH2 and pTRK160 (pBluescript::2.3-kb *lafAX* operon) were digested with *Sal*I and *Bgl*II or *Bam*HI, respectively. Both fragment and vector were purified from 0.8% agarose gels (wt/vol; 1× Tris-acetate-EDTA buffer, pH 8.0), using the GENE CLEAN II Kit (Bio 101, La Jolla, Calif.), and ligated with T4 ligase (New England Biolabs). The ligation mix was used to transform *E. coli* SURE (Stratagene). Seventeen Em^r transformants were analyzed with one, NCK718, containing the recombinant plasmid of expected size (pTRK386, 9.2 kb). Restriction analysis confirmed the presence of the cloned 2.3-kb *lafAX* operon fragment. The fidelity of pTRK386 was confirmed by electroporation into NCK65, a mutant of VPI 11088 with a chromosomal deletion eliminating the lactacin F operon from the genome (4, 22, 23), in which pTRK386 directed production of lactacin F.

RESULTS

Cloning the lactacin F operon into *C. piscicola*. *C. piscicola* LV17 is the wild-type producer of three carnobacteriocins, A, BM1, and B2 (designated $CbnAB^+$) (2, 28, 41). *C. piscicola* LV17C, a plasmidless derivative of LV17, is phenotypically bacteriocin negative ($CbnAB^-$); however, the structural gene for carnobacteriocin BM1 is located on the chromosome (2, 28). pTRK386 was introduced into LV17 and LV17C. As a negative control, pTRK201, containing truncated *lafA* (*lafA886*) (Fig. 1), was also electrotransformed into LV17C. Acquisition of the recombinant plasmids in the *Carnobacterium* transformants was confirmed by analysis of plasmid profiles (data not shown).

The bacteriocin-producing phenotypes of LV17(pTRK386) (*cbnAB*$^+$ *lafA*$^+$ *lafX*$^+$) and LV17C(pTRK386) (*lafA*$^+$ *lafX*$^+$) were investigated by using the lactacin F-sensitive (Laf^s) indicators, *Lactobacillus delbrueckii* subsp. *lactis* ATCC 4797 and *Lactobacillus helveticus* 87, and the carnobacteriocin indicator, *Carnobacterium divergens* LV13 (2). LV17 (*cbnAB*$^+$) did not inhibit strains ATCC 4797 or 87, indicating that these Laf^s strains are not sensitive to carnobacteriocins A, BM1, or B2 (Fig. 2A and B). LV17(pTRK386) (*cbnAB*$^+$ *lafA*$^+$ *lafX*$^+$) produces lactacin F and inhibits both Laf^s strains, while LV17C (pTRK386) (*lafA*$^+$ *lafX*$^+$) does not cause inhibition of ATCC 4797 and 87 (Fig. 2A and B). LV17(pTRK386) simultaneously produced $CbnAB^+$ inhibition of *C. divergens* LV13 at levels similar to that of the wild-type strain LV17 (Fig. 2C). LV17C, LV17C(pTRK201) (data not shown), and LV17C(pTRK386) are all $CbnAB^-$ and do not inhibit the Laf^s indicators (Fig. 2). These data indicated that the processing and export machinery of the carnobacteriocins is required for simultaneous production of active lactacin F peptides.

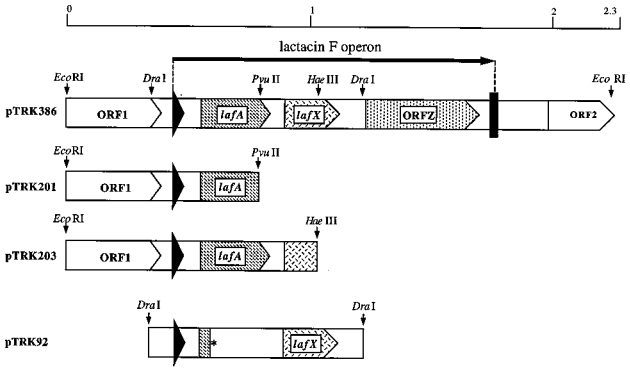

FIG. 1. The lactacin F operon and constructions cloned into *Carnobacterium* strains. DNA fragments subcloned into pTRKH2 (26) to construct pTRK386 and pTRK92 are shown. DNA fragments subcloned into pGKV210 (37) to construct pTRK201 and pTRK203 are also shown. *, frameshift mutation (4); ▶, promoter; ■, rho-independent terminator (10).

Cloning of components of the lactacin F operon into *C. piscicola*. The lactacin F complex is composed of two peptides, LafA and LafX (4, 10). The *lafA* and *lafX* genes were separately cloned into *C. piscicola* to determine if the individual peptides could be produced by the heterologous host. Strain LV17C was included as a negative control because the LV17C(pTRK386) transformant was unable to produce lactacin F.

The *lafA* gene, cloned into pTRK203 (*lafA*$^+$) (Fig. 1), was introduced into LV17 and LV17C. When LV17(pTRK203) (*cbnAB*$^+$ *lafA*$^+$) and LV17C(pTRK203) (*cbnAB lafA*$^+$) were assayed for LafA activity against *L. helveticus* 87 ($LafA^s$ and Laf^s) (4), bacteriocin production was not detected (Fig. 2B). However, when complemented with the LafX-producing *L. johnsonii* clone, NCK65(pTRK92), a zone of lactacin F inhibition was observed between the NCK65 and LV17 recombinants (Fig. 3). When LV17C(pTRK203) was plated next to NCK65(pTRK92), complementation of bacteriocin activity was not observed (Fig. 3). These data confirmed that the LafA peptide was processed and excreted by the carnobacteriocin's machinery; however, the levels appeared extremely low because LafA activity from LV17(pTRK203) alone could not be detected with *L. helveticus* 87 as the indicator (Fig. 2B).

The *lafX* gene, encoding the second peptide of the lactacin F complex, was cloned into pTRK92 (*lafX*$^+$) (Fig. 1) and transformed into LV17 and LV17C. When complemented with the LafA-producing *L. johnsonii* clone, NCK65(pTRK203), a large zone of lactacin F activity was observed between the *Lactobacillus* and *Carnobacterium* recombinant strains (Fig. 3), but complementation did not occur between NCK65(pTRK203) and LV17C(pTRK92) (Fig. 3). These data confirmed that *C. piscicola* LV17 can produce both LafA and LafX, but in the absence of the secretion machinery in LV17C, these peptides are not produced. Comparing the zones of complementation between recombinants $LafX^+$ NCK65(pTRK92) and $LafA^+$

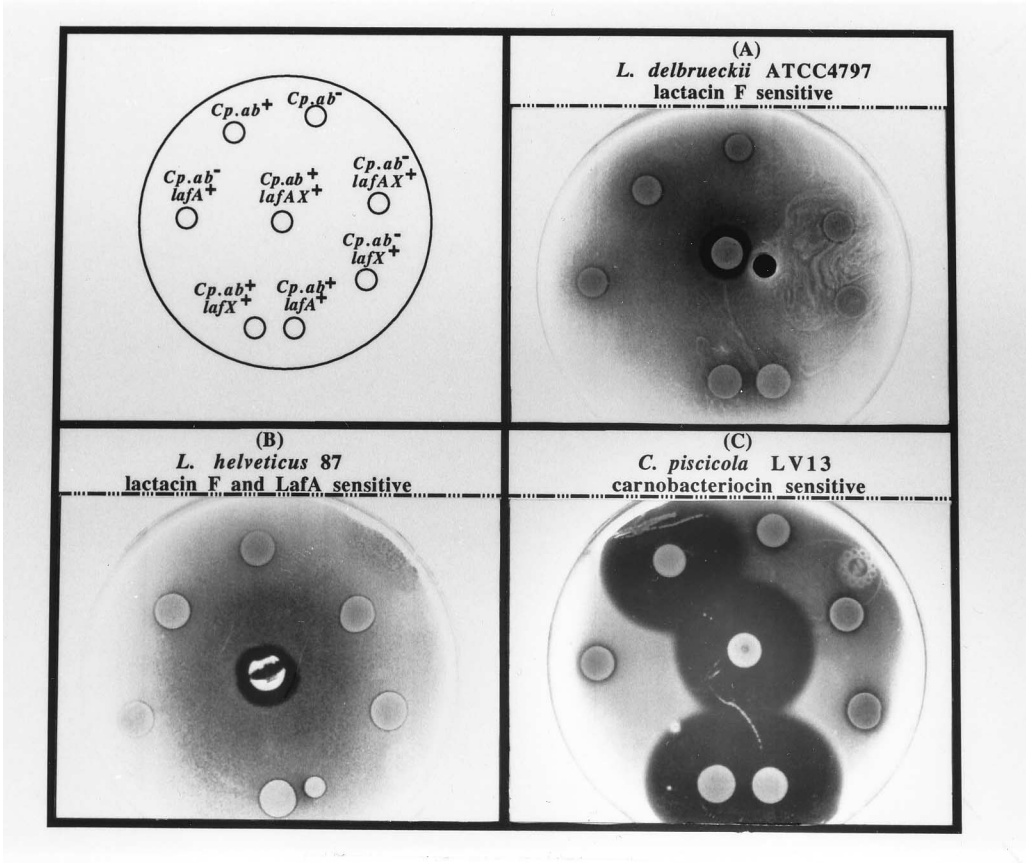

FIG. 2. Bacteriocin production by *C. piscicola* LV17 (CbnAB$^+$) and LV17C (CbnAB$^-$) and their *lafA*$^+$ and *lafX*$^+$ derivatives. Producers were plated onto APT medium and overlaid with indicators (designated in panels A to C) as described in Materials and Methods. Culture designations (top left panel): *Cp.ab*$^+$, LV17; *Cp.ab*$^-$, LV17C; *Cp.ab*$^-$ *lafA*$^+$, LV17C(pTRK203); *Cp.ab*$^+$ *lafA*$^+$ *lafX*$^+$, LV17(pTRK386); *Cp.ab*$^-$ *lafA*$^+$ *lafX*$^+$, LV17C(pTRK386); *Cp.ab*$^-$ *lafX*$^+$, LV17C(pTRK92); *Cp.ab*$^+$ *lafX*$^+$, LV17(pTRK92); and *Cp.ab*$^+$ *lafA*$^+$, LV17(pTRK203).

LV17(pTRK203) and also between LafA$^+$ NCK65(pTRK203) and LafX$^+$ LV17(pTRK92), the level of LafX production by *C. piscicola* LV17 appears to be greater than that of LafA (Fig. 3). However, in *L. johnsonii*, the levels of LafA and LafX production appear to be similar (Fig. 3). Even though LV17(pTRK203) and LV17(pTRK92) produce LafA and LafX, respectively (Fig. 3), there was no zone of lactacin F activity between these cultures in a homologous complementation assay (Fig. 2), suggesting that LafA production is low and a limiting factor in this case.

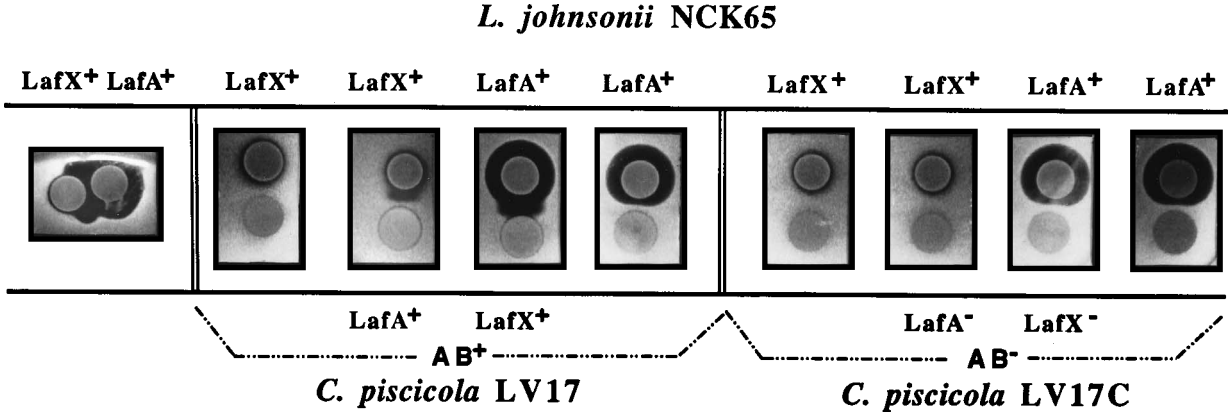

FIG. 3. Assays for complementation between *C. piscicola* and *L. johnsonii* clones. Cultures appearing in the top half of each rectangle are LafX- and LafA-producing *L. johnsonii* clones, NCK65(pTRK92) and NCK65(pTRK203), respectively. The cultures appearing in the bottom half of each rectangle are *C. piscicola* controls and clones with the phenotypes denoted. LV17 and LV17C with phenotypes LafA$^+$ and LafA$^-$, respectively, have been transformed with pTRK203, which contains the *lafA* gene. LV17 and LV17C with phenotypes LafX$^+$ and LafX$^-$, respectively, have been transformed with pTRK92, which contains the *lafX* gene. The indicator used in the overlay was *L. helveticus* 87, which is Laf^s and LafA^s.

The carnobacteriocin phenotypes of LV17(pTRK203) and LV17(pTRK92) were also assayed. In addition to producing LafA and LafX, respectively, the LV17 transformants simultaneously produced carnobacteriocins and inhibited the indicator strain at levels equivalent to those of the parental strain LV17. Therefore, concurrent production of the native carnobacteriocins and LafA, LafX, or both, did not retard maturation or excretion of the carnobacteriocins (Fig. 2C). LV17C(pTRK203) and LV17C(pTRK92) were $CbnAB^-$ as expected (Fig. 2C).

Inhibitory spectra of recombinants producing mixed components of two bacteriocin systems. Because LV17 produces LafA, LafX, and the lactacin F complex simultaneously with the native carnobacteriocins, the possibility that combined bacteriocin systems and/or components thereof could interact to form heterologous complexes was evaluated. New complexes might be effective against strains normally insensitive to the native bacteriocins. Therefore, LV17(pTRK386) ($CbnAB^+$ $LafAX^+$), LV17(pTRK203) ($CbnAB^+$ $LafA^+$), and LV17 (pTRK92) ($CbnAB^+$ $LafX^+$) were assayed for inhibitory activity against a variety of lactic acid bacteria. The indicators screened included *Lactobacillus plantarum*, *Lactococcus lactis*, *Listeria monocytogenes*, *Pediococcus pentosaceus*, and the other four lactacin F indicators (Table 1). Only the Cbn^s strains were inhibited by LV17 recombinant strains. Only Laf^s strains ATCC 4797 and 87 were inhibited by LV17(pTRK386) ($CbnAB^+$ $LafAX^+$). These data indicate that the heterologous peptide combinations did not increase or expand the inhibitory spectrum beyond those indicator strains examined in this study.

DISCUSSION

C. piscicola LV17 is capable of simultaneous production of multiple bacteriocins. LV17 naturally produces three different bacteriocins, carnobacteriocins A, BM1, and B2 (28, 41). When the lactacin F operon or components thereof were cloned into *C. piscicola* LV17, low levels of production of active LafA and/or LafX were observed. The native carnobacteriocins and the heterologous lactacin F peptides are produced concurrently, and expression of the non-native bacteriocin does not retard the level of carnobacteriocin production (Fig. 2). Consequently, LV17 is capable of producing five bacteriocins simultaneously. However, it appears that the lactacin F peptides are expressed at a lower level than the carnobacteriocins, suggesting that their bacteriocin precursors do not compete efficiently for the native processing and excretion recognition signals or that the transcription and translation signals are not proficiently read in this background. Expression of both the native and non-native bacteriocins appears to be dependent on the presence of the carnobacteriocin processing and export mechanisms, which are encoded on pCP40 and pCP49 (3, 28). LV17C, a plasmidless mutant of LV17 that does not produce carnobacteriocin, failed to produce active lactacin F after introduction of the *laf* operon.

The processing and secretion systems of pediocin PA-1 and lactococcin A, both class II bacteriocins, have been studied in detail. In both cases, the two gene products required to effect bacteriocin production show homology to the hemolysin export system of *E. coli* (18, 21, 35). The export of hemolysin is accomplished through an ABC transporter system, so named from the characteristic ATP-binding cassette common to one of the proteins involved (reviewed in reference 9). ABC transporters export numerous products, ranging from proteinaceous toxins to capsular polysaccharides. The hemolysin export system consists of three proteins, HlyB, HlyD, and TolC. HlyB is

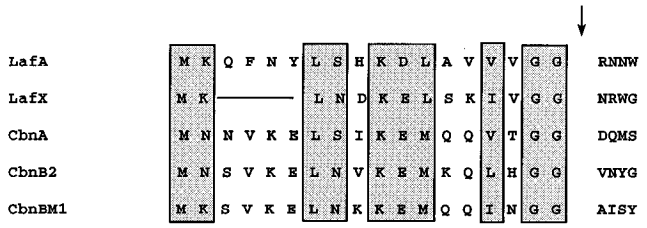

FIG. 4. Comparison of the leader peptide sequences of the bacteriocins produced by *L. johnsonii* VPI 11088 and *C. piscicola* LV17. The shaded areas indicate regions of homologous amino acid sequences that are conserved among all five bacteriocins. ↓, precursor cleavage site.

a large protein, with 707 amino acids, which has several transmembrane helices and a conserved ATP binding site. HlyD is a protein with 477 amino acids and one transmembrane helix. It is proposed that a dimer of HlyB becomes embedded in the inner membrane. A dimer of HlyD interacts with the HlyB complex and TolC such that a channel spanning the periplasmic space is formed. TolC is an outer membrane protein. HlyB and HlyD are part of the hemolysin operon, while TolC is not. Other components of the operon encode HlyC, which aids in toxin activation, and HlyA, the alpha-hemolysin structural gene. Like the case for hemolysin A, the genetic determinants for the transport and processing of many bacteriocins are located near the bacteriocin structural gene and may or may not be in the same operon (reviewed in reference 16). Additional bacteriocin systems which encode proteins homologous to HlyB and other ABC transporters include the following (reviewed in reference 9): colicin V and microcin B17 of *E. coli*, nisin and lactococin G of *L. lactis*, epidermin of *Staphylococcus epidermidis*, gallidermin of *Staphylococcus gallidermidis*, subtilin of *Bacillus subtilis*, and the hemolysin-bacteriocin of *E. faecalis*. Thus, there appears to be a dedicated processing and secretion system for these antimicrobial peptides which is common among all bacteriocin-producing strains. The heterologous expression of lactacin F in *C. piscicola* LV17 provides evidence that these systems are highly similar and potentially interchangeable.

The proteolytic cleavage site that releases the mature bacteriocin from the intracellular precursor is conserved among numerous bacteriocins (10, 13, 28; reviewed in reference 16). This conserved motif, $Gly^{-2}Gly^{-1}{**}Xaa^{+1}$, is predicted to break the alpha-helical structure of the N terminus of the precursor and provoke a beta-turn, such that the processing region is exposed to the action of a specific protease (19). However, site-directed mutations designed to disrupt the beta-turn conformation did not inhibit lactacin F activity as long as the Gly-Gly sequence was conserved (10). This indicates that processing specificity relies heavily on sequence recognition in this region. Comparison of the N-terminal sequences of LafA, LafX, and carnobacteriocins A, BM1, and B2 reveals a high degree of conservation and homology (Fig. 4): a hydrophobic residue at position −4; Leu or Met at position −7; an acidic (Asp or Glu) residue and Lys at positions −8 and −9, respectively; Ser or Asn at position −11; Leu at position −12; and Lys or Asn and Met at positions −17 and −18, respectively. LafX is the exception, with Lys and Met located at positions −13 and −14, respectively. The degree of sequence homology supports the importance of sequence recognition. Perhaps the class II bacteriocin precursors have the same requirements as the signal sequence of alpha-hemolysin, which has been proposed to have important contact residues required for processing and secretion (reviewed in reference 9). In the case of the

bacteriocins, these contact residues may be important for specific interactions with the processing peptidase.

C. piscicola LV17 expresses the lactacin F peptides at a low level relative to the carnobacteriocins. This may suggest that the subtle differences between the N-terminal extension sequences are such that the LafA and LafX precursors compete inefficiently for the native processing and excretion recognition signals. Alternatively, the *Lactobacillus* transcription and translation signals may not be proficiently read in the *C. piscicola* background. Unfortunately, little is known about the regulation of these bacteriocins. Sequence analysis of the area upstream of the *cbnB2* gene have identified possible −10 and −35 regions that are similar to *E. coli* σ^{70} and *B. subtilis* σ^{43} promoter sequences (28). The −10 promoter region of *cbnA* contains a TG sequence which is conserved in some gram-positive promoters (41). Analysis of the transcriptional start site and upstream promoter region of the lactacin F operon did not reveal any similarities to vegetative promoter sequences, and it has been suggested that the operon may be regulated (10). Further analysis is required to gain insight into the low levels of expression of the heterologous genes in *C. piscicola.*

Lactacin F and the carnobacteriocins are examples of class II bacteriocins which have several biochemical and structural characteristics in common. The mature forms of these peptides are predicted to form amphiphilic helices or beta sheets with variable hydrophobicity. It has been proposed that these amphiphilic helices and beta sheets interact with one another to form pores in the cytoplasmic membrane by means of the barrel stave mechanism (25). In the case of lactococcin G and lactacin F, two heterologous peptides interact with one another to form the poration complex and the complementary peptides are predicted to form transmembrane helices (1, 4, 24). Given the hydrophobic nature of LafA and LafX (17), interaction of these two peptides may be promoted by hydrophobic forces. Analysis of the amino acid sequences of carnobacteriocins A and BM1 by the method of Rao and Argos (29) predicts the formation of transmembrane helices between amino acids 40 to 67 and 43 to 60, respectively. These putative transmembrane helices occur in the hydrophobic C-terminal regions of both peptides. Because LV17 produces LafA, LafX, and the lactacin F complex simultaneously with the native carnobacteriocins, the possibility that combined bacteriocin systems and/or components thereof could interact in a novel way to form an active heterologous complex was investigated. Heterologous combinations of peptides did not increase the inhibitory spectra beyond that of lactacin F and carnobacteriocins. Several explanations are possible. The nature of the interaction of LafA and LafX is unknown, and peptide interactions may be highly specific, as is the case for the model proposed for cecropin, a cationic, antimicrobial peptide produced by silkworms (8). The model predicts that six cecropin peptides, each of which is in a helix-bend-helix motif, specifically interact with one another to form pores in lipid membranes (8). Finally, bacteriocin interactions with sensitive cells are also poorly understood, and the requirement for receptors varies between bacteriocins. Nisin, for example, acts on energized liposomes and does not require a protein receptor, whereas lactococcin A does (12, 18). If receptors are necessary for both the carnobacteriocins and lactacin F, receptor-bacteriocin interactions may be highly specific but not altered by peptide interactions between bacteriocins if these interactions occur.

Heterologous expression of bacteriocins has often included cloning of the processing and excretion system along with the bacteriocin structural gene. Examples of heterologous expression of both a bacteriocin and its export machinery have been illustrated in the production of pediocin PA-1 by *E. coli* (21) and epidermin by *Staphylococcus carnosus* (32, 33). In the lactacin F experiments reported here, the bacteriocin operon, which comprises the structural bacteriocin genes and open reading frame encoding the putative immunity protein, or only the individual bacteriocin genes, were introduced into the heterologous hosts. Other reports of this type of heterologous expression have included the production of lactacin F by *Leuconostoc gelidum* UAL187-22 (16, 17), helveticin J by *L. johnsonii* NCK64 (11, 15), and acidocin B by *L. plantarum* 80 (20, 27).

Insight into the heterologous expression of lactacin F by *C. piscicola* LV17 provides the basis for cloning other broad-range bacteriocins into fermentative lactic acid bacteria. Moreover, insight into bacteriocin processing and excretion could be exploited to produce other peptides of interest via this dedicated pathway. Ultimately, this could not only improve the safety and longevity of fermented dairy products but could also lead to the development of new and novel, value-added food products.

ACKNOWLEDGMENTS

This work was supported in part by the National Dairy Promotion and Research Board and the North Carolina Dairy Foundation. G. E. Allison is a recipient of the 1967 Science and Engineering Scholarship of the Natural Science and Engineering Research Council of Canada.

REFERENCES

1. **Abee, T., T. R. Klaenhammer, and L. Letellier.** 1994. Kinetic studies of the action of lactacin F, a bacteriocin produced by *Lactobacillus johnsonii* that forms poration complexes in the cytoplasmic membrane. Appl. Environ. Microbiol. **60:**1006–1013.
2. **Ahn, C., and M. E. Stiles.** 1990. Plasmid-associated bacteriocin production by a strain of *Carnobacterium piscicola* from meat. Appl. Environ. Microbiol. **56:**2503–2510.
3. **Ahn, C., and M. E. Stiles.** 1992. Mobilization and expression of bacteriocin plasmids from *Carnobacterium piscicola* isolated from meat. J. Appl. Bacteriol. **73:**217–228.
4. **Allison, G. E., C. Fremaux, and T. R. Klaenhammer.** 1994. Expansion of bacteriocin activity and host range upon complementation of two peptides encoded within the lactacin F operon. J. Bacteriol. **176:**2235–2241.
5. **Chopin, A., M.-C. Chopin, A. Moillo-Batt, and P. Langella.** 1984. Two plasmid-determined restriction and modification systems in *Streptococcus lactis*. Plasmid **11:**260–263.
6. **Daeschel, M. A., and T. R. Klaenhammer.** 1985. Association of a 13.6-megadalton plasmid in *Pediococcus pentosaceus* with bacteriocin activity. Appl. Environ. Microbiol. **50:**1538–1541.
7. **Dower, W. J., J. F. Miller, and C. W. Ragsdale.** 1988. High efficiency transformation of *Escherichia coli* by high voltage electroporation. Nucleic Acids Res. **16:**6127–6145.
8. **Durell, S. R., G. Raghunathan, and H. R. Guy.** 1992. Modeling the ion channel structure of cecropin. Biophys. J. **63:**1623–1631.
9. **Fath, M. J., and R. Kolter.** 1993. ABC transporters: bacterial exporters. Microbiol. Rev. **57:**995–1017.
10. **Fremaux, C., C. Ahn, and T. R. Klaenhammer.** 1993. Molecular analysis of the lactacin F operon. Appl. Environ. Microbiol. **59:**3906–3915.
11. **Fremaux, C., and T. R. Klaenhammer.** 1994. Helveticin J, a large heat-labile bacteriocin from *Lactobacillus helveticus*, p. 397–418. *In* L. De Vuyst and E. J. Vandamme (ed.), Bacteriocins of lactic acid bacteria: microbiology, genetics and applications. Blackie Academic & Professional, Glasgow, United Kingdom.
12. **Gao, F. H., T. Abee, and W. N. Konings.** 1991. Mechanism of action of the peptide antibiotic nisin in liposomes and cytochrome *c* oxidase-containing proteoliposomes. Appl. Environ. Microbiol. **57:**2164–2170.
13. **Håvarstein, L. S., H. Holo, and I. F. Nes.** 1994. The leader peptide of colicin V shares consensus sequences with leader peptides that are common among peptide bacteriocins produced by Gram-positive bacteria. Microbiology **140:** 2383–2389.
14. **Holo, H., Ø. Nilssen, and I. F. Nes.** 1991. Lactococcin A, a new bacteriocin from *Lactococcus lactis* subsp. *cremoris*: isolation and characterization of the protein and its gene. J. Bacteriol. **173:**3879–3887.
15. **Joerger, M. C., and T. R. Klaenhammer.** 1990. Cloning, expression, and nucleotide sequence of the *Lactobacillus helveticus* 481 gene encoding the bacteriocin helveticin J. J. Bacteriol. **172:**6339–6347.
16. **Klaenhammer, T. R.** 1993. Genetics of bacteriocins produced by lactic acid

bacteria. FEMS Microbiol. Rev. **12:**39–86.
17. **Klaenhammer, T. R., C. Ahn, and P. M. Muriana.** 1994. Lactacin F, a small hydrophobic heat-stable bacteriocin from *Lactobacillus johnsonii*, p. 377–396. *In* L. De Vuyst and E. J. Vandamme (ed.), Bacteriocins of lactic acid bacteria: microbiology, genetics and applications. Blackie Academic & Professional, Glasgow, United Kingdom.
18. **Kok, J., H. Holo, M. J. van Belkum, A. J. Haandrikman, and I. F. Nes.** 1993. Non-nisin bacteriocins in lactococci: biochemistry, genetics, and mode of action, p. 121–150. *In* D. Hoover and L. Steenson (ed.), Bacteriocins of lactic acid bacteria. Academic Press, Inc., San Diego, Calif.
19. **Kolter, R., and F. Moreno.** 1992. Genetics of ribosomally synthesized peptide antibiotics. Annu. Rev. Microbiol. **46:**141–163.
20. **Leer, R. J., J. M. B. M. van der Vossen, M. J. F. van Giezen, and P. H. Pouwels.** 1992. Molecular genetic analysis and phenotypic expression of a bacteriocin produced by *Lactobacillus acidophilus* M46. TNO Health Research Annual Report.
21. **Marugg, J. D., C. F. Gonzalez, B. S. Kunka, A. M. Ledeboer, M. J. Pucci, M. Y. Toonen, S. A. Walker, L. C. M. Zoetmulder, and P. A. Vandenbergh.** 1992. Cloning, expression, and nucleotide sequence of genes involved in production of pediocin PA-1, a bacteriocin from *Pediococcus acidilactici* PAC1.0. Appl. Environ. Microbiol. **58:**2360–2367.
22. **Muriana, P. M., and T. R. Klaenhammer.** 1987. Conjugal transfer of plasmid-encoded determinants for bacteriocin production and immunity in *Lactobacillus acidophilus* 88. Appl. Environ. Microbiol. **53:**553–560.
23. **Muriana, P. M., and T. R. Klaenhammer.** 1991. Cloning, phenotypic expression, and DNA sequence of the gene for lactacin F, an antimicrobial peptide produced by *Lactobacillus* spp. J. Bacteriol. **173:**1779–1788.
24. **Nissen-Meyer, J., H. Holo, L. S. Håvarstein, K. Sletten, and I. F. Nes.** 1992. A novel lactococcal bacteriocin whose activity depends on the complementary action of two peptides. J. Bacteriol. **174:**5686–5692.
25. **Ojcius, D. M., and J. D.-E. Young.** 1991. Cytolytic pore-forming proteins and peptides: is there a common structural motif? Trends Biochem. Sci. **16:**225–229.
26. **O'Sullivan, D. J., and T. R. Klaenhammer.** 1993. High- and low-copy-number *Lactococcus* shuttle cloning vectors with features for clone screening. Gene **137:**227–231.
27. **Pouwels, P. H., and R. J. Leer.** 1993. Genetics of lactobacilli: plasmids and gene expression. Antonie Leeuwenhoek **64:**85–107.
28. **Quadri, L. E. N., M. Sailer, K. L. Roy, J. C. Vederas, and M. E. Stiles.** 1994. Chemical and genetic characterization of bacteriocins produced by *Carnobacterium piscicola* LV17B. J. Biol. Chem. **269:**12204–12211.
29. **Rao, J. K. M., and P. Argos.** 1986. A conformational preference parameter to predict helices in integral membrane proteins. Biochim. Biophys. Acta **869:**197–214.
30. **Raya, R. R., C. Fremaux, G. L. De Antoni, and T. R. Klaenhammer.** 1992. Site-specific integration of the temperate bacteriophage ϕadh into the *Lactobacillus gasseri* chromosome and molecular characterization of the phage (*attP*) and bacterial (*attB*) attachment sites. J. Bacteriol. **174:**5584–5592.
31. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
32. **Schnell, N., G. Engelke, J. Augustin, R. Rosenstein, F. Gotz, and K.-D. Entian.** 1991. The operon-like organization of lantibiotic epidermin biosynthesis genes, p. 269–276. *In* G. Jung and H.-G. Sahl (ed.), Nisin and novel lantibiotics. ESCOM Science Publishers, Leiden, The Netherlands.
33. **Schnell, N., G. Engelke, J. Augustin, R. Rosenstein, V. Ungermann, F. Gotz, and K.-D. Entian.** 1992. Analysis of genes involved in the biosynthesis of lantibiotic epidermin. Eur. J. Biochem. **204:**57–68.
34. **Shaw, B. G., and C. D. Harding.** 1984. A numerical taxonomic study of lactic acid bacteria from vacuum-packed beef, pork, lamb and bacon. J. Appl. Bacteriol. **56:**25–40.
35. **Stoddard, G. W., J. P. Petzel, M. J. van Belkum, J. Kok, and L. L. McKay.** 1992. Molecular analyses of the lactococcin A gene cluster from *Lactococcus lactis* subsp. *lactis* biovar diacetylactis WM4. Appl. Environ. Microbiol. **58:** 1952–1961.
36. **Tagg, J. R., A. S. Dajani, and L. W. Wannamaker.** 1976. Bacteriocins of gram-positive bacteria. Bacteriol. Rev. **40:**722–756.
37. **van der Vossen, J. M. B. M., D. van der Lelie, and G. Venema.** 1987. Isolation and characterization of *Streptococcus cremoris* Wg2-specific promoters. Appl. Environ. Microbiol. **53:**2452–2457.
38. **von Heijne, G.** 1983. Patterns of amino acids near signal-sequence cleavage sites. Eur. J. Biochem. **133:**17–21.
39. **von Heijne, G.** 1986. A new method for predicting signal sequence cleavage sites. Nucleic Acids Res. **14:**4683–4691.
40. **Walker, D. C., and T. R. Klaenhammer.** 1994. Isolation of a novel IS*3* group insertion element and construction of an integration vector for *Lactobacillus* spp. J. Bacteriol. **176:**5330–5340.
41. **Worobo, R. W., T. Henkel, M. Sailer, K. L. Roy, J. C. Vederas, and M. E. Stiles.** 1994. Characteristics and genetic determinant of a hydrophobic peptide bacteriocin, carnobacteriocin A, produced by *Carnobacterium piscicola* LV17A. Microbiology **140:**517–526.