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A Signal Peptide Secretion-Dependent Bacteriocin from *Carnobacterium divergens*

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Divergicin A is a strongly hydrophobic, narrow-spectrum, nonlantibiotic bacteriocin produced by *Carnobacterium divergens* LV13. This strain of *C. divergens* contains a 3.4-kb plasmid that mediates production of, and immunity to, the bacteriocin. N-terminal amino acid sequencing of the purified divergicin A was used to locate the structural gene (*dvnA*). The structural gene encodes a prepeptide of 75 amino acids consisting of a 29-amino-acid N-terminal extension and a mature peptide of 46 amino acids. Directly downstream of *dvnA* there is a second open reading frame that encodes the immunity protein for divergicin A. Divergicin A has a calculated molecular mass of 4,223.89 Da. The molecular mass determined by mass spectrometry is 4,223.9 Da, indicating that there is no posttranslational modification of the peptide. The N-terminal extension of divergicin A has an Ala-Ser-Ala (positions −3 to −1) cleavage site and acts as a signal peptide that accesses the general export system of the cell (such as the *sec* pathway in *Escherichia coli*). This is the first bacteriocin of lactic acid bacteria to be reported that does not have dedicated maturation and secretion genes. Production of divergicin A was observed in heterologous hosts containing only the two genes associated with divergicin A production and immunity. Fusing alkaline phosphatase behind the signal peptide for divergicin resulted in the secretion of this enzyme in the periplasmic space and supernatant of *E. coli*.

Bacteriocins are antagonistic peptides or proteins that typically inhibit the growth of closely related bacteria. Klaenhammer (15) divided the bacteriocins of lactic acid bacteria (LAB) into four classes on the basis of their chemical, structural, and functional properties. The class I and II bacteriocins are the best characterized. They are small, heat-stable peptides that are inactivated by proteolytic enzymes of the intestinal tract. This makes them interesting as potential preservatives for foods. Class III and IV bacteriocins are not as well characterized and they are generally heat-labile, large proteins or protein conjugates, respectively. The class I bacteriocins are known as lantibiotics because they contain the unusual amino acids lanthionine and β-methyllanthionine. The lantibiotics are not exclusive to LAB, but they include nisin, which is an important food preservative produced by *Lactococcus lactis* subsp. *lactis* (8). Their production is characterized by extensive posttranslational modification of the gene product to produce the active peptide. Fusion of the N-terminal extension of subtilin in front of the structural component of nisin resulted in the production of nisin, suggesting the involvement of the N-terminal extension in the secretion of lantibiotics (17). Nisin has a polycistronic gene cluster that requires close to 10 kb of DNA to control its production, secretion, and posttranslational modification (16). Other LAB produce lantibiotics, such as carnocin UI49 produced by *Carnobacterium piscicola* (29), but these lantibiotics have not been extensively characterized.

Class II bacteriocins produced by LAB are the largest group that have been characterized. They are produced as prebacteriocins that comprise the precursor of the mature peptide with an 18- to 24-amino-acid N-terminal extension that is cleaved during secretion or maturation of the bacteriocin. They are minimally modified, apart from cleavage of the N-terminal extension at the Gly-Gly (positions −2 and −1) site. The role of the N-terminal amino acid extension of the class II bacteriocins has not been established, but it may be important in recognition of the prebacteriocin by the transport and maturation machinery of bacteriocin-dependent secretion systems. The N-terminal extensions of class II bacteriocins have marked homology in their hydrophobicity profiles (10, 13, 24). The class II bacteriocins, such as the lactococcins (28) and pediocin PA-1/AcH (21, 22) require dedicated secretion and maturation systems to produce the extracellular, mature bacteriocin. They require less genetic information than nisin for their production, but they generally have structural and immunity genes as an operon, with the secretion genes in close proximity in a relatively condensed gene package contained in approximately 3.5 to 4.5 kb of DNA (21, 31).

The focus of our research on bacteriocins of the meat-related LAB *Carnobacterium piscicola* (currently proposed as *Carnobacterium maltaromicus* [6]) and *Leuconostoc gelidum* (12, 24, 39) has been to characterize these bacteriocins with a view to enhancing their antibacterial spectrum through site-directed mutagenesis or production of two or more bacteriocins within gene cassettes. *C. piscicola* LV17 produces three bacteriocins (24, 39) and contains at least two independent secretion systems for the three bacteriocins, although the regulation of production of these bacteriocins is interrelated (27). In contrast, lactococcins A, B, and M share a common secretion system for all three bacteriocins (31). This indicates that the expression of multiple bacteriocins by one organism may require more than one secretion system and multiple immunities. There are no reports of immunity proteins capable of expressing immunity to more than one bacteriocin or of secretion proteins involved in the production of multiple, heterologous bacteriocins. As a result of these bacteriocin-dependent immunity and secretion genes, gene cassettes would be difficult to produce with class II bacteriocins because of the large amount of DNA required for immunity to, and production of, each bacteriocin. In this paper we report the purification and sequence of a novel bacteriocin, divergicin A, that functions as

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TABLE 1. Bacteria and plasmids used to study bacteriocin production by *C. divergens* LV13

Bacterial strain or plasmid	Description[a]	Source or reference
C. divergens		
LV13	*dvn*$^+$ *dvi*$^+$ (containing pCD3.4)	NCFB[b]
AJ	Dvn^s (indicator strain)	Laboratory isolate
C. piscicola		
LV17C	Dvn^s (indicator strain), plasmidless	2
LV17A	*cbnA* (containing pCP49), Bac$^+$ Dvn^s	2
LV17B	*cbnB2* and *cbnBM1* (containing pCP40)	2
UAL 26	Bac$^+$ Dvn^s	2
L. lactis subsp. *lactis*		
MG1363	Dvn^r, plasmidless	11
IL1403	Dvn^r, plasmidless	5
E. coli		
DH5α	F$^-$ *endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1* λ$^-$ *recA1 gyrA96 relA1* Δ(*argF-lacZYA*)UI69 ϕ80*dlac* ZΔM15	Bethesda Research Laboratories Life Technologies Inc.
MH1	MC1061 derivative; *araD139 lacX74 galU galK hsr hsm*$^+$ *strA*	4
Plasmids		
pCD3.4	*dvn*$^+$ *dvi*$^+$, 3.4 kb	This study
pCD4.4	pCD3.4 containing 1.0-kb *Eco*RI Cm^r gene of pGS30; Cm^r *dvn*$^+$ *dvi*$^+$, 4.4 kb	This study
pUC118	*lacZ′* Amp^r, 3.2 kb	34
pGS30	pUC7 containing 1.0-kb *Pst*I Cm^r gene of pC194; Cm^r, 3.7 kb	G. Venema[c]
pKM1	pUC7 containing 1.3-kb *Pst*I Km^r gene of pUB110; Km^r, 3.7 kb	G. Venema[c]
pGKV259	Em^r Cm^r 5.0 kb	33
pRW5.6	pGKV259 containing 514-bp *Eco*RV-*Acc*I fragment; Em^r *dvn*$^+$ *dvi*$^+$, 5.6 kb	This study
pRW6.0	pGKV259 containing divergicin signal peptide fused to alkaline phosphatase	This study

[a] *dvn*$^+$, divergicin production gene; *dvi*$^+$, divergicin immunity gene; Dvn^r, divergicin resistant; Dvn^s, divergicin sensitive; Bac$^+$, bacteriocinogenic; Em^r, erythromycin resistance; Cm^r, chloramphenicol resistance; Amp^r, ampicillin resistance; Km^r, kanamycin resistance.
[b] NCFB, National Collection of Food Bacteria, Reading, United Kingdom.
[c] Strain from the laboratory of G. Venema, Department of Genetics, University of Groningen, Haren, The Netherlands.

a signal peptide secretion-dependent bacteriocin, which does not require bacteriocin dedicated secretion machinery.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Carnobacteria were grown in APT broth (Difco Laboratories Inc., Detroit, Mich.) at 25°C, and *Escherichia coli* was grown in Luria-Bertani (LB) broth (26) on a rotary shaker at 250 rpm at 37°C. Solid media were prepared by adding 1.5% agar to the broth media; overlay media were prepared with 0.75% agar. Stock cultures of carnobacteria were maintained at 4°C in Cooked Meat Medium (Difco) and at −70°C in APT broth containing 40% (vol/vol) glycerol. *E. coli* strains were stored in LB broth with 40% (vol/vol) glycerol at −70°C. When appropriate, media were supplemented with antibiotics in the following concentrations: ampicillin and erythromycin at 200 μg/ml for *E. coli* and erythromycin at 10 and 5 μg/ml for broth and solid media, respectively, for carnobacteria.

Production and purification of divergicin. Partial purification of divergicin was done with a 1% inoculum of an overnight culture of *Carnobacterium divergens* LV13 in 2 liters of APT broth, which was maintained at pH 7.5 with 2 N NaOH by using a pH stat (Chem-Cadet; Cole Palmer) while being stirred gently at 25°C. The culture was adjusted to pH 5.6 with 5 N HCl and heated to 70°C for 35 min before centrifugation (9,000 × *g*, 5 min, 4°C). The bacteriocin in the supernatant was precipitated with ammonium sulfate (700 g/liter) by stirring for 24 h at 4°C and was harvested by centrifugation (9,000 × *g*, 20 min, 4°C). The precipitate was dissolved in 60 ml of 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) (Sigma Chemical Co., St. Louis, Mo.), pH 5.5 (fraction A). Ammonium sulfate (200 g/liter) was added to fraction A, and precipitated proteins were removed by centrifugation (9,000 × *g*, 20 min, 4°C). The clarified fraction A was loaded onto a 45-ml octyl-Sepharose CL4B chromatography column (Pharmacia, Uppsala, Sweden) equilibrated with 20% ammonium sulfate in 20 mM MES, pH 5.5. The column was washed with 1.5 volumes each of 20, 15, 10, 5, and 0% ammonium sulfate in 20 mM MES, pH 5.5, and then with 1.5 volumes each of water, 10% ethanol, and 70% ethanol. The active fraction was concentrated by rotary evaporation, resuspended in 6 M urea, and loaded onto a Sephadex G-75 column (2.5 by 120 cm; Pharmacia) equilibrated with 6 M urea. Elution was monitored by A_{228}. All fractions were assayed for bacteriocin activity by the spot-on-lawn test (2). The urea was dialyzed in 3,500-molecular-weight-cutoff dialysis tubing (Spectra Por; Spectrum Medical Industries Inc., Los Angeles, Calif.) and lyophilized.

Complete purification was achieved by using the method previously described by Hastings et al. (12), except that the culture was grown in 5 liters of APT broth supplemented with 1% glucose. After incubation for 20 h, the cells were removed by centrifugation and the culture supernatant was applied directly to an Amberlite XAD-8 column (4 by 40 cm; BDH Chemicals Ltd., Poole, England) that was washed with 3 liters of 0.05% trifluoroacetic acid (TFA) and 2 liters each of 20 and 35% ethanol in 0.05% TFA. The active fraction eluted with 50% ethanol in 0.05% TFA (1.5 liters) was concentrated by rotary evaporation, and 10% (5 ml) was loaded onto a Sephadex G-50 column (2.5 by 120 cm; Pharmacia) that had been equilibrated with 0.05% TFA. The active fractions were concentrated to 1 ml by rotary evaporation, applied in 100-μl portions to a C_4 column (Waters Delta-Pak; 10 by 200 mm; 15 μm particle size; 300 Å [30 nm] pore size flow rate, 1.5 ml/min; mobile phase, 0.05% TFA in water [A] and 95% ethanol in 0.05% TFA [B]), and eluted by a gradient method (first 50 to 63% solvent B in 7 min and then 63 to 64% in 6 min). Fractions were monitored for A_{218} and for activity against the indicator strain. The purities of the fractions were checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Bacteriocin production and immunity were tested by the deferred inhibition assay (2).

SDS-PAGE. Purified divergicin preparations were examined for the presence of contaminating proteins by using 15% polyacrylamide gels and 3 M Tris-HCl, pH 8.8. Electrophoresis was done at constant current of 20 mA as described by Laemmli (18). Gels were fixed in 50% (vol/vol) methanol–10% (vol/vol) acetic acid for 30 min and stained with Coomassie brilliant blue (Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada). To test for activity, the polyacrylamide gel was washed with water (twice with 1 liter each time) for 2.5 h. The gel was placed onto an APT plate and overlayered with soft APT agar inoculated with 1% of a sensitive indicator strain.

N-terminal amino acid sequence and amino acid analysis. Partially purified divergicin obtained by using octyl-Sepharose CL4B and Sephadex G-75 was subjected to SDS-PAGE, and the proteins were electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad) at 50 V for 3 h in blotting buffer (10 mM $NaHCO_3$, 3 mM Na_2CO_3 [pH 9.9] in 20% ethanol [9]). The blotted membrane was stained with 0.25% Coomassie brilliant blue in 40% methanol and destained with 50% methanol. The protein band corresponding to activity on the overlayered gel was excised and used for N-terminal amino acid sequencing by Edman degradation analysis on an automated gas phase sequencer (model 470A; Applied Biosystems, Foster City, Calif.) with on-line phenylthiohydantoin derivative identification by reversed-phase high-pressure liquid chromatography (HPLC) (Applied Biosystems model 120A). Amino acid analysis was done on a similarly excised band containing the peptide. The amino acids were derivatized with phenylisothiocyanate on an Applied Biosystems (model 420A) derivatizer and separated by HPLC (Applied Biosystems model 130A) with a C_{18} column. Data were recorded and analyzed on an Applied Biosystems (model 920A) data analyzer system.

Mass spectrometry. The mass spectra of divergicin were determined by Paul Semchuk of the Protein Engineering Centres of Excellence, University of Victoria, Victoria, British Columbia, Canada. Electrospray mass spectra were acquired on a Fisons VG Quatro instrument. Divergicin, which had been purified by HPLC, was introduced by direct infusion (1 μg/ml) in 50% aqueous acetonitrile containing 0.5% TFA through a sample loop. Fisons software was employed to determine the molecular mass from the envelopes of multiply charged peaks in the *m*/*z* spectra.

DNA isolation, manipulation, and sequence analysis. Large-scale plasmid preparation from *C. divergens* LV13 was done as previously described for *C. piscicola* LV17A (39). Other DNA manipulations were based on those described by Sambrook et al. (26). *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.) and restriction endonucleases and T4 DNA ligase (Promega, Madison, Wis.; Bethesda Research Laboratories, Burlington, Ontario, Canada; Boehringer Mannheim, Dorval, Quebec, Canada; and New England Biolabs, Mississauga, Ontario, Canada) were used according to the suppliers' recommended procedures. Stepwise deletion derivatives for sequencing were prepared by using the Erase-a-Base system (Promega), and DNA fragment recovery was done with Geneclean II (Bio 101 Inc., La Jolla, Calif.). Carnobacteria were transformed by resuspending a 3-h culture from a 2% inoculum in 1/10 of the volume of cold Milli-Q water and collecting the cells by centrifugation at 6,000 × *g*. The cells were washed twice with electroporation buffer (0.5 M sucrose, 2.5 mM $CaCl_2$) and resuspended in 1/40 of the original volume of electroporation buffer. The cells were electroporated with a Gene Pulser (Bio-Rad) by the method recommended by the supplier. Immediately following electroporation, 1 ml of APT containing 0.5 M sucrose was added, and the cells were incubated at room temperature for 3 h and plated onto appropriate selective media. Oligonucleotides prepared as sequencing and PCR primers were synthesized on an Applied Biosystems (model 391) PCR Mate synthesizer. Double-stranded DNA was sequenced by *Taq* DyeDeoxy Cycle sequencing on an Applied Biosystems (model 373A) sequencer. For amplification of the DNA encoding the mature part of alkaline phosphatase, primers KLR 179 (5′ GCGCAAGCTTCTGCTCGGACACCAGAAATGCCTGTT 3′) and KLR 180 (5′ GGCCAAGCTTGCCATTAAGTCTGGTTGCTA 3′) were used with the *E. coli* C_4F_1 (30) alkaline phosphatase gene as a template.

Assay for alkaline phosphatase. Cells from 1.5 ml of an overnight culture grown in LB broth were centrifuged (9,000 × *g*, 5 min, 25°C) and washed in an equal volume of STE (50 mM NaCl, 10 mM Tris [pH 8.0], 1 mM EDTA [pH 8.0]). The culture medium and periplasmic fractions were assayed for alkaline phosphatase. Periplasmic fractions were prepared by resuspending the washed cells in 0.5 ml of 20% sucrose with 50 μl of 0.5 M EDTA and 25 μl of lysozyme (10 mg/ml) and incubating at room temperature for 15 min. The samples were centrifuged (9,000 × *g*, 5 min, 25°C), and the supernatant was assayed for alkaline phosphatase activity (30) by A_{405}.

Nucleotide sequence accession number. The DNA sequence was submitted to GenBank (Los Alamos, N.Mex.) and was given the accession number L37791.

RESULTS

Production and purification of divergicin. *C. divergens* LV13 produces a bacteriocin, divergicin A, that is active against the indicator strains *C. piscicola* LV17C and *C. divergens* AJ. The amount of divergicin produced by *C. divergens* LV13 in APT broth at pH 7.5 and 25°C for 18 h is 2 mg/liter. Different media and pH conditions were tested for increased bacteriocin production. Divergicin was not produced in the semidefined medium CAA used by Hastings et al. (12). Of the media tested, only APT supported bacteriocin production. The best production was achieved at pH 6.5 with 1% glucose added to the APT broth. For purification of divergicin by ammonium sulfate precipitation followed by octyl-Sepharose chromatography, all of the activity was eluted in the 70% ethanol wash. With size exclusion chromatography (Sephadex G-75), most of the activity was detected in the second peak. This fraction was concentrated, subjected to SDS-PAGE, and blotted onto a polyvinylidene difluoride membrane. The band corresponding to activity on the SDS-PAGE gel that was overlayered with the sensitive *C. piscicola* LV17C indicator strain was excised from the membrane and used for Edman degradation and amino acid analysis. Divergicin is rapidly inactivated in acetonitrile, which was used in our initial HPLC purification protocol, but it was stable in ethanol. The use of ethanol as the carrier solvent enabled further purification of divergicin A and determination of its molecular mass. HPLC-purified bacteriocin for mass spectrometry was obtained by using the protocol of Hastings et al. (12) with gradients of ethanol and water containing TFA to elute the pure divergicin.

N-terminal sequence and amino acid analysis of divergicin. The excised bacteriocin band from the electroblotted polyvinylidene difluoride membrane enabled the following 21-amino-acid N-terminal sequence to be determined: Ala-Ala-Pro-Lys-Ile-Thr-Gln-Lys-Gln-Lys-Asn-X-Val-Asn-Gly-Gln-Leu-Gly-Gly-Met-Leu-Ala. This sequence matches the amino acid content derived from the nucleotide sequence and amino acid analysis except for the apparent absence of methionine in the amino acid analysis. Methionine was not detected in the amino acid analysis, probably because of the small amount of bacteriocin contained on the blotted membrane. The unidentified amino acid at position 12 was subsequently shown to be cysteine by interpretation of the nucleotide sequence (Fig. 1).

Mass spectrometry of divergicin. Mass spectral analysis by positive-ion fast atom bombardment with an electrospray interface gave multiply charged molecular ions (Fig. 2), which allowed the calculation of an average molecular mass of 4,223.9 ± 0.1 Da.

Identification and expression of the divergicin structural and immunity genes. The native plasmid pCD3.4 was completely sequenced (unpublished data), and three open reading frames (ORFs) were identified. The N-terminal amino acid sequence of divergicin was observed in the nucleotide translation product in the ORF containing 225 nucleotides. This ORF is contained in the 514-bp *Eco*RV-*Acc*I fragment of pCD3.4 (Fig. 1), starting with the alanine residue at position 30 of the structural gene for divergicin A (*dvnA*). This gene encodes a 75-amino-acid prepeptide consisting of a 29-amino-acid N-terminal extension and a 46-amino-acid bacteriocin. Immediately following the divergicin structural gene is a second ORF that encodes 56 amino acids that could be the protein for divergicin immunity. Two probable ribosome binding sites (GGAGG) for the divergicin structural gene and the possible divergicin immunity gene are located 9 and 13 bp, respectively, upstream of the initiator codons. Downstream of the second ORF (nucleotides 530 to 564) there is a 14-base inverted repeat with a 7-base loop that is a potential *rho*-independent terminator. A unique *Ssp*I site contained within the divergicin immunity gene (*dviA*) was used to insert a kanamycin resistance marker from pKM1 and to inactivate the immunity gene. Nucleotide sequencing of the immunity gene was done to confirm insertion into the *dviA* gene. The loss of immunity was accompanied by the strain still producing divergicin A, but growth of these organisms was poor.

The native plasmid pCD3.4 with a chloramphenicol resistance marker from pGS30 inserted into the *Eco*RI site (not shown) to produce pCD4.4 was used to transfer the plasmid to heterologous carnobacterial hosts, including *C. piscicola* LV17A, LV17B, LV17C, and UAL 26 (Table 1). Transformation of pCD4.4 into heterologous *C. piscicola* and *C. divergens* hosts resulted in chloramphenicol resistance as well as in production of, and immunity to, divergicin. To utilize the *Pst*I site immediately following the P59 promoter of pGKV259, the 514-bp *Eco*RV-*Acc*I fragment and the *Pst*I site of pGKV259 were blunt ended and the fragment was cloned into the *Pst*I site, resulting in the construct pRW5.6. The presence of the correct fragment and orientation for the expression of divergicin A was confirmed by restriction enzyme analysis. The presence of the structural and possible immunity genes for divergicin was also confirmed by nucleotide sequencing. Electroporation of the pRW5.6 construct into other *Carnobacterium* hosts resulted in full divergicin production and immunity as determined by deferred inhibition assays (Fig. 3). The "half moon" halo effect in the zones of inhibition shown in Fig. 3B

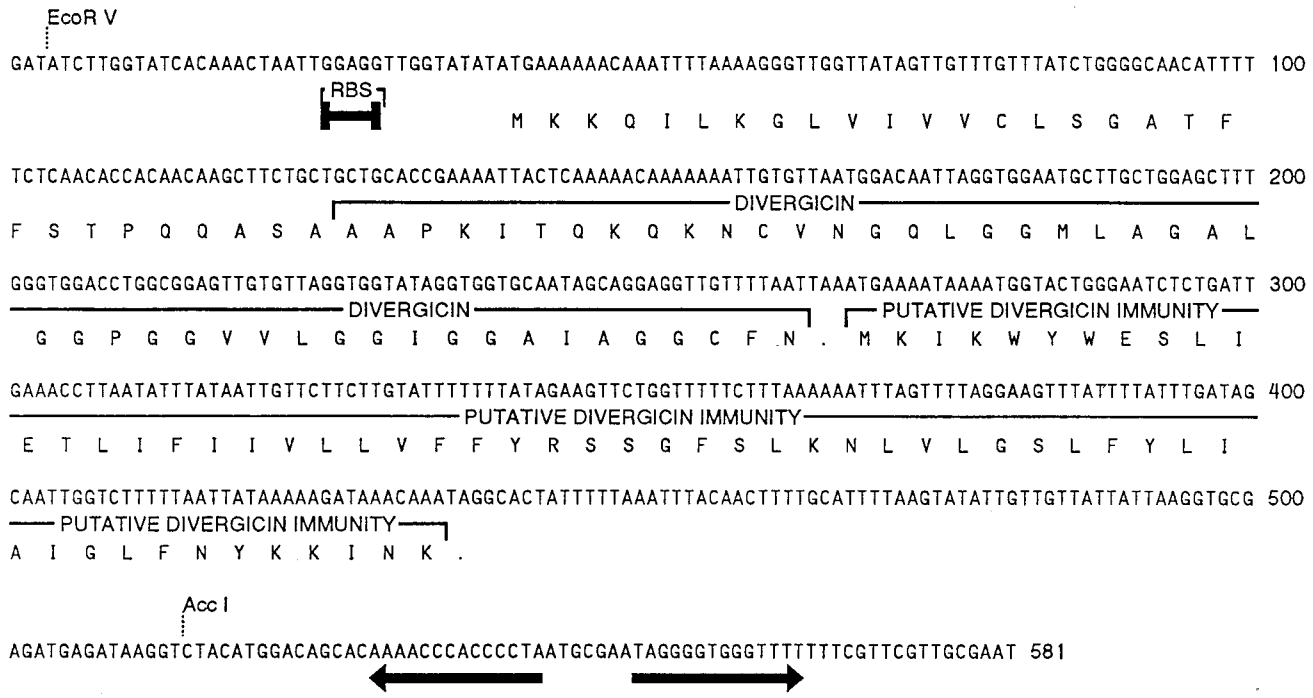

FIG. 1. Nucleotide sequence of a 581-bp fragment from pCD3.4, a native plasmid of *C. divergens* LV13. The deduced amino acid sequences for the precursor of divergicin A and its putative immunity protein are shown below the nucleotide sequence. A potential *rho*-independent termination site is indicated by reversed arrows on the nucleotide sequence, and the putative ribosome binding site (RBS) is underlined.

was created by spotting pronase E in close proximity to the producer organism, confirming that the inhibition was due to bacteriocin production. The pRW5.6 construct was also electroporated into two *Lactococcus* strains, MG1363 and IL1403. Expression was achieved in both of these strains, with more divergicin being produced by strain MG1363 than by strain IL1403. Divergicin immunity in the *Lactococcus* strains could not be tested because the host strains are normally resistant to divergicin.

Alkaline phosphatase fusion. PCR-generated DNA encoding the mature part of alkaline phosphatase with *Hin*dIII linking ends was inserted into the *Hin*dIII site of the divergicin signal peptide. The *dvnA* Ala-Ser-Ala cleavage site was regenerated by incorporation of these three residues into the PCR primer. The presence of phosphatase activity was screened by direct plating of the ligation mixture onto LB agar containing 5-bromo-4-chloro-3-indolyl phosphate and appropriate antibiotics. Blue colonies were selected, and the transformants were cultured in liquid medium. The periplasmic and culture medium fractions were assayed for phosphatase activity by a spectrophotometric method (30) that uses the hydrolysis of *p*-nitrophenylphosphate and the production of *p*-nitrophenol for quantitative assay. Activities (micromoles of *p*-nitrophenol per minute per milliliter of original culture) were as follows: *E. coli* MH1 supernatant, 4.37; MH1 periplasmic fraction, 0.18; MH1/pRW6.0 supernatant, 167.00; and MH1/pRW6.0 periplasmic fraction, 89.35. These data show that the alkaline phosphatase activity for the phosphatase fusion is located in the culture medium and the periplasm. The amount of phosphatase activity in the fusion construct compared with that in the control shows active production and secretion of phosphatase.

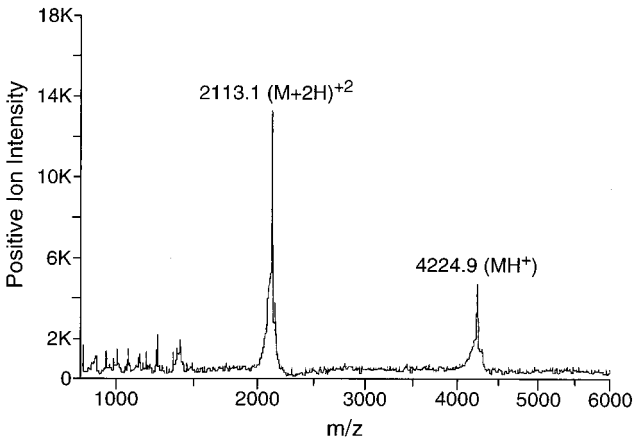

FIG. 2. Electrospray fast atom bombardment mass spectrum of divergicin A, showing multiply charged molecular ions from which the atomic mass was calculated.

DISCUSSION

We are interested in the development of a food grade vector suitable for use in our studies of bacteriocin production by LAB that were isolated from meats. The plasmid pCD3.4 in *C. divergens* LV13 was of interest because of its high copy number and the possibility that it was responsible for the production of divergicin A. Initially it was thought that the plasmid was too small to contain the genetic information for bacteriocin production or that bacteriocin production was controlled by genes on the plasmid and on the chromosome, as in the case of carnobacteriocin BM1 (25). The purification of divergicin A and the determination of its N-terminal sequence enabled the position of the structural gene for divergicin to be located on pCD3.4. From the precise agreement of the calculated and determined molecular masses of divergicin A of 4,223.89 and

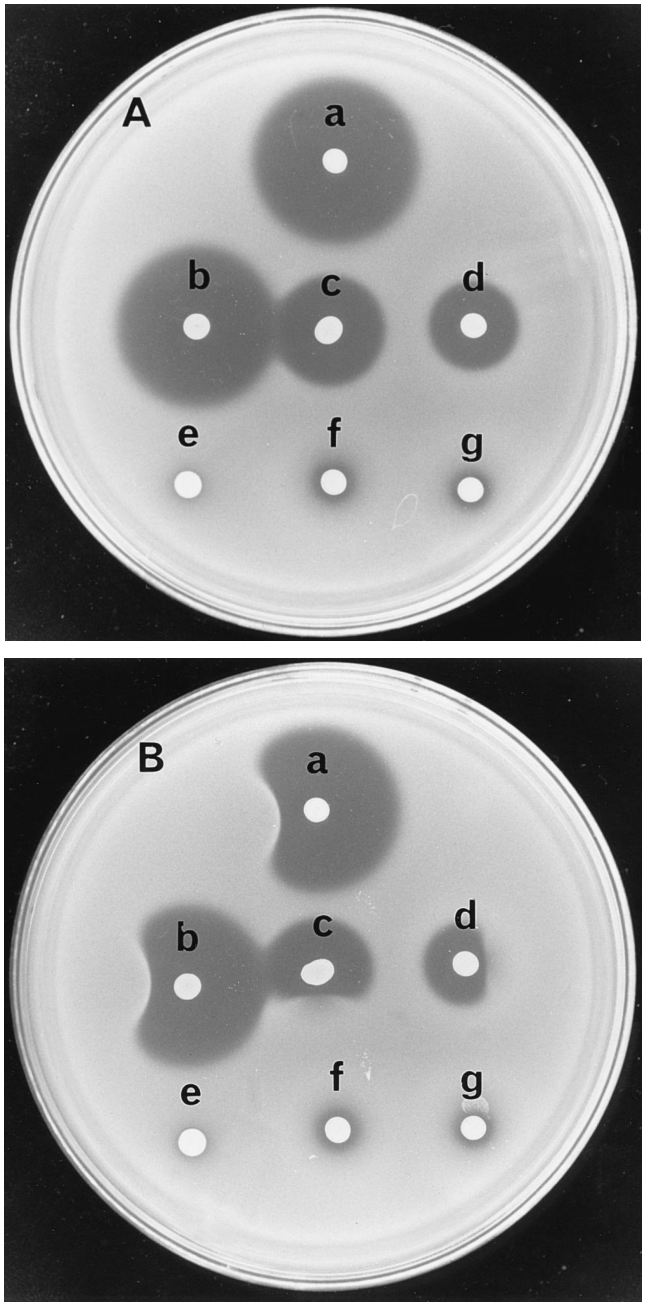

FIG. 3. Deferred inhibition of *C. divergens* AJ by homologous and heterologous hosts transformed with the divergicin A gene. (A) a, *C. divergens* LV13; b, *C. piscicola* LV17C containing pRW5.6; c, *L. lactis* MG1363 containing pRW5.6; d, *L. lactis* IL1403 containing pRW5.6; e, plasmidless *C. piscicola* LV17C; f, plasmidless *L. lactis* MG1363; g, plasmidless *L. lactis* IL1403. (B) Inactivation of divergicin A by pronase E. The producer strains are as described for panel A.

N-terminal extension of class I & II bacteriocins

Class I	
Nisin A	MSTKDFNLDLVSVSKKDSGASPR-
Class II	
Pediocin PA-1	MKKIEKLTEKEMANIIGG-
Leucocin A	MMNMKPTESYEQLDNSALEQVVGG-
Carnobacteriocin A	MNNVKELSIKEMQQVTGG-

Signal peptide of divergicin A

MKKQILKGLVIVVCLSGATFFSTPQASA-

FIG. 4. Amino acid sequence of the signal peptide of divergicin A compared with the sequences of the N-terminal amino acid extensions of well-characterized class I and class II bacteriocins of LAB. The sequences for nisin A, pediocin PA-1, leucocin A, and carnobacteriocin A are from references 16, 21, 12, and 39, respectively.

4,223.9 Da, respectively, it was evident that no posttranslational modifications occurred, such as a disulfide bridge between the cysteine residues at positions 12 and 44. The formation of disulfide bridges in mature bacteriocins has been observed for carnobacteriocins A and B2 (24, 39), leucocin A (12), and pediocin PA-1 (21).

Divergicin A has a novel amino acid sequence, N-terminal extension, and processing site. Its small size, hydrophobic nature and thermostability are indicative of a class II bacteriocin as defined by Klaenhammer (15). This class of bacteriocins includes pediocin PA-1; sakacins A and P; curvacin A; lactacin F; lactococcins A, B, G, and M; carnobacteriocins A, BM1, and B2; mesentericin Y105; and leucocin A-UAL 187. All of the class II bacteriocins for which the nucleotide sequence has been characterized are produced as precursors that contain an N-terminal extension with a Gly-Gly processing site at positions −1 and −2 of the cleavage site. These N-terminal extensions have marked homology in their amino acid sequences (10, 13, 15, 24). However, the N-terminal extension of divergicin has no homology with that of the class II bacteriocins, and, compared with other LAB bacteriocins, it has a unique processing site of Ala-Ser-Ala. No homology exists with the recently identified peptide antibiotic AS-48, produced by *Enterococcus faecalis*, which is postulated to contain a signal peptide in the N-terminal region of the immature antibiotic (20). The N-terminal extension of the divergicin structural gene resembles a signal peptide (Fig. 4), comparable to signal peptides of α- and β-amylases, alkaline phosphatase, outer cell wall proteins, β-lactamase, fimbriae, and proteases that access the *sec*-dependent pathway in bacteria (38). Signal peptides have three distinct domains referred to as the N, C, and H regions (23, 38). The N terminus region has at least one lysine residue in the first 6 to 8 amino acids, the H region contains 8 to 15 amino acids and has strong hydrophobicity, and the C region contains the cleavage site that consists of neutral amino acids with small side chains at positions −3 and −1, respectively (35, 36). The 29-amino-acid N-terminal region of divergicin complies with all of the Von Heijne rules for a signal peptide. The signal peptide for divergicin also contains a proline located two residues upstream of the Ala at position −3. This is thought to bend the signal peptide so that it can expose the cleavage site to the peptidase (37).

Class I and II LAB bacteriocins that have been characterized require a dedicated secretion apparatus that in most cases has been shown to be genetically linked to, or associated with, the bacteriocin structural and immunity genes. The possibility that all of the genetic information required for divergicin expression was contained on a small 3.4-kb plasmid seemed likely because transformation of the pCD3.4 plasmid into heterologous, bacteriocinogenic and nonbacteriocinogenic hosts resulted in divergicin production and immunity. The remaining 2.9-kb portion of pCD3.4 is too small to encode the different proteins necessary for secretion of the bacteriocin equivalent to those for pediocin PA-1, lactococcins A, B, and M, or nisin A. All of these bacteriocins rely on two or more large proteins for their activity, including an ATP binding cassette translocator protein (15).

Comparison of the N-terminal sequence with those of established signal peptides and the absence of additional secretory and maturation genes indicated that divergicin may be utilizing existing signal sequence-dependent general export pathways in the host. This was confirmed by cloning the dedicated genes for divergicin production and immunity present on the 514-bp *Eco*RV-*Acc*I fragment of pCD3.4 behind the P59 promoter of pGKV259. Production of divergicin A was also observed in heterologous hosts such as *C. piscicola* LV17C and *L. lactis* IL1403 and MG1363. *L. lactis* IL1403 contains a set of genes on the chromosome that are involved in production and secretion of lactococcins (28). However, production of divergicin in IL1403 was not greater than that observed in MG1363, indicating that a dedicated bacteriocin secretion machinery is probably not involved in divergicin production. Conclusive evidence that the N-terminal extension of divergicin acts as a signal peptide was achieved with the production of alkaline phosphatase in *E. coli* when the native signal peptide was replaced by that of divergicin. Divergicin requires as little as 0.5 kb for its production, whereas most bacteriocins require 3.5 to 10 kb for independent bacteriocin production. Although the spectrum of activity for divergicin A is narrow, the signal peptide creates interesting possibilities for secreting other bacteriocins via the *sec*-dependent pathway. With fusions between the signal peptide gene of divergicin and structural genes of other bacteriocins, it may be possible for bacteriocins of interest to be secreted without the specific secretion and maturation proteins.

Most LAB bacteriocins have a regular pattern of hydrophobic and hydrophilic domains. Divergicin A is a very hydrophobic molecule, with a calculated pI of 9.2 and only one small hydrophilic region. Several class II bacteriocins have been shown to contain a positively charged residue at the N terminus of the mature molecule. This would most likely inhibit secretion through the general export pathway (14). The class II bacteriocins have a common mode of action (1, 3, 19, 32). They are membrane-active compounds that cause cell permeabilization with loss of proton motive force and efflux of intracellular components. The immunity protein of divergicin has the unusual feature of being a small, very hydrophobic molecule and has an unusual topology because the second of the two transmembrane segments is flanked by positive charges. Divergicin A may have a different mode of action or immunity. Divergicin A contains six Gly-Gly paired residues in the mature peptide. The Gly-Gly motif is a feature of microcin B17, which is an inhibitor of DNA replication (7). The unique natures of the Ala-Ser-Ala processing site and the signal peptide indicate that divergicin A does not fit into established classes of bacteriocins. Further study will indicate whether divergicin A represents a fifth class of bacteriocins (15). The detection of a similar system in *E. faecalis* (20) represents another bacteriocin that may access the *sec* pathway of bacteria.

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