Characteristics and genetic determinant of a hydrophobic peptide bacteriocin, carnobacteriocin A, produced by *Carnobacterium piscicola* LV17A

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Carnobacteriocin A is a hydrophobic nonantibiotic bacteriocin that is detected early in the growth cycle of *Carnobacterium piscicola* LV17A and encoded by a 49 MDa plasmid. The bacteriocin was purified using hydrophobic interaction and gel filtration chromatography, and reversed-phase HPLC. Three different active peaks (A1, A2 and A3) were detected, but the purified samples had identical N-terminal amino acid sequences for the first 15 amino acids as determined by Edman degradation analysis. Only a 2.4 kb fragment of the *EcoRI* digest of the plasmid pCP49 hybridized with a 23-mer oligonucleotide probe derived from amino acids 5 to 13 of the amino acid sequence. The structural gene for carnobacteriocin A is located 600 base pairs into the 2.4 kb *EcoRI* fragment, but no other genetic information was detected on this unit. The structural gene includes an 18 amino acid N-terminal extension of the bacteriocin, ending with Gly-Gly residues in the –2, –1 positions with respect to the cleavage site. The bacteriocin consists of 53 amino acids that differ markedly from the majority of hydrophobic peptide bacteriocins characterized to date. Based on the amino acid sequence derived from the nucleotide sequence a molecular mass of 5052.85 Da was calculated. Mass spectrometric analysis showed that the molecular mass of the major component (A3) was 2 Da lower, thereby indicating the presence of a disulphide bridge between Cys 22 and Cys 51. Carnobacteriocin A2 has a similar structure except that Met 52 is oxidized to a sulfoxide, whereas A1 appears to be a mixture of peptides derived proteolytically from A3 or A2.

Keywords: bacteriocin, lactic acid bacteria, *Carnobacterium piscicola*, carnobacteriocin A, meat

INTRODUCTION

The lactic acid bacterium *Carnobacterium piscicola* LV17, isolated from the adventitious microflora of vacuum-packaged meat as an 'atypical *Lactobacillus*-type' organism (Shaw & Harding, 1984), produces heat-stable bacteriocins that are detected early in the growth cycle (Ahn & Stiles, 1990a, b). The parent strain contains three plasmids. Separation of the plasmids revealed that 'early' bacteriocin production is mediated by the 49 MDa

plasmid (Ahn & Stiles, 1992). Bacteriocins are proteinaceous, antimicrobial compounds that typically inhibit closely related bacteria (Klaenhammer, 1988). Nisin A produced by *Lactococcus lactis* subsp. *lactis* has been extensively studied and is used commercially as a food preservative (Delves-Broughton, 1990), but its applications are limited by its insolubility and instability at pH > 5. Interest in the use of bacteriocins for food preservation has increased with the discovery of many new active compounds produced by lactic acid bacteria (Klaenhammer, 1988). Some of these have been extensively characterized to reveal new 'classes' of bacteriocins, including heat-stable, low molecular mass, hydrophobic nonantibiotic compounds: lactacin F from *Lactobacillus acidophilus* (Muriana & Klaenhammer, 1991); lactococcin

Abbreviations: AU, (arbitrary) activity units; MeCN, acetonitrile; TFA, trifluoroacetic acid.

The GenBank accession number for the sequence reported in this paper is L23112.

A from *Lactococcus lactis* subsp. *cremoris* (Holo *et al.*, 1991); leucocin A-UAL187 from *Leuconostoc gelidum* (Hastings *et al.*, 1991); mesentericin Y105 from *Leuconostoc mesenteroides* subsp. *mesenteroides* (Hécharde *et al.*, 1992); pediocin PA-1 from *Pediococcus acidilactici* (Marugg *et al.*, 1992; Lozano *et al.*, 1992); and sakacin A from *Lactobacillus sake* (Holck *et al.*, 1992). All of these compounds, except for mesentericin Y105, have been shown to be ribosomally synthesized with an N-terminal extension to the structural peptide that is cleaved at a peptide bond adjacent to a common Gly-Gly (−2, −1) site.

Elucidation of the amino acid sequences of the widely reported pediocin PA-1/AcH (Motlagh *et al.*, 1992; Henderson *et al.*, 1992; Lozano *et al.*, 1992) revealed that these bacteriocins are the same compound. Exceptional similarity exists between leucocin A and mesentericin Y105, despite the different sources of the producer strains. They consist of 37 amino acids with variation only in amino acid residues 22 and 26, yet the activity spectra are strikingly different (Hastings *et al.*, 1991; Hécharde *et al.*, 1992; Y. Cenatiempo, personal communication). Similarly, there is considerable homology between the first 20 amino acids of leucocin A, pediocin PA-1, sakacins A and P, and curvacin A (Hastings *et al.*, 1991; Marugg *et al.*, 1992; Holck *et al.*, 1992; Tichaczek *et al.*, 1992). Many other bacteriocins may possess analogous sequences but lack of structural characterization limits the information that can be interpreted from reports of their occurrence. Complete elucidation of the amino acid sequence and the genetic determinants of bacteriocin activity can provide insight into the structure and function relationships and modes of action of these compounds. It is also a prerequisite for determination of three-dimensional structure by NMR spectrometry (Henkel *et al.*, 1992; Sailer *et al.*, 1993). In this study we describe the characteristics of carnobacteriocin A produced by *Carnobacterium piscicola* LV17A, including the complete amino acid sequence and the nucleotide sequence for the structural gene involved in synthesis of this bacteriocin.

METHODS

Bacteriology. The bacteriocin-producing organism *C. piscicola* LV17 was identified as a nonaciduric '*Lactobacillus*' isolated from vacuum-packaged meat by B. G. Shaw (AFRC Institute of Food Research, Langford, Bristol, UK). The parent strain contains three plasmids (Ahn & Stiles, 1990b). Strains LV17A and LV17A.17 are bacteriocinogenic (Bac⁺) and contain the 49 MDa plasmid or a 17 MDa religated Bg/II fragment of pCP49, respectively (Ahn & Stiles, 1992). Production of bacteriocin was monitored against *C. divergens* LV13 (from B. G. Shaw) by a spot-on-lawn technique (Ahn & Stiles, 1990b). Cultures were stored in Cooked Meat Medium (Difco) at 4 °C and at −70 °C in APT broth (Difco) at pH 6.5 containing 20% (v/v) glycerol. Stock cultures were subcultured in APT at 25 °C on two successive days before use in an experiment. Growth of *C. piscicola* LV17A for bacteriocin purification was done in semidefined medium (CAA) as described by Hastings *et al.* (1991). *Escherichia coli* strains were grown in Luria-Bertani (LB; Sambrook *et al.*, 1989) broth or agar at 37 °C, and stored at −70 °C in LB broth with 20% glycerol. The bacterial strains and plasmids used in this study are listed in Table 1.

Purification of carnobacteriocin A. The procedure was adapted from Sailer *et al.* (1993). The producer strain was grown at 25 °C in 2 litres of CAA adjusted to and maintained at pH 6.2 with sterile 1 M NaOH using a pH stat (Chem-Cadet, Cole Palmer) while being stirred gently under a steady flow of nitrogen (40 ml min^{−1}). After 16 h (NaOH consumption 150 ml) the supernatant fluids were collected by centrifugation at 8000 g for 20 min at 4 °C. The culture supernatant (2150 ml) was applied directly to an Amberlite XAD-8 column (4.5 × 45 cm; BDH Chemicals) washed with 2 litres of 0.1% trifluoroacetic acid (TFA), and eluted with successive 1 litre volumes of 20, 30, 45 and 80% (v/v) ethanol in 0.1% TFA. Active fractions were combined and concentrated to 30 ml using a rotary evaporator at 30 °C, mixed with the same volume of acetonitrile (MeCN) and loaded onto a Sephadex LH-60 column (5 × 25 cm) that had been equilibrated with 50% (v/v) MeCN in 0.1% aqueous TFA. The active fractions were pooled and concentrated to 5 ml by rotary evaporation. The concentrate was applied in 0.5 ml portions to a C₈ HPLC column (VYDEC, 10 × 250 mm, 10 µm, 300 Å, flow rate 3 ml min^{−1}, mobile phase: A, 0.1% TFA in water; B, 70% acetonitrile in 0.1% TFA) and eluted using a gradient method: first 43% to 54% B solvent in 4 min and then 54% to 61% B in 7 min. Fractions were monitored for absorbance at 218 nm and activity against the indicator strain. Purifications of separated active peptides were completed by isocratic elution on the same column (flow rate 3.5 ml min^{−1}, in 46%, 55% and 57% of B solvent for A1, A2 and A3, respectively).

Stability of carnobacteriocins A. The effects of pH, heat treatment, and selected organic solvents on the activity of purified carnobacteriocins A1 [1600 arbitrary units (AU) ml^{−1}], A2 (200 AU ml^{−1}), and A3 (400 AU ml^{−1}) were determined. Samples were suspended in the following buffers: pH 1.0 and 3.0 glycine/HCl (50 mM and 30 mM, respectively), pH 5.0 in citric acid/HCl (50 mM), pH 7.0 and 9.0 in Tris buffer (50 mM) adjusted with HCl. Samples at each pH were heated in a boiling water bath for 30 min. Residual activity was determined after 2 and 24 h at each pH level and immediately after heat treatment. To test effects of organic solvents, the carnobacteriocins were suspended in 50% (v/v) solutions of aqueous 0.1% TFA with acetone, acetonitrile, ethanol, ethyl acetate, methanol and tetrahydrofuran. Lyophilized samples were also dissolved in 0.1% TFA, stored at 5 °C for 14 d, and checked by HPLC. Degradation was estimated from the appearance of decomposed products and residual activity.

Determination of N-terminal amino acid sequence of carnobacteriocin A. Purified carnobacteriocins from *C. piscicola* LV17A were subjected to Edman degradation analysis on an automated gas-phase sequencer (Applied Biosystems model 470A) with on-line phenylthiohydantoin derivative identification by reversed-phase HPLC (Applied Biosystems model 120A).

Mass spectrometry of carnobacteriocins. All mass spectra were kindly acquired by Dr Robert B. Cody at Jeol USA (Peabody, MA) on a Jeol SX102A instrument using an electrospray interface for fast atom bombardment mass spectrometry (FAB MS) in the positive ion mode. Samples of A1, A2, and A3 obtained by HPLC purification were dissolved in 1:1 (v/v) aqueous methanol containing 2% (v/v) glacial acetic acid and were analysed by direct infusion through a sample loop. Jeol software was employed to determine molecular masses from the envelopes of multiply charged peaks in the *m/z* spectra.

SDS-PAGE. Purified carnobacteriocin preparations were examined using 20% (w/v) polyacrylamide gels and 3 M Tris/HCl,

Table 1. Bacteria and plasmids used to study bacteriocin production by *C. piscicola* LV17A

Bacterial strain or plasmid/phage type	Description*	Source or reference
<i>Carnobacterium piscicola</i>		
LV17A	Bac ⁺ Imm ⁺ containing pCP49	Ahn & Stiles (1990b)
LV17A.17	Bac ⁺ Imm ⁺ containing 17 MDa <i>Bgl</i> II religated fragment of pCP49	Ahn & Stiles (1992)
LV17B	Bac ⁺ Imm ⁺ containing pCP40	Ahn & Stiles (1990b)
<i>Carnobacterium divergens</i>		
LV13	Bac ^s (indicator strain)	B. G. Shaw, Langford, Bristol, UK
<i>Escherichia coli</i>		
MV 1193	$\Delta(lac-proAB) rpsL thr end A spcB15 hsdR4 \Delta(srl-recA) 306::Tn10 (Tet^r) F' [traD_{36} pro AB^+ lacI^q lacZ \Delta M15]$	Sambrook <i>et al.</i> (1989)
Plasmids		
pUC118	<i>lacZ'</i> Ap ^r , 3.2 kb	Vieira & Messing (1987)
pK194	<i>lacZ'</i> Km ^r , 2.4 kb	Jobling & Holmes (1990)
pRW5.9U	Ap ^r <i>cbnA</i> , 5.9 kb	This study
pRW4.4K	Km ^r <i>cbnA</i> , 4.4 kb	This study
Phage		
M13K07	Helper phage for pUC118	Sambrook <i>et al.</i> (1989)

* Bac⁺, production of carnobacteriocin A; Imm⁺, immunity to carnobacteriocin A.

pH 8.8, as described by Laemmli (1970). Electrophoresis was done at constant current of 20 mA. Gels were fixed in 5% (v/v) formaldehyde for 30 min and stained with Coomassie Blue (Bio-Rad).

DNA isolation, manipulation, and hybridization. Large-scale preparation of plasmid DNA from *C. piscicola* LV17A was done by a modification of the Klaenhammer (1984) method. The method was scaled up 10× using cells from 1 litre of culture grown in APT broth. The resuspended cell solution was carefully adjusted to pH 8.0 for lysozyme treatment, and to pH 7.0 after alkaline lysis. Plasmids were purified by CsCl density gradient ultracentrifugation (Sambrook *et al.*, 1989). Small- and large-scale plasmid isolation from *E. coli* was done according to Sambrook *et al.* (1989). Restriction endonucleases and T4 DNA ligase (Boehringer-Mannheim or Bethesda Research Laboratories) were used as recommended by the suppliers. DNA cloning and hybridization were done by methods described by Sambrook *et al.* (1989). Southern and colony blot hybridizations were done on Hybond N nylon membranes (Amersham), and DNA probes were labelled with [γ -³²P]ATP (Amersham) using T4 polynucleotide kinase (Pharmacia) or with digoxigenin UTP using terminal transferase (Boehringer-Mannheim). Hybridization and washes were done at 30 °C. A degenerate 23-mer oligonucleotide, RWO1 [5'-GAXGGXGTAA(CX)TAXGGXAA(AG)GG3'; X = bromouracil] was used to detect carnobacteriocin A gene(s) in all Southern and colony blot hybridizations. Oligonucleotides prepared as sequencing primers were synthesized on an Applied Biosystems 391 PCR Mate synthesizer.

Molecular cloning of carnobacteriocin A gene(s). Plasmid DNA from *C. piscicola* LV17A was digested with a range of restriction enzymes that have unique restriction sites within the multiple cloning site (MCS) of pUC118. A 2.4 kb *Eco*RI fragment that hybridized with the carnobacteriocin probe (RWO1) was cloned into pUC118 and screened by colony blot

hybridization for successful clones. Plasmid DNA from Bac⁺ clones was digested with *Eco*RI and the 2.4 kb *Eco*RI insert was eluted from the gel using a one-step method for isolation of DNA fragments (Heery *et al.*, 1990) and cloned into the MCS of pK194 (Jobling & Holmes, 1990). Positive pUC118 clones were used to produce single-stranded DNA for sequencing.

Nucleotide sequencing of plasmid DNA. Single- and double-stranded DNA was sequenced by *Taq* DyeDeoxy Cycle sequencing on an Applied Biosystems 373A sequencer. Single-stranded DNA was sequenced from the pUC118 clone (pRW5.9U) using specific sequence oligonucleotides and the universal primer of pUC118. The complementary strand was sequenced in the double-stranded form using the pK194 clone (pRW4.4K) as the sequencing template. Single-stranded DNA was produced by infecting the *E. coli* clone containing the pUC118 clone with M13K07 helper phage and incubating at 37 °C with rotary shaking (200 r.p.m.) for 75 min. Kanamycin (Km) was then added to give a final concentration of 75 µg ml⁻¹ and incubation was continued for 18 h. Supernatant containing the progeny phage was harvested by polyethylene glycol precipitation (Applied Biosystems Instruments recommended procedures). Large-scale preparation of single-stranded DNA was done by 50× scaling up of the same procedure. Opposite orientation nucleotide sequencing of the 2.4 kb *Eco*RI fragment was tested on existing positive clones. Opposite orientations were attempted by cutting the clone of pRW5.9U and religating the pool of fragments.

RESULTS

Purification and stability of A carnobacteriocins

Carnobacteria do not grow well below pH 5.0 and do not produce bacteriocin below pH 5.5 (Ahn & Stiles, 1990a). From the data in Fig. 1 it can be seen that with the pH held

Fig. 1. Bacteriocin production and activity [in arbitrary units (AU)] of carnobacteriocin A produced by *C. piscicola* LV17A with time at constant pH of 6.0 at 25 °C. □, AU ml⁻¹; ●, log c.f.u. ml⁻¹.

constant at 6.0 the greatest amount of bacteriocin activity was detected between 9 and 18 h of incubation at 25 °C. The bacteriocin is unstable and activity decreases rapidly during further incubation. Using the culture supernatant of *C. piscicola* LV17A grown at pH 6.5 for 18 h and the purification steps shown in Table 2, a total yield of 4.6×10^6 arbitrary activity units (AU) determined against *C. divergens* LV13 was obtained. A total of 1.9×10^5 AU (41 %) was recovered after purification by reversed-phase HPLC. Following this isolation protocol three extracellular active peptides were observed (Fig. 2) and separated by HPLC. The major product, carnobacteriocin A3, was separated with a yield of about 800 µg from 2 litres of culture broth. Carnobacteriocin A2 was obtained with a lower yield of 500 µg, whereas A1 proved to be a

Fig. 2. Elution pattern of carnobacteriocins A on a reversed-phase HPLC C-8 column (VYDEC) using 0.1% TFA/MeCN gradient. Peaks A1, A2, and A3 represent the active peaks obtained.

mixture of degradation products. Carnobacteriocins A1 and A2 also displayed lower specific activities. Gel electrophoresis indicated molecular masses of approximately 4000 Da (Fig. 3). Although partially purified carnobacteriocins A decomposed rapidly (possibly due to proteolysis), pure lyophilized samples of carnobacteriocins A2 and A3 showed no decrease in biological activity and gave clean mass spectra after storage for several months at 4 °C (Fig. 4). All three bacteriocins were unstable in solution for an extended period of time. During storage at 5 °C for 14 d in 0.1% TFA, A1 was completely degraded, A2 was the most stable, with 80 % of its activity remaining, and A3 retained only 10 % of its activity. None of the degradation products isolated by HPLC showed biological activity against *C. divergens* LV13 (data not shown). Data in Table 3 show that

Table 2. Purification of carnobacteriocins A1–A3 produced by *C. piscicola* LV17A at pH 6.5

Purification stage	Volume (ml)	Activity (AU ml ⁻¹)*	Total activity (AU)	Amount of protein† (mg ml ⁻¹)	Specific activity (AU mg ⁻¹)	Activity recovery (%)	Purification (-fold)
Supernatant	5800	0.8×10^3	4.64×10^6	3.3	2.43×10^2	100	1
XAD-8 fractions ‡	130	3.2×10^4	4.16×10^6	1.7	1.94×10^4	90	80
Sephadex LH-60 fractions‡	220	1.28×10^5	2.88×10^6	0.17	7.9×10^4	62	325
C-8 column fractions‡	300	0.64×10^5	1.92×10^6	0.08	8.24×10^4	41	339
RP-HPLC§							
A1	0.15	0.64×10^5	9.6×10^3	0.7	9.15×10^4	8.6	
A2	0.92	1.28×10^5	1.18×10^5	0.5	2.56×10^5		
A3	0.40	6.83×10^5	2.73×10^5	1.0	6.83×10^5		

* AU, activity units determined against *Carnobacterium divergens* LV13.

† Determined by method of Lowry as modified by Markwell using BSA as a standard (pure sample).

‡ Pooled active fractions were concentrated to the given volumes.

§ Active fractions were lyophilized and resuspended in 0.1% TFA.

Fig. 3. Polyacrylamide gel (20%) electrophoresis of carnobacteriocin A fixed with 5% formaldehyde and stained with Coomassie blue. Lanes: A, carnobacteriocin A3 (5 µl); B, carnobacteriocin A3 (3 µl); C, carnobacteriocin A3 (1 µl); D, blank; E, low molecular mass protein standards – 66, 45, 34.7, 24, 18.4, 14.3 kDa (Sigma); F, nisin; G, insulin; H, low molecular mass protein standards – 16949, 14404, 8159, 6124 and 2512 Da (Fluka, Biochemika).

carnobacteriocin A1 is relatively heat stable (100 °C for 30 min) compared with A2 and A3. The carnobacteriocins were most stable at pH 1.0 and stability generally decreased with increase in pH. They were stable in the organic solvents tested; in fact enhanced activity was noted in some cases after 24 h of storage in the solvents. Although the reason for this enhanced activity is uncertain, it may be due to the increase in the population of

Fig. 4. Electrospray FAB mass spectra (positive ion mode) of carnobacteriocins A2 and A3 showing multiply charged molecular ions from which atomic mass was calculated.

an active conformation in the distribution of possible three-dimensional structures caused by the interaction of the lipophilic peptide with organic solvents.

The ratios between the different bacteriocins during growth of the producer strain at 25 °C at pH 6.5 are

Table 3. Effect of pH, temperature and organic solvents (1:1 mixtures with 0.1% TFA) on the antimicrobial activity (AU ml⁻¹) of the purified carnobacteriocins A1, A2 and A3

Temp. °C...	A1			A2			A3		
	25		100	25		100	25		100
	2	24	0·5	2	24	0·5	2	24	0·5
0·1 % TFA (control)	1600	1600	1600	200	200	—	400	400	—
pH 1	800	400	400	200	200	200	400	400	0
pH 3	800	0	800	200	200	0	400	100	100
pH 5	800	100	200	< 100	0	0	400	100	0
pH 7	200	< 100	200	< 100	0	< 100	200	100	0
pH 9	100	0	0	100	100	100	400	< 100	0
Ethyl acetate	—	—	—	200	200	—	400	400	—
Acetonitrile	1600	1600	—	200	800	—	400	400	—
Tetrahydrofuran	—	—	—	200	400	—	400	400	—
Ethanol	1600	1600	—	200	800	—	400	1600	—
Methanol	1600	1600	—	200	400	—	400	1600	—
Acetone	—	—	—	200	400	—	400	200	—

Table 4. Ratio of carnobacteriocins produced by *C. piscicola* LV17A during fermentation at 25 °C in APT broth at pH 6.5

HPLC retention time... Incubation time (h)	Activity (AU ml ⁻¹)		
	A1 4 min	A2 19 min	A3 23 min
9	800	200	200
18	800	100	100
26	200	ND	ND
33	ND	ND	ND

ND, Not detected.

shown in Table 4. At the end of the exponential phase of growth the ratios of A1:A2:A3 were 4:1:1; this changed to 8:1:1 after another 18 h of incubation; and after a further 26 h, only A1 could be detected. This is unlike the stability observed with the purified bacteriocins suspended in 0.1% TFA.

Characterization of carnobacteriocins A

Mass spectral analysis using positive ion fast atom bombardment with an electrospray interface gave multiply charged molecular ions (Fig. 4) which allowed calculation of the average molecular masses as follows: for carnobacteriocin A3, 5050.8 ± 0.3 Da; for A2, 5067.0 ± 0.3 Da; and for the major component of A1, 4524.7 ± 0.4 Da. Edman degradation of purified samples revealed 16, 20 and 28 amino acids in the N-terminal sequences of samples A1, A2 and A3, respectively. The only difference detected between the three compounds was at amino acid residue 16. After sequencing of 16 to 28

Table 5. Amino acid analysis of carnobacteriocins

Amino acid	A2		A3	
	pmol	No. of residues in sequence	pmol	No. of residues in sequence
Asn/Asp	929	5	3028	5
Ala	1065	6	3120	6
Gly	2864	13	19911	13
Gln/Glu	361	2	1100	2
His	127	1	377	1
Ile	430	2	1280	2
Leu	1138	6	3231	6
Lys	846	4	2473	4
Met	195	1	293	1
Pro	—	—	1279	2
Ser	1027	6	3332	6
Thr	232	2	734	2
Tyr	238	2	686	2
Val	424	2	1314	2

amino acids, the accuracy of identification decreased due to background peaks that resulted in a lower confidence level for the identification of the amino acid sequence. The amino acid analyses of the A2 and A3 bacteriocins are given in Table 5. These amino acid analyses agree with the subsequent results derived from the nucleotide sequence data, except for the failure to detect proline for A2.

Isolation of carnobacteriocin A structural gene

Plasmid DNA (pCP49 and pCP17) was isolated from *C. piscicola* LV17A and LV17A.17 and digested with restriction enzymes compatible with the multiple cloning site of pUC118 (Fig. 5). A degenerate 23-mer oligo-

Fig. 5. Restriction maps of pCP49 and pCP17, a religated 25.5 kb *Bgl*II fragment of pCP49 from *C. piscicola* LV17A.

AAAAAAGAAAATTTTTCTCGATTATTTATTTACTAAATAAATAATGCTATGGTGAGCACATAGCTAAATATGAATATCACTATTACCGA 90
 ATTTTTATTTATAAGATATGCTGTGGTAAGGAGAAGCTACTTTTCCAAAATGAATATTTAATTTAGTTTCAATAGTTTTAACTTT 180
 GCACCCCAATCACTTTCTGAGCGAAAAATAAATATGAAAATCTTCTTTTAGGACATCAATCAGTAGTGTTCCAGGATATTTTGACTAAA 270
 TATCTATTTGGCATGATAGAATGTTCTTGTAACCTCAAATAAAATTTAAAGGAGTCTGATTAGATGAATAATGTAAAAGAGTTAAGTATT 360
 [RBS] M N N V K E L S I
 AAAGAAATGCAACAAGTTACTGGTGGAGACCAAATGTCAGATGGTGTAATTATGAAAAGGCTCTAGCTTATCAAAAGGTGGTGCCAAA 450
 K E M Q Q V T G G D Q M S D G V N Y G K G S S L S K G G A K
 TGTGGTTTAGGGATCGTCGGCGGATTAGCTACTATCCCTTCAGGTCCTTTAGGCTGGTTAGCCGGAGCAGCAGGTGTAATTAATAGCTGT 540
 C G L G I V G G L A T I P S G P L G W L A G A A G V I N S C
 ATGAAATAAAAACTCAAATAATTTGAATTTAGACTGTAGACCAAAGAGTTGGATTTAAGTTCCAACCTCTTTTGTCTTTACTATTTAGT 630
 M K .
 ATTTTAAATTAATAAAAAAGGCCTACTGCCTTTCAATAGTCTCGTAGCTAATTTCTTTGTTTATACTAGTTTAAAGTGGAACACTTT 720
 TTATGGATGTGATGGTTGAAAAGTACACCACTAATAACTCACAATAAGGAGATGAACCTATCTTTTATTGGAGGAATCAAGAGTGAAAA 810
 AATTAATGGATAAATGTACAATTAACGTCTAATTGAAAGTGGTCTTCTCAAAGGTCTGTTGCTAAAAAATTAACATGAATTGCCGCA 900
 GAGTTGCCAGATATTGGGTGGAATATCTTCAAGTAAAAGAACCGGTTTCACTTAATCCTTCGGATCCAATAAAAAAGGAACCTCATCAG 990
 TCTAAACCTACTTATATGAAAACCAATCACAGACCAATAAAGTACACTAAAGAAACGGATAATAGAATTGATGAATTATTAGAATTTGAT 1080
 TGTGATAAGGCAAAAACTGGACGGCATAAACAAAAAACTAATGACAATAAATATTCATGAAATCTTGATTTTCGAAAGTTTTGACATTG 1170
 GTGAAAGCACTATTTCATTCCTATGTTAGGAAGAAGGTTTCAGAAAATAAAGAAAGCCTTTATCAAGCAAATATATCCTTATTAATAAAAA 1260
 CTGAAAACGTCATCCCTATTGAGATATTTGTCTACAGTCTGAAACTAAAATTAATTTGAGTTTTTTTTTACTTCTTTACATACAACCTACT 1350
 AATAGAATTATTAAGCTTAGTGAACCTTAATACTTTTTGGATAATAGTATCAGTTAAGAATATTAATATTAGATTGGAAATATAGAAA 1438

Fig. 6. Single-stranded nucleotide sequence of a 1.4 kb region within the 2.4 kb *EcoRI* fragment of pCP17. The sequence shows one open reading frame commencing at nucleotide residue 334 encoding the structural gene for carnobacteriocin A. The underlined portion represents the 53 amino acid sequence of the mature carnobacteriocin A. No other open reading frame was detected. A potential rho-independent termination site is indicated by reversed arrows on the nucleotide sequence between residues 584 and 612. RBS, ribosome binding site.

nucleotide (RWO1) probe was derived from the identical sequence of amino acid residues 5 to 12 of the N-terminus of all three bacteriocins. Hybridization was observed with a 2.4 kb *EcoRI* fragment. No other hybridization signal was observed in the remaining *EcoRI* fragments of pCP49 or when the probe was hybridized with the genomic DNA and pCP40, another native plasmid contained in the wild-type strain of *C. piscicola* LV17 (Ahn & Stiles, 1990b). Shotgun cloning of an *EcoRI* restriction digest of pCP17 into the *EcoRI* site of pUC118 was performed and colonies were screened using alpha complementation. Colony blots were done to screen the white colonies for the correct insert. The positive clones were digested, Southern transferred and hybridized with the degenerate probe RWO1 to confirm the presence of the carnobacteriocin gene(s).

Nucleotide sequence of the carnobacteriocin A structural gene

The nucleotide sequence of a 1.4 kb segment of the 2.4 kb *EcoRI* fragment of pCP17 is shown in Fig. 6. Analysis of this sequence revealed the presence of only one open reading frame (ORF) with a probable promoter site. The N-terminal amino acid sequences of carnobacteriocins A2 and A3 matched the sequence derived from nucleotide sequencing. These data indicate that residue 16 in carnobacteriocin A1 is actually serine, as determined by Edman degradation analyses for carnobacteriocins A2 and A3. The first amino acid of the N-terminal sequence matches with the nineteenth amino acid derived from the nucleic acid sequence, so that the prepeptide contains 71 amino acids and is comprised of an N-terminal extension

consisting of 18 amino acids and the bacteriocin consisting of 53 amino acids. The probable ribosome-binding site (AGGAG) for the carnobacteriocin A (*cbnA*) structural gene has the optimal spacing of 9 bases upstream of the initiation codon (coordinate 319). A likely -10 promoter region (TAGAAT) has been identified at position 287 with a TG sequence one nucleotide ahead of the -10 region. Upstream of the -10 region is a potential -35 region located at base pair 262. Downstream of the structural gene there is an 11 base inverted repeat with a 7 base loop that is a potential rho-independent terminator. No significant second ORF was observed in the remaining 800 bases of the 2.4 kb fragment (see Fig. 6) and a total of 65 stop codons is present across the three possible reading frames. The ends of the 2.4 kb *EcoRI* fragment were identified by the *EcoRI* sites at each end of the fragment, along with the remainder of the M13/pUC multiple cloning site.

DISCUSSION

The technique for isolation of leucocin A-UAL 187 reported by Sailer *et al.* (1993) worked well for the purification of bacteriocins from *C. piscicola* LV17A. Three peaks (A1, A2 and A3) which had activity against the indicator strain were separated by HPLC. This suggests either that three different bacteriocins are independently produced by *de novo* protein synthesis, or that subsequent post-translational or chemical modifications of one or more bacteriocins occurs. N-terminal amino acid sequencing of the active substances revealed that at least the first 15 amino acids were identical in each of the three peptides. This result indicates that there is only one parent antibacterial compound (carnobacteriocin A) directly generated from a single structural gene, and that this peptide is subsequently post-translationally transformed to other active derivatives. This was confirmed by experiments with the 23-mer oligonucleotide probe which hybridized with a single restriction fragment. Initial mass spectra for the three compounds isolated by HPLC were ambiguous (not reported) because of relatively rapid decomposition of the samples to complex mixtures. Subsequently, more rigorous HPLC purification gave stable samples that afforded clean fast atom bombardment electrospray mass spectra for compounds A2 and A3, and showed that A1 was still an apparently inseparable mixture resulting from degradation at the C-terminal end of the parent bacteriocin. Comparison of the molecular mass of carnobacteriocin A3 (5050.8 ± 0.3 Da) calculated from the mass spectrum to that predicted from the nucleotide sequence (5052.8 Da) indicated that this parent bacteriocin contains a disulphide bridge between the two cysteines at positions 22 and 51 but is not otherwise modified. The difference of +16.2 Da in the molecular mass of A2 relative to A3 and the identical results from amino acid sequencing for the first 15 residues of both compounds suggests that carnobacteriocin A2 has the sulphur in methionine 52 oxidized to a sulfoxide. Similar oxidations have been confirmed by detailed structural analyses of carnobacteriocins BM1 and B1 produced by *C. piscicola* LV17B (unpublished data). Thus the actual amino

acid sequences of A3 and A2 are identical except for the presence of a methionine sulfoxide at position 52 in A2. Based on the mass spectra for A1, it is assumed that this is a breakdown product of A2 and A3.

Activity of carnobacteriocins A was rapidly lost during storage in buffered solutions at pH > 3, and the compounds are relatively unstable until completely purified. However, pure samples of A2 and A3 are completely stable for months in lyophilized form. The antimicrobial activity of carnobacteriocins A is relatively stable to organic solvents and heat, especially the breakdown mixture A1. Although a number of low molecular mass, heat-stable, nonlantibiotic, hydrophobic peptide bacteriocins have been purified and characterized from various lactic acid bacteria, carnobacteriocins A3 and A2 have a slightly larger molecular mass and show little amino acid sequence homology with other bacteriocins (Hécharde *et al.*, 1992; Henderson *et al.*, 1992; Holck *et al.*, 1992; Lozano *et al.*, 1992; Marugg *et al.*, 1992; Motlagh *et al.*, 1992; Tichaczek *et al.*, 1992), including carnobacteriocins BM1 and B2 produced by the wild-type *C. piscicola* LV17 (unpublished data).

Despite difficulties with stability of purified carnobacteriocin A, as many as 16 to 28 amino acids were determined in the N-terminal sequence for all three compounds. Detection of a single structural gene *cbnA* on the plasmid pCP49 coding for a serine residue at position 16 of the N-terminal amino acid sequence confirmed that carnobacteriocins A2 and A3 are products of the same gene. The possibility of the structural gene for carnobacteriocin A1 being on the chromosome was excluded by hybridizing the carnobacteriocin probe with the chromosomal DNA. It was concluded that carnobacteriocins A1, A2 and A3 have only one structural gene and that initial tentative identification of valine at position 16 of carnobacteriocin A1 resulted from difficulty in interpretation of the Edman degradation data of this apparently inseparable peptide mixture.

Production of more than one bacteriocin by a single organism is not unique. In the case of *C. piscicola* LV17, Ahn & Stiles (1992) demonstrated that bacteriocin production was mediated by different plasmids. In the case of lactococcins A, B, and M (van Belkum *et al.*, 1991, 1992) all three bacteriocins were shown to be produced by a 60 kb plasmid in *L. lactis* subsp. *cremoris* 9B4. Of interest for further study in *C. piscicola* LV17 is the fact that carnobacteriocins A and B are detected early and late in the growth cycle, respectively. It is not clear whether an 'early' bacteriocin, such as carnobacteriocin A or leucocin A-UAL187 (Ahn & Stiles, 1992; Hastings *et al.*, 1991) is attributable to production of highly active compounds that are detected early in the growth cycle, to differences in copy number of the plasmids promoting higher production, or whether there is genetic control that determines time of production relative to stage of the growth cycle.

Production of lactococcin A by *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* WM4 is controlled by a cluster of four genes contained within a 5 kb fragment of plasmid

DNA (Stoddard *et al.*, 1992). Upstream of the structural and immunity genes are three ORFs, two of which are implicated in secretion of the bacteriocin through their homology with proteins of signal sequence-independent secretion systems. A similar cluster of four genes from *P. acidilactici* in a 3.5 kb segment of plasmid DNA was associated with production and secretion of pediocin PA-1 in *E. coli* (Marugg *et al.*, 1992). In this case all genes are located immediately downstream of the structural gene in a relatively compact configuration; however, it was previously shown for this strain of *P. acidilactici* that immunity to its own bacteriocin is chromosomally mediated (Gonzalez & Kunka, 1987). The structural gene for carnobacteriocin A is located on the 2.4 kb *EcoRI* fragment of DNA on which only one ORF was identified, indicating a considerable separation of the structural gene from its immunity and secretion genes. Evidence that the immunity gene is on the same plasmid was obtained by cloning different fragments of DNA downstream of the structural gene of carnobacteriocin A into a plasmidless strain of *C. piscicola* LV17 (data not shown). Analysis of the genetic control of carnobacteriocin A production is proceeding.

Although carnobacteriocin A differs in molecular size and amino acid composition from other bacteriocins in this group, it is similar to other bacteriocins by having an N-terminal extension that terminates in Gly-Gly residues immediately prior to the cleavage site (Hastings *et al.*, 1991; Holo *et al.*, 1991; Marugg *et al.*, 1992; Muriana & Klaenhammer, 1991; Stoddard *et al.*, 1992; Van Belkum *et al.*, 1991, 1992). The role of the N-terminal extension in synthesis or secretion of the active bacteriocin has yet to be demonstrated.

In this fragment only one significant ORF was identified and it is the structural gene for carnobacteriocin A. Contained within this region just upstream of the start of the ORF is a probable ribosome-binding site spaced at the optimal number of base pairs upstream of the initiation codon for the carnobacteriocin A structural gene. The -10 promoter region contained a TG sequence upstream. This TG region is conserved in some Gram-positive promoters (Graves & Rabinowitz, 1986). Earlier work by C. Ahn (unpublished data) indicated that the structural and immunity genes on pCP49 are both on a 5.4 kb *XbaI*-*PstI* fragment. The orientation of the structural gene in the *EcoRI* fragment indicates that the adjacent *EcoRI*-*XbaI* fragment probably contains the immunity gene.

Carnobacteriocin A, as described in this and previous studies (Ahn & Stiles, 1990a, b, 1992), is an interesting bacteriocin because its early production during the growth of *C. piscicola* LV17 gives the producer organism the potential for an ecological advantage in mixed fermentations. The antibacterial spectrum includes many lactic acid bacteria, and also *Enterococcus* and *Listeria* spp. (Ahn & Stiles, 1990b). Cloning and transformation of the 25.5 kb *Bgl*/II fragment of pCP49 into bacteriocinogenic *C. piscicola* UAL 26 resulted in the cloned organism acquiring the antibacterial spectrum of both bacteriocins (Ahn & Stiles, 1992). The ability to produce the cloned

bacteriocin in a heterologous Bac⁺ host, with expansion of the activity spectrum of the host strain, encourages the further study of producing 'gene cassettes' of bacteriocins to broaden the antibacterial activity of a producer strain. The immunity and secretion genes have yet to be demonstrated. An understanding of the specific functions of the secretion proteins may allow secretion of several bacteriocins by a single secretion system.

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