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Purification and Amino Acid Sequences of Piscicocins V1a and V1b, Two Class IIa Bacteriocins Secreted by *Carnobacterium piscicola* V1 That Display Significantly Different Levels of Specific Inhibitory Activity

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Two bacteriocins produced by *Carnobacterium piscicola* V1 were purified and characterized. Piscicocin V1a (molecular mass = 4,416 Da) and piscicocin V1b (molecular mass = 4,526 Da) are nonlantibiotic, small, heat-stable antibacterial peptides. Piscicocin V1b is identical to carnobacteriocin BM1, while piscicocin V1a is a new bacteriocin. Its complete sequence of 44 amino acid residues has been determined. Piscicocin V1a belongs to the class IIa bacteriocins having the consensus YGNGV motif. These peptides inhibit various gram-positive bacteria, including *Listeria monocytogenes*. Piscicocin V1a is approximately 100 times more active than piscicocin V1b against indicator strains. However, the antagonistic spectrum is the same for both piscicocins. Comparison of these results with the analysis of the amino acid sequence and secondary structure predictions suggests that (i) the conserved N-terminal conserved domain is involved in the receptor recognition and therefore in an "all-or-none" response against target bacterial cells and (ii) the C-terminal variable and hydrophobic domain determines membrane anchoring and therefore the intensity of the antagonist response.

Converting sugars into organic acids by lactic acid bacteria (LAB) is a well-known fermentation process to improve the flavor and texture of some food products. LAB are also interesting for their ability to inhibit the growth of undesirable bacteria. This inhibitory activity is due to numerous metabolic events, including production of organic acids, hydrogen peroxide and carbon dioxide, nutrient depletion, decrease in redox potential, and synthesis of bacteriocins (22). The mechanism of bacteriocin secretion has attracted attention for potential applications in the preservation of food products. In this regard, it has been shown that LAB or their isolated bacteriocins may be useful in extending the shelf life of fish, meat, and dairy products (20, 22).

More interesting, LAB bacteriocins could provide, with other peptides from animal and plant sources, original properties in the search for new antimicrobial agents usable in human health care (18) or in plant breeding (4, 24). In this regard, LAB bacteriocins can serve as models in the design of new analogs and chimeric peptides (28) or in the elaboration of new noncovalent synergistic associations with other proteins and peptides (26, 37) in order to generate drugs with broader and improved antimicrobial activities or plants with enhanced resistance to microbial pathogens.

Bacteriocins are bacterial proteins or peptides acting against related bacterial strains, some of which are associated with food spoilage and food-borne illness, such as *Staphylococcus aureus* and *Listeria monocytogenes* (31). Four main types of proteinaceous compounds can be produced by LAB (23): membrane-active and heat-stable peptides, including lantibiotics (class I) and nonlantibiotics (class II), and higher-molecular-weight polypeptides, including proteins (class III) and lipo- and glycoproteins (class IV). Some nonlantibiotic membrane-active peptides display a narrow antibacterial spectrum, with always an antagonist effect towards *Listeria* spp. so that these peptides are often designated as anti-listerial bacteriocins (class IIa). Structure-function relationships of class IIa bacteriocins are worth studying, since the molecular features that provide such a narrow antagonistic activity are still obscure. Finally, such studies are also required to develop applications of LAB bacteriocins in food and in nonfood technology. Several class IIa bacteriocins have been fully sequenced, namely, leucocin A, produced by *Leuconostoc gelidum* (13); mesentericin Y105, produced by *Leuconostoc mesenteroides* (14); sakacin P (38) and sakacin A (15), produced by *Lactobacillus sake*; pediocin PA1, produced by *Pediococcus acidilactici* (27); and carnobacteriocin BM1 and carnobacteriocin B2, produced by *Carnobacterium piscicola* LV17B (33). These bacteriocins are characterized by a highly conserved half N-terminal domain flanked by a nonconserved hydrophobic C-terminal peptide of variable length. In a first attempt, it might be suggested that the N-terminal conserved amino acid sequence is involved in *Listeria* strain recognition. In this paper, we report on the isolation and characterization of two class IIa bacteriocins produced by *C. piscicola* V1, a LAB strain isolated from fish (32).

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TABLE 1. Indicator strains and corresponding culture conditions used in this work[a]

Indicator strain	Culture conditions[b]
Carnobacterium divergens NCDO 2763	Elliker, 30°C
Carnobacterium divergens V41 ENITIAA	Elliker, 30°C
Lactobacillus curvatus INRA Theix 674	Elliker, 30°C
Lactobacillus sake INRA Theix 110	Elliker, 30°C
Lactobacillus plantarum ATCC 14917	Elliker, 30°C
Leuconostoc mesenteroides ATCC 8293	Elliker, 30°C
Lactococcus lactis subsp. *cremoris* INRA CNRZ 117	Elliker, 30°C
Pediococcus acidilactici INRA Theix 716	Elliker, 30°C
Enterococcus faecalis ENITIAA 24	Elliker, 30°C
Listeria innocua F ENITIAA 15	Elliker, 37°C
Listeria monocytogenes Scott A DSV	Elliker, 37°C
Bacillus cereus ENITIAA 8	NB, 37°C
Clostridium tyrobutyricum ATCC 25755	RCM, 37°C
Clostridium perfringens NCTC 8798	RCM, 37°C
Clostridium sporogenes IPP 3679	RCM, 37°C
Staphylococcus aureus ENITIAA 2	NB, 30°C
Pseudomonas fluorescens ENITIAA 7	NB, 20°C

[a] Culture collections: ATCC, American Type Culture Collection, Rockville, Md.; DSV, Direction des Services Vétérinaires, Nantes, France; ENITIAA, École Nationale d'Ingénieurs des Techniques des Industries Agricoles et Alimentaires, Nantes, France; INRA CNRZ, Centre National de Recherches Zootechniques, Jouy en Josas, France; INRA Theix, Institut National de la Recherche Agronomique, Theix, France; IPP, Institut Pasteur, Paris, France; NCDO, National Collection of Dairy Organisms, Reading, United Kingdom; NCTC, National Collection of Type Cultures, London, United Kingdom.

[b] All of the media were supplied by Biokar (Beauvais, France). NB, nutrient broth; RCM, reinforced clostridia medium.

Piscicocin V1a is a new class IIa bacteriocin while piscicocin V1b is identical to carnobacteriocin BM1 produced by *C. piscicola* LV17B (33). Interestingly, these peptides exhibit significant differences in their inhibitory activities against target gram-positive bacteria which could be explained by some structural traits of the corresponding peptides. The objective of this work was, beyond describing a new class IIa bacteriocin, to provide a possible mode of action of these bacteriocins through sequence analysis, by structural studies using predictive algorithms, and by comparing some sequence characteristics with those of other membrane-active antimicrobial peptides and proteins.

MATERIALS AND METHODS

Bacterial strains and media. *C. piscicola* V1 is a nonmotile, avirulent, nonspore-forming, gram-positive, and facultatively anaerobic LAB which has been isolated from fish (32). The strain was stored at −80°C in MRS broth (7) containing 15% (vol/vol) glycerol. Before use, the strain was cultivated twice for 24 h at 30°C in MRS broth.

Bacteriocin activity against *L. mesenteroides* ATCC 8293, grown in Elliker broth, was monitored by serial 2-fold or 10-fold dilution as previously described (32). Activity was defined as the reciprocal of the highest dilution showing definite inhibition of the indicator lawn and was expressed as activity units (AU) per milliliter (2). *Listeria innocua* F and *L. monocytogenes* Scott A (École Nationale d'Ingénieurs des Techniques des Industries Agricoles et Alimentaires, Nantes, France) were also used as sensitive strains. Fifteen other bacterial species were tested (Table 1).

Production of the bacteriocin. Tween-free MRS medium prepared from basal ingredients was sterilized by autoclaving (15 min, 120°C) and was aseptically transferred to a bioreactor connected to an automatic pH and temperature controller (Set 2M; SGI, Toulouse, France). It was then inoculated with 2% (vol/vol) of an overnight culture of *C. piscicola* V1. pH was maintained at 6.5 with 6 M NaOH. The temperature was held at 20°C, and agitation was set at 80 rpm. Samples were removed at different time intervals for determination of optical density at 650 nm and antimicrobial activity.

Purification of piscicocins. A 28-h culture was centrifuged for 15 min at 20,000 × *g*. The active supernatant was filtered on a 0.22-μm-pore-size filter and treated for 10 min at 100°C to prevent bacteriocin proteolysis. Ammonium sulfate powder was gently added to the cell supernatant maintained at 4°C to obtain 60% saturation, and the mixture was stirred at 4°C for 4 h. After centrifugation for 1 h at 20,000 × *g*, a pellet was obtained which was resuspended in 25 mM ammonium acetate, pH 6.5, and loaded on a Sep-Pack C_{18} cartridge (Waters Millipore). The cartridge was washed with 30% 2-propanol in 25 mM ammonium acetate, pH 6.5, and the bacteriocin was eluted with 50% 2-propanol in 25 mM ammonium acetate, pH 6.5. After drying under reduced pressure (Speed-Vac; Savant), the bacteriocin fraction was partially dissolved in 0.1% trifluoroacetic acid (TFA). This active fraction was used for final purification by reverse-phase HPLC on a C18 Nucleosil column (250 by 4.6 mm). Elution was performed by using a linear gradient from 100% 0.1% TFA (solvent A) to 90% acetonitrile in 0.1% TFA (solvent B) in 65 min. Polypeptides detected by A_{220} were collected manually. After drying under reduced pressure and dissolution in 1 ml of deionized water, the aqueous polypeptide solutions were stored at −20°C. Protein content (in milligrams per milliliter), estimated by A_{220} (36), and antagonistic activity were determined at each step of the purification process.

Antimicrobial spectrum of piscicocins. The inhibitory activity of the bacteriocins produced by *C. piscicola* V1 was tested against several gram-positive bacteria and a gram-negative strain, *Pseudomonas fluorescens* (Table 1), by an agar spot test. Pure bacteriocin solution (10 μl, 1 μg/ml) was spotted onto the surface of soft agar which had been inoculated with 1 ml of a culture containing 10^6 cells of the indicator strain per ml. Plates were examined after overnight incubation.

Mode of action of piscicocins. Bacteriocin indicator strain cultures (*L. innocua* F and *L. mesenteroides*) were centrifuged (15 min, 6,000 × *g*) at the end of the log phase. Cells were washed in sterile saline water (NaCl, 9 g/liter) and resuspended in fresh Elliker medium so as to obtain about 10^8 CFU/ml. After addition of pure bacteriocins (1 μg/ml), the mixtures were incubated at 37°C. Viable counts were determined on Elliker agar, at selected intervals, after treatment for 1 h with pronase E. Cell lysis was estimated by measuring the optical density at 650 nm. Indicator cells with saline water in the place of the bacteriocin were used as experimental controls.

Amino acid composition and amino acid sequencing of piscicocins. HPLC-purified piscicocins were hydrolyzed in 6 N HCl under vacuum at 110°C for 24 h. The amino acids were derivatized with phenylisothiocyanate, and the PTC amino acids were separated by reverse-phase HPLC as previously described (8).

The N-terminal amino acid sequence of piscicocins was obtained by Edman degradation performed on a model 477 A gas phase sequencer (Applied Biosystems, Foster City, Calif.), equipped with an online 120A phenylthiohydantoin amino acid analyzer (Applied Biosystems). Cystine residues were reduced and alkylated to carboxyaminomethyl cysteine as described by Allen (1).

Mass spectrometry. Mass measurement of peptides resulting from HPLC was performed by using electrospray mass spectrometry (ESMS). ESMS was done on a VG Bio-Q quadrupole with a mass range of 4,000 Da (Bio-Tech, Manchester, United Kingdom) in the positive mode. The protein was dissolved in H_2O/CH_3CN (50/50 [vol/vol]) with 1% acetic acid at a concentration of about 5 pmol/μl (by volume); 10-μl aliquots were introduced into the ion source at a flow rate of 4 μl/min. Scanning was usually performed from $m/z = 500$ to $m/z = 1{,}500$ in 10s with the resolution adjusted so that the peak at $m/z = 998$ from horse heart myoglobin was 1.5 to 2 wide on the base. Calibration was performed by using the multiply charged ions produced by separate introduction of horse heart myoglobin (16,950.4 Da) (19).

RESULTS AND DISCUSSION

Production of the piscicocins. Piscicocin production was done in MRS without Tween, since it has been observed that this nonionic detergent interferes in the purification of bacteriocins (31a). However, the absence of Tween modifies neither the production of the bacteriocin nor the cell growth. As previously described (32), the best production of the bacteriocins was obtained at 20°C and pH 6.5.

Inhibitory activity increased slightly during the exponential phase of cell growth and sharply during the early stationary phase (Fig. 1). An activity of 3,200 AU/ml was recorded after 28 h of incubation; a value quite similar to this was previously obtained (32). In contrast to the data reported by Worobo et al. (39) and Quadri et al. (33), no decrease in inhibitory activity was observed on further incubation during the stationary phase.

Purification. Purification of piscicocins was achieved by using consecutive ammonium sulfate precipitation and reverse-phase chromatography (Sep-Pack followed by HPLC) (30). The inhibitory activity against growth and protein concentration have been recorded at each step of purification (Table 2). Precipitation with ammonium sulfate shows that the activity recovered in the pellet is higher than that present in the initial supernatant. Increased activity has been observed previously upon purification of lactocin S (29), lactacin F (30), and plan-

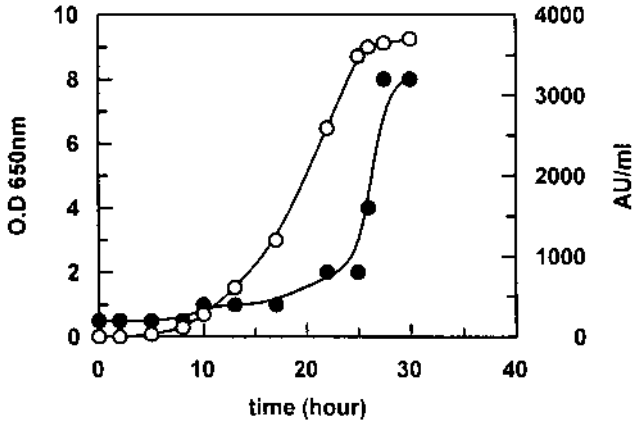

FIG. 1. Growth and production of bacteriocin by *C. piscicola* V1 at 20°C in MRS broth, without Tween, at pH 6.5. Symbols: ○, growth of *C. piscicola* (optical density at 650 nm); ●, bacteriocin activity (AU/ml).

taricin S (21). This increase can be due to either the elimination of an inhibitory substance or the effect of a high salt concentration on the aggregation state of bacteriocin.

The final separation by reverse-phase HPLC yielded two major peaks active against *L. mesenteroides* (Fig. 2). These compounds were called piscicocin V1a and piscicocin V1b, according to generally accepted nomenclature (31). Reverse-phase HPLC resulted in 1.6×10^6- and 1.6×1.0^4-fold increases in specific activity for piscicocin V1a and piscicocin V1b, respectively. The production of more than one bacteriocin by a single organism is not unique. This phenomenon has been reported several times: carnobacteriocin A produced by *C. piscicola* LV17A (39), plantaricin S and plantaricin T from *Lactobacillus plantarum* (21), and, especially interesting, carnobacteriocin BM1 and carnobacteriocin B2 produced by *C. piscicola* LV17B (33).

TABLE 2. Purification of piscicocins V1a and V1b[a]

Sample (vol [ml])	Total activity (AU)	Total protein (mg)	Sp act (AU/mg)	Yield (%)	Increase in sp act (fold)
Supernatant (20)	1.6×10^3	247	6.4	100	1
60% $(NH_4)_2SO_4$ precipitate (2)	1.3×10^4	2.40	5.5×10^3	800	859
Sep-Pack (0.2)	4×10^4	1.32	3×10^4	2,500	4.7×10^3
HPLC (reverse-phase C18)					
V1a (0.3)	3×10^5	0.03	1×10^7	18,700	1.6×10^6
V1b (0.2)	2×10^3	0.02	1×10^5	125	1.6×10^4

[a] Tests were performed against *L. mesenteroides* as described in Materials and Methods.

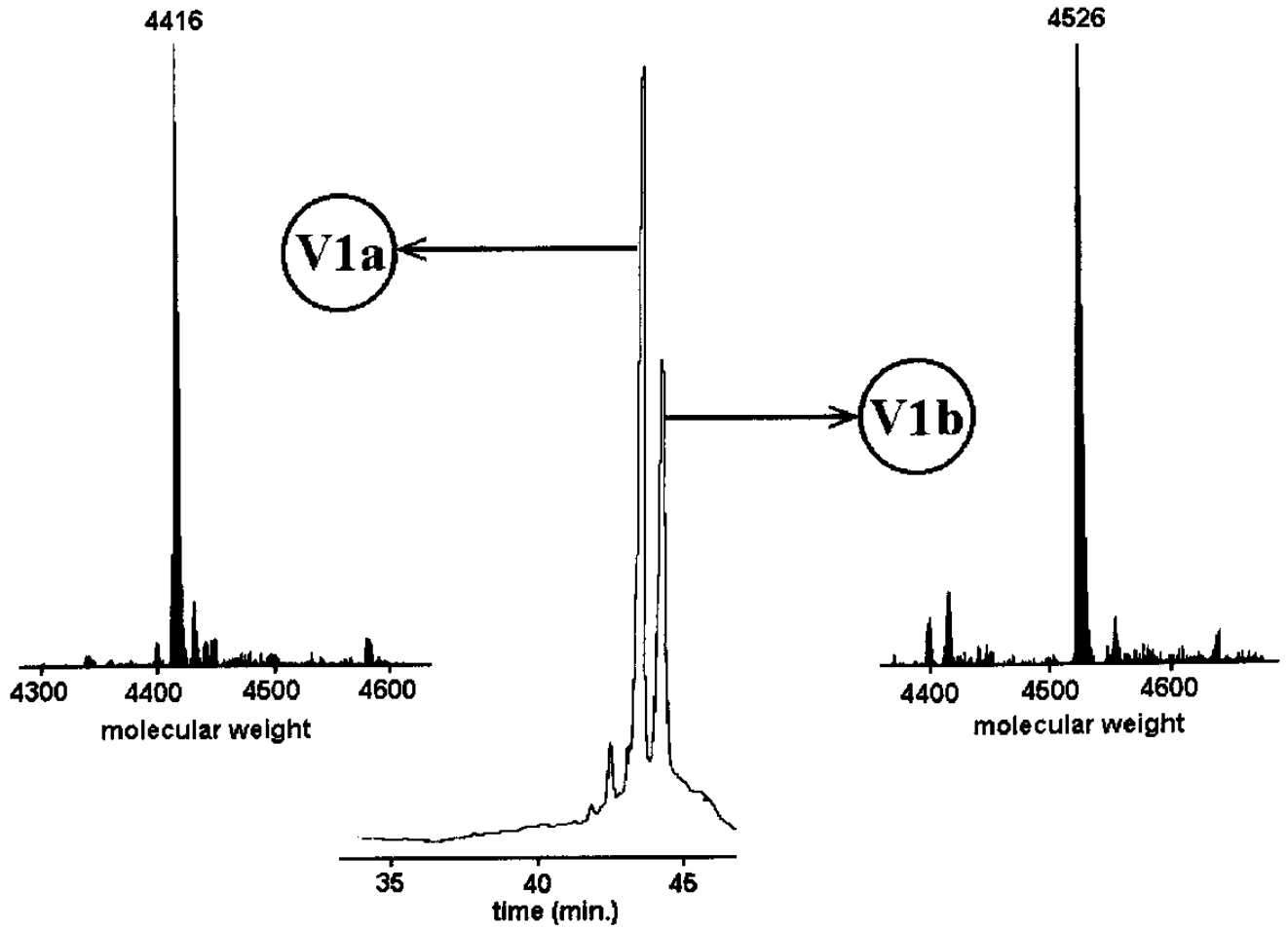

FIG. 2. Reverse-phase HPLC of piscicocins and their corresponding mass spectra.

TABLE 3. Indicator strains and their susceptibilities[a]

Indicator strain	Susceptibility (diam of halo [mm]) to:	
	V1a	V1b
Carnobacterium divergens	24	18
Carnobacterium divergens	30	20
Lactobacillus curvatus	20	6
Lactobacillus sake	23	14
Lactobacillus plantarum	24	18
Leuconostoc mesenteroïdes	30	24
Lactococcus lactis subsp. *cremoris*	—	—
Pediococcus acidilactici	22	18
Enterococcus faecalis	23	12
Listeria innocua F	22	15
Listeria monocytogenes Scott A	24	20
Bacillus cereus	—	—
Clostridium tyrobutyricum	—	—
Clostridium perfringens	—	—
Clostridium sporogenes	—	—
Staphylococcus aureus	—	—
Pseudomonas fluorescens	—	—

[a] Shown are susceptibilities to pure piscicocins V1a and V1b (concentration, 1 μg/ml) produced by *Carnobacterium piscicola* V1. Refer to Table 1 for details. —, no inhibition.

Finally, the most striking result is the high specific inhibitory activity of piscicocin V1a, which is approximately 100 times higher than that of piscicocin V1b (Table 3). Although it is impossible to compare the results obtained by different researchers since the target cells were different, it is noteworthy that specific inhibitory activities of both piscicocins are in the range generally observed from 10^5 to 10^8 AU/mg for LAB bacteriocins (15, 27, 33). However, such a high difference in the specific inhibitory activity, which is also observed for other sensitive bacteria (see below), has never been reported in the case of LAB producing more than one antagonistic peptide. For example, in the case of *C. piscicola* LV17B, carnobacteriocin BM1 is at best twice as active as carnobacteriocin B2 (33).

Bactericidal activity. To investigate whether the mode of action of the piscicocin V1a and piscicocin V1b on *L. mesenteroides* and *L. innocua* F was bactericidal, bacteriostatic, or bacteriolytic, purified bacteriocins were added to the sensitive cells and the viable count and optical density were determined versus time (Fig. 3). A rapid decline in viability was observed in the first minutes of contact in both cases, and further incubation did not result in further killing. However, a sharper decline was exhibited with piscicocin V1a, indicating that the latter is more active than piscicocin V1b. In the case of *L. mesenteroides*, there was a 5-log reduction in viability after 30 min of incubation for V1a and only a 2- to 3-log reduction for piscicocin V1b (Fig. 3A). Such a difference of 2 to 3 logs in viable cells was also observed with *L. innocua* F in the presence of piscicocins V1a and V1b (Fig. 3B). The optical density of the treated culture with piscicocins did not change significantly during the experiments. These results suggest that both piscicocins V1a and V1b acted bactericidally rather than bacteriolytically on the sensitive cells.

Spectrum of inhibitory activity. The antagonistic effect of both piscicocins was tested against various indicator strains, including LAB, listeriae, and other gram-positive strains. *P. fluorescens* was chosen to represent the gram-negative bacteria since it is the most common microorganism in adulterated foodstuffs (Table 3). Piscicocins display a reasonably wide spectrum of activity, and both spectra of activity are very close to each other. However, piscicocin V1a is more active than piscicocin V1b since in each case the diameter of the inhibited zone is larger in the presence of piscicocin V1a. Both antimicrobial substances are active against *L. monocytogenes*. Unlike previous reported results (32), *L. plantarum* and *Clostridium tyrobutyricum* were found, respectively, sensitive and nonsensitive to *C. piscicola* V1 bacteriocins. However, our previous data were obtained by using crude supernatant from culture media which could contain interfering substances. It is noteworthy that in spite of the higher specific antagonistic activity of piscicocin V1a, the inhibitory spectrum does not expand. This result is in agreement with the receptor-dependent antagonistic activity of class IIa bacteriocins (23).

Amino acid sequence of piscicocins. Ion spray mass spectrometry gives average molecular masses of 4,416 ± 0.3 and 4,526 ± 1.0 Da for piscicocin V1a and piscicocin V1b, respectively (Fig. 2). For piscicocin V1b, the first 15 amino acid residues, the molecular mass, and the amino acid composition (Table 4) are in agreement with the amino acid sequence of carnobacteriocin BM1 produced by *C. piscicola* LV17B (33). Amino acid sequencing of the entire peptide of piscicocin V1a has enabled us to determine a sequence of 44 amino acid

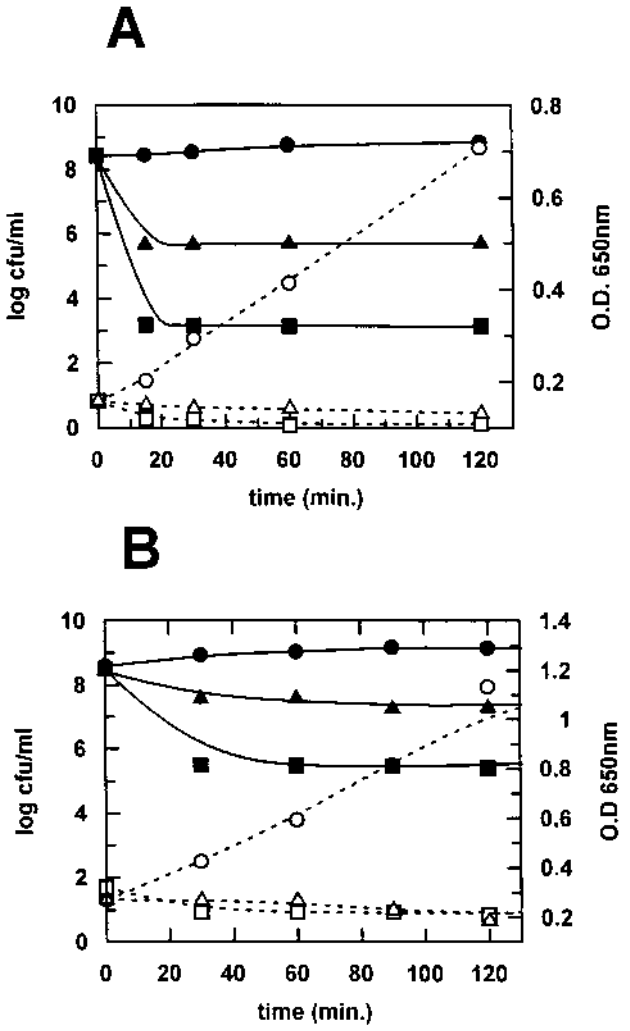

FIG. 3. Bactericidal effect of pure piscicocins (1 μg/ml) V1a and V1b on *L. mesenteroides* (A) and *L. innocua* F (B). Viable cell counts were determined by plating on Elliker agar: ■, V1a; ▲, V1b; ●, control. The optical density at 650 nm was measured: □, V1a; △, V1b; ○, control.

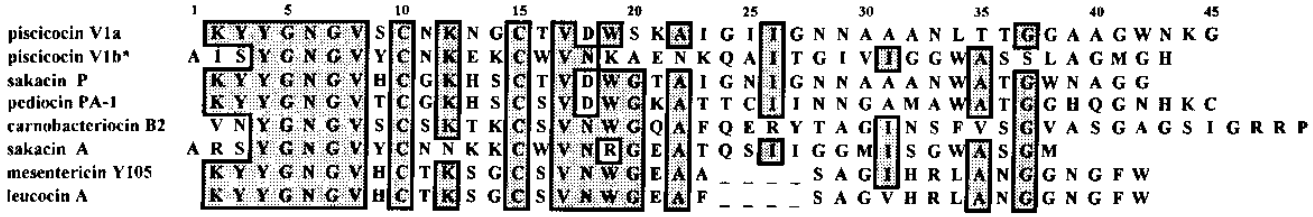

FIG. 4. Multiple sequence alignment of class IIa bacteriocins. Sequence of piscicocin V1b was given by homology with carnobacteriocin BM1 (36) on the basis of the first 15 N-terminal amino acid residues, mass spectrum, and amino acid composition (see text for details).

residues (Fig. 4) containing two blank cycles. Amino acid sequencing of reduced and alkylated bacteriocin clearly showed that the blank cycles were due to the presence of cysteine. The −2-Da difference existing between the molecular mass obtained from mass spectrometry and the calculated mass from the sequence could be due to the presence of a disulfide bridge between two cysteine residues at positions 9 and 14. Finally, the amino acid sequence is in agreement with the amino acid composition (Table 4).

The purification and characterization of piscicocin V1a adds a new member to the group of class IIa bacteriocins from lactic acid bacteria (23). Piscicocin V1a has a high degree of homology with other class IIa bacteriocins, including mesentericin Y105 (14), leucocin A (13), pediocin PA-1 (25), sakacin P, curvacin A (38), and carnobacteriocin B2 (33) (Fig. 4). Piscicocin V1a displays the highest homology—about 60%—with the sequence of sakacin P produced by *L. sake.*

Observed homologies existing among the amino acid sequence of these bacteriocins and their spectrum of activity are often used to explain structure-function relationships. Among others, the conserved N-terminus motif is considered to be involved in the action against *Listeria* strains. However, many other bacteriocins without structural homologies with class IIa peptides are active against *L. monocytogenes*. This has been highlighted for both lantibiotic peptides such as nisin (16) and nonlantibiotic peptides such as curvaticin FS47 from *Lactobacillus curvatus* (10) and pediocin L50 from *P. acidilactici* (6). Furthermore, the class IIa peptides are also active against other gram-positive bacteria. In fact, the N-terminal conserved domain of this peptide family could act at the level of specific recognition or bactericidal activity against certain sensitive bacteria (14, 23). Sequence alignment shows that the consensus N-terminal sequence of this class of bacteriocins is not limited—as generally presented—to the YGNGV motif. The N-terminal consensus motif is larger and lies over almost half of the total amino acid sequence. In this regard, it is possible to expand the consensus sequence previously defined by Quadri et al. (33) in the following: $YGNGVX_1CX_2(K/N)X_3X_4CX_5V(N/D)(W/K/R)X_7(A/N)$, wherein X_i (i = 1 to 7) represents variable amino acid residues. Inside brackets, residues with low variability are in large capitals and can be replaced by residues represented as small capitals.

TABLE 4. Amino acid composition of piscicocins V1a and V1b determined by amino acid analysis and sequencing

Amino acid	Composition[a]			
	Piscicocin V1a		Piscicocin V1b	
	From sequence	From amino acid analysis[b]	From sequence	From amino acid analysis[b]
Asp/Asn	8	7–8	4	4
Gln/Glu	—	1	3	3
Ser	2	2	3	3
Gly	9	9	8	7–8
His	—	—	1	1
Arg	—	—	—	—
Thr	3	2–3	1	1
Ala	5	5	4	4
Pro	—	—	—	—
Tyr	2	1–2	2	1–2
Val	2	2	2	2–3
Met	—	—	1	1
Cys	2	ND	2	ND
Ile	3	2	4	3
Leu	1	2	1	2
Phe	—	—	—	—
Lys	4	4–5	4	4
Trp	2	ND	1	ND

[a] —, not found; ND, not determined.
[b] Nearest integers.

Secondary structure prediction of piscicocins. In the middle of the N-terminal consensus sequence a disulfide bond is found. This covalent bond stabilizes a loop which is predicted as a β-turn by the algorithm described by Chou and Fasman (5). Such a turn is predicted for the YGNGV consensus sequence. A hydrophilic sequence follows the β-turn region. This sequence is shorter in the case of piscicocin V1a (6 residues from Thr-15 to Lys-21) than for piscicocin V1b (9 residues from Trp-16 to Gln-24). Generally this sequence is composed of 9 residues or more in the case of carnobacteriocin B2 (Fig. 4). By the Chou and Fasman algorithm, this hydrophilic region is predicted as a helix with a hydrophobic moment (μH) of about 0.5 for both piscicocins. The combination of hydrophobicity and hydrophobic moment maps these helices near the region of peptides interacting with the membrane surface (9).

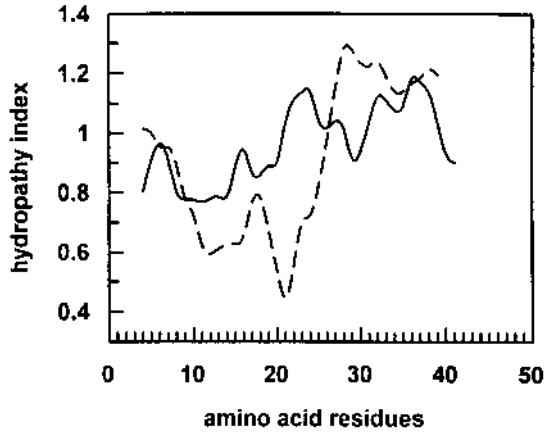

FIG. 5. Hydropathy index as a function of amino acid position according to the method described by Rao and Argos (37). ——, piscicocin V1a; – – –, piscicocin V1b.

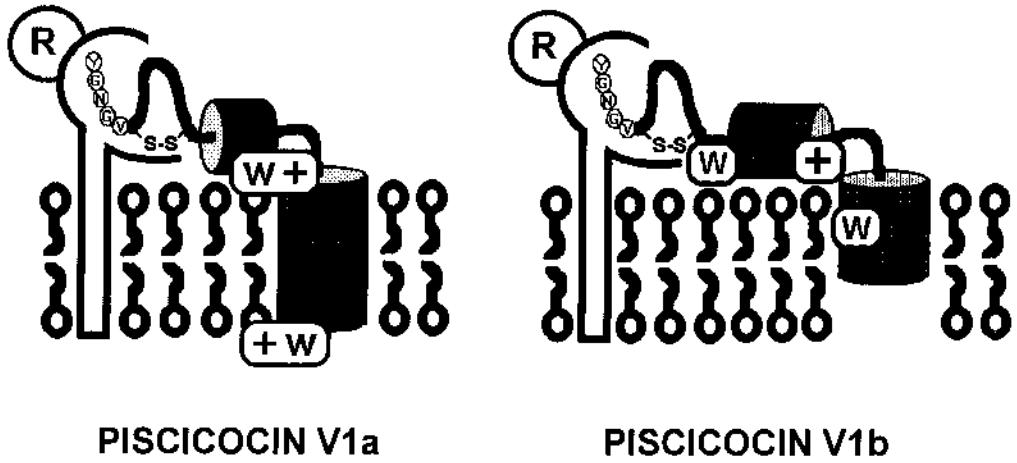

FIG. 6. Model for piscicocin insertion in lipid bilayers (see text for details). W and + represent tryptophan and interfacial lysine residues, respectively.

The C-terminal domain is characterized by a higher hydrophobicity than the N-terminal consensus peptide, and it could be responsible for the insertion of bacteriocins in the membrane of target cells and their membranotoxicity (23). This domain is generally predicted as containing unordered structure, and β-sheet for piscicocin V1b or α-helix for piscicocin V1a, by the Chou-Fasman method (5). Other predictive methods predict unordered and α-helix in this domain for both piscicocins (results not shown). This hydrophobic C-terminal sequence of piscicocins is not predicted as a transmembrane helix according to the membrane-buried helix parameters defined by Rao and Argos (34) (Fig. 5). However, it is noteworthy that the length of the hydrophobic zone is higher in piscicocin V1a (about 20 residues) than in piscicocin V1b (about 15 residues). Similar results are obtained with other amino acid hydrophobicity scales (results not shown). Considering that at least 20 amino acid residues are required for a helix to cross the nonpolar portion of the bilayer, piscicocin V1a, in contrast to piscicocin V1b, should easily form a transmembrane channel in targeted bacterial membranes. Furthermore, at both extremities of the hydrophobic C-terminal domain of piscicocin V1a basic (Lys-20 and Lys-43) and aromatic amino acid residues are found (Trp-18 and Trp-41). For piscicocin V1b, only a basic residue is found at the N-terminal extremity of the hydrophobic domain (Lys-23) and the tryptophan residues are located either in the middle of (Trp-34) or far from (Trp-16) the hydrophobic domain. In membrane proteins, aromatic and basic residues are generally found at the interface between the polar and nonpolar portions of the lipid bilayer and should strengthen the stability of the helix. Especially, tryptophan, which is capable of forming hydrophobic and hydrogen bonds with the hydrophilic and hydrophobic parts, of a membrane lipid bilayer, is suspected to be essential for correct inside-outside orientation of membrane proteins during their transfer in bilayers (35). Tryptophan is often essential in the membranotoxic effect of peptides and proteins (11) and more generally in the interaction of proteins with membrane lipids (3, 17). The differences in the length of the C-terminal hydrophobic domain and in the position of lysine and tryptophan residues nearby could be responsible for differences in the translocation of the bacteriocins into the membrane and therefore in the corresponding specific activity.

On the basis of this predictive structural study, we propose a model for the insertion of piscicocins in lipid bilayers which could explain the difference in specific activities against target cells. In this model the N-terminal hydrophilic and conserved region is outside the lipid membrane. The β-turn region exposes the highly conserved YGNGV motif which could be recognized by the probable membrane receptor. The hydrophilic and slightly amphiphilic helix ensures anchoring at the membrane surface of the lipid bilayer and the C-terminal hydrophobic domain forms a helix which is anchored in the hydrophobic core of the lipid bilayer. Tryptophan and lysine residues as well as the length of the hydrophobic helix could play a major role in anchoring properly the peptide in the hydrophobic lipid core and in governing its stability in the membrane environment (Fig. 6). The receptor would allow a correct positioning of the peptide on the target membrane, a situation resembling that suggested for signal peptides (12).

Conclusion. The results obtained in this work suggest that the length and amphiphilicity-hydrophobicity of class IIa bacteriocins could play a major role in their specific antibacterial activity. Tryptophan residues found in the C-terminal hydrophobic peptide could play a major role in this specific inhibitory activity as in other membrane-active peptides and proteins. However, differences in the specific antagonistic properties do not expand the antimicrobial spectrum of piscicocins V1a and V1b (Table 1). The same results were obtained for other LAB strains producing more than one peptide similar in structure (21, 33, 39). These results are in agreement with a receptor-mediated activity of class IIa bacteriocins in which (i) the N-terminal highly conserved domain is involved in the receptor recognition and therefore in an "all-or-nothing" response, i.e. the inhibitory spectrum, against target bacterial cells and (ii) the C-terminal sequence is involved in the interaction with the membrane lipids and therefore modulates the specific inhibitory activity.

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