

Comparative Studies of Class IIa Bacteriocins of Lactic Acid Bacteria

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Four class IIa bacteriocins (pediocin PA-1, enterocin A, sakacin P, and curvacin A) were purified to homogeneity and tested for activity toward a variety of indicator strains. Pediocin PA-1 and enterocin A inhibited more strains and had generally lower MICs than sakacin P and curvacin A. The antagonistic activity of pediocin-PA1 and enterocin A was much more sensitive to reduction of disulfide bonds than the antagonistic activity of sakacin P and curvacin A, suggesting that an extra disulfide bond that is present in the former two may contribute to their high levels of activity. The food pathogen *Listeria monocytogenes* was among the most sensitive indicator strains for all four bacteriocins. Enterocin A was most effective in inhibiting *Listeria*, having MICs in the range of 0.1 to 1 ng/ml. Sakacin P had the interesting property of being very active toward *Listeria* but not having concomitant high levels of activity toward lactic acid bacteria. Strains producing class IIa bacteriocins displayed various degrees of resistance toward noncognate class IIa bacteriocins; for the sakacin P producer, it was shown that this resistance is correlated with the expression of immunity genes. It is hypothesized that variation in the presence and/or expression of such immunity genes accounts in part for the remarkably large variation in bacteriocin sensitivity displayed by lactic acid bacteria.

Many lactic acid bacteria (LAB), including members of the genera *Lactococcus*, *Lactobacillus*, *Carnobacterium*, *Enterococcus*, and *Pediococcus*, are known to secrete small, ribosomally synthesized antimicrobial peptides called bacteriocins (26, 29, 34). Some of these peptides undergo posttranslational modifications (class I bacteriocins), whereas others are not modified (class II bacteriocins) (29, 34). Class II bacteriocins contain between 30 and 60 residues and are usually positively charged at a neutral pH. Studies of a large number of class II bacteriocins have led to subgrouping of these compounds (29, 34). One of the subgroups, class IIa, contains bacteriocins that are characterized by the presence of YGNG and CXXXXCXV sequence motifs in their N-terminal halves as well as by their strong inhibitory effect on *Listeria* (e.g., 3, 4, 22, 23, 27, 28, 31, 38, 45) (Fig. 1). Because of their effectiveness against the food pathogen *Listeria*, class IIa bacteriocins have potential as antimicrobial agents in food and feed.

Class IIa bacteriocins act by permeabilizing the membrane of their target cells (1, 5, 6, 9, 10, 26, 28). The most recent studies on the mode of action of these bacteriocins indicate that antimicrobial activity does not require a specific receptor and is enhanced by (but not fully dependent on) a membrane potential (9, 28). Little is known about bacteriocin structure, and unravelling the relationships between structure and function is one of the great challenges in current bacteriocin research. A logical starting point for structure-function studies is a thorough study of the differences in activity and target cell specificity between naturally occurring homologous bacteriocins. A few such studies have been described, but these suffer from either a very limited number of tested indicator strains or the use of culture supernatants instead of purified bacteriocins

(3, 4, 17, 45). The use of purified bacteriocins for comparative analyses is absolutely essential, since it is becoming increasingly evident that bacteriocin producers produce more than one bacteriocin (4, 8, 38, 48; this study).

In the present study, the activities of four pure class IIa bacteriocins (pediocin PA-1, enterocin A, curvacin A, and sakacin P) (Fig. 1) were tested against a large number of LAB as well as several strains of the food pathogen *Listeria monocytogenes*. The bacteriocins were purified from their respective producer strains by use of an optimized purification protocol yielding highly pure samples. The contribution of disulfide formation was assessed and found to be important for activity. The effects of the purified bacteriocins on (noncognate) class IIa bacteriocin-producing strains are described, and the implications of our findings for immunity and resistance are discussed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Lactobacillus* spp., *Pediococcus* spp., *Enterococcus* spp., *Leuconostoc* spp., and *Carnobacterium* spp. were grown in MRS broth (Oxoid, Unipath Ltd., Basingstoke, Hampshire, England) at 30°C. Lactococci were grown in M17 medium (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% (wt/vol) glucose at 30°C. *Listeria* spp. were grown on brain heart infusion medium (Difco) at 30°C, and *Clostridium* spp. were grown on both thioglycolate medium (Oxoid) and reinforced clostridial medium (Oxoid) at 37°C. For cultivating *Clostridium* spp., the Anaerogen system for anaerobic incubation (Oxoid) was used.

Bacteriocin purification and concentration determination. Pediocin PA-1, enterocin A, sakacin P, and curvacin A were purified from the supernatants of early-stationary-phase cultures of *Pediococcus acidilactici* (35), *Enterococcus faecium* CTC492 (3), *Lactobacillus curvatus* LTH1174 (45), and *Lactobacillus sake* LTH673 (45), respectively. In terms of bacteriocin production, *E. faecium* CTC492 is identical to *E. faecium* T136 (8). The bacteriocins were purified to homogeneity essentially as described previously (3, 35, 45). The method consists of ammonium sulfate precipitation followed by three chromatography steps (ion exchange, hydrophobic interaction, and reversed phase). The final, reversed-phase chromatography step (with a fast protein liquid chromatography system supplied by Pharmacia-LKB, Uppsala, Sweden) was repeated two or three times to ensure maximum purity. In contrast to the previously described method, very large volumes of washing buffer (up to 40 times the column volume) were used

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	1	10	20	30	40	Reference
Pediocin PA-1	KYYGNGVTCGKHSC	SVDWGKATTCI	INNGAMAWATGGH	QGNHCK		23, 32
Enterocin A	TTHSGKYYGNGVYCT	TKNKC	TVDWAKATTCI	AGMSIGGFLGGAIPGK		3
Sakacin P	KYYGNGVHCGKHSC	TVDWGT	AIGNIGNNAAANWATGGNAGWNK			25, 45
Curvacin A	ARSYGNVYCNKKCWVNR	GEATQSI	IGGMISGWASGLAGM			45
Piscicolin 126	KYYGNGVSCNKNCGC	TVDWSKAIGI	IGNNAAANLTTGGAAGWNK			27
Leucocin A	KYYGNGVHCTKSGCS	VNWGEAF	SAGVHRLANGGNGFW			22
Mesentericin Y105	KYYGNGVHCTKSGCS	VNWGEAAS	SAGIHLRLANGGNGFW			19
Carnobacteriocin B2	VNYGNGVSCSKTKCS	VNWQAQFQERY	TAGINSFVSGVASGAGSIRRP			38
Carnobacteriocin BM1	AISYGNVYCNKEKCV	WNKAENKQAITG	IIVGGWASSLAGMGH			38
Bavaricin MN	TKYYGNGVYxNSKKx	WVDWQAAGGIGQ	TVVxGWLGAIPGK			28
Bacteriocin 31	ATYYGNGLYCNKQKC	WVDWNKASREIGK	IIVNGVWQHGPWAPR			47
Enterocin P	ATRSYGNVYCNNSKC	WVNWGEAKENI	AGIVISGWASGLAGMGH			11
Consensus :	<u>Y</u> <u>Y</u> <u>G</u> <u>N</u> <u>G</u> <u>V</u> <u>.</u> <u>C</u> <u>.</u> <u>K</u> <u>.</u> <u>.</u> <u>C</u> <u>.</u> <u>V</u> <u>.</u> <u>W</u> <u>.</u> <u>A</u> <u>.</u> <u>.</u> <u>.</u> <u>I</u>					

FIG. 1. Sequence alignment of class IIa bacteriocins. Residue numbering is according to the sequence of pediocin PA-1. Cysteine residues are printed in boldface; the two known class IIa bacteriocins with four cysteine residues are in the upper group. No attempt was made to optimize the alignment in the C-terminal halves of the peptides. Piscicolin 126 is identical to pisciocin V1a (4). Carnobacteriocin BM1 most probably is identical to pisciocin V1b (4). Sakacin P most probably is identical to bavaricin A (30). Curvacin A is identical to sakacin A (2). The consensus sequence includes residues conserved in at least 8 of the 12 sequences shown; 100% conserved residues are underlined.

for the washing steps during ion-exchange and hydrophobic-interaction chromatography. This small change in the protocol contributed considerably to purity.

The purity of the bacteriocins was assessed by 10 steps of Edman degradation with a model 477A sequencer (Applied Biosystems, Foster City, Calif.). Purity was assessed by comparing the yields of the expected amino acids with the yields of other amino acids (that would result from contaminating peptides) in each degradation step. On the basis of these analyses, purity was estimated to be >98% for sakacin P, curvacin A, and pediocin PA-1 and about 95% for enterocin A.

Initially, the concentration of the purified bacteriocins was assessed by two methods: measuring UV absorption at 280 nm and amino acid composition analysis. For the former method, molar extinction coefficients were calculated from the contributions of individual amino acid residues by use of the program PCGENE (IntelliGenetics Inc., Geneva, Switzerland). The differences between the results of the two methods were on the order of 10% for all four bacteriocins (data not shown). The simplest of the two methods (measuring UV absorption at 280 nm) was therefore used for further routine concentration determinations.

The purified bacteriocins were stored at -20°C and used within 1 month of purification. The bacteriocins retained ≥90% of their activity during this period, as indicated by the results of weekly activity assays with a standard set of three indicator strains.

Bacteriocin assay. Bacteriocin activity was quantified with a microtiter plate assay (21, 24). For the comparative studies, a standardized procedure was used; indicator cells were always derived from a fresh overnight culture and diluted 400 times before use. The subsequent incubation time was 18 to 20 h in all assays. The activities of the four bacteriocins toward a specific strain were always determined in a single assay. The MICs given represent the bacteriocin concentration needed to obtain 50% inhibition of growth. The values are the averages of three independent measurements, which gave standard deviations on the order of 25% of the values. Bacteriocin activity is expressed in units, one bacteriocin unit being the amount of bacteriocin required to reduce the growth of the indicator strain by 50% under the conditions of the assay (200- μ l culture volume).

Disulfide bonds. Disulfide bond formation was assessed by determining the molecular masses of the purified bacteriocins by electrospray ionization mass spectrometry and by determining the number of free thiol groups as described by Ellman (15). In the latter method, the molar extinction coefficient used for 2-nitro-5-thiobenzoate was 14,150 M⁻¹ cm⁻¹. A sample containing a peptide with only one cysteine was included as a positive control.

To test the effect of the reduction of disulfide bonds on bacteriocin activity, bacteriocin assays in which dithiothreitol (DTT; final concentration, 10 mM) was added to the culture medium in the microtiter plate wells were conducted. These experiments were conducted with indicator strains whose growth was not inhibited by the presence of 10 mM DTT alone.

Induction of bacteriocin production. Under certain conditions, bacteriocin-negative (Bac⁻) cultures of the sakacin P producer *L. sake* LTH673 can be obtained, as described previously (7, 13). These cultures have a stable Bac⁻ phenotype, but the bacteriocin-positive (Bac⁺) phenotype may be restored by adding the pheromone needed for the expression of bacteriocin-related genes (13). In the present study, Bac⁻ and Bac⁺ cultures of *L. sake* LTH673 were obtained by making two identical 100-fold dilutions of a Bac⁻ overnight culture. To one of the two dilutions, the appropriate pheromone was added to a final concentration of 50 ng/ml (7, 13). The addition of the pheromone led to the

transcription of genes involved in bacteriocin production, as documented previously (7).

Sequence alignments. Sequence identities and similarities between immunity proteins were determined with the program PALIGN, which is part of the PCGENE software package (33). The genetic code matrix (16) with default gap opening and extension penalties was used. These settings kept the numbers of insertions and deletions equal to or below five for all pairwise sequence alignments. The following groups of amino acids were defined as similar: A, S, and T; D and E; N and Q; R and K; I, L, M, and V; and F, Y, and W.

RESULTS AND DISCUSSION

Class IIa bacteriocin producers produce more than one bacteriocin. Recent studies of bacteriocin producers suggest that it may be common for LAB to produce more than one bacteriocin. For example, *E. faecium* CTC492 not only produces the class IIa bacteriocin enterocin A but also produces enterocin B, which does not belong to class IIa. These two bacteriocins were shown to have different inhibitory spectra (8, 14). Another example of the production of multiple bacteriocins by LAB is illustrated by Table 1. The data in Table 1 show that during purification of sakacin P, inhibitory activity toward some strains was lost, whereas inhibitory activity toward other strains was retained (Table 1). The lost activity could be recovered by pooling the supernatant and the pellet obtained in the ammonium sulfate precipitation step at the start of the

TABLE 1. Purification of bacteriocins from *L. sake* LTH673 and assessment of yields with various indicator strains

Indicator strain	Total activity, 10 ⁻⁵ bacteriocin units, of:		Yield (%) ^b
	Culture supernatant	Purified sakacin P ^a	
<i>Lactobacillus coryneformis</i> NCDO 2740	6.6	3.4	52
<i>L. sake</i> NCDO 2714	76	4.1	5.4
<i>Lactobacillus</i> sp. strain LMG 2804	8.5	0.09	1.1

^a Ten steps of Edman degradation were performed and indicated that the material contained >98% pure sakacin P.

^b The dramatic indicator strain-dependent variation in yield showed that antimicrobial activity toward some strains had been lost, whereas activity toward other strains had been retained (see the text for details).

TABLE 2. Activities of purified class IIa bacteriocins toward various indicator strains

Indicator strain ^b	Bac ^c	Sensitivity (MIC, µg/ml) ^d to:			
		Pediocin PA-1	Curvacin A	Sakacin P	Enterocin A
<i>Lactobacillus curvatus</i>					
LMG 2353		>2.0	>1.0	>2.0	>2.0
LMG 2371		0.020	0.15	>0.5	0.017
NCFB 2739 B		>2.0	>2.0	>2.0	>0.5
<i>L. plantarum</i>					
NCDO 1869		1.5	>2.0	>2.0	0.40
LMG 2352		0.050	>2.0	>2.0	0.12
LMG 2362	+ ^d	0.96	>2.0	>2.0	>0.5
LMG 2362	- ^d	0.58	>2.0	>2.0	0.31
LMG 2375		0.017	>2.0	>0.5	0.40
DSM 20174		0.0094	>2.0	>0.5	0.015
NCDO 1193 B		>2.0	>2.0	>2.0	>0.5
<i>L. sake</i>					
NCDO 2714		0.0004	0.0011	0.0037	0.0001
LMG 2380	+	>1.0	1.1	>0.5	0.035
LMG 2799		0.093	0.31	0.42	0.011
<i>L. casei</i>					
NCDO 161		0.32	>2.0	>2.0	>2.0
NCDO 2713		>2.0	>2.0	>2.0	>0.5
NCDO 1857		0.0084	0.98	>2.0	0.0059
NCDO 2743		>2.0	>2.0	>2.0	>0.5
ATCC 334 B		>2.0	>2.0	>2.0	>0.5
<i>L. coryneformis</i>					
NCDO 2740		0.0005	0.0037	0.0052	0.0003
NCDO 2741		0.011	0.050	0.0049	0.0066
<i>Lactobacillus</i> sp. strain					
LMG 2003		0.0009	0.91	0.023	0.0026
NCDO 2503		0.016	0.27	>0.5	0.36
LMG 2333	+	0.0008	0.0040	0.0086	0.0016
<i>Pediococcus acidilactici</i>					
NCDO 1859		0.0028	>2.0	>2.0	0.080
NCDO 521		0.29	>2.0	>2.0	>2.0
NCDO 1851		0.0039	1.8	>2.0	0.0068
<i>P. pentosaceus</i>					
LMG 2001	(+) ^e	0.0030	>2.0	>2.0	0.0062
NCDO 559		0.0086	0.98	0.72	0.021
NCDO 1850		>2.0	1.8	>2.0	0.55
LMG 2366		>2.0	>2.0	>2.0	>2.0
LMG 2722		0.0004	0.54	0.15	0.0005
<i>Enterococcus faecalis</i> NCDO 581		0.0007	0.0020	0.0057	0.0013
<i>E. faecium</i> NCDO 942		>2.0	>2.0	>2.0	>0.5
<i>Carnobacterium piscicola</i>					
LMG 2332	+ ^f	0.0002	0.0021	0.0009	0.0006
NCDO 2764		0.0027	0.017	0.0015	0.0017
<i>C. divergens</i> NCDO 2306		0.0041	0.023	0.0033	0.00004
<i>Leuconostoc mesenteroides</i> NCDO 529		1.4	>2.0	>2.0	>2.0
<i>L. cremoris</i> LMG 2724		0.025	>2.0	>2.0	>1.0
<i>Leuconostoc</i> sp. strain NCDO 543		0.0009	>2.0	0.70	>1.0
<i>Lactococcus lactis</i>					
Nine different strains	+ ^g	>2.0	>2.0	>2.0	>0.5
LMG 2070		0.17	>2.0	>0.5	>0.5
LMG 2095	+	>2.0	0.91	>2.0	>0.5
<i>Clostridium tyrobutyricum</i> (11 different strains)		>2.0	>2.0	>2.0	>0.5
<i>C. butyricum</i> NCDO 855A		0.035	>2.0	0.083	0.0021
<i>Listeria monocytogenes</i>					
LMG 2650		0.0047	0.049	0.0073	0.0008
LMG 2651		0.0029	0.028	0.0036	0.0006
LMG 2652		0.0050	0.030	0.0068	0.0004
LMG 2653		0.0050	0.049	0.0068	0.0008
LMG 2800		0.0062	0.069	0.0083	0.0011
LMG 2801		0.0033	0.040	0.0034	0.0005
LMG 2802		0.0013	0.028	0.0034	0.0002
<i>L. innocua</i> LMG 2654		0.0031	0.026	0.0057	0.0003
<i>L. ivanovii</i> LMG 2803		0.0006	0.0092	0.0002	<0.0001

^a Values preceded by a greater-than sign indicate the highest bacteriocin concentration tested.

^b Strains were from our laboratory collection (LMG) or from well-known culture collections: ATCC, American Type Culture Collection, Rockville, Md.; NCDO and NCFB, National Collection of Food Bacteria, Reading, United Kingdom.

^c +, the indicator strain is known to produce at least one bacteriocin; -, the indicator strain is capable of producing bacteriocins but has lost the Bac⁺ phenotype.

^d This strain produces several bacteriocins that do not belong to class IIa; production can be controlled in a manner similar to that in the sakacin P producer (see the text) (12).

^e This strain produces pediocin A, a high-molecular-weight "bacteriocin" (80 kDa) (37).

^f This strain produces the lantibiotic carnobacteriocin U149 (44).

^g These strains included strains producing nisin and lactococcin A.

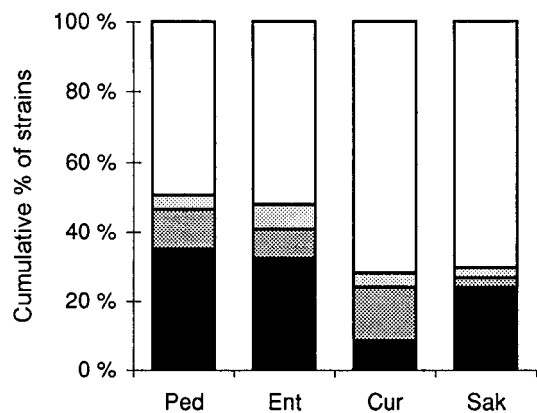


FIG. 2. Sensitivity of indicator strains. Strains are categorized according to MICs (Table 2): black, <10 ng/ml; dark grey, 10 to 100 ng/ml; light grey, 100 to 500 ng/ml; white, >500 ng/ml. Ped, pediocin PA-1; Ent, enterocin A; Cur, curvacin A; Sak, sakacin P.

purification protocol (data not shown). Thus, in addition to sakacin P, *L. sake* LTH673 produces at least one other bacteriocin. This other bacteriocin(s) has not yet been characterized, but transcription studies have shown that at least one gene encoding a hitherto-uncharacterized bacteriocin-like peptide is expressed in addition to the sakacin P structural gene (7). The data in Table 1 show how erroneous results would have been obtained if the inhibitory spectrum of sakacin P had been assessed with culture supernatants.

Inhibitory spectra. The activities of the bacteriocins toward various indicator strains are shown in Table 2 and Fig. 2. The results show that pediocin PA-1 and enterocin A are generally more active than sakacin P and curvacin A. Pediocin PA-1 and enterocin A also have a broader inhibitory spectrum (Fig. 2). The magnitude of the difference in activity between pediocin PA-1 and enterocin A on the one hand and sakacin P and curvacin A on the other hand varies widely with the indicator strain used. The latter represents a general phenomenon illustrated by Table 2: when LAB were used as indicator strains, the activity ratio between the various bacteriocins differed from species to species and even from strain to strain. More consistent results were obtained when listeriae were used as indicator strains: enterocin A was 5 to 10 times more active than sakacin P and pediocin PA-1, which were 5 to 10 times more active than curvacin A.

Interestingly, sakacin P had modest activity toward LAB but was almost as effective as enterocin A and pediocin PA-1 against listeriae. Because of this combination of high antilisterial activity and a narrow inhibitory spectrum (Fig. 2), sakacin

P is perhaps the most promising of the tested bacteriocins for use in LAB fermentations that are prone to *Listeria* infections.

For analyzing possible synergistic inhibitory effects of class IIa bacteriocins, the activity of every possible one-to-one combination of two of the purified bacteriocins was measured at maximum total concentrations of approximately 1 μ g/ml. No synergistic effects were observed (results not shown).

Role of disulfide bonds. Mass spectrometry analysis showed that the molecular masses of purified pediocin PA-1, enterocin A, curvacin A, and sakacin P were 4.5, 4.7, 2.2, and 3.2 Da, respectively, lower than those expected when cysteine residues were assumed to be in a reduced state. Considering the standard deviation in the mass determinations (\sim 1 Da), these data are in accordance with the notion that all cysteine residues are oxidized and are involved in disulfide bonds. In accordance with this observation, the Ellman assay did not reveal any free cysteine residues in the bacteriocins, whereas it did reveal free cysteine residues in a one-cysteine-containing control peptide (results not shown). Thus, pediocin PA-1 and enterocin A indeed contain two disulfide bonds, whereas sakacin P and curvacin A contain one. It was previously shown that the two disulfide bonds in pediocin PA-1 are formed between Cys9 and Cys14 and between Cys24 and Cys44 (23).

Interestingly, the two most active bacteriocins in this study were the ones with two disulfide bonds, suggesting a correlation between these two properties. To investigate the contribution of disulfide bond formation to bacteriocin activity, three indicator strains whose growth was not inhibited by DTT were selected for testing bacteriocin activity under reducing conditions. As shown in Table 3, DTT dramatically reduced the activities of pediocin PA-1 and enterocin A, whereas the activities of sakacin P and curvacin A were only moderately reduced. Thus, in the presence of DTT, the two-disulfide-bond-containing bacteriocins pediocin PA-1 and enterocin A were no longer generally more potent than the one-disulfide-bond-containing bacteriocins sakacin P and curvacin A.

These results suggest that the high levels of activity of pediocin PA-1 and enterocin A may be due at least in part to the extra disulfide bond present in the C-terminal region. The effect of DTT on the activity of sakacin P and curvacin A suggests that the disulfide bond between fully conserved Cys9 and Cys14 is important but not crucial for activity. This conclusion is in accordance with previous studies on carnobacteriocin B2 (in which the bond between Cys9 and Cys14 is not formed) (38) and leucocin A (which retains considerable activity after reduction and modification of the cysteines) (22). Remarkably, quite opposite results have also been reported. In one study, pediocin PA-1 was found to lose all of its activity upon reduction (10). Furthermore, the activity of mesentericin Y105 was shown to be reduced at least 2,000-fold upon modification or mutation of Cys9 and Cys14 (18). At present, we

TABLE 3. Effect of reduction on antimicrobial activity^a

Strain	Sensitivity to:							
	Pediocin PA-1		Enterocin A		Curvacin A		Sakacin P	
	Standard	+ DTT	Standard	+ DTT	Standard	+ DTT	Standard	+ DTT
<i>L. sake</i> NCDO 2714	0.4	45 (0.9)	0.1	19 (0.5)	1.1	4.5 (24)	3.7	29 (13)
<i>Lactobacillus</i> sp. strain LMG 2333	0.8	8.2×10^2 (0.1)	1.6	1.9×10^2 (0.9)	4.0	48 (8.3)	8.6	54 (16)
<i>P. pentosaceus</i> LMG 2722	0.4	3.2×10^2 (0.1)	0.5	84 (0.6)	5.4×10^2	$>20 \times 10^2$ (<27)	1.5×10^2	5.4×10^2 (28)

^a The standard microtiter plate assay was used, with or without the addition of 10 mM DTT (final concentration) to each well. No growth inhibition occurred in control cultures without bacteriocin but with 10 mM DTT. Sensitivity is reported as MIC in nanograms per milliliter. Values in parentheses indicate the percentages of antimicrobial activity under reducing conditions relative to that under standard (nonreducing) conditions.

TABLE 4. Bacteriocin sensitivity of LAB producing class IIa bacteriocins

Strain	Reference	Bacteriocin produced	Sensitivity (MIC, µg/ml) to:			
			Pediocin PA-1	Curvacin A	Sakacin P	Enterocin A
<i>L. sake</i> LTH673	7, 45	Sakacin P	>1.0	0.63	>0.7	0.021
<i>L. sake</i> LTH673	7, 45	Bac ⁻ phenotype ^a	0.020	0.037	0.057	0.0056
<i>P. acidilactici</i>	35	Pediocin PA-1	>1.0	>2.0	>0.7	>0.6
<i>L. curvatus</i> LTH1174	45	Curvacin A ^b	0.12	>2.0	0.39	0.061
<i>L. sake</i> Lb706	2	Sakacin A ^b	>1.0	>2.0	>0.7	>0.6
<i>E. faecium</i> CTC492	3	Enterocin A	>1.0	>2.0	>0.7	>0.6

^a See the text for details.

^b Sakacin A is identical to curvacin A; the cognate immunity proteins are highly identical (2, 46).

have no explanation for the apparent inconsistencies among these results.

Several authors have discussed possible structural models for a class IIa bacteriocin in a membrane environment (4, 9, 17, 20, 28). The bacteriocins are unstructured in watery solutions, whereas they adopt a partly helical structure in more hydrophobic environments (14, 18, 20, 42). It has been suggested that the C-terminal half of class IIa bacteriocins forms a hydrophobic or amphiphilic transmembrane α helix, permitting the formation of a so-called "barrel-stave" (36) poration complex (4, 17, 29). Obviously, this simple structural model is incompatible with the presence of a disulfide bond formed by cysteine residues located at the beginning and end of this putative transmembrane helix (as would be the case for pediocin PA-1 and enterocin A). Recent studies of the three-dimensional structure of leucocin A (leucocin A has only the Cys9-Cys14 disulfide bond) (Fig. 1) show that the most C-terminal portion of this bacteriocin has a rather extended (nonhelical) structure, folding back onto a preceding helical region that comprises residues 17 to 31 (20). Extrapolating these observations to enterocin A and pediocin PA-1, one may speculate that the extra disulfide bond in these two bacteriocins stabilizes their structures by covalently coupling the C-terminal residue to a cysteine in the helical region.

Immunity. LAB producing class IIa bacteriocins generally were not particularly sensitive to noncognate class IIa bacteriocins, but there was a tendency for the producers of the less potent sakacin P and curvacin A to be sensitive to the more

potent pediocin PA-1 and enterocin A (Table 4). Remarkably, *L. sake* Lb706 and *L. curvatus* LTH1174, which are known to produce identical bacteriocins, displayed different behaviors in terms of bacteriocin sensitivity.

These results do not permit discrimination between insensitivity (the cause of which is not known) and immunity provided by a specific immunity protein. To obtain this discrimination, we exploited the fact that the expression of genes involved in the production of sakacin P can be controlled (7, 13). Cultures of *L. sake* LTH673 which have a stable Bac⁻ phenotype and in which the transcription of bacteriocin-related genes (including the *spiA* gene, encoding immunity for sakacin P) is switched off can be obtained. As shown in Table 4, *L. sake* LTH673 Bac⁻ cells were sensitive to all four bacteriocins. *L. sake* LTH673 Bac⁺ cells, which were obtained after induction of a Bac⁻ culture with an appropriate pheromone and which are known to express the *spiA* gene (7), were much less sensitive. Within the time frame of the experiments, spontaneous development of insensitivity to class IIa bacteriocins was not observed. Thus, the low sensitivity or immunity of Bac⁺ cells of *L. sake* LTH673 to various (noncognate) class IIa bacteriocins is correlated with the expression of the sakacin P gene cluster (including *spiA*).

Table 5 shows that sequence similarities between proteins that (putatively) provide immunity to class IIa bacteriocins are generally low but that most of the proteins do clearly resemble several others. A certain similarity between the proteins was also suggested by secondary structure predictions (40, 41), which indicated that all 12 proteins shown in Table 5 were

TABLE 5. Sequence similarities between proteins that (putatively) provide immunity to class IIa bacteriocins^a

Immunity protein	% Identity or similarity to:											
	CarBM1	SakA	CarB2	Orf- β 3	SakP	LeuA	MesY105	EntA	OrfY	PedPA-1	EntP	Bac31
CarBM1		49	19	17	18	20	20	19	24	25	44	50
SakA (2)	65		19	19	17	21	24	21	22	18	47	42
CarB2 (39)	31	33		26	23	11	12	15	18	5	19	24
Orf- β 3	31	31	37		46	28	22	27	22	21	20	22
SakP (25)	25	22	34	62		18	18	12	17	17	23	17
LeuA (49)	38	32	21	38	32		73	38	40	11	22	21
MesY105	35	32	20	41	29	81		40	36	9	20	22
EntA	31	31	19	40	19	50	47		27	20	14	17
OrfY	35	29	27	36	26	51	45	39		14	17	16
PedPA-1 (50)	35	39	19	27	28	20	18	31	23		19	22
EntP	59	58	33	31	31	35	32	25	28	30		52
Bac31	66	54	31	32	30	30	32	29	21	34	67	

^a Sequence identity (upper-right triangle) and similarity (lower-left triangle) between proteins that (putatively) provide immunity to class IIa bacteriocins are shown. References are given for proteins for which the immunity function has been demonstrated through functional studies. The proteins displayed are the (putative) immunity proteins for carnobacteriocin BM1 (CarBM1) (38), carnobacteriocin B2 (CarB2) (38, 39), sakacin A (SakA) (2), sakacin P (SakP) (25), leucocin A (LeuA) (22, 49), enterocin A (EntA) (3), mesentericin Y105 (MesY105) (19), pediocin PA-1 (PedPA-1) (32, 50), enterocin P (EntP) (11), and bacteriocin 31 (Bac31) (47). Other putative immunity proteins shown are the products of *orfY* (see the text) and of *orf- β 3* (an open reading frame that follows the genes encoding carnobacteriocin B2 and the CarB2 immunity protein) (38).

largely α helical but were devoid of transmembrane helices (14). Studies with *L. sake* LTH673 indicated that, despite the low degree of sequence homology, the immunity proteins shown in Table 5 provide (partial) immunity to various class IIa bacteriocins.

Interestingly, the sakacin P producer *L. sake* LTH673 contains at least one more putative immunity gene (7) (*orfY* in Table 5), which is transcribed in Bac⁺ cells only (14). This immunity gene is not coupled to a cognate bacteriocin gene (7). In addition to *L. sake* LTH673, several other LAB seem to contain immunity genes that are not directly associated with a gene encoding a cognate bacteriocin. Copies of the sakacin P immunity gene have been detected in the chromosome of the sakacin A producer *L. sake* Lb706 and, most importantly, in the chromosome of an *L. sake* strain that does not produce any bacteriocin at all (25). The producer of carnobacteriocins BM1 and B2 contains at least three (putative) immunity genes (38, 39). In addition to putative immunity genes for the two class IIa bacteriocins that are produced (designated CarBM1 and CarB2 in Table 5), a third putative immunity gene is present downstream of the structural gene for carnobacteriocin B2 (the protein is designated Orf- β 3 in Table 5). Studies of transcription have indicated that the *orf- β 3* gene is cotranscribed with the genes encoding carnobacteriocin B2 and its cognate immunity protein (43).

Extrapolating the above-mentioned observations, one may speculate that LAB from genera containing class IIa bacteriocin producers generally possess one or more immunity genes for class IIa bacteriocins. These genes may show various degrees of homology and may be expressed to various extents. These suggestions could provide an explanation for the remarkable variation in bacteriocin sensitivity that is observed for closely related LAB strains.

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