Mutational analysis of the role of charged residues in target-cell binding, potency and specificity of the pediocin-like bacteriocin sakacin P

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The significance of charged residues for the target-cell binding, potency and specificity of pediocin-like bacteriocins has been studied by site-directed mutagenesis of sakacin P. Most of the charged residues are located in the N-terminal half, which is thought to mediate the initial binding of these bacteriocins to target cells through electrostatic interaction. All the mutated peptides in which the net positive charge was reduced by one (by replacing a charged residue with threonine) exhibited reduced binding to target cells and a 2–15-fold reduction in potency. The least deleterious of these mutations was the removal of the positive charge in position 8 (H8T). This mutation was, in fact, less deleterious than the conservative His to Lys mutation, indicating that the positive charge in position 8 *per se* is not of major importance. Somewhat more deleterious was the removal of positive charges at the N- and C-terminal ends (K1T, K43T). Most deleterious was the elimination of the positive charge at positions 11 and (but to a lesser extent) 12, demonstrating the importance of the cationic patch in the middle of the N-terminal half of pediocin-like bacteriocins. Mutated peptides in which the net positive charge was increased by one were also constructed. Some of these exhibited increased cell binding and a potency that was the same as (44K, i.e. an extra positive charge at the C-terminus), or somewhat greater (T20K) than, that of sakacin P, whereas others (0K, i.e. an extra positive charge at the N-terminus) had reduced potency. Sakacin P contains only one negatively charged residue (Asp17). This negative charge and its orientation in space were crucial for activity, since the Asp to Asn mutation and (especially) the conservative Asp to Glu mutation were deleterious. Mutations that made the peptide less cationic had, overall, less effect on the potency toward the *Carnobacterium piscicola* strain than on the potency toward the three other strains tested, whereas the opposite was the case for mutations that made the peptide more cationic. Thus, charged residues in the N-terminal half may – apparently via the initial electrostatic binding of the bacteriocin to target cells – influence the target-cell specificity.

Keywords: antimicrobial peptides, mutagenesis, lactic acid bacteria

INTRODUCTION

Many Gram-positive bacteria produce antimicrobial peptides, generally termed bacteriocins. These peptides are usually cationic, less than 50 amino acid residues long, contain an amphiphilic or hydrophobic region, and often kill their target cells by permeabilizing the cell

membrane (Nes *et al.*, 1996, 2002; Nissen-Meyer *et al.*, 1997). Antimicrobial peptides with these characteristics are also produced by plants and a wide variety of animals, including humans, and are thus widely distributed in nature (Nissen-Meyer & Nes, 1997). The cationic character of antimicrobial peptides presumably facilitates interactions with negatively charged bacterial

phospholipid-containing membranes and/or acidic cell walls, whereas their amphiphilic/hydrophobic character enables membrane permeabilization.

The pediocin-like bacteriocins constitute a dominant and well-studied group of antimicrobial peptides produced by lactic acid bacteria. The peptides are of considerable interest because of their antilisterial activity. The group contains at least 17 bacteriocins, of which pediocin PA-1 (Biswas *et al.*, 1991; Henderson *et al.*, 1992; Marugg *et al.*, 1992; Nieto Lozano *et al.*, 1992), leucocin A UAL-187 (Hastings *et al.*, 1991), mesentericin Y105 (Hécharde *et al.*, 1992), sakacin P (Tichaczek *et al.*, 1992) and curvacin A (identical to sakacin A; Tichaczek *et al.*, 1992; Holck *et al.*, 1992) were the first to be identified. All pediocin-like bacteriocins are cationic, contain between 35 and 50 amino acid residues, permeabilize target-cell membranes, and have very similar primary structures, but they differ markedly with respect to their target-cell specificities (Chen *et al.*, 1997a, b; Chikindas *et al.*, 1993; Eijsink *et al.*, 1998; Fimland *et al.*, 1996, 1998, 2000; Johnsen *et al.*, 2000; Nes *et al.*, 2002).

Based on their primary structures, the peptide chains of pediocin-like bacteriocins may be divided roughly into two regions: a hydrophilic, cationic and highly conserved N-terminal half, and a less conserved, hydrophobic and/or amphiphilic C-terminal half (Fimland *et al.*, 1996). NMR studies indicate that the N-terminal region and the middle region form, respectively, a three-stranded antiparallel β -sheet supported by a conserved disulphide bridge and an amphiphilic α -helix (Fregeau Gallagher *et al.*, 1997; Wang *et al.*, 1999; H. H. Hauge, personal communication). NMR studies indicate also that the remaining C-terminal stretch is relatively unstructured. However, the fact that insertion of a C-terminal disulphide bridge into sakacin P by site-directed *in vitro* mutagenesis rendered the bacteriocin activity less temperature dependent indicates that the C-terminal stretch has a rather extended structure and folds back onto the helical part when it inserts into the target-cell membrane (Fimland *et al.*, 2000).

It has been proposed that the well-conserved cationic N-terminal half mediates the initial binding of pediocin-like bacteriocins to target cells through electrostatic interactions (Chen *et al.*, 1997a) and that the hydrophobic or amphiphilic C-terminal half penetrates into the hydrophobic part of the target-cell membrane, thereby mediating membrane leakage (Fimland *et al.*, 1996; Miller *et al.*, 1998a). The hydrophobic/amphiphilic C-terminal half also appears (in part) to determine the target-cell specificity, since hybrid bacteriocins containing N- and C-terminal regions from different pediocin-like bacteriocins have antimicrobial spectra similar to the bacteriocin from which the C-terminal region is derived (Fimland *et al.*, 1996). Moreover, the wild-type bacteriocins and variants constructed by site-directed *in vitro* mutagenesis of C-terminal regions often differed in their antibacterial spectra (Fimland *et al.*, 2000). The fact that 15-mer fragments from the C-terminal half of pediocin PA-1, but not fragments from

the N-terminal half, inhibit pediocin PA-1 to a greater extent than they inhibit other closely related pediocin-like bacteriocins also suggests that the C-terminal half contains important specificity determinants (Fimland *et al.*, 1998). In the present study, the possibility that the N-terminal half of the pediocin-like bacteriocins also may influence the target-cell specificity has been investigated by the use of site-directed *in vitro* mutagenesis. We have studied the pediocin-like bacteriocin sakacin P, focusing in particular on the role of charged residues because of their apparent importance for the initial binding of pediocin-like bacteriocins to target cells.

METHODS

Bacterial strains, plasmids and media. Sakacin P and mutated variants of sakacin P were produced by, and purified from, a two-plasmid bacteriocin expression system developed recently (Axelsson *et al.*, 1998; Fimland *et al.*, 2000). This system is based on the use of pSAK20 and pSPP2 introduced into the bacteriocin-deficient strain *Lactobacillus sake* Lb790. pSAK20 and pSPP2 confer resistance to, respectively, chloramphenicol and erythromycin. pSPP2 is a pLPV111-based *Escherichia coli*-*Lactobacillus* shuttle vector in which a bacteriocin gene and its cognate immunity gene have been placed under the control of a bacteriocin-specific promoter derived from the sakacin A producer *L. sake* Lb706 (Axelsson *et al.*, 1998). pSAK20 is a pVS2-based (von Wright *et al.*, 1987) plasmid that contains the *orf4sapKRTE* operon from *L. sake* Lb706 (Axelsson & Holck, 1995; Axelsson *et al.*, 1998). The *orf4sapKRTE* operon contains genes encoding proteins necessary for activation of the bacteriocin-specific promoters and for processing and secretion of the prebacteriocins (Axelsson & Holck, 1995; Axelsson *et al.*, 1998; Nes & Eijsink, 1999).

Epicurian Coli XL-1 Blue supercompetent cells (Stratagene) were used for cloning of all mutated pSPP2 plasmids, and plasmids with desired mutations were transformed into *L. sake* Lb790/pSAK20.

E. coli was grown at 37 °C in LB medium (Difco) with vigorous agitation, whereas the lactic acid bacteria were grown at 30 °C without agitation. The indicator strains used in the bacteriocin assays were *L. sake* NCDO 2714 (type strain), *Lactobacillus coryneformis* subsp. *torquens* NCDO 2740, *Enterococcus faecalis* NCDO 581 and *Carnobacterium piscicola* U149 (Stoffels *et al.*, 1992). *C. piscicola* U149 was grown in M17 (Oxoid) supplemented with glucose and Tween 80 at final concentrations of 0.4% (w/v) and 0.1% (v/v), respectively. The other lactic acid bacteria were grown in MRS (Oxoid). For agar plates, the media were solidified by adding 1.5% (w/v) agar. The selective antibiotic concentrations used were as follows: 150 µg erythromycin ml⁻¹ for *E. coli*; 10 µg erythromycin ml⁻¹ and 10 µg chloramphenicol ml⁻¹ for normal growth of plasmid-containing *L. sake* Lb790; and 2 µg erythromycin ml⁻¹ and 5 µg chloramphenicol ml⁻¹ for initial selection of *L. sake* Lb790/pSAK20 transformed with pSPP2 variants.

Purification of sakacin P and mutated variants of sakacin P. Sakacin P and its mutated variants were purified to homogeneity from 400 ml cultures by ammonium sulphate precipitation followed by cation-exchange chromatography, hydrophobic interaction chromatography and reverse-phase chromatography as described previously (Nieto Lozano *et al.*, 1992). The primary structures of the peptides were confirmed by determining their molecular masses with a Voyager-DE RP matrix-assisted laser desorption ionization time-of-flight mass

spectrometer (Perseptive Biosystems) using α -cyano-4-hydroxycinnamic acid as the matrix. Typically, the errors in the masses that were determined were less than 1 Da. The purities of the bacteriocins were verified to be greater than 90% by analytical reverse-phase chromatography by using a μ RPC SC 2.1/10 C₂/C₁₈ column (Amersham Biosciences) in the SMART chromatography system (Amersham Biosciences).

The concentrations of purified bacteriocins were determined by measuring UV absorption at 280 nm, and the values were converted to protein concentrations by using molar absorption coefficients calculated from the contributions of individual amino acid residues.

Bacteriocin assay. The potencies of the bacteriocins were measured by using a microtitre plate assay system, essentially as described previously (Nissen-Meyer *et al.*, 1992). Each well of a microtitre plate contained 200 μ l culture medium with bacteriocin fractions at twofold dilutions and an indicator strain at an OD₆₁₀ of about 0.01. The microtitre plate cultures were incubated overnight (12–16 h) at 30 °C, after which growth of the indicator strain was measured spectrophotometrically at 610 nm with a microtitre plate reader. The MIC was defined as the concentration of bacteriocin that inhibited growth of the indicator strain by 50%.

Plasmid isolation and transformation. Plasmids were isolated from *E. coli* and *L. sake* Lb790 by using the Wizard Plus SV Minipreps DNA-purification system (Promega). To ensure lysis of *L. sake*, lysozyme and mutanolysin were added to the cell-resuspension solution included in the Wizard Plus SV Minipreps kit to final concentrations of 5 mg ml⁻¹ and 15 U ml⁻¹, respectively.

Chemocompetent Epicurian Coli XL-1 Blue supercompetent cells were transformed according to the protocol provided with the Quick Change site-directed mutagenesis kit (Stratagene). *L. sake* Lb790/pSAK20 was transformed by electroporation using a Gene Pulser and Pulse Controller unit (Bio-Rad) as described previously (Aukrust *et al.*, 1995). *L. sake* Lb790/pSAK20 cells were made competent by growth in MRS broth supplemented with 1.5% (w/v) glycine. The cells were thereafter washed with 1 mM MgCl₂ and, subsequently, with 30% (w/v) polyethylene glycol 1500 (molecular mass range 1300–1600 Da) prior to electroporation.

Site-directed mutagenesis and DNA sequencing. Mutations in the sakacin P gene cloned in pSPP2 were made by using the Quick Change site-directed mutagenesis kit (Stratagene). The PCRs were performed with a Gene-Amp 2400 PCR system (Perkin Elmer) by using *PfuTurbo* DNA polymerase (Stratagene). The 50 μ l reaction mixtures each contained about 40 ng plasmid template, 125 ng each oligonucleotide primer (Eurogentec), each deoxynucleoside triphosphate (Stratagene) to a final concentration of 0.05 mM, and 2.5 U *PfuTurbo* DNA polymerase. After a 1 min hotstart at 95 °C, 16 cycles of the following programme were run: denaturation for 30 s at 95 °C, primer annealing for 1 min at 50 °C, and polymerization for 8 min at 68 °C. The PCR product was digested for 1 h at 37 °C with restriction enzyme *DpnI* (Stratagene) to eliminate the original template and thereby increase mutation efficiency. The DNA sequences of the mutated plasmids were verified by automated DNA sequence determination by using an ABI Prism 377 DNA sequencer and an ABI Prism Ready Reaction dye terminator cycle sequencing kit (Perkin Elmer).

Target-cell-binding studies. Binding of sakacin P and sakacin P variants to target cells was measured in a manner similar to that described by Atrih *et al.* (2001) and Bhunia *et al.* (1991). *L. sake* target cells from a 100 ml overnight culture were pelleted, washed in 50 ml MRS, and finally suspended in 20 ml

MRS; 0.5 ml of this cell suspension was then incubated for 20 min at 20 °C with 0.5 ml culture supernatant containing sakacin P or a mutated variant of sakacin P (about 1 μ g ml⁻¹). The cells were then pelleted and the amount of sakacin P or mutated variant of sakacin P that bound to the target cells was determined by comparing the amount of bacteriocin activity in this supernatant with the amount that was present before exposure to the target cells.

RESULTS AND DISCUSSION

Production and purification of sakacin P and mutated variants of sakacin P

The sequences of 17 pediocin-like bacteriocins (including sakacin P) and an overview of the 14 mutated variants of sakacin P that were constructed are shown in Fig. 1(a, b). We have, in this study, particularly focused on charged residues because of the apparent importance of electrostatic interactions in the initial binding of pediocin-like bacteriocins to target cells (Chen *et al.*, 1997a). Thus, nearly all the sakacin P variants have a slightly altered net charge compared to sakacin P, which contains one negatively (Asp17) and five positively charged residues (Lys1, Lys11, Lys43, His8 and His12), and these are, with one exception (Lys43, the C-terminal residue), all clustered in the N-terminal half of the peptide (Fig. 1b). Four indicator strains (*L. sake* NCDO 2714, *E. faecalis* NCDO 581, *L. coryneformis* subsp. *torquens* NCDO 2740, *C. piscicola* UI49) were used to determine the effects that the mutations had on potency and target cell specificity.

Between 10 and 100 μ g sakacin P and mutated variants of sakacin P were purified from 400 ml cultures. The last reverse-phase chromatography step gave a major symmetrical absorbance peak that contained a peptide with bacteriocin activity and the expected molecular mass (determined by mass spectrometry). Analysis of the peptide fraction by analytical reverse-phase chromatography revealed one major absorbance peak, verifying the purity of the peptide.

Effect on activity of mutations that render sakacin P less cationic

By replacing the positively charged residues with threonine, a set of sakacin P variants (K1T, H8T, K11T, H12T and K43T; Fig. 1b) were constructed in which the net positive charge was reduced by about one. All of these peptides had reduced potency compared to sakacin P, the reduction varying between two- and 15-fold depending on which indicator strain was used in the bacteriocin assay and on where in the peptide the charge was altered (Table 1, Fig. 2). The least deleterious mutation was the His to Thr substitution at position 8 (H8T). This non-conservative mutation, which completely eliminates the positive charge at position 8, was in fact somewhat less deleterious than the conservative His to Lys mutation (H8K), which only slightly accentuates the positive charge at position 8. The former mutation reduced the potency by only two- to threefold, whereas the latter reduced it by three- to fivefold (Table

(a)

Pediocin PA-1: KY**Y**NGV**T**CGKHSCSV**D**WGKA**T**TCI**I**NGAMAWATGGH**Q**GN**H**KC
Coagulin: KY**Y**NGV**T**CGKHSCSV**D**WGKA**T**TCI**I**NGAMAWATGGH**Q**GT**H**KC
Sakacin P: KY**Y**NGV**H**CGKHSC**T**VDWGT**A**IGN**I**GNNAANWATGGNAGW**N**K
Piscicolin: KY**Y**NGV**S**CNKNGCTVDW**S**KA**I**GI**I**GNNAANLTGGAGW**N**KG
Mundticin: KY**Y**NGV**S**CNKKGCSVDW**S**KA**I**GI**I**GNNAANLTGGAGW**S**K
Sakacin 5X: KY**Y**NG**L**SCNKSGCSVDW**S**KA**I**SI**I**GNNAVANLTGGAGW**S**K
Leucocin C: K**N**YNGV**H**CT**K**KGCSVDWGYAW**T**NIANN**S**VMNGLTGGNAGW**H**N
Bacteriocin 31: AT**Y**YNG**L**YCN**K**QKCWVDW**N**KAS**R**E**I**G**K**I**I**VNGWV**Q**HGPW**A**P
Curvacin A: AR**S**YNGV**Y**CNN**K**KCWVNR**G**EAT**Q**SI**G**GMISGWASGL**A**GM
Carnobacteriocin BM1: AISYNGV**Y**CNN**K**ECWV**N**KAEN**K**QAITG**I**VIGWASSLAGM**G**H
Enterocin P: AT**R**SYNGV**Y**CNN**S**KCWVNW**G**EAKEN**I**AGIVISGWASGLAGM**G**H
Enterocin A: TT**H**SGKY**Y**NGV**Y**CT**K**NKCTVDW**A**KAT**T**CIAGMSIGGFLGGAI**P**G**K**C
Divergin V41: TK**Y**YNGV**Y**CNS**K**KCWVDW**Q**ASG**C**IG**Q**TVVGWLGGAI**P**G**K**C
Carnobacteriocin B2: VN**Y**NGV**S**CS**K**TKCSVN**W**GQAF**Q**ERYTAGINSFVSGVASGAGS**I**GRR**P**
Leucocin A: KY**Y**NGV**H**CT**K**SGCSVN**W**GEAF**S**AGV**H**RLANGGNG**F**W
Mesentericin Y105: KY**Y**NGV**H**CT**K**SGCSVN**W**GE**A**ASAG**I**HRLANGGNG**F**W
Plantaricin C19: KY**Y**NG**L**SC**S**KK**G**CTVNW**Q**AFSCGV**N**RVATAG**H**K**K**...

(b)

	0	1	8	11	12	17	20	43	44																																
	↓	↓	↓	↓	↓	↓	↓	↓	↓																																
Sakacin P:	K	Y	Y	N	G	V	H	C	G	K	H	S	C	T	V	D	W	G	T	A	I	G	N	I	G	N	N	A	A	N	W	A	T	G	G	N	A	G	W	N	K
0K	K																																								
K1T	T																																								
H8T																																									
H8K																																									
K11E																																									
K11T																																									
H12T																																									
H12K																																									
D17E																																									
D17N																																									
T20K																																									
K43T																																									
44K																																									
0K+H8K	K																																								

Fig. 1. (a) Sequences of 17 pediocin-like bacteriocins and (b) an overview of the 14 mutated sakacin P variants that were constructed. Charged residues are represented as black boxes in (a). References for the sequences are as follows: pediocin PA-1, Henderson *et al.* (1992), Marugg *et al.* (1992); coagulin, Le Marrec *et al.* (2000); sakacin P, Tichaczek *et al.* (1994); piscicolin (same as piscicolin V1a), Jack *et al.* (1996), Bhugaloo-Vial *et al.* (1996); mundticin, Bennik *et al.* (1998); sakacin 5x, Vaughan *et al.* (2001); leucocin C, G. Fimland and others (unpublished); bacteriocin 31, Tomita *et al.* (1996); curvacin A (same as sakacin A), Holck *et al.* (1992), Tichaczek *et al.* (1993); carnobacteriocin BM1, Quadri *et al.* (1994); enterocin P, Cintas *et al.* (1997); enterocin A, Aymerich *et al.* (1996); divercin V41, Métivier *et al.* (1998); carnobacteriocin B2, Quadri *et al.* (1994); leucocin A, Hastings *et al.* (1991); mesentericin Y105, Fleury *et al.* (1996); and plantaricin C19 (not the complete sequence), Atrih *et al.* (2001).

1, Fig. 2). Neither mutation had a marked effect on the target-cell specificity (i.e. the mutations affected the four strains to the same extent; Fig. 2). Thus, it appears that the positive charge at position 8 *per se* is not of major importance for the binding of the peptide to target cells. This is consistent with the fact that the positive charge in position 8 is the one in sakacin P which is least conserved among the pediocin-like bacteriocins; only four of the 17 pediocin-like bacteriocins shown in Fig. 1(a) have a positive charge at position 8.

Somewhat more deleterious was the removal of positive charges at the N- and C-terminal ends of the peptide. This was expected, since these positive charges are quite well conserved in the pediocin-like bacteriocins (Fig.

1a). Moreover, analysis of pediocin AcH variants that were generated by random mutagenesis also indicated that the positive charges at the terminal ends are important for activity (Miller *et al.*, 1998b). The two mutations (K1T, K43T) had nearly identical effects: they reduced the potency toward the *C. piscicola* strain by two- to threefold and the potency toward the *L. sake*, *E. faecalis* and *L. coryneformis* strains by between four- and sixfold (Table 1, Fig. 2). They also altered the target-cell specificity somewhat, since the effect on the *C. piscicola* strain was considerably less than that on the three other strains (Table 1, Fig. 2).

Most deleterious was the elimination of a positive charge at positions 11 and (but to a lesser extent) 12. The

Table 1. Potency of bacteriocin variants toward various indicator strains

Bacteriocin potency was measured as described in Methods. The indicator strains used in the bacteriocin assay were *L. sake* NCDO 2714 (type strain), *L. coryneformis* subsp. *torquens* NCDO 2740, *E. faecalis* NCDO 581 and *C. piscicola* UI49. The MIC is the concentration of bacteriocin that inhibited growth of the indicator strain by 50%. The values and the standard deviations are the results of 10 measurements.

Bacteriocin variant	MIC (nM)			
	<i>L. sake</i>	<i>E. faecalis</i>	<i>L. coryneformis</i>	<i>C. piscicola</i>
Sakacin P	0.21 ± 0.03	0.44 ± 0.11	0.41 ± 0.06	0.48 ± 0.11
Neutral variants				
H8K	0.63 ± 0.11	1.6 ± 0.3	2.0 ± 0.4	1.3 ± 0.3
H12K	0.18 ± 0.01	0.58 ± 0.10	0.65 ± 0.07	0.48 ± 0.17
D17E	1.7 ± 0.3	5.5 ± 1.4	6.9 ± 0.8	9.0 ± 1.6
Less cationic variants				
K1T	1.1 ± 0.25	2.4 ± 0.7	2.5 ± 0.5	1.4 ± 0.4
H8T	0.67 ± 0.07	0.92 ± 0.18	1.0 ± 0.2	1.4 ± 0.3
K11T	1.9 ± 0.2	5.9 ± 1.5	5.9 ± 1.1	2.5 ± 0.4
K11E	5.7 ± 0.6	15 ± 3	15 ± 3	3.6 ± 0.5
H12T	1.0 ± 0.2	3.3 ± 0.6	3.5 ± 0.6	0.84 ± 0.16
K43T	0.87 ± 0.09	2.0 ± 0.4	2.1 ± 0.4	1.1 ± 0.3
More cationic variants				
0K	0.65 ± 0.20	2.5 ± 0.5	2.2 ± 0.4	7.8 ± 1.2
0K + H12K	0.54 ± 0.18	3.4 ± 1.0	3.1 ± 0.5	8.4 ± 1.3
D17N	0.75 ± 0.23	2.9 ± 0.5	2.9 ± 0.3	11 ± 2
T20K	0.15 ± 0.01	0.25 ± 0.06	0.20 ± 0.04	0.40 ± 0.15
44K	0.22 ± 0.01	0.62 ± 0.12	0.57 ± 0.9	0.80 ± 0.16

Lys to Thr mutation at position 11 (K11T) reduced the potency toward the *C. piscicola* strain by about fivefold and the potency toward the three other strains by nine- to 15-fold (Table 1, Fig. 2). The corresponding mutation at position 12 (H12T) reduced the potency toward the *C. piscicola* strain by about twofold and the potency toward the three other strains by five- to ninefold (Table 1, Fig. 2). Even more deleterious was the introduction of a negative charge in this region by a Lys to Glu mutation at position 11 (K11E), in contrast to earlier studies showing that this mutation in pediocin AcH increased the potency by twofold (Miller *et al.*, 1998b). The Lys to Glu mutation in sakacin P reduced the potency toward *C. piscicola* by eightfold and the potency toward the three other strains by 30–40-fold (Table 1, Fig. 2). In contrast, an accentuation of the region's cationic character by a His to Lys mutation at position 12 (H12K) had no marked detrimental effects (Table 1, Fig. 2). These results clearly demonstrate the importance of this cationic patch. Its importance is perhaps not surprising, considering that all 17 bacteriocins have at least one positively charged residue (in fact, 11 of them have two positively charged residues) at position 11, 12 and/or 13 (Fig. 1a). Consequently, the cationic character of this region is expected to be particularly important for bacteriocin action, perhaps through involvement in the initial electrostatic interactions between the bacteriocin and the target cell. This conclusion is in agreement with

a study by Chen *et al.* (1997a), in which intrinsic tryptophan fluorescence was used to investigate the binding of pediocin PA-1 and its fragments to target membranes. The study indicated that the initial binding step primarily involved electrostatic interactions between two positively charged residues at positions 11 and 12 and negatively charged phospholipid head groups in the target membrane (Chen *et al.*, 1997a). The three mutations (K11T, H12T and K11E) in this region that altered potency also altered the target-cell specificity, since the effect on the *C. piscicola* strain was considerably less than that on the three other strains (Table 1, Fig. 2).

Effect on activity of mutations that render sakacin P more cationic

Mutated variants of sakacin P that were more cationic than sakacin P were constructed by replacing a neutral residue with a positively charged one (T20K) or by adding an extra positive residue at the N- or C-terminal end (0K, 0K + H12K, 44K). One of these peptides, T20K, was somewhat more potent than sakacin P (Table 1, Fig. 2). A charged residue at position 20 thus appears to be beneficial, consistent with the fact that seven of 17 pediocin-like bacteriocins have a positively charged residue (Lys) in this position. The addition of an extra positive charge at the C-terminal end (44K), resulting in

Fig. 2. Fold reduction in the potencies of the various mutated sakacin P variants (as calculated from the data in Table 1), where the fold reduction in potency is defined as the MIC for the peptide divided by the MIC for sakacin P. The peptide has the same potency as sakacin P when the fold reduction in potency is equal to one (indicated by the dashed line) and is more active than sakacin P if it is less than one (below the dashed line). The four indicator strains that were used are as indicated in the figure.

a peptide with two C-terminal lysine residues, had no marked effects on either the potency or specificity. In contrast, the addition of an extra positive charge at the N-terminal end (0K), resulting in a peptide with two N-terminal lysine residues, reduced the potency by three- to 18-fold depending on which indicator strain that was tested (Table 1, Fig. 2). This mutation also altered the target-cell specificity, but in this case the potency toward the *C. piscicola* strain was reduced more than that toward the three other strains (Table 1, Fig. 2). The doubly mutated peptide 0K+H12K had the same potency and specificity as the mutated peptide 0K, consistent with results showing that the His to Lys mutation at position 12 had no marked effects (Table 1, Fig. 2).

A variant of sakacin P (D17N) that was more cationic than sakacin P was also constructed by replacing the negatively charged aspartate residue in position 17 with the structurally related, but neutral, asparagine residue. This mutation was deleterious: it reduced the potency to the same extent and had nearly the same effect on the specificity as the 0K and the 0K+H12K mutations (Table 1, Fig. 2). Interestingly, the conservative mutation (D17E) in which the negatively charged aspartate residue

was replaced with the structurally related and negatively charged glutamate residue – thus extending the negative charge from the peptide backbone by one carbon atom – was even more deleterious (Table 1, Fig. 2). Thus, both the negative charge at position 17 and its exact location in space are apparently crucial for the bacteriocin to be fully active. This suggests that the negative charge at position 17 interacts in a structurally specified and restricted manner with a (positively charged) group on the target cell or on the peptide itself. The importance of this negatively charged residue is also suggested by the fact that it is conserved in 10 of the 17 pediocin-like bacteriocins, and by the fact that five of the seven bacteriocins that do not have an aspartate at position 17 have, instead, a negatively charged glutamate at position 20 (Fig. 1a).

The more cationic variants of sakacin P have greater affinity for target cells and are inhibited by salts to a greater extent than the less cationic variants

The more cationic variants of sakacin P had greater affinity for the target cells than the less cationic ones (Fig. 3), demonstrating the importance of electrostatic interactions for the initial binding of pediocin-like

Fig. 3. Percentage of sakacin P and mutated sakacin P variants that bound to the *L. sake* target cells. Black bars represent variants that are more cationic than sakacin P, the shaded bar represents sakacin P, and white bars represent variants that are less cationic than sakacin P. The values shown are means, with standard deviations of three determinations. The assay conditions were as described in Methods.

bacteriocins to target cells. However, the locations of the positive charges are clearly also important, since some of the peptides (0K, 0K + H12K) that were more cationic than sakacin P had less affinity for target cells than did sakacin P. The importance of the positive charge in position 11 and the lesser importance of the one at position 8 were demonstrated also by the binding studies, the affinity being reduced to the greatest extent upon removal of the charge at position 11. Removal of the charge at position 8 had less effect on the affinity. Two of the more cationic peptides (T20K, 44K) had greater affinity for target cells than did sakacin P (Fig. 3), and these were the ones that had a potency that was the same as (44K), or somewhat greater (T20K) than, that of sakacin P.

Consistent with earlier studies (Atrih *et al.*, 2001; Bhunia *et al.*, 1991), the binding of the peptides to target cells was reduced upon reducing the electrostatic interactions by increasing the ionic strength by adding NaCl (0.3 M) or $MgCl_2$ (50 mM; results not shown). These salts also inhibited the activity of sakacin P and all the sakacin P variants; their MIC values increased by between two- and 100-fold, depending on the salt, the salt concentration, the indicator strain and the peptide (results not shown). The sakacin P variants (44K, T20K, D17N, 0K, 0K + H12K) that were more cationic than sakacin P were inhibited to a greater extent (their MIC values increased 20–40-fold in the presence of 50 mM $MgCl_2$) than sakacin P (its MIC increased 15-fold in the presence of 50 mM $MgCl_2$), whereas those that were less cationic (K11E, K11T, K43T, H8T, K1T, H12T) were inhibited to a lesser extent (their MIC values increased 5–10-fold in the presence of 50 mM $MgCl_2$) – apparently because the electrostatic interactions are more pronounced for the most-cationic peptides.

The net charge influences the target-cell specificity

We have previously reported that residues in the C-terminal halves of pediocin-like bacteriocins (in part) determine the target-cell specificity. The present results indicate that residues in the N-terminal halves also may influence the specificity. The N-terminal halves are hydrophilic and ionic and may influence specificity via the initial electrostatic binding of the bacteriocins to target cells. In contrast, the C-terminal halves are hydrophobic or amphiphilic and may influence the target-cell specificity through interactions with the hydrophobic part of the membrane. Both types of interactions must be optimal in order for a bacteriocin to exert its toxic effect, and may consequently have an effect on the specificity.

Interestingly, mutations (K1T, K11T, K11E, H12T, K43T) that made the peptides less cationic than sakacin P had lesser effects on the potency toward the *C. piscicola* strain than on the potency toward the three other strains, whereas the opposite was the case for mutations (0K, 0K + H12K, D17N) that made the peptide more cationic (Table 1, Fig. 2). The latter mutations had the least effect on the potency toward the *L. sake* strain. There were three exceptions: the His to Thr mutation at position 8 (H8T), the Thr to Lys mutation at position 20 (T20K), and the addition of a Lys residue at the C-terminal end (44K). Although these three mutations altered the net charge, they had no marked effects on the specificity – probably because these alterations had no gross detrimental effects on the potencies of the peptides. The charges that appear to be of most importance are clearly the ones in the positive patch in the middle of the N-terminal half – in agreement with the results of Chen *et al.* (1997a) – and the negative charge at position 17, although the positive charges at the N- and C-termini also influence the target-cell binding, potency and specificity.

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