Mutational Analysis of the Role of Tryptophan Residues in an Antimicrobial Peptide†

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ABSTRACT: Antimicrobial peptides belonging to the pediocin-like family of bacteriocins (class IIa bacteriocins) produced by lactic acid bacteria contain several tryptophan residues that are highly conserved. Since tryptophan residues in membrane proteins are often positioned in the membrane−water interface, we hypothesized that Trp residues in bacteriocins could be important determinants of the structure of membrane-bound peptides and of anti-microbial activity. To test this hypothesis, the effects of mutating each of the 3 tryptophan residues (Trp18, Trp33, and Trp41) in the 43-residue pediocin-like bacteriocin sakacin P were studied. Trp18 and Trp33 are located at each end of an amphihilic α-helix, whereas Trp41 is near the end of an unstructured C-terminal tail. Replacement of Trp33 with the hydrophobic residues Leu and Phe had marginal effects on activity, whereas replacement with the more polar Tyr and Arg reduced activity 10−20 and 500−1000 times, respectively, indicating that Trp33 and the C-terminal part of the helix interact with the hydrophobic core of the membrane. Any mutation of Trp18 and Trp41 reduced activity, indicating that these two residues play unique roles. Substitutions with other aromatic residues were the least deleterious, indicating that both Trp18 and Trp41 interact with the membrane−water interface. The suggested locations of the three Trp residues are compatible with a structural model in which the helix and the C-terminal tail form a hairpin-like structure, bringing Trp18 and Trp41 close to each other in the interface, and placing Trp33 in the hydrophobic core of the membrane. Indeed, the deleterious effect of the W18L and W41L mutations could be overcome by stabilizing the hairpin-like structure by introduction of a disulfide bridge between residues 24 and 44. These results provide a basis for a refined structural model of pediocin-like bacteriocins and highlight the unique role that tryptophan residues can play in membrane-interacting peptides.

Bacteriocins are proteinaceous compounds with antimicrobial activity that are produced by bacteria. Many bacteriocins are relatively short peptides that kill target-cells by membrane permeabilization (*1*). One important group of antimicrobial peptides consists of the pediocin-like (class IIa) bacteriocins produced by lactic acid bacteria (*2*). Today, at least 20 members of this group are characterized (*3, 4*; see also Figure 1 for references). They consist of 37−48 residues, contain an YGNGV/L sequence-motif, are cationic, and display anti-listeria activity (*4*; Figure 1). The fact that they act on cell membranes (*1, 24*) makes them useful for peptide−membrane interaction studies.

Pediocin-like bacteriocins are unstructured in watery solutions, but become partly structured in the presence of trifluoroethanol or membrane-mimicking entities such as micelles or negatively charged liposomes (*14, 16, 25*−*27*). The three-dimensional structures of the two pediocin-like bacteriocins leucocin A and carnobacteriocin B2 have been analyzed by nuclear magnetic resonance (NMR)[1] spectroscopy, using membrane-mimicking conditions (*25, 26*). These studies have left some doubt as to the structure of the hydrophilic N-terminal region (residues 1−16/17) of the peptides, since leucocin A and carnobacteriocin B2 yielded quite different results in this region despite more than 70% sequence identity. Leucocin A displayed a well-structured three-strand β-sheet (supported by the 9−14 disulfide bridge; Figure 1), whereas the N-terminal region in carnobacteriocin B2 was disordered. The NMR studies clearly indicated that both bacteriocins contain an amphiphilic α-helix starting at residue 17/18 and ending at residue 31 (leucocin A) or 39 (carnobacteriocin B2). In both bacteriocins, the C-terminal tail was found to be unstructured. Recent NMR analysis of sakacin P indicates that the structure of this bacteriocin resembles that of leucocin A (H. H. Hauge, personal communication).

Information on the role and position of the apparently unstructured C-terminal tail may be deduced from studies dealing with the second disulfide bridge that is present in

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[1] Abbreviations: CD, circular dichroism; DOPG, dioleoylphosphatidylglycerol; MALDI-TOF, matrix-assisted laser desorption time-of-flight; MIC, minimal inhibition concentration; NMR, nuclear magnetic resonance; LB, Luria−Bertani; OD, optical density; TFA, trifluoroacetic acid.

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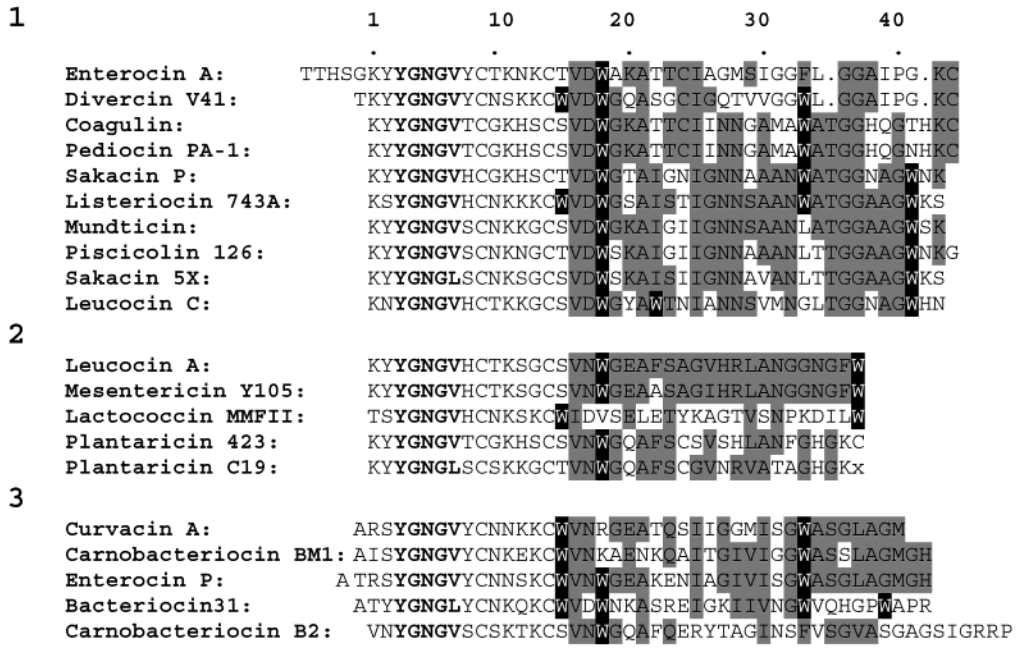

FIGURE 1: Multiple sequence alignment of pediocin-like bacteriocins, highlighting the YGNGV/L motif, tryptophan residues (black boxes), and also conserved residues in the C-terminal half of the peptides. Residues in the C-terminal parts are in a gray box if they occur in at least 4 (group 1) or 3 (groups 2 and 3) of the sequences. The following residues were considered similar: I = L = V; E = D; K = R; Ser = Thr. Group 1: enterocin A (*5*), divercin V41 (*6*), coagulin (*7*), pediocin PA-1 (*8*), sakacin P (*9*), listeriocin 743 (*10*), mundticin (*11*), piscicolin 126 (*12*), sakacin 5x (*13*), leucocin C (*13, 14;* Fimland, G., Sletten, K., and Nissen-Meyer, J., unpublished results). Group 2: leucocin A (*15*), mesentericin Y105 (*16*), lactococcin MMFII (*17*), plantaricin 423 (*18*), and plantaricin C19 (*19*). Group 3: curvacin A (*20*), carnobacteriocin BM1 (*21*), enterocin P (*22*), bacteriocin 31 (*23*), and carnobacteriocin B2 (*21*). The pediocin-like bacteriocins contain a disulfide bond between the conserved Cys9 and Cys14 residues. Some group 1 (and putatively group 2) bacteriocins also have a disulfide bond between Cys24 and the cysteine residue at the C-terminal end.

the C-terminal region in some pediocin-like bacteriocins (e.g., pediocin PA-1, see Figure 1). This C-terminal disulfide bridge connects the C-terminal end with the central α-helical region, thereby creating what we will call a "hairpin" structure in this report. Whether or not this hairpin-like spatial organization occurs in all pediocin-like bacteriocins remains to be seen. It is known that the activity and stability of sakacin P, that naturally contains only the 9−14 disulfide bridge, can be increased by introducing a C-terminal disulfide bridge (*28*). This suggests that at least sakacin P and pediocin PA-1 have approximately the same structure when they are in their active conformations.

It is generally assumed that the amphiphilic α-helical part and the C-terminal tail are involved in interactions with the hydrophobic part of the cell membrane and may thus be important for membrane permeabilization (*11*, *29*, *30*). The deleterious effect of introducing hydrophilic amino acids in this region supports this notion (*31*−*33*). The hydrophilic N-terminal *β*-sheet region of the bacteriocins is considered an important mediator for interactions between the peptide and the target-cell membrane, and positive charges in this region contribute to membrane binding (*34*, *35*). Even small modifications in the YGNGV motif or reduction of the conserved 9−14 disulfide bond are deleterious with respect to activity (*16*, *31*, *32*, *36*).

It is known from studies on membrane proteins and model peptides that tryptophan residues preferentially occur in the membrane−water interface, particularly in the region near the lipid carbonyls (*37*−*40*). Interestingly, pediocin-like bacteriocins contain conserved tryptophan residues in the 15−18 region, at position 33, and/or near the C-terminus (see Figure 1). One may hypothesize that these Trp residues contribute to correct positioning and structuring of the membrane-bound bacteriocin and, thus, to anti-microbial activity. To investigate this hypothesis, we have studied the effects of replacing Trp18 or Trp33 or Trp41 in the 43-residue bacteriocin sakacin P (Figure 1), by various other amino acids. Further insights were obtained by combining Trp → Xxx mutations with mutations aimed at introducing the second disulfide bridge in sakacin P as well as by studying the effects of "reciprocal" mutations in pediocin PA-1.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Media. All bacteriocins were produced using a previously developed system for heterologous bacteriocin expression in the bacteriocin-deficient strain *Lactobacillus sake* Lb790 (*41*). The system is based on two plasmids, pSAK20 and pSPP2 or pPED2, conferring resistance to, respectively, chloramphenicol and erythromycin. pSPP2/pPED2 are pLPV111 (*41*) based *E. coli*−*Lactobacillus* shuttle vectors in which a bacteriocin gene and its cognate immunity gene have been placed under control of a bacteriocin-specific promoter derived from the sakacin A producer *L. sake* Lb706 [see (*42*) for details]. pSAK20 is a pVS2 (*43*) based plasmid that contains the *orf4sapKRTE* operon from *Lactobacillus sake* Lb706 (*41*, *42*). The products of *orf4* and the *sapK* and *sapR* genes are necessary for activation of the bacteriocin-specific promoters on pSPP2/pPED2, whereas the products of the *sapT* and *sapE* genes ensure processing and secretion of the prebacteriocins encoded by these plasmids (*41*, *42*). This system permitted routine production of correctly processed, secreted (mutant) bacteriocins, which could be purified using well-established

methods for purification of these peptides from lactic acid bacteria (see below).

Epicurian coli XL1-Blue Supercompetent cells (Stratagene) were used for the cloning of all mutated pSPP2/pPED2 plasmids. pSPP2/pPED2 derivatives containing desired mutations were transformed into *L. sake* Lb790/pSAK20.

E. coli was grown at 37 °C in LB medium (Difco) with vigorous agitation. The indicator strains used in the bacteriocin assays were *L. sake* NCDO 2714 (type strain), *L. coryneformis* subsp. *torquens* NCDO 2740, *Enterococcus faecalis* NCDO 581, and *Carnobacterium piscicola* UI49 (*44*). *C. piscicola* UI49 was grown in M17 (Oxoid) supplemented with glucose and Tween 80 to final concentrations of 0.4% (w/v) and 0.1% (v/v), respectively. The other lactic acid bacteria were grown in MRS (Oxoid). Lactic acid bacteria were grown at 30 °C without agitation. For agar plates, adding 1.5% (w/v) agar solidified the media. Selective antibiotic concentrations used were 150 μg/mL erythromycin for *E. coli* and 10 μg/mL erythromycin and 10 μg/mL chloramphenicol for normal growth of plasmid containing *L. sake* Lb790, and 2 μg/mL erythromycin and 5 μg/mL chloramphenicol for initial selection of *L. sake* Lb790/pSAK20 transformed with pSPP2 or pPED2 variants.

Purification of Sakacin P and Mutant Bacteriocins. Wild-type and mutant bacteriocins were purified to homogeneity from 300 to 400 mL cultures by applying the bacteria culture directly on a cation exchanger followed by reverse-phase chromatography, as described previously (*45*). To confirm that the recombinant lactobacilli had correctly produced and processed the bacteriocins, molecular masses of the isolated peptides were determined by mass spectrometry, using a MALDI-TOF Voyager-DERP mass spectrometer (Perseptive Biosystems) with α-cyano-4-hydroxy-cinnamic acid as matrix. Typically, the error margin in the determined masses was below 1 Da. The mass spectrometry analyses confirmed that all cysteine residues in the bacteriocin variants presented in this study were in the disulfide form. The purity of bacteriocins was verified to be greater than 90% by analytical reverse-phase chromatography using a μRPC SC 2.1/10 C_2/C_{18} column (Amersham Bioscience) in the SMART chromatography system (Amersham Bioscience).

The concentration of purified bacteriocins was determined by measuring UV absorption at 280 nm, which was converted to protein concentration using molecular extinction coefficients, calculated from the contributions of individual amino acid residues. Pediocin-like bacteriocins are unstructured in watery solutions (*25*−*27*), and it has previously been shown that the UV method is sufficiently reliable for concentration determination (*36*).

Bacteriocin Assay. Bacteriocin activity was measured using a microtiter plate assay system, essentially as described previously (*46*). Each well of a microtiter plate contained 200 μL of culture medium with bacteriocin fractions at 2-fold dilutions and the indicator strain at an OD_{610} of about 0.01. The microtiter plate cultures were incubated overnight (14−16 h) at 30 °C, after which growth of the indicator strain was measured spectrophotometrically at 610 nm with a microtiter plate reader. The minimal inhibitory concentration (MIC) was defined as the concentration of bacteriocin that inhibited growth of the indicator strain by 50%. The MIC values presented are the result of at least 3 independent measurements. Standard deviations were less than 50% of the value in all cases.

Plasmid Isolation and Transformation. All plasmid isolations from *E. coli* and *L. sake* Lb790 were done 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 and 15 units/mL, respectively.

Epicurian coli XL1-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 the Bio-Rad Gene Pulser (Bio-Rad Laboratories) as described previously (*47*). *L. sake* Lb790/pSAK20 cells were made competent by growth to an OD_{600} of 0.6 in MRS broth supplemented with 1.5% (w/v) glycine. The cells were thereafter washed with 1 mM $MgCl_2$ and, subsequently, with 30% (w/v) PEG 1500 [poly(ethylene glycol), molecular weight range 1300−1600] prior to electroporation.

Site-Directed Mutagenesis and DNA Sequencing. Mutations in the sakacin P gene cloned in pSPP2 were made using the Quick Change Site-Directed Mutagenesis Kit (Stratagene). The PCR reactions were run on a GeneAmp PCR System 2400 (Perkin-Elmer) using *Pfu Turbo* DNA polymerase (Stratagene). The 50 μL reaction mixtures contained about 40 ng of plasmid template, 125 ng of each oligonucleotide primer (Eurogentec), dNTPs (Stratagene) to a final concentration of 0.05 mM for each nucleotide, and 2.5 units of *Pfu Turbo* DNA polymerase. After 1 min at 95 °C, 16 cycles of the following program were run: denaturation (30 s at 95 °C), primer annealing (1 min at 50 °C), and polymerization (8 min at 68 °C). The PCR product was digested 1 h at 37 °C with restriction enzyme *Dpn*I (Stratagene) to eliminate the original template and thereby increase mutation efficiency. The DNA sequence of mutant plasmids was verified by automated DNA sequence determination, using an ABI PRISM 377 DNA sequencer and the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer).

RESULTS

Production and Purification of Pediocin-like Bacteriocins and Their Mutants. Figure 1 shows the amino acid sequences of 20 pediocin-like bacteriocins, highlighting their relatively well-conserved tryptophan residues. The figure shows a putative subgrouping of the bacteriocins on the basis of sequence similarities in the C-terminal parts. Sakacin P and pediocin PA-1, the bacteriocins mutated in this study, belong to the same group and have highly similar sequences. The bacteriocin variants made in this study are presented in Figure 2. Each of the three tryptophan residues in sakacin P was replaced with aromatic (Phe, Tyr), hydrophobic (Leu), and hydrophilic positive (Arg, His) or neutral (Asn) residues. To determine whether the deleterious effect of replacing tryptophan residues could be overcome by introducing the 24−44 disulfide bridge that is naturally present in pediocin PA-1 (Figure 1), such a bridge was introduced into some of the sakacin P mutants in which one of the tryptophan residues had been replaced. "Reciprocal" mutations were introduced

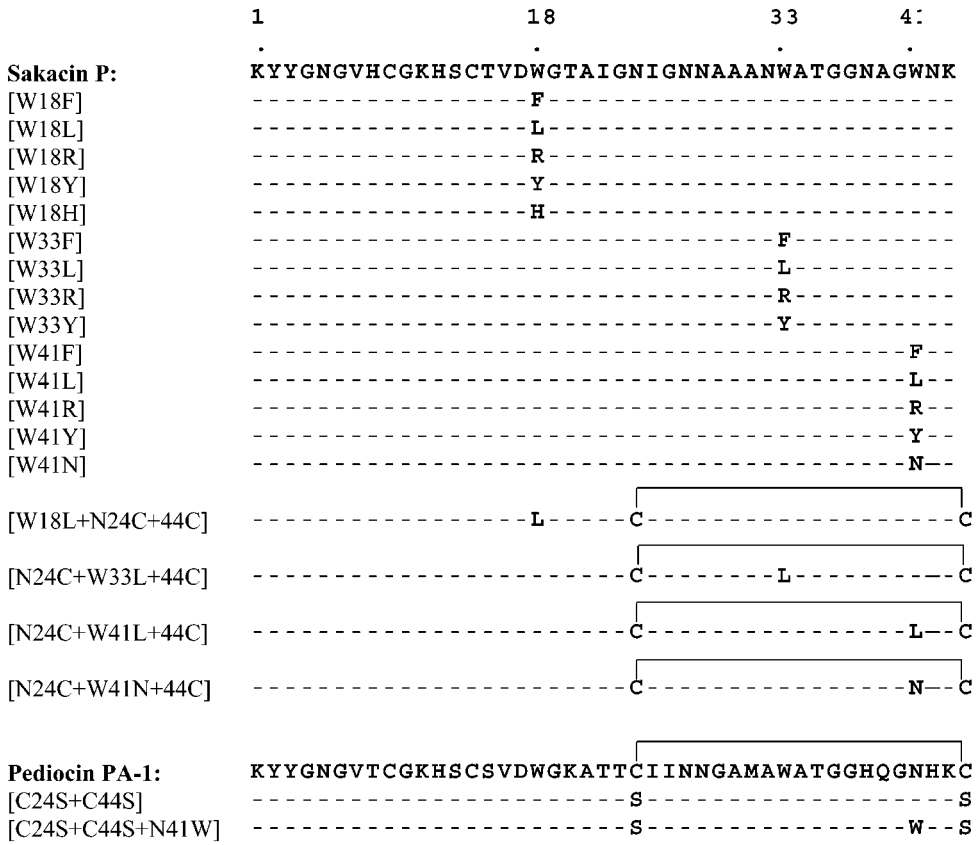

FIGURE 2: Overview of sakacin P and pediocin PA-1 mutants constructed and characterized in this study. The presence of C-terminal disulfide bridges is indicated.

in pediocin PA-1 by removing the 24−44 disulfide bridge and introducing a tryptophan residue near the C-terminal end (Figure 2).

The standard purification procedure, starting with 300−400 mL of culture medium, yielded 50−200 μg of sakacin P, pediocin PA-1, and the bacteriocin variants. The purity of the purified peptides was at least 90% as judged by analytical chromatography. The molecular masses of the purified peptides as determined by MALDI-TOF mass spectrometry were in agreement with the calculated molecular masses.

MIC values of bacteriocin variants were assessed using four different indicator strains (*L. sake* NCDO 2714, *L. coryneformis* subsp. *torquens* NCDO 2740, *E. faecalis* NCDO 581, and *C. piscicola* UI49). Use of several indicator strains is necessary to detect general trends in mutational effects, since such effects in some cases may be indicator strain dependent (*28*, *35*).

Mutational Effects. Most of the Trp → Xxx mutations in sakacin P reduced anti-microbial activity considerably (Table 1). It is important to note, though, that the effect of certain types of Trp replacements (Trp → Leu, Trp →Phe, and Trp → Tyr) was strongly dependent on the position of the mutated residue (18, 33, or 41). This indicates that the three Trp residues in sakacin P interact with different environments in the target cell membrane.

The replacement of any of the three tryptophans in sakacin P with a hydrophilic neutral (Asn) or positive (Arg, His) residue was deleterious in all cases. The MIC values for these mutants (W18R, W18H, W33R, W41R, W41N) were 500−10 000-fold higher than that for wild-type sakacin P (Table 1), indicating that none of the three Trp residues interacts with a hydrophilic environment.

More complex results were obtained when the tryptophans were substituted with an aliphatic and hydrophobic (Leu), an aromatic and hydrophobic (Phe), or an aromatic and weakly hydrophilic (Tyr) residue. The hydrophobic residues leucine and phenylalanine could both replace Trp33 without major loss of activity, the MIC values for the W33L and W33F mutants being only 1.5−4-fold higher than for sakacin P (Table 1). Replacement of Trp33 with the somewhat more hydrophilic tyrosine was more deleterious, yielding a 10−30-fold increase in MIC value (Table 1). These results show that activity depends on hydrophobicity but not on aromaticity at position 33 and indicate that residue 33 interacts with a "regular" hydrophobic environment, such as the interior of a membrane.

In contrast to Trp33, Trp18 and Trp41 could not be replaced with hydrophobic residues without a great loss of activity. The MIC values for the W18L and W41L mutants were, respectively, 300−1000- and 3000−5000-fold higher than for sakacin P, while the MIC values for the W18F and W41F mutants were, respectively, 20−70- and about 200-fold higher (Table 1). Interestingly, the aromatic and hydrophobic Phe and even the aromatic and weakly hydrophilic Tyr were more favorable for bacteriocin activity than the aliphatic hydrophobic Leu, especially at position 41 (Table 1). This indicates that bacteriocin activity in part depends on the aromaticity of the residues at positions 18 and 41, and not just on hydrophobicity.

Table 1: Activity of Bacteriocin Variants toward Various Indicator Strains[a]

bacteriocin variant	MIC (nM)[b]			
	L. sake	*L. coryneformis*	*C. piscicola*	*E. faecalis*
sakacin P	0.2	0.5	0.2	0.3
Trp18 sakacin P mutants				
[W18L]	70	110	280	250
[W18F]	10	15	5	20
[W18Y]	20	50	30	60
[W18R]	230	200	230	230
[W18H]	800	7000	2500	3000
Trp33 sakacin P mutants				
[W33L]	0.4	1.3	0.3	0.7
[W33F]	0.4	2	0.8	0.9
[W33Y]	1.5	8	5	4
[W33R]	200	400	100	250
Trp41 sakacin P mutants				
[W41L]	600	>2000	750	900
[W41F]	50	70	50	60
[W41Y]	25	100	75	40
[W41N]	700	>1300	930	1000
[W41R]	300	>2000	1100	>2000
Trp + S−S bridge sakacin P mutants				
[W18L+N24C+44C][c]	0.6	1.2	0.5	0.8
[N24C+W33L+44C][c]	0.2	0.5	0.2	0.3
[N24C+W41L+44C]	26	180	3.2	6.3
[N24C+W41N+44C]	7.3	50	15	16
pediocin PA-1	0.2	0.1	0.5	0.2
Trp pediocin PA-1 mutants				
[C24S+C44S][d]	40	47	62	65
[C24S+N41W+C44S][d]	45	21	70	32

[a] Bacteriocin activity was measured as described under Materials and Methods. The indicator strains used in the bacteriocin assay were *L. sake* NCDO 2714 (type strain), *L. coryneformis* subsp. *torquens* NCDO 2740, *C. piscicola* UI49, and *E. faecalis* NCDO 581. [b] The minimal inhibition concentration (MIC) is the concentration of bacteriocin that inhibited growth of the indicator strain by 50%. The values are the results of at least 3 independent measurements, and standard deviations were less than 50% of the value. [c] These variants also contained the mutation G23T; it has previously been shown that this mutation has only marginal effects on bacteriocin activity (*28*). [d] To avoid artifacts due to methionine oxidation, these variants also contained the mutation M31L; it has previously been shown that this mutation has only marginal effects on bacteriocin activity (*33*).

The W41Y and W41F mutants were equally active, whereas the W18F mutant was 3−6 times more active than W18Y. It thus appears that hydrophobicity is somewhat more important at position 18 than at position 41. This is consistent with the observation that the W18L mutant is more potent than the W41L mutant (Table 1).

The results described so far indicated that hydrophobicity is important at position 33, while aromaticity is relatively more important at positions 18 and 41. This led us to hypothesize that Trp33 inserts into the hydrophobic core of the target membrane, whereas Trp18 and -41 interact with the membrane−water interface. We then noted that pediocin-like bacteriocins belonging to group 1 that contain the extra 24−44 disulfide bridge lack Trp at position 41, which again led to the hypothesis that Trp41 stabilizes the same kind of "hairpin" structure as the 24−44 disulfide bond (*28*; illustrated in Figure 3). This hypothesis was tested by introducing the 24−44 disulfide bond in sakacin P mutants lacking one of the Trp residues. Indeed, introduction of the 24−44 disulfide bridge in sakacin P mutants W18L, W41L, and W41N increased bacteriocin activity quite considerably, thus compensating in part for the activity drop caused by mutation of Trp18 and -41. The W18L+N24C+44C mutant was nearly as potent as sakacin P and more than 100-fold more potent than the W18L mutant (Table 1). Similarly, the N24C+W41L+44C and N24C+W41N+44C mutants were about 100-fold more potent than the W41L and W41N mutants, respectively (Table 1). As a control, the 24−44 disulfide bridge was also introduced into the (quite active) W33L mutant. This resulted in a small increase in bacteriocin activity, making the N24C+W33L+44C mutant as active as wild-type sakacin P (Table 1).

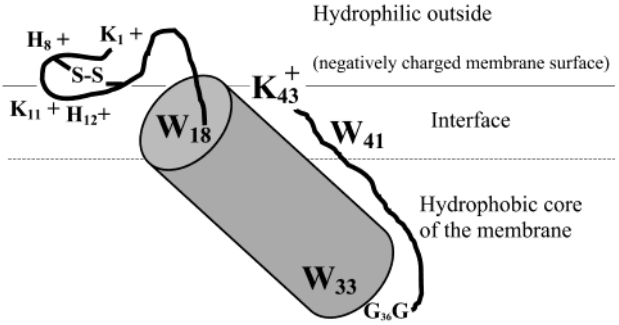

FIGURE 3: Putative model for the interaction of sakacin P with the target cell membrane. See text for details. Note that the extra disulfide bridge in pediocin PA-1 involves residue 24 and an extra C-terminal residue at position 44.

Tryptophan in position 41 along with Trp18 thus clearly help to stabilize the hairpin-like structure in group 1 bacteriocins that lack a C-terminal bridge. "Reciprocal" mutations in pediocin PA-1 showed, however, that insertion of a tryptophan in position 41 in pediocin PA-1 could not compensate for the removal of its C-terminal disulfide bridge (pediocin mutants C24S+C44S and C24S+N41W+C44S in Table 1). Thus, there are more activity-determining differences between the C-terminal parts of sakacin P and pediocin PA-1 than just the presence of Trp41 in the former and the presence of the C-terminal disulfide bridge in the latter. This is in line with previous observations indicating that the

C-terminal part of pediocin PA-1 is less well adapted to the lack of the 24−44 disulfide bridge than the C-terminal part of sakacin P (*28*).

DISCUSSION

Although the pediocin-like bacteriocins overall have very similar sequences, there is enough diversity in their C-terminal halves to enable subclassification of these peptides into at least 3 groups (Figure 1). Because of the sequence differences, one cannot exclude that bacteriocins belonging to different groups differ in terms of three-dimensional structures of their C-terminal halves. On the basis of available NMR data (*25*, *26*; H. H. Hauge, personal communication) and the observation that an activity-enhancing 24−44 disulfide bridge is formed in sakacin P variants and pediocin PA-1 (*28*), it is apparent that the C-terminal regions of at least some pediocin-like bacteriocins adopt the hairpin-like structure shown in Figure 3. To achieve this structure, the mobile C-terminal tail must fold back onto the central helical region. The present results indicate that there are at least two structural elements that group 1 pediocin-like bacteriocins may use to position their C-terminal tail: a disulfide bond between residues 24 and 44 or a tryptophan residue at position 41 (possibly in combination with Trp18). The fact that the deleterious effects of the Trp18 and Trp41 to leucine mutations in sakacin P were to a large extent overcome by introducing a disulfide bond between residues 24 and 44 shows that Trp41 (together with Trp18) is involved in the stabilization of the hairpin-like structure, and it explains why the six group 1 bacteriocins (sakacin P, listeriocin 743A, mundticin, piscicolin 126, sakacin 5x, and leucocin C; Figure 1) that do not contain a C-terminal disulfide bridge have a tryptophan residue in position 41.

Trp41 may stabilize the hairpin-like structure by positioning itself together with Trp18 in the interface between the hydrophobic core of the membrane and the aqueous phase, as illustrated in Figure 3. The fact that aromaticity at positions 18 and 41 was much more important than hydrophobicity clearly indicates that these two residues are positioned in the interface. It is conceivable that the two residues are located in each other's vicinity, which could give rise to an additional interaction for stabilizing the active bacteriocin structure. It has been suggested on the basis of computer modeling that the central α-helical region has an oblique orientation at the hydrophobic−hydrophilic membrane interface at an angle of 30−55°, depending on the bacteriocin (*11*). As illustrated in Figure 3, such an angle would allow positioning of both Trp18 and Trp41 in the interface. An oblique orientation (in contrast to a perpendicular orientation) of the peptide might possibly by itself interfere sufficiently with the proper orientation and close packing of membrane hydrocarbon chains to cause membrane permebilization.

Trp33 could successfully be replaced by an aliphatic and hydrophobic leucine residue. This is consistent with the fact that the residue at position 33 varies naturally among group 1 pediocin-like bacteriocins (Leu, Phe, Gly; Figure 1). It has been shown that the F33S mutant of carnobacteriocin B2 is inactive (*32*), and that mutants of pediocin PA-1 in which hydrophilic residues were introduced near position 33 (positions 26, 31, 34, and 36) had greatly reduced activity (*31*, *33*). Together, these results suggest that Trp33 is located in the hydrophobic core of the membrane (Figure 3). Interestingly, using fluorescence, Chen et al. (*34*) have shown that Trp18 and Trp33 in pediocin PA-1 interact with the membrane, and that one of these residues penetrates more deeply into the lipid bilayer than the other. Our results concerning Trp18 and Trp33 in sakacin P are consistent with the observations made by Chen et al. (*34*), and indicate that Trp33 is the residue that most deeply penetrates the lipid bilayer.

The hairpin structural model (Figure 3) may apply to all pediocin-like bacteriocins that belong to group 1, as they all have either a C-terminal disulfide bridge or a tryptophan residue in position 41 that—together with Trp18—may position the bacteriocins correctly in the membrane and stabilize the hairpin structure. The last or next to the last residue is positively charged in all these bacteriocins and may further stabilize the structure by interacting with negatively charged lipid phosphate groups close to the aqueous phase (*39*). Indeed, it has been shown that replacement of Lys43 in sakacin P by threonine reduces bacteriocin activity 4-fold (*35*).

It is known that pediocin-like bacteriocins bind to micelles and to simple membranes (e.g., DOPG liposomes), while becoming partly structured (*14*, *16*, *25*−*27*, *34*). Binding seems to be mediated by electrostatic interactions involving the N-terminal half of the bacteriocin (*34*, *35*) and not by specific interactions with a receptor (*1*, *2*, *4*). Binding to lipid II has been shown for the two lantibiotics nisin and epidermin, but could not be demonstrated for the lantibiotics pep5 and epilancin 7 (*48*, *49*, *50*). The binding to lipid II thus appears to be specific for nisin and epidermin, and it is apparently due to a homologous "ring structure" region shared by nisin and epidermin (*50*). Theoretically it is possible that the mutational effects observed here are not due to changes in the positioning and structural stability of the membrane-bound bacteriocin but to changes that directly affect a (stereospecific) interaction between the structured bacteriocin and a receptor. However, our results indicate that this explanation is not very likely. It is difficult to conceive that the compensation of W18L or W41L by the 24−44 disulfide bridge is due to the fact that the disulfide bridge has similar interactions with a receptor molecule as Trp18 or Trp41. It is much more likely that the compensatory effect is due to the fact that the disulfide bridge and Trp18 or Trp41 make similar contributions to the structure of the membrane-bound bacteriocin.

Whether or not the bacteriocins classified as group 2 and 3 adopt similar structures as depicted in Figure 3 remains to be seen. Bacteriocins in group 3 (Figure 1) lack both a C-terminal disulfide bridge and (except for bacteriocin 31) a tryptophan residue near the C-terminal end. The C-terminal parts of group 2 bacteriocins differ from those of group 1 bacteriocins in that they are clearly shorter. Interestingly, though, the C-terminal sequences in groups 1 and 2 display some remarkable similarities. Analogous to group 1 bacteriocins, the C-terminal parts of group 2 bacteriocins either contain a second disulfide bridge (between residues 24 and 37 in plantaricin 423 and, putatively, plantaricin C19) or contain a C-terminal tryptophan residue (Trp37 in leucocin A, mesentericin Y105, and lactococcin MMFII). It has previously been shown that Trp37 is essential for mesentericin Y105 activity (*16*). These observations suggest that

group 2 bacteriocins adopt a similar hairpin-like structure as group 1 bacteriocins (Figure 3). The central α-helix in the group 2 bacteriocin leucocin A starts with Asn17 and ends with Asn31 (*25*) and is followed by two glycine residues (at positions 32 and 33) that may render enough structural flexibility to allow the C-terminal tail to fold back. Interestingly, this double glycine motif is also present in the C-terminal tail of all group 1 bacteriocins (at positions 36 and 37; Figure 1). If indeed both subclasses adopt a similar structure and if our speculation about the role of the double glycine motif is correct, the main structural difference between group 1 and group 2 pediocin-like bacteriocins lies in the length of the α-helical part.

In conclusion, the present study shows that all tryptophan residues in sakacin P are important for bacteriocin activity but that their contribution varies in terms of magnitude and character. Clearly, Trp41 is the most crucial, presumably because it contributes to stabilizing an important structural feature, namely, the folding back of the C-terminal tail onto the central helical part. The large effect of any mutation at position 41 shows that tryptophan can play a unique role in an anti-microbial peptide, presumably because of this residue's special ability to interact favorably with the interfacial region in the membrane.

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