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A C-Terminal Disulfide Bridge in Pediocin-Like Bacteriocins Renders Bacteriocin Activity Less Temperature Dependent and Is a Major Determinant of the Antimicrobial Spectrum

GUNNAR FIMLAND,[1]* LINE JOHNSEN,[1] LARS AXELSSON,[2] MAY B. BRURBERG,[3,4] INGOLF F. NES,[4] VINCENT G. H. EIJSINK,[4] AND JON NISSEN-MEYER[1]

Department of Biochemistry, University of Oslo, Oslo,[1] and MATFORSK, Norwegian Food Research Institute,[2] Norwegian Crop Research Institute,[3] and Department of Chemistry and Biotechnology, Agricultural University of Norway,[4] Ås, Norway

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Several lactic acid bacteria produce so-called pediocin-like bacteriocins that share sequence characteristics, but differ in activity and target cell specificity. The significance of a C-terminal disulfide bridge present in only a few of these bacteriocins was studied by site-directed mutagenesis of pediocin PA-1 (which naturally contains the bridge) and sakacin P (which lacks the bridge). Introduction of the C-terminal bridge into sakacin P broadened the target cell specificity of this bacteriocin, as illustrated by the fact that the mutants were 10 to 20 times more potent than the wild-type toward certain indicator strains, whereas the potency toward other indicator strains remained essentially unchanged. Like pediocin PA-1, disulfide-containing sakacin P mutants had the same potency at 20 and 37°C, whereas wild-type sakacin P was approximately 10 times less potent at 37°C than at 20°C. Reciprocal effects on target cell specificity and the temperature dependence of potency were observed upon studying the effect of removing the C-terminal disulfide bridge from pediocin PA-1 by Cys→Ser mutations. These results clearly show that a C-terminal disulfide bridge in pediocin-like bacteriocins contributes to widening of the antimicrobial spectrum as well as to higher potency at elevated temperatures. Interestingly, the differences between sakacin P and pediocin PA-1 in terms of the temperature dependency of their activities correlated well with the optimal temperatures for bacteriocin production and growth of the bacteriocin-producing strain.

Many bacteria are known to produce ribosomally synthesized antimicrobial polypeptides called bacteriocins. Bacteriocins produced by gram-positive bacteria are usually membrane-permeabilizing cationic peptides with less than 50 amino acid residues (27, 29). These bacteriocins may be divided into two classes. Class I bacteriocins, termed lantibiotics, contain modified residues, whereas class II bacteriocins do not. One group of class II bacteriocins (frequently called class IIa) consists of the so-called "pediocin-like bacteriocins," produced by a variety of lactic acid bacteria. These bacteriocins are characterized by high antilisterial activity, by the presence of a YGNGV motif and a disulfide bridge in their N-terminal halves, and by the fact that they apparently kill cells by permeabilizing the target cell membrane (9, 10). The first pediocin-like bacteriocins that were identified and thoroughly characterized were pediocin PA-1 (7, 20, 24, 28), leucocin A-UAL 187 (16), mesentericin Y105 (19), and sakacin P and curvacin A (21, 33). Today, at least nine other pediocin-like bacteriocins have been isolated and characterized (4–6, 11, 22, 23, 25, 31, 32, 34). Despite similarities in their primary structures, the pediocin-like bacteriocins have different target cell specificities (12).

Based on their primary structures, pediocin-like bacteriocins may roughly be divided into two regions: a hydrophilic, cationic, and highly conserved N-terminal half and a less-conserved hydrophobic and/or amphiphilic C-terminal half (13). It has been proposed that the well-conserved cationic N-terminal half mediates the initial binding of these bacteriocins to target cells through electrostatic interactions (8) and that the hydrophobic or amphiphilic C-terminal half penetrates into the hydrophobic part of the target cell membrane, thereby mediating membrane leakage (13, 26). The hydrophobic or amphiphilic C-terminal half also appears (in part) to mediate target cell specificity, since hybrid bacteriocins containing N- and C-terminal regions from different pediocin-like bacteriocins have antimicrobial spectra similar to that of the bacteriocin from which the C-terminal region is derived (13).

In addition to the conserved disulfide bridge in the N-terminal half, a few pediocin-like bacteriocins contain a second disulfide bridge, located in the C-terminal half (Fig. 1). Comparative studies of natural bacteriocins have led to the suggestion that this second disulfide bridge is an important determinant of bacteriocin activity (12). Here, we present the results of site-directed mutagenesis studies of pediocin PA-1 (two disulfide bridges) and sakacin P (one disulfide bridge), aimed at analyzing the contribution of the second disulfide bridge to the potency, target cell specificity, and temperature dependency of activity.

Production and purification of sakacin P, pediocin PA-1, and their mutants. Figure 1 gives an overview of the mutants that were made in this study. In pediocin PA-1, the C-terminal disulfide bridge was removed by replacing cysteine residues in the C-terminal half with serine (ped[C24S+C44S]; Fig. 1). A C-terminal disulfide bridge was introduced in sakacin P, by introducing cysteine residues at the positions indicated by alignment with pediocin PA-1. Thus, one cysteine residue was added to the C terminus (position 44), whereas another cysteine was introduced to replace an asparagine at position 24 (sak[N24C+44C]; Fig. 1). Since there is a conspicuous sequence difference between sakacin P and pediocin PA-1 at the

* Corresponding author. Mailing address: Department of Biochemistry, University of Oslo, Post Box 1041, Blindern, 0316 Oslo, Norway. Phone: 47-22 85 66 32. Fax: 47-22 85 44 43. E-mail: gunnar.fimland@biokjemi.uio.no.

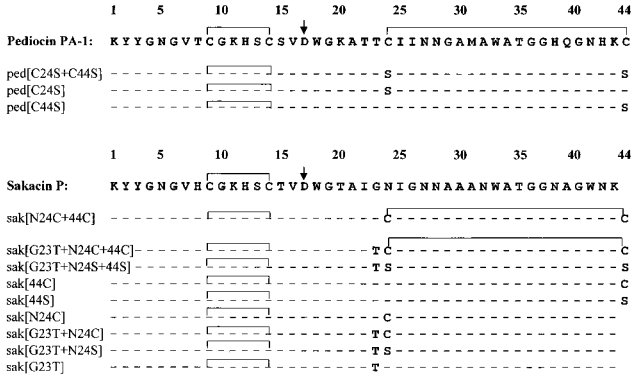

FIG. 1. An overview of bacteriocin mutants. Disulfide bridges are indicated. Assignment of the disulfide bridges is based on studies by Henderson et al. (20) and on results presented in this study. The arrows indicate the unique aspartate residue in sakacin P and pediocin PA-1, which was used for studies with endoproteinase Asp-N.

position preceding residue 24 (Gly23 and Thr23, respectively), a second disulfide variant of sakacin P was constructed in which Gly23 was changed into Thr (sak[G23T+N24C+44C]; Fig. 1). In addition, a series of control mutants was made, as shown in Fig. 1 (ped[C24S], ped[C44S], sak[G23T+N24S+44S], sak[44C], sak[44S], sak[N24C], sak[G23T+N24C], sak[G23T+N24S], and sak[G23T]).

With the exception of wild-type pediocin PA-1, which was purified from its natural producer (*Pediococcus acidilactici* LMG2351 [28]), all bacteriocins were produced by using a recently developed system for bacteriocin expression in the bacteriocin-deficient strain *Lactobacillus sake* Lb790 (3). The system is based on the use of two plasmids: pSAK20 and pSPP2 (for production of sakacin P and variants) or pPED2 (for production of pediocin PA-1 variants). Mutations in the sakacin P and pediocin PA-1 genes, cloned in pSPP2 and pPED2, respectively, were made by using the Quick Change site-directed mutagenesis kit (Stratagene). The DNA sequence of the mutant plasmids was verified by automated DNA sequence determination with an ABI PRISM 377 DNA sequencer and the ABI Prism dye terminator cycle sequencing Ready Reaction kit (Perkin-Elmer). *Epicurian Coli* XL1-Blue Supercompetent cells (Stratagene; grown at 37°C in Luria-Bertani medium [Difco] with vigorous agitation) were used for the cloning of all mutated pSPP2 and pPED2 plasmids. pSPP2 and pPED2 derivatives containing the desired mutations were transformed into *L. sake* Lb790/pSAK20 by electroporation as described previously (1). All plasmid isolations from *Epicurian Coli* and *L. sake* Lb790 were done by using the Wizard Plus SV Minipreps DNA purification system (Promega). *L. sake* cells were treated with lysozyme and mutanolysin (5 mg/ml and 15 U/ml, respectively) before lysis. The selective antibiotic concentrations used were 150 μg of erythromycin per ml for *E. coli*, 10 μg (each) of erythromycin and chloramphenicol per ml for normal growth of plasmid-containing *L. sake* Lb790, and 2 μg of erythromycin per ml and 5 μg of chloramphenicol per ml for initial selection of *L. sake* Lb790/pSAK20 transformed with pSPP2 or pPED2 variants.

Wild-type and mutant bacteriocins were purified to homogeneity from 400- or 800-ml cultures by ammonium sulfate precipitation followed by cation-exchange, hydrophobic interaction, and reverse-phase chromatography as described previously (30). Routinely, between 10 and 100 μg of sakacin P, pediocin PA-1, and mutant bacteriocins was purified from 400-ml cultures. To check that the recombinant lactobacilli had correctly produced and processed the bacteriocins, molecular masses of the isolated peptides were determined by mass spectrometry with a matrix-assisted laser desorption ionization–time of flight Voyager-DE RP mass spectrometer (Perseptive Biosystems). The purity of bacteriocins was verified to be greater than 90% by analytical reverse-phase chromatography with a μRPC SC 2.1/10 C_2/C_{18} column (Pharmacia Biotech) in the SMART chromatography system (Pharmacia Biotech). The concentration of purified bacteriocins was determined by measuring UV A_{280}, which was converted to protein concentration with molecular extinction coefficients calculated from the contributions of individual amino acid residues.

In the last reverse-phase chromatography step, sakacin P, pediocin PA-1, and mutants that did not contain cysteine residues in their C-terminal region (the sakacin P mutants sak[G23T], sak[G23T+N24S], sak[44S], and sak[G23T+N24S+44S] and the pediocin PA-1 mutant ped[C24S+C44S]; Fig. 1) gave one major, almost symmetrical absorbance peak that contained a peptide with bacteriocin activity and the expected molecular weight (determined by mass spectrometry). The mutants that contained only one cysteine residue in the C-terminal region (the sakacin P mutants sak[N24C], sak[44C], and sak[G23T+N24C] and the pediocin PA-1 mutants ped[C24S] and ped[C44S]; Fig. 1) gave more complex absorbance profiles containing several—often asymmetrical—absorbance peaks, presumably because of stability problems and incorrect formation of disulfide bridges (including intermolecular disulfide bridges). For each of these mutants, the fraction with the most bacteriocin activity was collected. Mass spectrometry confirmed that these fractions contained the expected mutant bacteriocins.

For sakacin P mutants containing two new cysteine residues (sak[N24C+44C] and sak[G23T+N24C+44C]; Fig. 1) the final reverse-phase chromatography step yielded three peaks of approximately equal size, each containing a peptide with the expected molecular mass. The contents of these three fractions were further studied by mass spectrometry analyses of the fragments obtained after treatment with endoproteinase Asp-N. This protease cleaves sakacin P in front of its unique Asp residue at position 17 that is located between the two N-terminal and the two C-terminal cysteine residues (Fig. 1). The results (not shown) revealed that the last peptide to elute from the column had correct disulfide bridges (that is bridges between 9 and 14 and between 24 and 44, analogous to bridge formation found in natural pediocin PA-1 [20]). The specific activity of this peptide was at least 100 times greater than that of the two other peptides. Interestingly, the activity of the two lesser active peptides increased greatly and became similar to that of the initially most active peptide, upon exposure to small amounts of dithiothreitol (DTT) (2 to 3 mM) during the bacteriocin assay. Thus, the two variants that appeared to display incorrect disulfide bridges could be transformed into active bacteriocins under conditions that promoted both structuring (i.e., the presence of target cell membranes [15, 17, 18]) and disulfide exchange (the presence of low concentrations of DTT). The activity of all three peptides was reduced at higher DTT concentrations (above 5 mM).

Production of pediocin PA-1 with the expression system also yielded peptides with incorrect disulfide bridges. In contrast, production of pediocin PA-1 by its wild-type producer and production of bacteriocin variants with only two cysteine residues (e.g., wild-type sakacin P and ped[C24S+C44S]) with the expression system were unproblematic. Taken together, the results indicate that a protein present in the natural producer, but not in *L. sake* Lb790/pSAK20/pPED2, helps to generate

the correct disulfide bridges in bacteriocins containing four cysteines. The secretion machinery used in our heterologous expression system is derived from a strain that produces sakacin A, a pediocin-like bacteriocin with only one disulfide bridge (2). It is thus tempting to speculate that the secretion machinery present in the natural pediocin PA-1 producer is to some extent adapted to generate the correct disulfide bridges in four cysteine-containing pediocin-like bacteriocins.

Mutational effects. Six indicator strains were used for testing the various bacteriocins. When assayed at 20 and 30°C, pediocin PA-1 and sakacin P had similar potencies toward four of these strains (*L. coryneformis* subsp. *torquens* NCDO 2740, *L. sake* NCDO 2714, *Enterococcus faecalis* NCDO 581, and *Carnobacterium piscicola* UI49; Table 1). Two of the strains, *P. acidilactici* NCDO 1859 and *Pediococcus pentosaceus* FBB63B, were at least 100 times more sensitive to pediocin PA-1 than to sakacin P (Table 1), and these two strains were therefore useful for detecting mutations which make sakacin P more like pediocin PA-1. Pediocin PA-1 and sakacin P also differed in the temperature dependency of their activity. Pediocin PA-1 had nearly the same potency at 20, 30, and 37°C, whereas sakacin P was about 10 times more potent at 20 and 30°C than at 37°C (Table 1 and Fig. 2). Thus, measurement of the temperature dependency of the activity of mutant bacteriocins provided an additional way of evaluating whether a mutation had made the one bacteriocin more like the other.

The pediocin PA-1 mutant, ped[C24S+C44S], which lacks the C-terminal disulfide bridge, had lost much of its potency against the two *Pediococcus* strains that were sensitive to wild-type pediocin PA-1, but relatively insensitive to sakacin P (Table 1). Its activity toward the four other strains was also reduced, but not nearly to the same extent, especially not at lower temperatures. In contrast to wild-type pediocin PA-1, ped[C24S+C44S] was much less active at 37°C than at 20°C (Table 1 and Fig. 2). Thus, the removal of the C-terminal disulfide bridge in pediocin PA-1 rendered pediocin PA-1 more sakacin-like with respect to both the target cell specificity and the temperature dependence of activity.

The complementary mutation in sakacin P, the introduction of a C-terminal disulfide bridge, clearly rendered sakacin P more similar to pediocin PA-1. When tested at 20 and 30°C, sak[N24C+44C] and sak[G23T+N24C+44C] had approximately the same potency as sakacin P against the four indicator strains that were sensitive to both sakacin P and pediocin PA-1. However, these two mutants had become more potent toward the two *Pediococcus* strains that were relatively resistant to sakacin P, but sensitive to pediocin PA-1 (Table 1). Moreover, in contrast to wild-type sakacin P, these two mutants had about the same potency at 37°C as at 20°C for four of the five strains tested (Table 1). Thus, also in terms of the temperature dependency of activity, the introduction of the extra disulfide bridge had made sakacin P more similar to pediocin PA-1. Differences in activity between sak[N24C+44C] and sak[G23T+N24C+44C] were small, the latter being slightly more active toward most of the strains and at most of the temperatures tested. The control mutants, sak[G23T+N24S+44S] and sak[G23T+N24S], in which serine instead of cysteine residues were introduced into sakacin P, displayed similar potency, target cell specificity, and temperature dependency of activity to wild-type sakacin P (Table 1 and Fig. 2). Also the mutants sak[44S] and sak[G23T] had similar potencies to wild-type sakacin P (Table 1).

Mutant bacteriocins with only one cysteine residue in the C-terminal half had reduced activities and were more unstable than those that had either none or two cysteine residues in this region. The sak[44C], sak[N24C], and sak[G23T+N24C] mu-

TABLE 1. Activity of bacteriocin variants toward various indicator strains at various temperatures[a]

Bacteriocin variant	MIC (nM) at incubation temp[b]:															
	L. coryneformis			*L. sake*			*E. faecalis*			*C. piscicola*			*P. pentosaceus*			*P. acidilactici*		
	20°C	30°C	37°C	20°C	30°C	37°C	20°C	30°C	37°C	20°C	30°C	37°C	20°C	30°C	37°C	20°C	30°C	37°C
Pediocin PA-1	0.1	0.1	0.2	0.3	0.2	0.3	0.2	0.2	0.2	0.7	0.7	0.1	0.1	0.2	3	2	10	
ped[C24S+C44S]	0.4	2	30	3	15	250	3	7	100	3	10	30	100	>500	1,000	>2,000	>2,000	
Sakacin P	0.2	0.4	7	0.1	0.3	2	0.2	0.2	2	0.1	0.2	6	40	≥100	>400	>400	>400	
sak[N24C+44C]	2	2	3	0.2	0.2	0.2	0.3	0.2	0.3	0.2	0.2	2	2	7	30	30	>300	
sak[G23T+N24C+44C]	0.7	0.7	0.7	0.1	0.1	0.1	0.3	0.1	0.2	0.2	0.1	0.5	1	5	7	10	90	
sak[G23T+N24S+44S]	0.2	0.3	4	0.2	0.5	8	0.1	0.2	1	0.1	0.2	10	60	>250	600	≥1,000	≥1,000	
sak[G23T+N24S]	0.2	0.7	10	0.5	1	30	0.2	0.2	2	0.1	0.2	30	120	1,000	≥1,000	≥1,000	≥1,000	
sak[44S]	NT[c]	0.3	NT	NT	0.1	NT	NT	0.3	NT	NT	0.1	NT	30	NT	NT	NT	NT	
sak[G23T]	NT	0.2	NT	NT	0.3	NT	NT	0.3	NT	NT	0.1	NT	20	NT	NT	>2,000	NT	

[a] Results obtained for mutants containing three cysteine residues in total are not included in the table, but are discussed in the text. Bacteriocin activity was measured with a microtiter plate assay system, essentially as described previously (28). The microtiter plate cultures were incubated overnight (12 to 16 h) at the indicated temperature, after which growth of the indicator strain was measured spectrophotometrically at 610 nm with a microtiter plate reader. The indicator strains used in the bacteriocin assays were *L. coryneformis* subsp. *torquens* NCDO 2740, *L. sake* NCDO 2714 (type strain), *Enterococcus faecalis* NCDO 581, *C. piscicola* UI49, *P. pentosaceus* FBB63B, and *P. acidilactici* NCDO 1859. *C. piscicola* UI49 was grown in M17 (Oxoid) supplemented with glucose and Tween 80 to final concentrations of 0.4% (wt/vol) and 0.1% (vol/vol), respectively. The other lactic acid bacteria were grown in MRS (Oxoid).

[b] Bacteriocin activities are presented as MICs. Here the MIC is the concentration that inhibited growth of the indicator strain by 50% under the conditions of the assay. The values were the result of at least three independent measurements, and standard deviations were less than 50% of the values.

[c] NT, not tested.

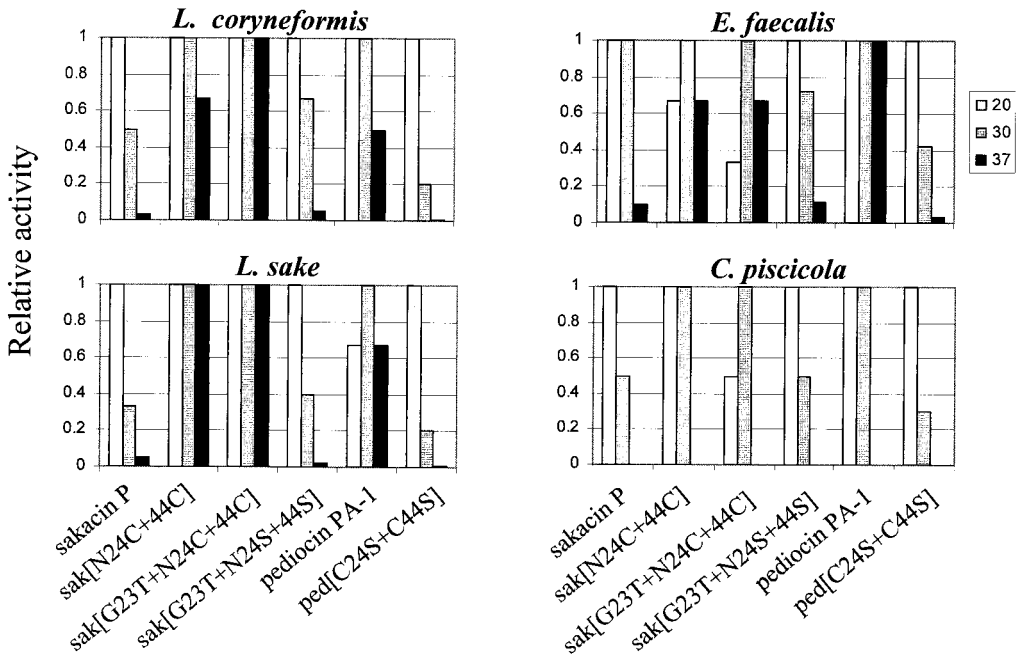

FIG. 2. Temperature sensitivity of bacteriocin activity. The bars represent relative bacteriocin activities at 20°C (open bars), 30°C (shaded bars), and 37°C (solid bars). For each combination of bacteriocin and indicator strain, the highest activity was set to 1.

tants were only about 1/10 as active as the sakacin P (results not shown). The two single pediocin PA-1 mutants, ped[C24S] and ped[C44S], were somewhat less active than the double mutant, ped[C24S+C44S] (results not shown). Miller et al. (26) previously reported that ped[C24S] and ped[C44S] are completely inactive. The difference between our observations and those of Miller et al. (26) may be caused by the fact that Miller et al. (26) conducted their assays with unpurified mutant bacteriocins in culture supernatants of overproducing *E. coli* strains. These conditions are clearly different from those used in the present study and may have promoted problems with stability and/or incorrect disulfide formation. It is our experience that the three-cysteine mutants (in contrast to the wild-type bacteriocins or mutants with two or four cysteines) lose activity after a few weeks of storage at 4°C in 0.1% trifluoroacetic acid and 20% 2-propanol and that it is not possible to recover the activity by exposing the mutants to small amounts (1 to 3 mM) of DTT. Concomitant with loss of activity, the molecular weight increased by 48 (determined by mass spectrometry), suggesting that the thiol group on cysteine was oxidized to sulfonic acid. A component with twice the expected molecular weight was also observed, suggesting some dimerization through the formation of intermolecular disulfide bridges.

Figure 3 shows that there exists a correlation between the temperature dependence of growth and bacteriocin production on the one hand and the temperature dependence of bacteriocin activity on the other hand. At temperatures above 30°C, the activity of sakacin P was reduced considerably (Table 1 and Fig. 2) and so were the growth of the bacteriocin-producing strain and production levels of the bacteriocin (Fig. 3). In contrast, pediocin PA-1 was fully active at 37°C (Table 1 and Fig. 2), a temperature at which both bacteriocin production and the growth of the producing strain were at their maximum (Fig. 3). The properties of the bacteriocins thus seem to be well adapted to the ecological niche that the producer strain is likely to grow in.

Nuclear magnetic resonance studies of the pediocin-like bacteriocins leucocin A-UAL 187 (15) and carnobacteriocin B2 (35) have shown that the middle part of the bacteriocins is likely to form an alpha-helix upon interaction with a target cell

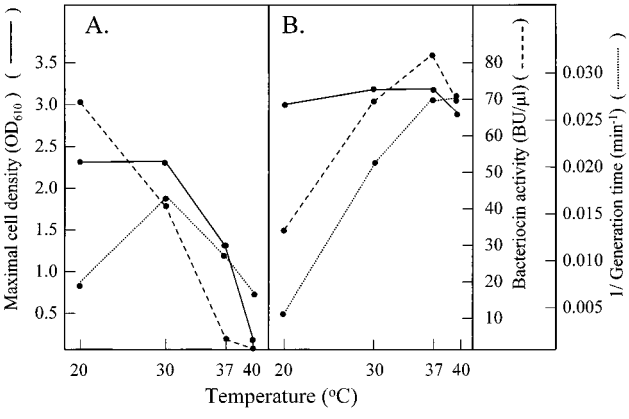

FIG. 3. Bacteriocin production and growth of natural producer strains. Growth of the wild-type sakacin P producer *L. sake* LTH673 (A) and of the wild-type pediocin PA-1 producer *P. acidilactici* (B) was monitored at various temperatures while simultaneously monitoring bacteriocin levels in the culture supernatants. Growth is represented by the generation times that could be calculated from the growth curves and by the maximal cell densities that were obtained. Bacteriocin concentrations are expressed in bacteriocin units (BU) per microliter and represent the highest levels observed while monitoring growth. One BU was defined as the amount of bacteriocin required to reduce the growth of the indicator strain by 50% in the bacteriocin assay described above. Bacteriocin activities were determined with standard bacteriocin assays (see reference 28 and Table 1). The assay temperature was 30°C, and *L. sake* NCDO 2714 was used as the indicator strain.

membrane. The remaining C-terminal residues were found to be relatively unstructured. If one extrapolates these observations to pediocin PA-1, the conclusion would be that the C-terminal residues must fold back onto the helical part, thus allowing formation of the 24–44 disulfide bridge. Introduction of the 24–44 disulfide bridge left the activity of sakacin P toward a number of indicator strains at 20 and 30°C largely unchanged. It is therefore not likely that formation of the 24–44 disulfide bridge in sakacin P required major structural rearrangements, indicating that pediocin PA-1 and sakacin P must have quite similar structures. Interestingly, sakacin P seems to be better adapted to a situation without the 24–44 disulfide bridge than pediocin PA-1 (compare ped[C24S+C44S] with sak[G23T+N24S+44S]; Table 1). Other structural characteristics of pediocin PA-1, which are adapted to the presence of the second disulfide bridge, apparently become unfavorable in a situation where the C-terminal disulfide bridge is not present.

Our results suggest that the connection between the helical part and the following stretch of C-terminal residues provided by the 24–44 disulfide bridge is of major importance for the target cell specificity of pediocin-like bacteriocins. The relative importance of the 24–44 bridge is further illustrated by the fact that control mutations that did affect the C-terminal part of sakacin P, but not disulfide formation (sak[44S], sak[G23T], sak[G23T+N24S], and sak[G23T+N24S+44S]), had only marginal effects on bacteriocin activity. The presence of the 24–44 disulfide bridge manifested itself in widening of the antimicrobial spectrum at lower temperatures and generally increased activity at higher temperatures.

This study is one of the first examples of a site-directed mutagenesis analysis aimed at unraveling the role of specific structural elements in bacteriocins produced by lactic acid bacteria. It has been suggested that the C-terminal half of pediocin-like bacteriocins is an important determinant of target cell specificity (13, 14), and our results pinpoint a specific C-terminal structural element that plays a major role in determining this specificity. It is important to note that the mutational effects observed in this study are truly strain-specific and that they do not reflect general increases or decreases in potency. This is especially apparent for sakacin P mutants tested at 20 or 30°C in that the effect of the introduction of the disulfide bridge on bacteriocin activity was marginal for four of the indicator strains, whereas activity toward two other strains (the two pediococci) was increased drastically. In addition to widening the antimicrobial spectrum, the C-terminal disulfide bridge clearly contributed to reducing the temperature sensitivity, and the two sakacin P variants that contain the C-terminal disulfide bridge (sak[N24C+44C] or sak[G23T+N24C+44C]) represent the first (semi-) rationally designed variants of pediocin-like bacteriocins with increased potencies.

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