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The Pediocin PA-1 Accessory Protein Ensures Correct Disulfide Bond Formation in the Antimicrobial Peptide Pediocin PA-1

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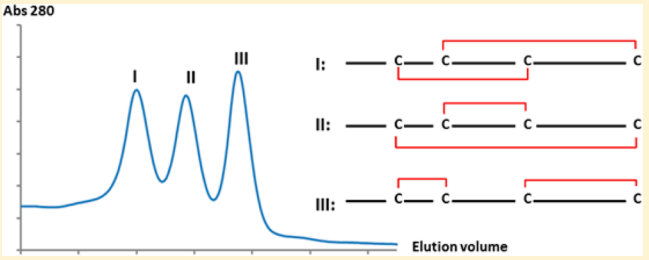
Supporting Information

ABSTRACT: Peptides, in contrast to proteins, are generally not large enough to form stable and well-defined three-dimensional structures. However, peptides are still able to form correct disulfide bonds. Using pediocin-like bacteriocins, we have examined how this may be achieved. Some pediocin-like bacteriocins, such as pediocin PA-1 and sakacin P[N24C+44C], have four cysteines. There are three possible ways by which the four cysteines may combine to form two disulfide bonds, and the three variants are expected to be produced in approximately equal amounts if their formation is random. Pediocin PA-1 and sakacin P[N24C+44C] with correct disulfide bonds were the main products when they were secreted by the pediocin PA-1 ABC transporter and accessory protein, but when they were secreted by the corresponding secretion machinery for sakacin A, a pediocin-like bacteriocin with one disulfide bond (two cysteines), peptides with all three possible disulfide bonds were produced in approximately equal amounts. All five cysteines in the pediocin PA-1 ABC transporter and the two cysteines (that form a CxxC motif) in the accessory protein were individually replaced with serines to examine their involvement in disulfide bond formation in pediocin PA-1. The Cys86Ser mutation in the accessory protein caused a 2-fold decrease in the amount of pediocin PA-1 with correct disulfide bonds, while the Cys83Ser mutation nearly abolished the production of pediocin PA-1 and resulted in the production of all three disufide bond variants in equal amounts. The Cys19Ser mutation in the ABC transporter completely abolished secretion of pediocin PA-1, suggesting that Cys19 is in the proteolytic active site and involved in cleaving the prebacteriocin. Replacing the other four cysteines in the ABC transporter with serines caused a slight reduction in the overall amount of secreted pediocin PA-1, but the relative amount with the correct disulfide bonds remained large. These results indicate that the pediocin PA-1 accessory protein has a chaperone-like activity in that it ensures the formation of the correct disulfide bond in pediocin PA-1.



When proteins fold, cysteine residues that form structure-stabilizing disulfide bonds become spatially positioned adjacent to each other and may combine correctly to form disulfide bonds. In contrast to proteins, peptides are generally not large enough to fold into well-defined stable three-dimensional structures, but peptides may nevertheless also correctly combine cysteine residues and form disulfide bonds. In this study, we have examined how this may be achieved, using pediocin-like (class IIa) bacteriocins that contain four cysteine residues.

The pediocin-like bacteriocins make up a group of closely related ribosomally synthesized antimicrobial peptides produced by lactic acid bacteria.[1] There has been much interest in these bacteriocins because of their potent anti-*Listeria* activity, and more than 20 different pediocin-like bacteriocins have now been identified and sequenced (Figure 1). They have a high degree of sequence similarity but differ somewhat with respect to the bacteria they kill.[1] They are synthesized as prepeptides with a leader sequence that is cleaved off during export by a dedicated ABC transporter.[1,2] The genes encoding this ABC transporter and its accessory protein, whose exact function is not known, are either in the same operon as the genes encoding the prebacteriocin and its immunity protein or in a separate operon near the bacteriocin operon.[2]

The pediocin-like bacteriocins function as membrane-interacting ligands that induce cell death upon specifically binding to a target cell membrane protein (i.e., the bacteriocin receptor), which has been shown to be the membrane-embedded IIC and IID subunits of the mannose phosphotransferase system.[3−5] Upon binding with the bacteriocin, the receptor presumably alters its conformation in a manner that leads to membrane leakage and cell death.[5] The immunity protein that protects the bacteriocin producer from being killed by its own bacteriocin senses this change in conformation and binds to the permease and thereby blocks membrane leakage.[5]

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* Enterocin A:          TTHSGKYYGNGVYCTKNKCTVDWAKATTCIAGMSIGGFL-GGAIPG-KC
* Divercin V41:             TKYYGNGVYCNSKKCWVDWGQASGCIGQTVVGGWL-GGAIPG-KC
* Divergicin M35:           TKYYGNGVYCNSKKCWVDWGTAQGCID--VVIGQLGGGIPGKGKC
* Coagulin:                  KYYGNGVTCGKHSCSVDWGKATTCIINNGAMAWATGGHQGTHKC
* Pediocin PA-1:             KYYGNGVTCGKASCSVDWGKATTCIINNGAMAWATGGHQGNHKC
  Sakacin P:                 KYYGNGVHCGKHSCTVDWGTAIGNIGNNAAANWATGGNAGWNK
` Sakacin P[N24C+44C]        KYYGNGVHCGKHSCTVDWGTAIGCIGNNAAANWATGGNAGWNKC
  Listeriocin 743A:          KSYGNGVHCNKKKCWVDWGSAISTIGNNSAANWATGGAAGWKS
  Mundticin:                 KYYGNGVSCNKKGCSVDWGKAIGIIGNNSAANLATGGAAGWSK
  Mundticin L:               KYYGNGLSCNKKGCSVDWGKAIGIIGNNSAANLATGGAAGWKS
  Mundticin KS:              KYYGNGVSCNKKGCSVDWGKAIGIIGNNSAANLATGGAAGWKS
  Piscicolin 126:            KYYGNGVSCNKNGCTVDWSKAIGIIGNNAAANLTTGGAAGWNKG
  Sakacin 5X:                KYYGNGLSCNKSGCSVDWSKAISIIGNNAVANLTTGGAAGWKS
  Leucocin C:                KNYGNGVHCTKKGCSVDWGYAWTNIANNSVMNGLTGGNAGWHN
  Avicin A:                  TYYGNGVSCNKKGCSVDWGKAISIIGNNSAANLATGGAAGWKS
  Leucocin A:                KYYGNGVHCTKSGCSVNWGEAFSAGVHRLANGGNGFW
  Mesentericin Y105:         KYYGNGVHCTKSGCSVNWGEAASAGIHRLANGGNGFW
  Leucocin B:                KYYGNGVHCTKSGCSVNWGEAFSAGVHRLANGGNGFW
  Lactococcin MMFII:         TSYGNGVHCNKSKCWIDVSELETYKAGTVSNPKDILW
* Sakacin G:                 KYYGNGVSCNSHGCSVNWGQAWTCGVNHLANGGHGVC
* Plantaricin 423:           KYYGNGVTCGKHSCSVNWGQAFSCSVSHLANFGHGKC
* Plantaricin C19:           KYYGNGLSCSKKGCTVNWGQAFSCGVNRVATAGHGKx
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Figure 1. Amino acid sequence alignment of some pediocin-like bacteriocins. All pediocin-like bacteriocins contain an YGNGV motif (marked in bold letters) and two conserved cysteine residues (gray background) in the N-terminal half. Some pediocin-like bacteriocins, marked with an asterisk, contain two additional cysteine residues (gray background). Most pediocin-like bacteriocins do not contain these two additional cysteine residues but instead contain a conserved tryptophan residue (black background) at the C-terminal end. Sakacin P[N24C+44C], marked with an apostrophe, is a mutant of sakacin P that contains two additional cysteine residues at positions 24 and 44.

The pediocin-like bacteriocins contain between 35 and 50 residues and are unstructured in water but become structured in membrane-mimicking environments.[6−8] Their receptor-mediated mode of action thus seems to involve so-called "membrane catalysis"[9,10] in that insertion into the target cell membrane induces the correct structure and orientation of the bacteriocins and thereby allows them to bind efficiently to their receptor. The three-dimensional structures of four pediocin-like bacteriocins in membrane-mimicking environments have been analyzed by nuclear magnetic resonance spectroscopy.[6−8,11] In all pediocin-like bacteriocins, the well-conserved N-terminal half appears to form a three-stranded antiparallel β-sheet-like structure that is stabilized by a disulfide bond (Figure 2). Some of the pediocin-like bacteriocins, such as pediocin PA-1, have an additional disulfide bond in the C-terminus that stabilizes a hairpinlike structure consisting of a central α-helix followed by a somewhat extended C-terminal tail that folds back on the helix (Figure 2). Other pediocin-like bacteriocins, such as sakacin P, lack this additional disulfide bond. In this case, the hairpinlike structure is stabilized by two tryptophan residues (one in the middle of the peptide and one near the C-terminal end) that are positioned in the membrane interface (Figure 2).

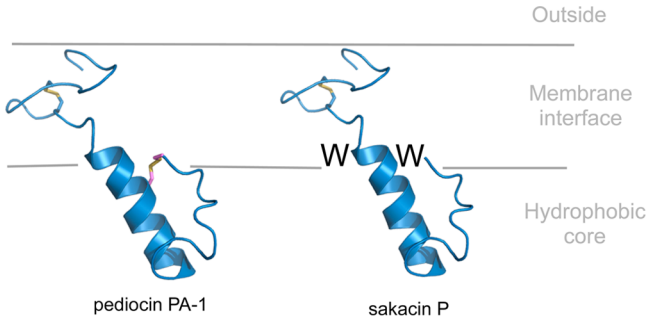

Figure 2. Three-dimensional structure and orientation in the target cell membrane of pediocin-like bacteriocins that form a hairpinlike structure in their C-terminal half. The hairpinlike structure is stabilized by either a second disulfide bond (colored pink and yellow, such as in pediocin PA-1) or the positioning of two tryptophan residues in the membrane interface (marked with W, such as in sakacin P).

There are three possible ways by which four cysteine residues may combine to form two disulfide bonds (Figure 3). One would expect that the three possible variants would be produced in approximately equal amounts if the formation of disulfide bonds was random in the pediocin-like bacteriocins, such as pediocin PA-1, that contain two disulfide bonds. Pediocin PA-1 is, however, produced almost exclusively with correctly formed disulfide bonds in the natural pediocin PA-1-producing bacteria. This study indicates that the pediocin PA-1 accessory protein has a chaperone-like activity in that it ensures that the correct disulfide bonds are formed in pediocin PA-1.

MATERIALS AND METHODS

Plasmids, Bacterial Strains, and Growth Conditions. All plasmids with their characteristics are summarized in Table 1. *Lactobacillus sakei* Lb790 containing pGF10, pMC117, or pMC117 with mutations in the accessory protein or ABC transporter genes was grown in MRS medium with 10 μg/mL erythromycin at 30 °C without being shaken. When the strain instead contained the two-plasmid system for the production of pediocin PA-1 (pSAK20/pPed2) or sakacin P[N24C+44C] [pSAK20/pSPP2(N24C+44C)], chloramphenicol and erythromycin were added to the growth medium, both at a final concentration of 10 μg/mL. *Pediococcus acidilactici* LMGT2351 (the natural pediocin PA-1 producer) and the indicator strain *Enterococcus faecalis* NCDO 581 were grown in MRS at 30 °C without being shaken. *Escherichia coli* DH5α was grown in LB medium at 37 °C while being shaken.

Site-Directed Mutagenesis and Transformation. Mutations in the genes encoding the ABC transporter and accessory protein of pediocin PA-1 were introduced using the pMC117 plasmid as a template and the QuikChange site-directed mutagenesis kit (Stratagene), according to the protocol. The

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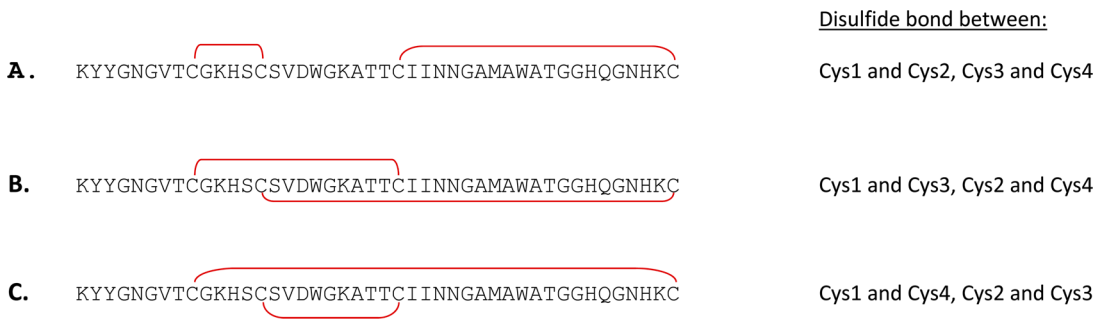

Figure 3. In peptides containing four cysteine residues, there are three possible ways these cysteine residues may combine to form two disulfide bonds. (A) Amino acid sequence of pediocin PA-1 with the correctly connected cysteine residues. (B and C) Two possible pediocin PA-1 peptides with incorrect disulfide bonds. The four cysteine residues in sakacin P[N24C+44C] may connect in the same three ways. The cysteine residues are numbered from the N-terminal end.

Table 1. Plasmids Used in This Study

plasmid	description	ref
pMC117	pMG36e-derived plasmid containing the pediocin PA-1 operon, *pedABCD* (encoding pediocin PA-1, immunity protein, accessory protein, and ABC transporter, respectively). Erythromycin resistance marker	32
pGF10	pMC117-derived plasmid. The genes encoding pediocin PA-1 and its immunity protein have been replaced with the sakacin P structural gene (with mutations N24C+44C) and the sakacin P immunity gene. The plasmid contains the genes encoding the pediocin PA-1 accessory protein and the pediocin PA-1 dedicated ABC transporter. Erythromycin resistance marker	6
pSAK20	pVS2-derived plasmid containing *orf4sapKRTE*, the genes necessary for activation, export, and processing of presakacin A. Chloramphenicol resistance marker	33
pPed2	pLPV111-derived plasmid containing the genes encoding pediocin PA-1 and its immunity protein. The pediocin-PA1 leader contains a K2N mutation. Erythromycin resistance marker	34
pSPP2(N24C +44C)	pLPV111-derived plasmid containing the genes encoding sakacin P (with mutations N24C+44C) and its immunity gene. Erythromycin resistance marker	14

PCR product was introduced into competent *E. coli* DH5α cells, and transformants were selected on LB plates containing 150 μg/mL erythromycin. Plasmids were isolated using the NucleoSpin Plasmid Kit (Macherey-Nagel), and the correct mutations were confirmed by DNA sequencing. Electrocompetent *L. sakei* Lb790 cells were prepared, and electroporation was performed according to the method described by Aukrust et al.[12]

Production and Purification of Bacteriocins. Pediocin PA-1 and sakacin P[N24C+44C] were produced using either the pediocin PA-1 or the sakacin A secretion machinery (ABC transporter and accessory protein). When *L. sakei* Lb790 contained plasmid pMC117 or pGF10, pediocin PA-1 or sakacin P[N24C+44C], respectively, was secreted by the pediocin PA-1 secretion machinery. When the peptides were secreted by the sakacin A secretion machinery, *L. sakei* Lb790 contained a two-plasmid expression system [pSAK20 and pPed2 for production of pediocin PA-1 or pSAK20 and pSPP2(N24C+44C) for production of sakacin P[N24C +44C]].

The two bacteriocins were purified from 0.5 L overnight cultures, as described previously.[13] Overnight cultures were applied on a SP Sepharose Fast Flow (GE Healthcare) cation-exchange column, equilibrated with buffer A [20 mM sodium phosphate buffer (pH 6)]. The column was then washed with buffer A before the peptides were eluted with 40 mL of buffer A containing 1 M NaCl. Trifluoroacetic acid (TFA) and 2-propanol were added to the eluent to final concentrations of 0.1% (v/v) and 5% (v/v), respectively. The eluent was then applied on a reverse phase column (Resource RPC 3 mL, GE Healthcare), and the bacteriocin was eluted using a linear 2-propanol gradient with 0.1% (v/v) TFA.

To estimate the heights of the absorbance peaks obtained after reverse phase chromatography, Unicorn software (version 5.31, GE Healthcare) was used. The software performed an automated baseline correction to remove any background before the height of each absorbance peak was calculated.

The peptide concentrations were determined by measuring the absorbance at 280 nm and using the calculated extinction coefficients (ε_{280}) for pediocin PA-1 and sakacin P[N24C +44C], which were 13940 and 19630 M^{-1} cm^{-1}, respectively.

Bacteriocin Activity Assay. The antimicrobial activity of the bacteriocin fractions was assayed in a similar manner as previously described.[13,14] Overnight cultures of the indicator strain were diluted 1:200, and 200 μL of the cell suspension together with the bacteriocin in 2-fold dilutions was added to each well in a 96-well microtiter plate. The plates were incubated overnight at 30 °C before the inhibition of growth was measured spectrophotometrically at 600 nm (Tecan plate reader). When specified, dithiothreitol (DTT) was added to the assays at a final concentration of 3 mM. The MIC (minimal inhibitory concentration) was defined as the amount of bacteriocin that inhibited growth by 50%.

In-Gel Protein Digestion. Coomassie-stained gel slices containing the three pediocin PA-1 fractions were washed and destained as previously described.[15] Digestion steps with trypsin (Sigma-Aldrich) at 37 °C overnight were performed as described previously,[15] except alkylation and reduction were not conducted. Dried samples were frozen at −80 °C and redissolved in 0.1% formic acid prior to liquid chromatographic tandem mass spectrometric (LC−MS^2) analyses.

Reverse Phase LC−MS^2 Analysis of Proteolytic Peptides. Nanoflow LC−MS and MS^2 analyses (nano-LC−MS^2) of proteolytic peptides were performed using an Agilent 1200 series capillary HPLC system with a corresponding autosampler, column heater, and integrated switching valve coupled via a nanoelectrospray ion source to an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen,

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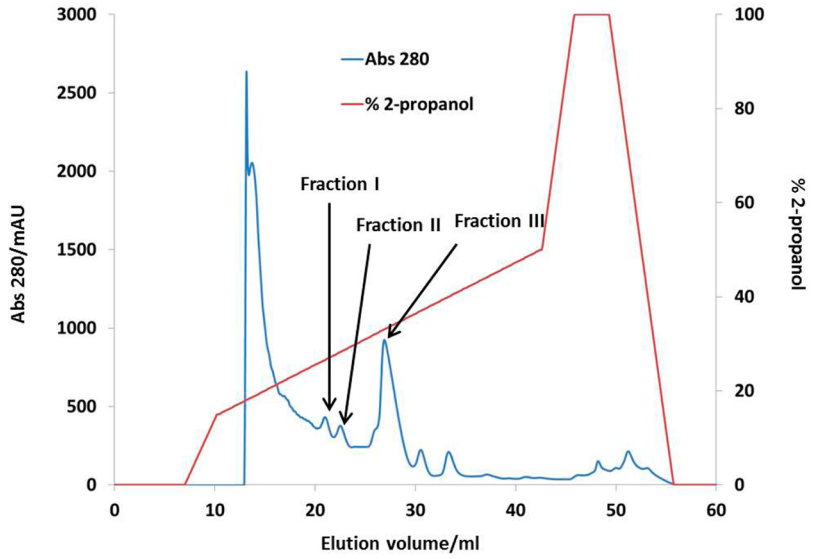

Figure 4. Reverse phase chromatogram obtained after purification of pediocin PA-1 from the natural producer *P. acidilactici* LMGT2351. Fractions I–III contained a peptide with the molecular weight of pediocin PA-1, but high antimicrobial activity was observed only in fraction III.

Germany). Five microliters of peptide sample was separated with a gradient from 5 to 55% acetonitrile with 0.1% formic acid over 60 min at a flow rate of 0.2 μL min^{-1}. The extraction column, C_{18} reverse phase column, and HPLC settings were as previously described.[16] LTQ-Orbitrap settings, including ionization, were as previously described.[16]

Analysis of MS Data. Mass spectrometric data were analyzed and manually verified using Xcalibur version 2.0.7 (Thermo Scientific), and deconvolution was performed with the built-in Xtract algorithm. Deconvoluted peptide masses were reported as monoprotonated [M + H$^+$].

RESULTS AND DISCUSSION

Pediocin PA-1 Produced by *P. acidilactici* LMGT2351. Purification of pediocin PA-1 from a natural pediocin PA-1 producer (*P. acidilactici* LMGT2351) resulted in one major absorbance peak after reverse phase chromatography, and the corresponding fraction [fraction III (Figure 4)] contained pediocin PA-1 as judged by mass spectrometry and exhibited high antimicrobial activity. Two smaller absorbance peaks that eluted at lower 2-propanol concentrations were also obtained (Figure 4), and mass spectrometry revealed that the two corresponding fractions (fractions I and II) also contained pediocin PA-1, although very little antimicrobial activity was detected in these two fractions. However, addition of 3 mM DTT in the antimicrobial activity assay resulted in a >2-fold increase in activity for fractions I and II, but a 16-fold decrease in activity for fraction III (Table 2). The increase in activity for the first two fractions upon addition of DTT indicates that these two fractions contained pediocin PA-1 with the wrongly connected disulfide bonds, as activity was gained under conditions that promote disulfide exchange (in the presence of DTT) and structuring of the peptide (in the presence of a target cell membrane). To further verify that all three fractions contained pediocin PA-1 but with different disulfide bonds, the three purified peptides were cleaved with trypsin and the resulting fragments were analyzed by mass spectrometry. Mass spectrometry revealed that the peptides in fractions I and II were in fact pediocin PA-1 with wrongly connected cysteine residues (see the Supporting Information for more details).

Table 2. Differences in Activity[a] of the Three Fractions When 3 mM DTT Was Added to the Assay

	fold difference[b] in activity upon addition of 3 mM DTT		
producer strain	fraction I	fraction II	fraction III
P. acidilactici LMGT2351	>+8	>+2	−16
Lb790/pMC117	>+2	>+2	−16
Lb790/pGF10	NF[c]	NF[c]	−8
Lb790/pSAK20/pPed2	>+2	+2	−16
Lb790/pSAK20/pSPP(N24C+44C)	+4	+4	−2
Lb790/pMC117-pedD[C19S]	NF[c]	NF[c]	NF[c]
Lb790/pMC117-pedD[C312S]	>+2	>+2	−16
Lb790/pMC117-pedD[C498S]	>+8	>+2	−16
Lb790/pMC117-pedD[C599S]	>+2	NC[d]	−16
Lb790/pMC117-pedD[C646S]	>+4	+2	−16
Lb790/pMC117-pedC[C83S]	+4	+4	−8
Lb790/pMC117-pedC[C86S]	>+2	>+2	−16

[a]Pediocin PA-1 with correct disulfide bonds has a MIC of 1.8 nM (standard deviation of three independent measurements of 0.7 nM), and sakacin P[N24C+44C] with correct disulfide bonds has a MIC of 0.4 nM (standard deviation of 0.1 nM) tested against the indicator strain *En. faecalis* NCDO 581 in the absence of DTT. [b]When DTT is added to the assay, a positive difference corresponds to an increased activity (i.e., lower MIC) whereas a negative difference corresponds to a decreased activity (i.e., higher MIC). The values are the average of three independent assays. [c]No fractions were obtained after reverse phase purification. [d]No change in activity was observed when DTT was added to the assay.

Fraction I contained pediocin PA-1 with disulfide bonds between Cys1 and Cys3 and between Cys2 and Cys4, while fraction II contained pediocin PA-1 with disulfide bonds between Cys1 and Cys4 and between Cys2 and Cys3 (Figure 3B,C; see also Figures S1 and S2 of the Supporting Information). Fraction III contained pediocin PA-1 with the correct disulfide bonds between Cys1 and Cys2 and between Cys3 and Cys4 (Figure 3A; see also Figures S1 and S2 of the Supporting Information). Analysis of pediocin PA-1 isolated from three independent overnight cultures of *P. acidilactici* LMGT2351 indicates that the natural producer of pediocin PA-

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Table 3. Average Heights of the Three Absorbance Peaks and the Corresponding Percents of Each Peak Obtained after Reverse Phase Purification of Pediocin PA-1 or Sakacin P[N24C+44C]

producer strain	mutation in PedC or PedD[a]	peptide produced	average height of absorbance peak I (mAU)[b]	average height of absorbance peak II (mAU)[b]	average height of absorbance peak III (mAU)[b]	% of each peak I	II	III
P. acidilactici LMGT2351	none	natural pediocin PA-1 producer	50 ± 30	40 ± 20	800 ± 100	6	4	90
Lb790/pMC117	none	pediocin PA-1	200 ± 20	180 ± 20	1100 ± 70	14	12	74
Lb790/pGF10	none	sakacin P [N24C +44C]	*c*	*c*	390 ± 10			100
Lb790/pSAK20/ pPed2	*d*	pediocin PA-1	86 ± 7	90 ± 10	49 ± 6	38	40	22
Lb790/pSAK20/ pSPP(N24C +44C)	*d*	sakacin P [N24C +44C]	74 ± 4	75 ± 3	90 ± 10	31	31	38
Lb790/pMC117-pedD[C19S]	PedD[C19S]	pediocin PA-1	*e*	*e*	*e*			
Lb790/pMC117-pedD[C312S]	PedD[C312S]	pediocin PA-1	70 ± 40	60 ± 40	500 ± 200	11	10	79
Lb790/pMC117-pedD[C498S]	PedD[C498S]	pediocin PA-1	96 ± 5	92 ± 6	690 ± 20	11	10	79
Lb790/pMC117-pedD[C599S]	PedD[C599S]	pediocin PA-1	30 ± 10	20 ± 10	250 ± 100	10	7	83
Lb790/pMC117-pedD[C646S]	PedD[C646S]	pediocin PA-1	120 ± 100	110 ± 100	700 ± 450	13	12	75
Lb790/pMC117-pedC[C83S]	PedC[C83S]	pediocin PA-1	5[f]	5[f]	5[f]	33	33	33
Lb790/pMC117-pedC[C86S]	PedC[C86S]	pediocin PA-1	200 ± 50	190 ± 30	600 ± 100	20	19	61

[a]PedC, pediocin PA-1 accessory protein; PedD, pediocin PA-1 ABC transporter. [b]The heights are given as the average heights of the absorbance peaks in three or four independent reverse phase purifications ± the standard deviation. [c]Only one absorbance peak was observed. [d]The recombinant strain contains the ABC transporter and accessory protein of sakacin A. [e]No absorbance peaks were obtained. [f]Absorbance peaks were observed from only one of four overnight cultures.

1 secretes ~90% pediocin PA-1 with correctly connected disulfide bonds and only 10% pediocin PA-1 with the incorrect disulfide bonds (Table 3). If the formation of disulfide bonds was random, one would expect equal amounts of the three different combinations. Clearly, there is a system in the natural producer that ensures that the correct disulfide bonds are formed.

The Pediocin PA-1 Secretion Machinery, but Not the Sakacin A Machinery, Secretes Pediocin PA-1 and Sakacin P[N24C+44C] with the Correctly Connected Cysteine Residues. When pediocin PA-1 was produced heterologously in *L. sakei* Lb790/pMC117 containing the pediocin PA-1 secretion machinery (ABC transporter and accessory protein), ~74% of the produced pediocin PA-1 contained the correct disulfide bonds (Table 3; see also Table S2 and Figure S3 of the Supporting Information). The natural pediocin PA-1 producer and the recombinant *L. sakei* strain produced nearly the same amount of pediocin PA-1 with the correct disulfide bonds as judged by the heights of the absorbance peaks obtained after purification of three independent overnight cultures of both strains (Table 3). However, the heights of the absorbance peaks corresponding to fractions I and II (containing pediocin PA-1 with the wrong cysteine connections) are ~4 times higher in the recombinant strain (Table 3). Thus, the total level of production of pediocin PA-1 (with both correct and incorrect disulfide bonds) in the recombinant *L. sakei* strain is much higher than in the natural producer. The difference in production of pediocin PA-1 cannot be explained by differences in cell density between the two strains as the cell density (estimated by the OD at 600 nm) was almost twice as high for the *P. acidilactici* strain. A possible explanation is that the higher level of production of pediocin PA-1 in the recombinant strain poses an increased pressure on the secretion system, thereby leading to an increased level of secretion of pediocin PA-1 with incorrect disulfide bonds. Moreover, *L. sakei* Lb790/pGF10 containing the pediocin PA-1 secretion machinery produced sakacin P[N24C+44C] with the correct disulfide bonds, indicating that the pediocin PA-1 secretion machinery is able to recognize other pediocin-like bacteriocins that contain four cysteine residues and secrete the peptide with the correctly connected cysteine residues. In this case, only one absorbance peak was obtained after reverse phase chromatography and the corresponding fraction contained a peptide with the expected molecular weight and with high antimicrobial activity (Table 3). The antimicrobial activity in this fraction was reduced 8-fold when DTT was added to the assay (Table 2). The total level of production of sakacin P[N24C+44C] was lower than the level of production of pediocin PA-1 when these peptides were expressed heterologously in *L. sakei* Lb790 containing the pediocin PA-1 secretion machinery (both recombinant strains reached approximately the same cell density) and might explain why only correct sakacin P[N24C+44C] was secreted.

On the other hand, when pediocin PA-1 was produced heterologously in *L. sakei* Lb790/pSAK20/pPed2 containing the secretion machinery (ABC transporter and accessory protein) belonging to sakacin A, a pediocin-like bacteriocin with only two cysteine residues, three similar absorbance peaks were obtained after reverse phase chromatography (Table 3). Mass spectrometry of the three corresponding fractions revealed that all three fractions contained a peptide with the same molecular weight that is expected for pediocin PA-1, but

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Biochemistry 2015, 54, 2967−2974

high antimicrobial activity was observed in only fraction III. Upon addition of 3 mM DTT to the assay, the activity increased >2-fold for fractions I and II but decreased 16-fold in fraction III (Table 2). Similar results were also obtained when *L. sakei* Lb790/pSAK20/pSPP(N24C+44C) containing the sakacin A secretion machinery was used to produce sakacin P[N24C+44C] (Tables 2 and 3; see also the chromatogram in Figure S3 of the Supporting Information). This is consistent with previous observations.[14] These results indicate that the formation of the two disulfide bonds is random, and that the sakacin A secretion machinery is not capable of efficient production of pediocin PA-1 or sakacin P[N24C+44C] with the correct disulfide bonds.

Cys to Ser Mutations in the Pediocin PA-1 Secretion Machinery. The ABC transporter (PedD) and accessory protein (PedC), which are dedicated to pediocin PA-1 export, have five and two cysteine residues, respectively. The cysteine residues in the ABC transporter are found at positions 19, 312, 498, 599, and 646, whereas the cysteine residues in the accessory protein are found in the proximity of each other at positions 83 and 86. To examine the involvement of these cysteine residues in disulfide bond formation, these cysteine residues were individually exchanged with serine residues, and the effect on pediocin PA-1 expression and disulfide bond formation was analyzed.

Cys to Ser Mutations in the ABC Transporter, Where Cys19 Is Part of the Proteolytic Active Site. No absorbance peaks were obtained after reverse phase chromatography, and no activity was detected in the supernatant of the recombinant strain when Cys19 in the ABC transporter was replaced with Ser (Table 3). This was likely caused by the abolishment of the proteolytic active site, and thus, the producer was not able to cleave the prebacteriocin and subsequently export the mature bacteriocin across the membrane. This is in agreement with previous observations that the replacement of Cys13 with Ala in the active site of the lactococcin G ABC transporter caused a loss of proteolytic activity.[17] On the basis of sequence similarities between the lactococcin G and pediocin PA-1 ABC transporters, Cys19 was suggested to be part of the proteolytic site in the pediocin PA-1 ABC transporter.[17]

None of the other Cys to Ser mutations (at positions 312, 498, 599, and 646) in the pediocin PA-1 ABC transporter had a marked effect on the relative amounts of pediocin PA-1 with the three possible disulfide connections, but the overall level of production of bacteriocin was slightly lower than that of the recombinant strain containing nonmutated pMC117 as judged by the heights of the absorbance peaks (Table 3). Three absorbance peaks were detected after reverse phase purification. The three corresponding fractions contained a peptide with the molecular weight of pediocin PA-1, but only fraction III had high antimicrobial activity. As observed for the natural pediocin PA-1 producer, addition of 3 mM DTT in the activity assay increased the activity in fractions I and II, whereas the activity in fraction III decreased (Table 2). Approximately 80% of the pediocin PA-1 secreted by these mutants contained the correct disulfide bonds, which is the same percentage observed for the recombinant strain containing nonmutated pMC117 (Table 3). These results indicate that the ABC transporter is not directly involved in the formation of the correct disulfide bonds.

Cys to Ser Mutations in the Accessory Protein Reduced the Amount of Pediocin PA-1 with Correct Disulfide Bonds. In contrast to the Cys to Ser mutations in the ABC transporter, the individual replacement of Cys83 or Cys86 with a serine residue in the pediocin PA-1 accessory protein had an effect on the ratio of pediocin PA-1 produced with the correct and incorrect disulfide bonds. Mutating Cys83 to Ser resulted in an almost complete loss of pediocin PA-1 secretion. From three of four overnight cultures grown, no absorbance peaks were observed after purification on a reverse phase column. From the fourth culture, three equally small absorbance peaks were observed (Table 3; see also Figure S3 of the Supporting Information for the reverse phase chromatogram). All three corresponding fractions contained a peptide with the expected mass of pediocin PA-1, but antimicrobial activity was observed in only fraction III. However, activity was obtained in the first two fractions when a small amount of DTT (3 mM) was added to the assay (Table 2). Mutating Cys86 to Ser also affected pediocin PA-1 production. This mutation resulted in a somewhat lower overall level of production of pediocin PA-1, but the relative amount of pediocin PA-1 with the wrong disulfide bonds was larger than that of the recombinant strain containing nonmutated pMC117. Judged by the heights of the absorbance peaks, the amount of pediocin PA-1 produced with correct disulfide bonds was almost reduced 2-fold, whereas the amount of pediocin PA-1 with incorrect disulfide bonds was approximately the same in the two strains (Table 3; see also Figure S3 of the Supporting Information for the reverse phase chromatograms).

Interestingly, the two cysteine residues in the pediocin PA-1 accessory protein are found in a so-called CxxC active site motif present in thiol−disulfide oxidoreductases.[18] The cysteine residues in these sites are separated by any two amino acids (denoted x). Furthermore, the N-terminal cysteine in this motif (i.e., Cys83 in the pediocin PA-1 accessory protein) has a lowered pK_a value that allowed this cysteine to initiate a nucleophilic attack on the substrate disulfide bond.[19] The subsequent attack by the C-terminal cysteine in the CxxC motif releases the reduced product. This is in accordance with our observations as the Cys83Ser mutation had a more dramatic effect on disulfide formation than the Cys86Ser mutation. A thiol−disulfide oxidoreductase has been shown to be important for the production of sublancin 168 in *Bacillus subtilis*, a lantibiotic that also contains two disulfide bonds. This oxidoreductase is encoded in the lantibiotic operon along with SunT, the dedicated ABC transporter.[20] The accessory proteins of some other pediocin-like bacteriocins that contain two disulfide bonds [coagulin,[21] plantaricin 423,[22] and sakacin G (GenBank entry ACB72722.1)] are highly similar to the pediocin PA-1 accessory protein, and they all contain a CxxC motif. For two of the pediocin-like bacteriocins with two disulfide bonds (divergicin M35 and plantaricin C19), the bacteriocin operons have not been sequenced. The putative accessory protein of enterocin A, however, does not contain a CxxC motif,[23] and no gene encoding an accessory protein has been identified in the divercin V41 operon, possibly because of an unusual genetic organization.[24] In general, putative accessory proteins for pediocin-like bacteriocins that contain only one disulfide bond do not contain a CxxC motif, with only a few exceptions, such as the accessory protein of avicin A[25] and sakacin P.[26] The lack of a CxxC motif in the accessory proteins of some pediocin-like bacteriocins suggests that the accessory protein also has other important functions. The production of both sakacin A and leucocin A (pediocin-like bacteriocins that contain only two cysteine residues) requires the presence of the accessory proteins SapE[27] and LcaD,[28] respectively. A gene encoding an accessory protein is also found in operons for

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Biochemistry 2015, 54, 2967−2974

many bacteriocins that do not contain cysteine residues (such as lactococcin G,[29] plantaricin EF,[30] plantaricin JK,[30] and lactococcin A[31]).

Taken together, these results may indicate that the pediocin PA-1 accessory protein is important for the formation of the correct disulfide bonds in pediocin PA-1, and that the protein thus has a chaperone-like activity.

■ ASSOCIATED CONTENT

Supporting Information

Experimental data of the mass spectrometry analysis of fragments obtained after trypsin cleavage of the purified pediocin PA-1 fractions, a table of the heights of absorbance peaks observed in the reverse phase chromatograms, reverse phase chromatograms obtained after purification of pediocin PA-1 or sakacin P(N24+44C) from recombinant strains *L. sakei* Lb790/pMC117, *L. sakei* Lb790/pMC117-pedC[C83S], *L. sakei* Lb790/pMC117-pedC[C86S], and *L. sakei* Lb790/pSAK20/pSPP(N24C+44C). The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00164.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ABC, ATP binding cassette; DTT, dithiothreitol; MIC, minimal inhibitory concentration; PCR, polymerase chain reaction; PedC, pediocin PA-1 accessory protein; PedD, pediocin PA-1 ABC transporter; TFA, trifluoroacetic acid.

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