Mutational Analysis of Residues in the Helical Region of the Class-IIa Bacteriocin Pediocin PA-1

Helén Sophie Haugen*, Gunnar Fimland† and Jon Nissen-Meyer*

*Department of Molecular Biosciences, University of Oslo, P.O.Box 1041 Blindern, 0316 Oslo, Norway

†Present address: Xellia Pharmaceuticals AS, Harbitzalléen 3, P.O.Box 158, Skøyen, 0212 Oslo, Norway

*Email corresponding author: h.s.haugen@imbv.uio.no

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A 15-mer fragment that is derived from the helical region in the C-terminal half of pediocin PA-1 inhibited the activity of pediocin PA-1. Of thirteen other pediocin-like (hybrid) bacteriocins, only the hybrid bacteriocin Sak/Ped was markedly inhibited by the 15-mer fragment. Sak/Ped was the only one of these bacteriocins which had a sequence (in the C-terminal helical-containing half) identical to that of the 15-mer fragment, indicating that the fragment inhibits pediocin-like bacteriocins in a sequence dependent manner. By replacing (one at a time) all 15 residues in the fragment with Ala or Leu, five residues (K1, A2, T4, N8, and A15) were identified as being especially important for the inhibitory action of the fragment. The results suggest that the corresponding residues (K20, A21, T23, N27 and A34) in pediocin PA-1 might be involved in interactions between pediocin PA-1 and its receptor. To characterize the environment surrounding these five residues when pediocin PA-1 interacts with target cells, these residues were replaced (one at a time) with a hydrophobic large (Leu) residue, a hydrophilic charged (Asp or Arg) residue, and a small (Ala or Gly) residue. The results revealed that residues A21 and A34 are in a spatially constrained environment, since replacement with a small (Gly) residue was the only substitution that did not markedly reduce the bacteriocin activity. The positive charge in K20 and the polar amide group in N27 appeared to interact with electronegative groups, since replacing these two residues with a positive (Arg) residue was well tolerated while replacement with a negative (Asp) residue was detrimental to the bacteriocin activity. K20 was in a less constrained environment than N27, since replacing K20 with a large hydrophobic (Leu) residue was tolerated fairly well, and to a greater extent than N27. T23 seemed to be in an environment that was not restricted with respect to size, polarity and charge, since replacements with a large (Leu) and small (Ala) hydrophobic residue, and a hydrophilic negative (Asp) residue were tolerated fairly well (2- to
6-fold reduction in activity). Moreover, replacement of T23 with a large positive (Arg) residue resulted in wild-type or better than wild-type activity.
INTRODUCTION

Membrane-permeabilizing, cationic, ribosomally synthesized antimicrobial peptides (AMPs) are produced by a wide variety of organisms, from bacteria to humans. When produced by bacteria, such peptides are generally termed bacteriocins and they are synthesized together with a cognate immunity protein that renders the bacteriocin-producing bacteria immune toward their own bacteriocins (6, 8, 13, 26-28). Bacteriocins produced by “food grade” lactic acid bacteria (LAB) have especially been the focus of extensive studies because of their potential application as nontoxic food preservatives and therapeutic agents for gastrointestinal infections. The LAB bacteriocins nisin and pediocin PA-1 are in fact used as bio-preservatives (4, 6), and the potential of LAB bacteriocins in medical applications is exemplified by results showing that oral intake of bacteriocin-producing LAB protects mice from lethal doses of *Listeria monocytogenes* (5).

There are two main classes of LAB AMPs: the lanthionine-containing (class-I) bacteriocins, and the non-lanthionine-containing (class-II) bacteriocins (6, 8, 13, 26-28). Class-II bacteriocins are heterogeneous and may further be divided into four groups: a) the pediocin-like (class-IIa) bacteriocins, b) the two-peptide (class-IIb) bacteriocins, c) the cyclic (class-IIc) bacteriocins and d) the non-pediocin one-peptide linear (class-IId) bacteriocins (6). The pediocin-like (class-IIa) bacteriocins, of which more than 20 have been identified, are perhaps the class-II bacteriocins that are best characterized (8, 13, 27). The interest in these bacteriocins is due to their anti-listerial activity combined with the fact that they are produced by food grade bacteria.

All pediocin-like bacteriocins have similar sequences, especially in their N-terminal half, which contains a disulfide bridge and a common YGNGV/L “pediocin-box” motif (8, 13, 27). Their well conserved cationic and rather hydrophilic N-terminal half (about 18 residues) forms an S-shaped β-sheet like structure, which is followed by a hinge and a
somewhat more hydrophobic and diverse helix-containing C-terminal half (14, 16, 33, 35). The hinge enables the N-terminal β-sheet like region and the C-terminal helix-containing region to move relative to each other and may thus allow the more hydrophobic C-terminal half to extend into the hydrophobic part of target cell membranes with the cationic and rather hydrophilic N-terminal half remaining in the hydrophilic exterior (8, 13, 27).

The membrane-embedded part (the C and/or D subunits) of the mannose phosphotransferase permease has been shown to act as a receptor or target molecule for the pediocin-like bacteriocins (7). Furthermore, the immunity proteins that protect cells from being killed by pediocin-like bacteriocins interact indirectly with their cognate bacteriocins through the same mannose permease (7). The helix-containing C-terminal half – being the membrane-penetrating part of pediocin-like bacteriocins (10, 27) – is thought to interact with the membrane-embedded part of the permease. Consistent with the notion that the C-terminal half is involved in receptor recognition are results showing that the C-terminal half is involved in determining the target-cell specificity of pediocin-like bacteriocins and is the half that is recognized by the cognate immunity protein (9, 20). The immunity proteins apparently recognize the C-terminal half of their cognate bacteriocins indirectly, by interacting with the receptor-bacteriocin complex (7).

A 15-mer fragment whose sequence is identical to a sequence in the C-terminal helical region of pediocin PA-1 inhibits the activity of pediocin PA-1 (11). It has been speculated that the fragment binds to the receptor and thus functions as a competitive inhibitor of pediocin-like bacteriocins that have sequence similarity to pediocin PA-1 in the helical region. We have in this study determined the ability of the 15-mer fragment and mutated variants of the fragment to inhibit wild type, hybrid and mutated pediocin-like bacteriocins. Residues that seem to be particularly important for the inhibitory action of the fragment – and thus possibly for the interaction between pediocin PA-1 and its receptor – have thereby been identified, and
the effect on bacteriocin activity of altering these residues in pediocin PA-1 has been determined.
MATERIALS AND METHODS

Bacterial strains, media, plasmids and production of peptides.

*Escherichia coli* DH5α was used for production and isolation of the plasmids before transferring plasmids to *Lactobacillus sake* Lb790. The *E. coli* strain was grown at 37°C in LB medium, either with vigorous agitation in liquid media or on agar plates solidified by adding 2% (wt/vol) agar. All lactic acid bacteria were grown without agitation in liquid medium or on agar plates solidified by adding 1.5% (wt/vol) agar. *Carnobacterium piscicola* UI 49 was grown at 30°C in M17 medium (Oxoid) supplemented with tween 80 (Sigma-Aldrich) and glucose (Sigma-Aldrich) to final concentrations of 0.1% and 0.4%, respectively. Lactic acid bacteria producing wild type and hybrid bacteriocins were grown at at 30°C in MRS medium (Oxoid), while lactic acid bacteria producing pediocin PA-1 mutants were grown at 20°C in either MRS medium or (for mutants A21L, A21G, T23A, N27L, N27R, A34L and A34R) in MRS medium supplemented with 4g/l yeast extract (Merck).

Three wild type producer strains were used in this study: *Lactobacillus curvatus* LTH1174 producing curvacin A (31), *Leuconostoc mesenteroides* 6 producing leucocin A (34) and *Pediococcus acidilactici* LMG 2351 producing pediocin PA-1 (24). Wild type sakacin P, enterocin A and mutant pediocin PA-1 variants were produced by the two-plasmid heterologous expression systems *L. sake* Lb790(pSAK20/pSSP2), *L. sake* Lb790(pSAK20/pEI), and *L. sake* Lb790(pSAK20/pPED2), respectively, as described earlier (3). Wild type strains were not used for the production of sakacin P and enterocin A because purification was problematic due to protease degradation (in the case of sakacin P) and the production of more than one bacteriocin by the wild type strain (in the case of enterocin A). Selective antibiotic concentrations of 2 µg/ml erythromycin and 5 µg/ml chloramphenicol were used for these two-plasmid systems. As described previously (20), all hybrid bacteriocins except Ped[1-21]/Sak[21-43] were produced by *L. sake* Lb790 transformed with...
a plasmid containing a gene encoding the hybrid bacteriocin and genes coding for proteins needed for secretion of and immunity to the hybrid bacteriocin. A sakacin P mutant with an inserted C-terminal disulfide bridge (Sak P Mut) was produced by *L. sake* Lb790 transformed with a plasmid containing the mutant bacteriocin gene as described previously (33). A selective antibiotic concentration of 10 µg/ml erythromycin was used when producing the hybrid bacteriocins, whereas concentrations of 2 µg/ml erythromycin and 5 µg/ml chloramphenicol were used when producing the mutant bacteriocins. These systems permitted routine production of correctly processed and secreted wild type, hybrid and mutant bacteriocins, which could be purified using well-established methods for purification of these peptides from LAB (see below).

The hybrid bacteriocin Ped[1-21]/Sak[21-43] and the 15-mer fragments derived from the wild type bacteriocin sequences were synthesized by standard methods of solid-phase multiple peptide synthesis with an F-moc strategy as described previously (11). Pediocin PA-15-mer fragments containing point mutations were synthesised by GenScript using their FlexPeptide™ technology.

**Plasmid isolation, preparation of competent cells, and cell transformation.**

Plasmids were isolated from *E. coli* DH5α and LAB using the Nucleo Spin® Plasmid Kit (Macherey-Nagel). To ensure lysis of LAB, lysozyme was added to Resuspension Buffer A1 included in the Nucleo Spin® Plasmid Kit to a final concentration of 5 mg/ml. For preparation of competent *E. coli* DH5α cells, the cells were cultured in LB medium to an OD$_{600}$ of about 0.3. The culture was cooled on ice for 10 min, and the cells were then washed with ice cold 0.1 M CaCl$_2$, and suspended in ice cold 0.1 M CaCl$_2$ containing 15% (vol/vol) glycerol. The cells were ready for transformation after incubation on ice for 45 min and transformed by use of heat shock (42°C, 90 seconds). *L. sake* Lb790/pSAK20 cells were made competent as described in (2). Briefly, the cells were cultured to an OD$_{600}$ of between
0.5 and 0.6 in MRS broth containing 10 µg/ml chloramphenicol and 2% (wt/vol) glycine, and
thereafter washed with 1 mM MgCl₂ and then with 30% (wt/vol) polyethylene glycol 1500
(molecular weight range 1,300-1,600). The cells were then transformed by electroporation
using a Gene Pulser and Pulse Controller unit (Bio-Rad Laboratories) as described previously
(2). L. sake Lb790 cells were made competent and transformed using the same method, but no
selective antibiotics were used for initial growth.

**Purification of wild type, hybrid and mutant bacteriocins.**

Wild type, hybrid and mutant bacteriocins were purified to homogeneity from 500 or
1000 ml cultures by applying the bacteria culture directly on a cation exchanger followed by
reverse-phase chromatography, as described previously (32). Briefly, the cells in 500 ml over
night cultures were applied to a 5-6 ml SP Sepharose Fast Flow (GE Healthcare) cation
exchange column that had been equilibrated with 50 ml 20 mM sodium phosphate buffer, pH
6. The column was then washed with 100 ml of the sodium phosphate buffer and the peptides
were subsequently eluted with 40 ml of the sodium phosphate buffer supplemented with NaCl
to a final concentration of 1 M. Trifluoroacetic acid (TFA) and 2-propanol were added to the
eluent to final concentrations of 0.1 and 5% (vol/vol), respectively, and the eluent was then
applied to a reverse phase column (Resource RPC, GE Healthcare). The peptides were eluted
from the reverse phase column (equilibrated with dH₂O containing 0.1% (vol/vol) TFA) with
a linear 2-propanol (containing 0.1% (vol/vol) TFA) gradient. 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 an Ultraflex
MALDI TOF/TOF (Bruker Daltonics, Bremen, Germany) instrument, in positive reflectron
mode, with α-cyano-4-hydroxy-cinnamic acid as matrix. In addition, the pediocin PA-1
mutants were checked for correct disulfide bridge formation using a bacteriocin assay (see
below for general description of assay) with and without 2 mM 1,4-dithiothreitol (DTT)
added to the indicator strain, as described previously (12). Bacteriocins with correct disulfide bridges have high antimicrobial activity and show a marked decrease in activity upon treatment with DTT. Bacteriocins with incorrect disulfide bridges have low antimicrobial activity and show a marked increase in activity upon the same DTT treatment.

The purity of bacteriocins was verified to be greater than 80% by analytical reverse-phase chromatography using a µRPC SC 2.1/10 C2/ C18 column (GE Healthcare) on the SMART chromatography system (GE Healthcare), except for the pediocin PA-1 mutants K20L and K20A which were judged to have purity of 50% or more. 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.

**Bacteriocin assay.**

Bacteriocin activity was measured using a microtiter plate assay system, essentially as described previously (25). 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$_{600}$ of about 0.01. For inhibition assays containing different peptide fragments, the fragments were added together with the indicator strain to a final concentration of 10 µM. The following strains were used as indicator strains: *C. piscicola* UI 49, *Lactobacillus coryniformis* subsp. *torquens* NCDO 2740, *Enterococcus faecalis* NCDO 581, and *L. sake* NCDO 2714. The microtiter plate cultures were incubated overnight (10-15 h) at 30°C for all strains except the *C. piscicola* strain, which was incubated for approximately 8 h, after which growth of the indicator strains was measured spectrophotometrically at 600 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.
RESULTS

The Ped-15-mer fragment inhibits bacteriocins in which the helical-containing C-terminal half stems from pediocin PA-1.

Consistent with earlier results (11), the Ped-15-mer fragment, whose sequence is identical to a sequence in the helical region in the C-terminal half of pediocin PA-1 (see Figure 1A), inhibited the bacteriocin activity of pediocin PA-1 (Table 1). Although the fragment also inhibited other tested pediocin-like bacteriocins, especially enterocin A and curvacin A, the inhibition was clearly less efficient (Table 1). To determine whether the inhibition depended on the sequence in the helical-containing C-terminal half of these bacteriocins, we tested how well the fragment inhibited hybrid bacteriocins containing N- and C-terminal domains from different pediocin-like bacteriocins. Eight different hybrid bacteriocins (Sak[1-19]/Ped[16-44], Ped[1-21]/Sak[21-43], Cur[1-18]/Sak[18-43], Sak[1-19]/Cur[20-41], Ent[1-23]/Sak[14-43], Sak[1-18]/Ent[19-47], Leu[1-16]/Sak[16-43], and Sak[1-19]/Leu[18-37]) derived from five different wild-type bacteriocins (pediocin PA-1, sakacin P, enterocin A, curvacin A, and leucocin A) were tested (20). The Sak[1-19]/Ped[16-44] hybrid consists of the N-terminal half of sakacin P (residues 1 to 19) and the C-terminal half of pediocin PA-1 (residues 16-44). Residues 16 to 19 in sakacin P and pediocin PA-1 are identical (Figure 1B). The terminology for the other hybrid peptides is analogous (see Figure 1B). Interestingly, only the hybrid bacteriocin (Sak[1-19]/Ped[16-44]) in which the helical-containing C-terminal half stemmed from pediocin PA-1 was consistently inhibited by the Ped-15-mer fragment with the same or greater efficiency as pediocin PA-1 (Table 1).

The fragments Ent-15-mer, Cur-15-mer, Sak-15-mer and Leu-15-mer (see Figure 1A for more detailed specification of these fragments) were also synthesized and tested for their ability to inhibit bacteriocin activity. These five fragments correspond to the Ped-15-mer
fragment, but were derived from enterocin A, curvacin A, sakacin P, and leucocin A, respectively (Figure 1A). Somewhat surprisingly, none of these fragments inhibited any of the tested bacteriocins effectively, highest inhibition being about a 2 fold inhibition of enterocin A by the Ent-15-mer fragment (results not shown).

Residues in the Ped-15-mer fragment which are important for its inhibitory activity.

The Ped-15-mer fragment was mutated and then assayed for its ability to inhibit pediocin PA-1 in order to identify residues that are especially important for the fragment’s inhibitory activity. All 15 residues in the Ped-15-mer fragment were (one at a time) replaced with an alanine or leucine residue. The results revealed that there were five residues in the Ped-15-mer fragment where replacements with an alanine or a leucine residue markedly reduced the inhibitory activity of the fragment (Table 2). These residues were K1, A2, T4, N8, and A15, which correspond, respectively, to K20, A21, T23, N27 and A34 in pediocin PA-1.

Effect on bacteriocin activity upon altering K20, A21, T23, N27 and A34 in pediocin PA-1.

The results obtained with the Ped-15-mer fragment and variants of this fragment (Tables 1 and 2) suggest that the Ped-15-mer fragment acts as a competitive inhibitor by binding to the bacteriocin receptor. The results suggest moreover that the K20, A21, T23, N27 and/or A34 residues in pediocin PA-1 may be involved in interactions between pediocin PA-1 and its receptor. It was consequently of interest to characterize the environment surrounding each of these five residues by determining the effect replacements of these residues had on the bacteriocin activity. All five residues were individually replaced with a hydrophobic large (leucine) and a hydrophilic negatively (aspartate) or positively (arginine) charged residue (Table 3). In addition, K20, T23 and N27 were replaced with a hydrophobic small (alanine) residue, whereas A21 and A34 were replaced with a glycine residue. A total of 19 different pediocin PA-1 variants were thus produced and purified, and analyzed by mass spectrometry.
to confirm that correct residue replacements had been made. The activities of all purified
mutants were then determined using four different indicator strains, and the activity relative to
that of wild-type pediocin PA-1 was calculated (Table 3). Four different strains were used in
order to detect general trends in mutational effects, as such effects may in some cases be
indicator strain dependent (12, 17, 23). With just a few exceptions, the effect of the various
residue replacements on bacteriocin activity did not depend on the indicator strain (Table 3).
Exceptions were the A21L, N27L, and A34R mutations. The A21L and N27L mutations were
somewhat less detrimental when assayed using the L. coryniformis and L. sake strains than
when assayed using the E. faecalis and C. piscicola strains (Table 3). The A34R mutation was
less detrimental when assayed against the L. sake strain than when assayed against the three
other strains (Table 3).

Replacing the positively charged (Lys) residue in position 20 with another positively
charged (Arg) residue resulted in activity as good as or better than that of wild-type pediocin
PA-1 (Table 3). In contrast, introducing a negatively charged (Asp) residue was detrimental,
causing a 20- to 30-fold reduction in activity (Table 3). It thus seems that K20 interacts with
an electronegative group. The environment surrounding K20 seems not to be highly
hydrophilic or sterically restricted, since replacing K20 with a large (Leu) or small (Ala)
hydrophobic residue was surprisingly well tolerated, causing only a 2- to 4-fold reduction in
the activity (Table 3). The neighbouring residue, A21, seemed to be in a much more restricted
environment, as replacement with a hydrophilic positively (Arg) or negatively (Asp) charged
residue was highly detrimental (≥ 100-fold reduction in activity; Table 3). Moreover,
replacement with a large hydrophobic (Leu) residue reduced the activity 3- to 20-fold
(depending on the target strain) and replacement with the small Gly residue reduced it 3- to 7-
fold (Table 3). T23 seemed in turn to be in a less restrictive environment, since replacement
of this hydrophilic residue with a large (Leu) or small (Ala) hydrophobic residue or a
negatively charged (Asp) residue reduced the activity only 2- to 6-fold. Moreover, replacement with a large positively charged (Arg) residue resulted in activity as good as or better than that of wild-type pediocin PA-1 (Table 3).

The hydrophilic amide group in Asn at position 27 seemed to be involved in an interaction with an electronegative group, since replacement with a large positively charged (Arg) residue did not markedly reduce the activity (0.5- to 3-fold reduction, Table 3), whereas replacement with a negatively charged Asp residue was detrimental (10- to 30-fold reduction in activity; Table 3). Replacement with a small hydrophobic (Ala) residue was well tolerated (about the same effect as replacement with an Arg residue), while replacement with a large hydrophobic (Leu) residue was more detrimental (5- to 20-fold reduction in the activity, Table 3). The Ala residue in position 34 seemed to be in a relatively hydrophobic environment, as replacement with a hydrophilic positively (Arg) or negatively (Asp) charged residue was highly detrimental, resulting in 10- to more than a 100-fold reduction in activity (Table 3). Replacement with the small Gly residue did not significantly reduce the activity (Table 3).
The Ped-15-mer fragment, whose sequence is identical to a sequence in the helix in the C-terminal half of pediocin PA-1, clearly inhibited pediocin PA-1. The fragment also inhibited the Sak/Ped hybrid whose N-terminal half is from sakacin P and C-terminal helical-containing half is from pediocin PA-1. The 12 other tested hybrids and wild-type bacteriocins, none of which had a C-terminal half identical to that in pediocin PA-1, were not inhibited to the same extent. An earlier study has shown that, of all possible 15-mer fragments that can be derived from pediocin PA-1, only the Ped-15-mer fragment and the 4 adjacent fragments (i.e. fragments covering residues 18 to 32, 19 to 33, 21 to 35, and 22 to 36) inhibit pediocin PA-1 (11). The four adjacent fragments, however, inhibit pediocin PA-1 less efficiently than the Ped-15-mer fragment (11). Also, the pediocin-like bacteriocins enterocin CRL35 and leucocin A have been shown to be inhibited by fragments that are derived from the (putative) helical region in these bacteriocins (29, 36). In contrast, fragments derived from the non-helical N-terminal part of enterocin CRL35 and the pediocin-like bacteriocin carnobacteriocin B2 did not inhibit these bacteriocins (29, 36). Taken together, the results indicate that peptide fragments that inhibit pediocin-like bacteriocins do so in a specific and sequence-dependent manner in the sense that their sequence must be derived from a sequence in the helical region of the C-terminal half of these bacteriocins.

The helix-containing C-terminal half is an important target-cell specificity determinant in these bacteriocins, since hybrid bacteriocins constructed by joining N- and C-terminal halves from different pediocin-like bacteriocins have target-cell specificities similar to the bacteriocin from which the C-terminal half is derived (9, 20). The membrane-penetrating C-terminal half is thus thought to interact with the membrane-embedded part (the C and/or D subunits) of the mannose phosphotransferase permease that acts as the receptor for the pediocin-like bacteriocins (7). The specific and sequence dependent manner by which the
Ped-15-mer fragment inhibits bacteriocin activity suggests that the fragment acts as a competitive inhibitor by binding to the bacteriocin receptor, and that this binding may involve the five residues (K1, A2, T4, N8 and A15) identified as being most essential for the fragment’s inhibitory activity. The corresponding residues (K20, A21, T23, N27 and A34) in pediocin PA-1 are thus expected to be involved in the binding of pediocin PA-1 to its receptor. Interestingly, four of these residues (K20, T23, N27 and A34) are positioned on the same side of the membrane penetrating α-helix, and might thus create a receptor-interacting stretch along one side of the helix.

Four of the five residues (K20, A21, N27 and A34) seemed to be in a restricted environment, as expected if they are involved in specific interactions with a receptor. Especially A21 and A34 seemed to be in a highly restricted environment due to spatial constraints and hydrophobic surroundings, as replacement with a small neutral (Gly) residue was the only mutation that was fairly well tolerated. Both the positive charge in K20 and the polar amide group in N27 appeared to interact with electronegative groups, since replacing these two residues with a positive Arg residue was well tolerated while replacement with a negative Asp residue was detrimental. For the amide group in N27, it thus seems that it is the −NH₂ with a positive dipole rather than the electronegative =O that is involved in the electrostatic interaction. K20 seemed to be in a somewhat less restricted environment than N27, since K20 tolerated replacement with a large hydrophobic (Leu) residue fairly well, and to a greater extent than N27. In contrast to K20, A21, N27 and A34, the Thr residue at position 23 seemed to be in a remarkably unrestricted environment, the reduction in activity being only about 2- to 6-fold irrespectively of whether T23 was replaced by a large (Leu) or a small (Ala) hydrophobic residue, or a hydrophilic negatively charged (Asp) residue. Moreover, wild-type or better than wild-type activity was obtained upon replacement with a large positively charged (Arg) residue. Consequently, T23 does not seem to be involved in
receptor interactions that are of great importance for bacteriocin activity. The fact that introducing an Arg residue at either position 20, 23 or 27 resulted in a somewhat better than wild-type activity against some of the indicator strains suggests that even more potent bacteriocin variants might be constructed by introducing Arg residues at all 3 positions.

The sensitivity of different indicator strains to pediocin PA-1 varies considerably, presumably in part due to the sequence diversity in the mannose phosphotransferase permease. The extent a residue in pediocin PA-1 is involved in binding the bacteriocin to the permease might consequently be indicator strain dependent. The effect of mutations involving A21, N27 and A34 seemed indeed to show some indicator strain dependency. The A21L and N27L mutations were somewhat less detrimental when assayed against the *L. coryniformis* and *L. sake* strains than when assayed against the *E. faecalis* and *C. piscicola* strains, whereas the A34R mutation was less detrimental when assayed against the *L. sake* strain than when assayed against the three other strains.

It should be noted that residues not identified as being essential for the fragment’s inhibitory activity might also interact with the receptor, since some of the residue replacements were conservative and may consequently not have a marked detrimental effect on the fragment’s inhibitory effect on pediocin PA-1. This might for instance be the case for replacements involving I6. The conservative replacement I6L increased the fragment’s inhibitory effect on pediocin PA-1, whereas the less conservative I6A replacement caused a slight reduction in the fragments inhibitory effect (Table 2). Also the N9L replacement reduced the fragment’s inhibitory effect (Table 2). These two residues (i.e. I6 and N9) correspond to residues I25 and N28 in pediocin PA-1, which are on the same side of the membrane penetrating helix as residue A21, which was clearly essential for the fragment’s inhibitory activity (Table 2). These three residues could, in a similar manner as K20, T23, N27 and A34, create a receptor-interacting stretch along one side of the helix.
Somewhat surprisingly, the Ent-15-mer, Cur-15-mer, Sak-15-mer and Leu-15-mer fragments derived, respectively, from enterocin A, curvacin A, sakacin P, and leucocin A (see Figure 1 A), did not inhibit any of the bacteriocins efficiently. These fragments may perhaps not associate with and/or penetrate into the target-cell membrane as efficiently as the Ped-15-mer fragment and may thereby be unable to efficiently interfere with bacteriocin-receptor interactions. The Ped-15-mer fragment is cationic (net charge of one) and contains a tryptophan residue. The fragment might thus associate more readily with membranes than the anionic Cur-15-mer fragment (net charge of minus one), the neutral Sak-15-mer fragment (no net charge), and the Ent-15-mer and Leu-15-mer fragments that both lack a membrane-interacting tryptophan residue. An alternative explanation for their inefficient inhibition of bacteriocin activity is that these fragments might not to a sufficient extent cover the relevant helical region. The structure of curvacin A reveals indeed that its 13-mer C-terminal helix stretches from Gly29 to the C-terminus (16), and thus only the last 7 residues in the Cur-15-mer fragment span this helical region. Screening all possible 15-mer fragments that can be derived from enterocin A, curvacin A, sakacin P, and leucocin A may be required in order to identify 15-mers that efficiently inhibit these bacteriocins.

Interactions between the helical-containing C-terminal half of pediocin-like bacteriocins and the membrane-embedded subunits of the mannose permease do not exclude additional bacteriocin-permease interactions. The fact that the fragments alone are not toxic suggests that additional interactions may indeed be required to obtain a bactericidal effect. More importantly, a recently published study revealed that residues in an extracellular loop in the C subunit of the permease along with residues in a flanking trans-membrane segment are required for target-cell-sensitivity to all of the tested pediocin-like bacteriocins (21). Residues in other unidentified parts of the C and/or D subunits seemed, however, also to be needed to obtain maximal sensitivity toward some of the tested bacteriocins, such as sakacin P and
enterocin P, but perhaps not pediocin PA-1 (21). The extracellular loop of the C-subunit might possibly be an interaction site for the more hydrophilic and relatively conserved β-sheet like N-terminal part of these bacteriocins, whereas trans-membrane segments of the permease might be interaction sites for the bacteriocin’s more diverse and hydrophobic helical-containing C-terminal half that is especially important for the relative potencies different pediocin-like bacteriocins have toward different sensitive target-cells.

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role of charged residues in target-cell binding, potency and specificity of the pediocin-


TABLE 1. Inhibition of bactericidal activities of pediocin-like bacteriocins and hybrid variants of these bacteriocins due to the presence of the Ped-15-mer fragment.

<table>
<thead>
<tr>
<th>Bacteriocin variant</th>
<th>Fold inhibition&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E. faecalis</td>
</tr>
<tr>
<td>Pediocin PA-1</td>
<td>16 ± 8</td>
</tr>
<tr>
<td>Sakacin P</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td>Curvacin A</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>Enterocin A</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Leucocin A</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Sakacin P mutant&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Sak/Ped</td>
<td>20 ± 6</td>
</tr>
<tr>
<td>Ped/Sak</td>
<td>ND</td>
</tr>
<tr>
<td>Cur/Sak</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>Sak/Cur</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Ent/Sak</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Sak/Ent</td>
<td>7 ± 3</td>
</tr>
<tr>
<td>Leu/Sak</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td>Sak/Leu</td>
<td>2 ± 1</td>
</tr>
</tbody>
</table>

<sup>a</sup>Fold inhibition is the minimal inhibitory concentration (MIC) of the bacteriocin (or hybrid bacteriocin) measured in the presence of 10 µM of the Ped-15-mer fragment divided by the MIC of the bacteriocin (or hybrid bacteriocin) measured in the absence of the fragment. The inhibition values presented are the result of at least 3 independent measurements. The MIC for the wild-type bacteriocins in the absence of fragment were all between 0.07 and 3 nM (depending on the indicator cell) and those for the hybrid bacteriocin were all between 0.02 and 14 nM. The indicator strains were: E. faecalis NCDO 581, L. coryniformis subsp. torquens NCDO 2740, L. sake NCDO 2714, and C. piscicola UI 49.
See Figure 1 for sequence of the hybrid bacteriocins. The terminology for the hybrid bacteriocins in the Table is the same as in Figure 1, except that the numbering indicating the positions where the N- and C-terminal regions were combined has not been included.

ND indicates not determined. Reliable values for fold inhibition were not obtained since the MIC values obtained in the absence of fragment were too high (i.e. the potency was too low).

The sakacin P mutant has an inserted C-terminal disulphide bridge; see Figure 1 for more detailed specification of this mutant bacteriocin.
TABLE 2. Inhibition of pediocin PA-1 by Ped-15-mer fragments in which residues have been replaced with either an alanine or a leucine residue

<table>
<thead>
<tr>
<th>Residue which is replaced&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Fold inhibition&lt;sup&gt;b&lt;/sup&gt;</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alaine replacement</td>
<td>Leucine replacement</td>
</tr>
<tr>
<td>K1</td>
<td>4 ± 1</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>A2</td>
<td>WT£</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>T3</td>
<td>70 ± 30</td>
<td>80 ± 30</td>
</tr>
<tr>
<td>T4</td>
<td>≥ 100</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>C5</td>
<td>≥ 100</td>
<td>≥ 100</td>
</tr>
<tr>
<td>I6</td>
<td>55 ± 25</td>
<td>≥ 100</td>
</tr>
<tr>
<td>I7</td>
<td>70 ± 20</td>
<td>≥ 100</td>
</tr>
<tr>
<td>N8</td>
<td>13 ± 4</td>
<td>7 ± 3</td>
</tr>
<tr>
<td>N9</td>
<td>60 ± 30</td>
<td>20 ± 10</td>
</tr>
<tr>
<td>G10</td>
<td>80 ± 30</td>
<td>40 ± 20</td>
</tr>
<tr>
<td>A11</td>
<td>WT£</td>
<td>60 ± 20</td>
</tr>
<tr>
<td>M12</td>
<td>40 ± 10</td>
<td>65 ± 15</td>
</tr>
<tr>
<td>A13</td>
<td>WT£</td>
<td>70 ± 30</td>
</tr>
<tr>
<td>W14</td>
<td>90 ± 30</td>
<td>80 ± 30</td>
</tr>
<tr>
<td>A15</td>
<td>WT£</td>
<td>14 ± 6</td>
</tr>
</tbody>
</table>

<sup>a</sup> The residues in the Ped-15-mer fragment which are replaced with alanine and leucine residues are designated by their one-letter abbreviations and a number indicating their position in the fragment, starting from the N-terminus. The first residue (K1) in the fragment corresponds to residue 20 (K20).
in pediocin PA-1, and so on, the last residue (A15) in the fragment corresponding to residue 34
(A34) in pediocin PA-1.

Fold inhibition is the minimal inhibitory concentration (MIC) of pediocin PA-1 measured in the
presence of 10 µM of mutant Ped-15-mer fragment divided by the MIC of pediocin PA-1 measured
in the absence of fragment. The inhibition values presented are the result of at least 3 independent
measurements using *C. piscicola* UI 49 as indicator strain. The MIC for pediocin PA-1 in the
absence of fragment was 0.3 ± 0.1 nM.

WT indicates the wild-type Ped-15-mer fragment (i.e alanine replaced with alanine) and it resulted
in a 60 ± 20 fold inhibition.
TABLE 3. Relative MIC values of the pediocin PA-1 variants.

<table>
<thead>
<tr>
<th>Position</th>
<th>Indicator strain</th>
<th>Mutations with the corresponding relative MIC values&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Leu</td>
</tr>
<tr>
<td>K20</td>
<td>E. faecalis</td>
<td>4.2 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>L. coryniformis</td>
<td>5.7 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>L. sake</td>
<td>3.7 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>C. piscicola</td>
<td>2.3 ± 0.6</td>
</tr>
<tr>
<td>A21</td>
<td>E. faecalis</td>
<td>21 ± 8</td>
</tr>
<tr>
<td></td>
<td>L. coryniformis</td>
<td>3.5 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>L. sake</td>
<td>3.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>C. piscicola</td>
<td>17 ± 3</td>
</tr>
<tr>
<td>T23</td>
<td>E. faecalis</td>
<td>5.9 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>L. coryniformis</td>
<td>3.4 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>L. sake</td>
<td>2.7 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>C. piscicola</td>
<td>4.2 ± 1.0</td>
</tr>
<tr>
<td>N27</td>
<td>E. faecalis</td>
<td>15 ± 6</td>
</tr>
<tr>
<td></td>
<td>L. coryniformis</td>
<td>5.8 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>L. sake</td>
<td>5.4 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>C. piscicola</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>A34</td>
<td>E. faecalis</td>
<td>30 ± 8</td>
</tr>
<tr>
<td></td>
<td>L. coryniformis</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>L. sake</td>
<td>26 ± 8</td>
</tr>
<tr>
<td></td>
<td>C. piscicola</td>
<td>15 ± 2</td>
</tr>
</tbody>
</table>

The relative MIC values are the MIC of the mutant peptide divided by the MIC of wild-type pediocin PA-1. Each relative MIC is the result of at least 3 independent measurements. The MICs for wild-type pediocin PA-1 were between 0.1 and 0.5 nM (depending on the indicator cell). The L. coryniformis and L. sake strains were 2 to 5 times more sensitive to pediocin PA-1 than the E. faecalis and C. piscicola strains.

ND indicates not determined. Replacement with a Gly residue was only done when replacement with an Ala residue was not relevant due to the presence of an Ala residue in pediocin PA-1. The A34L mutation was not analyzed because we were unable to produce this variant, despite several attempts.

WT indicates wild-type bacteriocin, and the relative MIC is thus 1.
FIG. 1. (A) Amino acid sequences of the wild-type pediocin-like bacteriocins used in this study. Regions where the sequences are identical to the sequence in pediocin PA-1 are in white with black background. The brackets above and below the sequences indicate the regions from which the 15-mer fragments were derived. The Ped-15-mer, Sak-15-mer and Leu-15-mer fragments span residues 20 to 34 in, respectively, pediocin PA-1, sakacin P, and leucocin A, whereas the Ent-15-mer fragment spans residues 25 to 34 in enterocin A and the Cur-15-mer fragment spans residues 21 to 35 in curvacin A. (B) Amino acid sequences of the hybrid bacteriocins used in this study. The Sak[1-19]/Ped[16-44] hybrid consists of the N-terminal half of sakacin P (residues 1 to 19) and the C-terminal half of pediocin PA-1 (residues 16-44). Residues 16 to 19 in sakacin P and pediocin PA-1 are identical and are in white with black background. The terminology and use of white with black background is analogous for the other hybrid bacteriocins. Note that there is a conservative mutation, V17I, in the Cur[1-18]/Sak[18-43] hybrid. (C) Amino acid sequences of the sakacin P variant. This variant has a disulfide bridge in its C-terminal half, since two cysteine residues have been introduced in sakacin P, in positions 24 and 44, both indicated in white with black background. References for the sequences are as follows: pediocin PA-1 (18, 22, 24), enterocin A (1), curvacin A (19, 31), sakacin P (30), and leucocin A (15).