Determination of Essential and Variable Residues in Pediocin PA-1 by NNK Scanning†

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Received 24 June 2005/Accepted 11 November 2005

Pediocin PA-1 is an antimicrobial peptide (called bacteriocin) that shows inhibitory activity against the food-borne pathogen *Listeria monocytogenes*. To elucidate which residue(s) is responsible for this function, the antimicrobial activities of pediocin PA-1 mutants were evaluated and compared. Each of the 44 native codons was replaced with the NNK triplet oligonucleotide in a technique termed NNK scanning, and 35 mutations at each position were examined for antimicrobial activities using a modified colony overlay screening method. As a consequence, the functional responsibility of each residue was estimated by counting the number of active mutants, allowing us to identify candidate essential/variable residues. Activity was abrogated by many of the mutations at residues Y2, G6, C9, C14, C24, W33, G37, and C44, indicating that these residues may be essential. In contrast, activity was retained by almost all versions harboring mutations at K1, T8, G10, S13, G19, N28, and N41, indicating that these are functionally redundant residues. Sequence analysis revealed that only the wild type was active and 14 and 11 substitutions were inactive at G6 and C14, respectively, while 12 and 11 substitutions were active and 2 and 0 substitutions were inactive at T8 and K1, respectively. These findings suggest that NNK scanning is effective for determining essential and variable residues in pediocin PA-1, leading to an elucidation of structure-function relationships and to improvements in the antimicrobial function efficiently by peptide engineering.

Various antimicrobial peptides, called bacteriocins, are produced by lactic acid bacteria to kill or inhibit the growth of closely related species (1). Bacteriocins are thought to have promise for use as safe food preservatives (7). For example, nisin is currently used in more than 40 countries as an antimicrobial additive (41), and pediocin PA-1 is currently being investigated in this context, as it has a strong inhibitory effect on the food-borne pathogen *Listeria monocytogenes* (2, 38). Pediocin PA-1, composed of 44 residues, is secreted by *Pedococcus acidilactici* (23, 29). More than 20 members of the pediocin-like (class IIa) bacteriocins have been identified to date (16). These peptides contain a consensus YGNGV motif and can be structurally divided into a highly conserved hydrophilic N-terminal domain and a relatively variable hydrophobic C-terminal domain (14). Nuclear magnetic resonance structure (18, 42, 44) and circular dichroism spectral (17, 27, 45) analyses have suggested that the N-terminal domain has a three-stranded β -sheet structure, while the C-terminal domain is folded into an amphiphilic α -helical structure. It is thought that the bacteriocins bind to bacteria via electrostatic interactions (4, 28) and then insert into the bacterial membrane via their amphiphilic α -helical region, subsequently forming a membrane pore that triggers dissipation of the membrane electrochemical potential (3) and/or nutrient leakage from within the bacterial cell (5, 22). A mannose phosphotransferase system (PTS) permease (EII_t^{Man}) has been proposed as a target

molecule specific for the recognition of target bacteria by class IIa bacteriocins (11, 21, 37).

Previous studies have sought to identify the residues responsible for the antimicrobial activities of class IIa bacteriocins by using various mutants generated by site-directed mutagenesis (13, 16, 26, 28). The cysteine residues of pediocin PA-1 (13) and mesentericin Y105³⁷ (17), the tryptophan residues of sakacin P (16), and the charged residues of sakacin P (28) were shown to play significant roles in the antimicrobial activities of these peptides. Replacement of the methionine residue of pediocin PA-1 had only a minor effect on the antimicrobial activity, except when it was replaced with a hydrophilic negatively charged aspartic acid (26). Furthermore, studies of the C-terminal 15-mer region of pediocin PA-1 revealed that the fragment interfered with the inhibitory activity of the native peptide (15). More recent studies have investigated multiple mutants with mutations at different residues (30, 33, 36). Six mutants with mutations at 6 different residues of carnobacteriocin B2 (36), 10 with mutations at 8 different residues of mesentericin Y105 (33), and 17 with mutations at 14 different residues of pediocin PA-1 (30) were generated by random PCR mutagenesis. These studies allowed evaluation of the functional importance of 15 specific residues, i.e., K1, N5, C9, K11, C14, W18, C24, I26, M31, A34, G37, N41, H42, K43, and C44, in the case of pediocin PA-1. However, since this type of mutagenesis depends on accidental errors made by *Taq* DNA polymerase, the mutational positions were random and the types of substituted amino acids examined were limited. No previous work has systematically examined all residues of the class IIa bacteriocins for functional importance or redundancy.

The use of the NNK oligonucleotide was first developed to facilitate the construction of phage libraries covering all 20

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† Supplemental material for this article may be found at <http://aem.asm.org/>.

amino acids without library enlargement (10, 39). For the present study, specific pediocin PA-1 mutants were generated by replacement of each native codon with the NNK triplet oligonucleotide, and the antimicrobial activity of each mutant was examined to investigate the functional importance of each codon. In this way, NNK scanning allowed the generation and analysis of a wider variety of mutants than those examined in previous studies. The results provided the functional responsibility of each residue spanning the whole pediocin PA-1 molecule, leading to the identification of essential/variable residues as a consequence. This study sheds light on the structure-function relationships of pediocin PA-1 and on improving its function efficiently in a future approach.

MATERIALS AND METHODS

Bacterial strains, culture media, and growth conditions. *Escherichia coli* DH5 α was used as the cloning host strain, and *E. coli* JE5505 was used as the bacteriocin-leaky host strain. *E. coli* cells were cultured at 37°C in Luria-Bertani (LB) medium supplemented with ampicillin (100 μ g ml⁻¹) as needed. *Listeria monocytogenes* ATCC 19115, *Listeria seeligeri* ATCC 35967, *Pediococcus pentosaceus* JCM2026, *Pediococcus pentosaceus* JCM5890, *Pediococcus acidilactici* IAM1233, *Lactobacillus plantarum* JCM1149, and *Lactobacillus sake* IAM1900 were used as the indicator strains. *P. pentosaceus*, *P. acidilactici*, *L. plantarum*, and *L. sake* were cultivated in MRS broth (Difco Laboratories) at 30°C, while *L. monocytogenes* and *L. seeligeri* were cultivated in brain heart infusion broth (Oxoid) at 30°C.

Construction of pFLAG-pediocin PA-1 plasmid. The oligonucleotides (InF and InR) and primers (ExtF, ExtR, pUCHinF, and pUCEoR) used to construct the pediocin PA-1-encoding fragment were as follows: InF, 5'-TGACC TGCGG CAAAC ATAGC TGCAG CGTGG ATTGG GGCAA AGCGA CCACC TGCAT TATT-3'; InR, 5'-CTGAT GGCCG CCGGT CGCCC ACGCC ATCGC GCCGT TGTTA ATAAT GCAGG TGGTC GCTT-3'; ExtF, 5'-CGGCC GGAAT TCAAA TATTA TGGCA ACGGC GTGAC CTGCG GCAAA CATAG C-3'; ExtR, 5'-CGGCC GGGAT CCTTA GCATT TATGG TTGCC CTGAT GGCCG CCGGT CGCCC-3'; pUCHinF, 5'-GGGAA GCTTA AATAT TATGG CAACG GCGTG ACC-3'; and pUCEoR, 5'-GGGGA ATTCT TAGCA TTTAT GGTTC CCCTG ATG-3'. Ten picomoles each of InF and InR was mixed with a solution containing 2 mM of Mg²⁺, a 0.2 mM concentration (each) of mixed deoxynucleoside triphosphates (dNTPs), and 2.5 units of ExTaq DNA polymerase (Takara Bio) in a final volume of 50 μ l. This reaction mix was subjected to 10 cycles of 1 min at 95°C and 1 min at 50°C and then soaked at 72°C for 10 min. The resulting hybridized product (1 μ l) was then subjected to PCR in a final volume of 50 μ l containing 25 pmol each of ExtF and ExtR, 2 mM of Mg²⁺, a 0.2 mM concentration (each) of mixed dNTPs, and 2.5 units of ExTaq DNA polymerase. The PCR amplification conditions were as follows: 3 min at 95°C followed by 25 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C, with a final soak for 10 min at 72°C. Cycling was performed in an i-Cycler machine (Bio-Rad). The resulting PCR product was digested with BamHI and EcoRI (Toyobo) and then ligated into pUC18. DNA sequencing was used to confirm the amplified fragment (Applied Biosystems 3730 DNA analyzer). A second round of PCR was performed in a final volume of 50 μ l containing 0.02 μ g of the cloned plasmid, 25 pmol each of pUCHinF and pUCEoR, 2 mM of Mg²⁺, a 0.2 mM concentration (each) of mixed dNTPs, and 2.5 units of ExTaq DNA polymerase, using the above PCR conditions. The resulting PCR product was digested with HindIII and EcoRI and cloned into the pFLAG-ATS expression vector (Sigma). DNA sequencing was used to confirm the cloned pediocin PA-1 coding sequence, and the vector is hereafter called pFLAG-pediocin PA-1.

Construction of mutagenized pFLAG-pediocin PA-1 plasmids. Mutations were introduced into the pediocin PA-1-encoding fragment by PCRs using primers with partial random sequences (see the supplemental material for a primer list). For the introduction of mutations from K1 (the lysine at the first residue from the N-terminal end) to T22, primers named K1mut to T22mut were designed to encode pediocin PA-1 containing an EcoRI site and the mixed oligonucleotide NNK. For example, the Y3mut primer sequence was as follows: 5'-GGGAA GCTTA AATAT NNKGG CAACG GCGTG-3'. Likewise, for the introduction of mutations from T23 to C44, primers named T23mut to C44mut were designed to introduce a HindIII recognition site and the mixed oligonucleotide MNN. For example, the K43mut primer sequence was as follows: 5'-GGGGA ATTCT TAGCA MNNAT GGTTC CCCTG-3'. PCRs were performed in a final volume of 50 μ l containing 25 pmol of pUCEoR and any one

of the K1mut to T22mut primer sets or pUCHinF and any one of the T23mut to C44mut primer sets, along with 2 mM of Mg²⁺, a 0.2 mM concentration (each) of mixed dNTPs, and 2.5 units of ExTaq DNA polymerase. PCRs were performed as described above, and the products were cloned into pFLAG-ATS.

Determination of antimicrobial activity. The antimicrobial activities of pediocin PA-1 and its substituted mutants were determined by the colony overlay method, essentially as previously described (31). Briefly, plates containing 50 to 200 CFU *E. coli* in 5 ml of 0.8% LB soft agar were overlaid with 3 ml 0.8% LB soft agar and then incubated at 30°C for 21 h. On the following day, the plates were overlaid with 5 ml of 0.8% melted MRS soft agar containing 1 mM IPTG (isopropyl- β -D-thiogalactopyranoside) and the indicator strain cultured to an optical density at 600 nm of $\sim$ 0.05. After incubation at 30°C for 15 h, zones of indicator strain growth inhibition could be visualized. Alternatively, single *E. coli* colonies containing the desired expression vector were picked with toothpicks and touched lightly onto the surfaces of 2.5% LB agar plates. The plates were incubated at 37°C for 5 h and then overlaid with 5 ml of 1.2% melted MRS soft agar containing 1 mM IPTG and the appropriate indicator strain. After the agar solidified, 5 ml of 1.2% melted soft agar was poured on each plate, and the plates were incubated at 30°C for 15 h for the development of growth inhibition zones. The peptide activities were assessed by measurement of the inhibition zone diameter. Since triplet oligonucleotides encoded by NNK comprise 32 possible ($4 \times 4 \times 2$) codons, 35 mutants were evaluated per residue.

Nucleotide sequence accession number. The sequence of the synthetic pediocin PA-1 was deposited in DDBJ under accession number AB218926.

RESULTS

Construction of plate assay system. To express pediocin PA-1 in *E. coli*, oligonucleotides were designed to encode pediocin PA-1, using the preferred codon usage of *E. coli* (34). The synthesized DNAs were cloned downstream of the OmpA secretion signal sequence of pFLAG-ATS. The constructed pFLAG-pediocin PA-1 was then transformed into the periplasmic leaky strain, *E. coli* JE5505, an *lpo* mutant that reportedly lacks both the free and bound forms of murein lipoprotein (24, 40) and has been shown to leak periplasmic enzymes into the culture medium (40). Thus, *E. coli* JE5505 harboring pFLAG-pediocin PA-1 was anticipated to produce and secrete antimicrobial peptides into the medium under the control of IPTG. In contrast to a previous report that showed growth inhibition circles even without IPTG (31), those of *P. pentosaceus* JCM2026 were formed only in the presence of IPTG in our experiment, probably because of the difference in the vector used (data not shown). We confirmed that *E. coli* JE5505 containing pFLAG-pediocin PA-1 displayed antimicrobial effects against various bacterial species, i.e., *L. monocytogenes* ATCC 19115, *L. seeligeri* ATCC 35967, *P. pentosaceus* JCM2026, *P. pentosaceus* JCM5890, *P. acidilactici* IAM1233, *L. plantarum* JCM1149, and *L. sake* IAM1900. Some of these tested indicator strains, including *L. monocytogenes*, showed semitransparent inhibitory circles with vague boundaries, while others, such as *P. pentosaceus* JCM5890, showed inhibitory circles that were too small for accurate measurement ($\sim$ 2 mm). The inhibitory circles against *P. pentosaceus* JCM2026 showed the clearest boundaries and had a measurable diameter ($\sim$ 6 mm). Therefore, *P. pentosaceus* JCM2026 was used as the indicator strain in the following experiments.

Although the peptide activity could be evaluated by measuring the diameter of the circle in the above-mentioned system, there was some question of reproducibility, as the size of the growth inhibition circle appeared to vary depending on whether a particular colony formed closer to the bottom or to the top of the agar layer. Thus, the activity assays were performed by individual

FIG. 1. Activities of representative NNK mutants with substitutions at C24 (A) and H42 (B). The antimicrobial activities were examined using a modified colony overlay screening assay with *P. pentosaceus* JCM2026. The mutant numbers are indicated under the colonies. As an internal control, the activity of wild-type pediocin PA-1 was assayed two times per plate (WT). The assay was performed on 35 mutant colonies per residue.

inoculation of *E. coli* colonies by use of a toothpick, which allowed standardization of the distance to the indicator strain growth layer. This modification improved the standard deviation of the inhibition circle size from 16% to 4% ($n = 10$).

NNK scanning of pediocin PA-1. Examples of the antimicrobial activities of mutants in which an NNK codon was introduced in place of the native C24 and H42 residues are shown in Fig. 1. For C24, 1 of 35 mutants exhibited nearly wild-type activity, while the remaining 34 tested mutants showed no detectable activity (Fig. 1A). In contrast, many of the H42 mutants exhibited antimicrobial activities, with some near wild-type levels (Fig. 1B).

NNK scanning was individually performed across all 44 pediocin PA-1 residues, and the number of mutants showing detectable activity was determined from the N terminus to the C terminus (Fig. 2). The values for Y2, G6, C9, C14, C24, W33, G37, and C44 were less than three (Fig. 2, solid bars), so these residues were examined as possible essential residues. All mutants which exhibited antimicrobial activities with mutations at these residues were sequenced (Table 1). Since NNK oligonu-

cleotides encode all 20 types of amino acids, it can be assumed that the wild-type sequence is represented in some of the mutant sets. Indeed, the wild-type sequence showed up at all eight residues. In addition, it turned out that we could not obtain mutants that showed activity with mutations at the G6, C9, C14, C24, and C44 residues. In contrast, Y2, W33, and G37 could be replaced by other amino acids, although the mutant activities were lower than that of the native peptide. For example, the Y2H mutant (in which tyrosine was replaced with histidine) showed 30 to 50% relative activities, while the W33L and G37A mutants displayed 50 to 70% relative activities. NNK encodes all 20 types of amino acids theoretically, but it was not clear how many types showed up in 35 tested mutants. To examine this matter, all mutants with mutations at C14 and G6 were sequenced (Fig. 3A and B). The results revealed some bias towards specific amino acids; for example, the C14 mutants contained seven leucine variants and only one glycine mutant. However, despite this bias, the results revealed that the antimicrobial activity was abrogated by the substitution of 11 different amino acids at C14 and 14 different amino acids at G6.

FIG. 2. Numbers of active mutants obtained by NNK scanning. The numbers of colonies showing detectable activities for the 35 tested mutants are shown for residues from the N terminus to the C terminus of pediocin PA-1. Data for residues that resulted in <3 active mutants are shown with solid bars, while those for residues resulting in >29 active mutants are shown with hatched bars.

TABLE 1. Analysis of mutants which showed detectable activities with substitutions at Y2, G6, C9, C14, C24, W33, G37, and C44 and which did not show any detectable activities with substitutions at K1, T8, G10, S13, G19, N28, and N41

Residue	No. of mutants showing activity	Mutant residue(s)	Relative activity ^a (%)
C14	1	C	>90
C24	1	C	>90
G37	2	G	>90
		A	50–70
C44	2	C	>90
Y2	3	Y	>90
		H	30–50
G6	3	G	>90
C9	3	C	>90
W33	3	W	>90
		L	50–70
K1	34	ND ^b	<10
N28	32	ND ^b	<10
T8	31	D, P	<10
G19	30	C, H	<10
N41	30	ND ^b	<10
G10	29	C, P, Q	<10
S13	29	C, P	<10

^a Relative activity was defined as the ratio of the diameter of the circle formed by the wild type to that formed by the mutant, multiplied by 100.

^b ND, residues at which the stop codon was introduced or the peptide was damaged by probable PCR error.

In contrast to the eight required residues, the NNK scanning analysis also identified residues at which almost all mutations resulted in mutants with some antimicrobial activity (Fig. 2). Residues K1, T8, G10, S13, G19, N28, and N41 had scores of >29 (Fig. 2, hatched bars) and were thus analyzed as presumptive variable residues. Mutants not showing antimicrobial activities with mutations at these variable residues were also analyzed. Of these mutants, three showed conversion of the native amino acid to cysteine (G19, G10, and S13; Table 1) and three showed conversion of the native residue to proline (T8, G10, and S13). Since NNK can encode a single stop codon, it is also possible that truncation mutants accounted for some of the inactive mutations at the variable residues. Indeed, the N41 mutant showing no activity was found to be a truncation mutant. Moreover, some of the nonfunctional mutants with mutations at N28 and N41 showed deletion of the native bases, and a K1 mutant showed damage at other residues, perhaps introduced by PCR error. Finally, the T8D, G10Q, and G19H mutants did not show any activity, perhaps due to residue-specific effects. For mutational analysis of representative variable residues, K1 and T8 were selected for analysis of all 35 mutants (Fig. 3C and D). As seen with the essential residues, there was some bias; specific mutations such as K1V and T8F were seen much more frequently than other mutations in these variable residues. Overall, antimicrobial activity was seen for 11 different mutations at K1 and 12 different mutations at T8 (neither of the scanning pools included the wild-type sequence). In addition, replacement with serine seemed to confer a relatively high activity, whereas replacement with phenylalanine and glycine conferred middling to low activities at both K1 and T8.

DISCUSSION

Previous studies reported only 24 types of substituted mutants for 15 different residues in pediocin PA-1 (13, 26, 30), creating a need for a more widespread functional screening of the other residues. However, the use of site-directed mutagenesis to introduce all 20 amino acids over all 44 residues would be prohibitively laborious. Alanine scanning (9) could be used, but this substitution is limited to alanine alone, perhaps leading to biased results for residues relatively similar to alanine. To overcome these issues, we generated pediocin PA-1 mutants by individual substitution of NNK oligonucleotides in place of each native codon. The introduction of NNK instead of NNN as a codon avoided the generation of two of three stop codons and improved codon usage to theoretically cover all 20 amino acids (10, 39). However, only 35 mutants per residue were evaluated in this work, covering 11 to 15 different amino acids. Moreover, although NNK provides the most equitable codon distribution (10), codon degeneration remains an issue, and there may be amino acid bias. Indeed, phenylalanine replaced T8 in 8 of the 35 tested mutants. If a biased active mutant emerged for a given residue, the mutant score would be overestimated, while the emergence of a biased inactive mutant would lead to underestimation of this value. However, although the values depicted in Fig. 2 may not strictly represent the functional importance of each residue, this work allowed the identification of eight candidate essential residues. These included four cysteine residues previously reported as essential (30), indicating that the NNK scanning method was effective for estimating functional responsibility.

In terms of evaluating bacteriocin activity, liquid dilution methods such as the microtiter plate assay system (35) are considered relatively exact but may be prohibitively laborious for the evaluation of large numbers of samples. Thus, the previously reported colony overlay screening technique (31) was modified to reduce the variability in results and then applied to this study. It is possible that the activity might have been affected by diffusion constants, peptide solubility, pH conditions, or secreted peptide amounts. However, mutational analysis of W33 in sakacin P using the microtiter plate assay system revealed that the W33L mutant showed 38 to 67% activity, while the other mutants showed much lower activities (16). Although future studies using liquid dilution may be necessary to confirm our present findings, the consistency between the previous findings and our present results indicates that the overlay method is effective for evaluating the activities of antimicrobial peptides.

Here we compared the functional responsibilities of the residues, leading to the identification of eight candidate essential residues and seven candidate variable residues. To assess the importance of the identified essential and variable residues in related peptides, we performed multiple sequence alignments (Table 2) among all group 1 pediocin-like bacteriocins previously classified by Fimland et al. (16). With the exception of C24, seven of the eight essential residues identified in our study were highly conserved across the examined sequences. Interestingly, of the seven residues identified as being variable in pediocin PA-1, two (K1 and N28) were highly conserved in the other sequences. However, other bacteriocins such as lactococcin MMF II contained threonine instead of lysine at

FIG. 3. Activity distributions for mutants with substitutions at C14 (A), G6 (B), K1 (C), and T8 (D). The relative activities of all 35 mutants were calculated, and the mutants were ranked by these values. The amino acid type for each mutant is shown on the horizontal axis. *, residues at which the stop codon was introduced or the peptide was damaged by probable PCR error.

TABLE 2. Multiple sequence alignment among group 1 pediocin-like bacteriocins classified by Fimland et al. (16)^a

Bacteriocin	Sequence																																															
Pediocin PA-1.....					K	Y	Y	G	N	G	V	T	C	G	K	H	S	C	S	V	D	W	G	K	A	T	T	C	I	I	N	N	G	A	M	A	W	A	T	G	G	H	Q	G	N	H	K	C
Enterocin A.....	T	T	H	S	G	K	Y	Y	G	N	G	V	Y	C	T	K	N	K	C	T	V	D	W	A	K	A	T	T	C	I	A	G	M	S	I	G	G	F	L	G	G	A	I	P	G	K	C	
Divercin V41.....					T	K	Y	Y	G	N	G	V	Y	C	N	S	K	K	C	W	V	D	W	G	Q	A	S	G	C	I	G	Q	T	V	V	G	G	W	L	G	G	A	I	P	G	K	C	
Coagulin.....					K	Y	Y	G	N	G	V	T	C	G	K	H	S	C	S	V	D	W	G	K	A	T	T	C	I	I	N	N	G	A	M	A	W	A	T	G	G	H	Q	G	T	H	K	C
Sakacin P.....					K	Y	Y	G	N	G	V	H	C	G	K	H	S	C	T	V	D	W	G	T	A	I	G	N	I	G	N	N	A	A	N	W	A	T	G	G	N	A	G	W	N	K		
Listeriocin 743A.....					K	S	Y	G	N	G	V	H	C	N	K	K	K	C	W	V	D	W	G	S	A	I	S	T	I	G	N	N	S	A	A	N	W	A	I	G	G	A	A	G	W	K	S	
Mundticin KS.....					K	Y	Y	G	N	G	V	S	C	N	K	K	G	C	S	V	D	W	G	K	A	I	G	I	I	G	N	N	S	A	A	N	L	A	T	G	G	A	A	G	W	K	S	
Piscicollin 126.....					K	Y	Y	G	N	G	V	S	C	N	K	N	G	C	T	V	D	W	S	K	A	I	G	I	I	G	N	N	A	A	N	L	T	T	G	G	A	A	G	W	N	K	G	
Sakacin X.....					K	Y	Y	G	N	G	L	S	C	N	K	S	G	C	S	V	D	W	S	K	A	I	S	I	I	G	N	N	A	V	A	N	L	T	T	G	G	A	A	G	W	K	S	
Leucocin C.....					K	N	Y	G	N	G	V	H	C	T	K	K	G	C	S	V	D	W	G	Y	A	W	T	N	I	A	N	N	S	V	M	N	G	L	T	G	G	N	A	G	W	H	N	
Essential residues.....					Y					G				C				C																														
Variable residues.....					K									T		G		S					G																									

^a The candidate essential residues and candidate variable residues identified in this study are shown below the alignment. Residues that are the same as those in pediocin PA-1 are shown in bold. The peptide sequences were derived from the database under the following accession numbers: enterocin A, X94181; divercin V41, AJ224003; coagulin, AF300457; sakacin P, X75081; listeriocin 743A, AF330821; mundticin KS, AB066267; piscicollin 126, AF275938; sakacin X, AY206863; and leucocin C, P81053.

the N-terminal end (accession number P83002), suggesting that the K1 residue substitution could retain activity (discussed below). The N28 residue varied slightly depending on whether the peptides formed one disulfide bridge, such as the case in sakacin P, or two disulfide bridges (i.e., enterocin A and divercin 41), suggesting that N28 is variable depending on the number of disulfide bridges formed in a particular bacteriocin. Future work will be required to examine this hypothesis, perhaps by introducing NNK in place of N28 in sakacin P versus enterocin A (or other members of the different disulfide bridge-containing bacteriocins).

Our results revealed an essential function for residue G6, which is located within the conserved YGNVG motif. The replacement of G6 with 14 different amino acids caused a functional loss at G6. In contrast, mutations at other positions of the conserved motif, such as G4T, N5Y, and V7M, retained antimicrobial activity (data not shown), suggesting that G6 is likely to play an especially important role within the YGNVG motif. GNGV is predicted to take on a β -turn structure (3, 42); it matches the type I' consensus sequence with nearly 80% homology, suggesting that this motif may take on a type I' β -turn structure in the peptide (30). When the homology values of all predicted mutants were calculated on the basis of the type I' consensus sequence (25; data not shown), the results revealed that the replacement of G6 with any other amino acid drastically decreased the value from 79% to 42%. Thus, any G6 substitution is likely to destroy the β -turn, leading to activity loss. Future work, such as nuclear magnetic resonance structure or circular dichroism spectral (17, 27, 45) analyses of inactive mutants will be required to determine whether similar structural explanations account for the functional requirements of the Y2, W33, and G37 residues.

Within the variable residues, mutants harboring cysteine substitutions at G10, S13, and G19 did not show any antimicrobial activity. This is possibly because the added cysteine caused inefficient formation of the C9-C14 and C24-C44 disulfide bonds, which are considered to be essential for activity (5, 12, 32). Antimicrobial activity was detected in 11 different mutants containing substitutions at K1. This was unexpected, as K1 is a highly conserved residue and because positively charged residues at the N terminus are reportedly important for recognizing and binding the target bacterial membrane (4, 28). However, a previous study showed that positively charged

residues such as K11 and H12 combine with K1 to form a positively charged patch in sakacin P (N24C + 44C) (42), suggesting that there might be functional redundancy in these positively charged residues. For T8, 12 substitutions were active and 2 substitutions (proline and aspartic acid) were inactive. This residue is predicted to form part of the antiparallel β -sheet structure (27). Proline and aspartic acid are considered strong β -sheet breakers, so their substitution might cause structural disruption and a loss of activity (6). This notion is further supported by the observation that replacement of T8 with valine, isoleucine, and tyrosine, which are thought to favor β -sheet structures, conferred >75% activity, suggesting that T8 substitutions are tolerated if they maintain the β -sheet structure.

Recently, the emergence of strains with resistance against class IIa bacteriocins and nisin has caused great concern regarding the use of these peptides as food preservatives (8, 19, 20, 43). The NNK scanning analysis reported herein provides systematic information on the functional responsibility of each residue spanning the whole pediocin PA-1 molecule and allowed the elucidation of essential and variable residues within this bacteriocin. This work may thus form the basis for more effective antimicrobial peptide engineering and the development of improved peptides capable of acting on resistant strains.

ACKNOWLEDGMENTS

We thank Y. Husimi, K. Nishigaki, H. Gotoh, N. Nemoto, M. Nakayama, T. Aita, L. Futatsugi, Y. Ishijima, Y. Honda, H. Nakajima, and M. Sekine for useful discussions and National Institute of Genetics and the IAM Culture Collection for providing bacterial strains. We also thank S. Butler for a critical reading of the manuscript.

This work was performed as part of the Rational Evolutionary Design of Advanced Biomolecules (REDS) Project, Saitama Prefecture Collaboration of Regional Entities for the Advancement of Technological Excellence, supported by JST.

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