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Synthetic Porcine Lactoferricin with a 20-Residue Peptide Exhibits Antimicrobial Activity against *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*

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Lactoferricins are positively charged, highly basic peptides that are generated upon gastric pepsin cleavage of various lactoferrins. In the past decade, there has been active investigation of the key antimicrobial segments of the various shorter synthetic bovine and human lactoferricins, but not in porcine lactoferricin. These studies have demonstrated the distinct solution structures of lactoferricin in bovine and human and established the multifunctional nature of the antibacterial, antifungal, antiendotoxin, and antiviral activities of lactoferricins. However, the protective effects of porcine lactoferricins have yet to be elucidated. In the present study, a series of synthetic derivatives of porcine, bovine, and human lactoferricins with 20-residue and 9-residue peptides were prepared to investigate their antimicrobial nature. We found that the 20-residue porcine lactoferricin (LFcin P-20) displayed antimicrobial activity against *Escherichia coli* ATCC25922, *Staphylococcus aureus* ATCC25923, and *Candida albicans* ATCC14053. The minimal inhibitory concentrations and minimal bactericidal concentrations of LFcin P-20 ranged from 12 to 25 μM when tested in bacteria and fungi. LFcin P-20 was 4 times more effective than human lactoferricin (LFcin H-20), but slightly less effective than bovine lactoferricin (LFcin B-20).

KEYWORDS: Porcine lactoferricin; antimicrobial activity; synthetic peptide; minimal inhibitory concentration (MIC); minimal bactericidal concentration (MBC)

INTRODUCTION

Lactoferrin (LF) has antimicrobial properties, activates gene transcription, regulates iron absorption, and promotes the production of macrophages, granulocytes, and neutrophils (*1*−*3*). Lactoferrin is a cationic protein exhibiting bacteriostatic and bactericidal functions that contribute to both systemic and mucosal immune defense (*4*). It serves as a scavenger of free iron and acts to deprive microorganisms of that essential nutrient (*5*). Lactoferrin destabilizes the outer membrane of Gram-negative bacteria by inducing the release of lipopolysaccharides (LPSs) from their cell walls, which leads to depolarization of the cytoplasmic membrane (*6*). LF is also capable of reducing LPS-induced proinflammatory cytokine release by monocytes, as well as blocking the LPS priming of neutrophils for superoxide production (*7*).

Lactoferricin, which is generated upon gastric pepsin cleavage of LF, has been demonstrated to have a much higher antimicrobial efficacy than intact lactoferrin (*8*). Lactoferricin is composed of a cationic distorted antiparallel β-sheet joined by a disulfide bridge (*9*). LF crystallography has revealed that this peptide structure forms a loop with a cationic charge at the tip. Lactoferricin derived from bovine LF is active against a wide range of Gram-negative and Gram-positive bacteria, fungi, protozoa, and tumors (*10*−*13*). Bovine lactoferricin (LFcin B) consists of 25 amino acid residues (17−41 in bovine LF). It has been demonstrated that several synthetic shorter derivatives of only 11−15 core residues of bovine lactoferricin, which were devoid of the disulfide bond, also exhibited strong bactericidal activity (*14*, *15*). Moreover, spectroscopic studies have shown that the core hexapeptide of LFcin B caused similar membrane disturbances in model membranes (*6*, *16*).

Porcine lactoferrin is secreted in the colostrum at a higher level than other mammalian lactoferrin (*17*, *18*). The structure and functions of bovine and human LF have been well characterized (*1*, *19*), but not porcine LF or porcine lactoferricin (LFcin P). In a previous study, we generated an expression system using recombinant methylotrophic yeast, *Pichia pastoris*, which harbored the porcine LF gene driven by the inducible promoter of the alcohol oxidase 1 gene (*AOX1*) and the yeast

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Table 1. Amino Acid Sequence and Molecular Mass for Synthetic Lactoferricins from Different Species

lactoferricin	location	molecular mass (g/mol)	amino acid sequence[a]
LFcin P-20	18–37	2590.1	K*C*RQWQSKIRRTNPIF*C*IRR
LFcin B-20	18–37	2448.1	K*C*RRWQWRMKKLGAPSIT*C*V
LFcin H-20	19–38	2433.9	K*C*FQWQRNMRKVRGPPVS*C*I
LFcin P-9	20–28	1257.5	RQWQSKIRR
LFcin B-9	20–28	1374.7	RRWQWRMKK
LFcin H-9	21–29	1293.5	FQWQRNMRK

[a] Amino acid sequences are listed in the direction from the NH_2 terminus to the COOH terminus.

α-mating factor signal peptide (*17*). Significant levels of secreted porcine LF were achieved in culture medium of the yeast transformants. The recombinant porcine LF was found to be similar in glycosylation pattern and antimicrobial function to natural LF derived from sow milk (*17*). It has been demonstrated that piglets that consume sufficient colostral milk exhibit enhanced resistance to diarrhea, respiratory tract infections, infection-induced wheezing, and anemia after weaning (*20*−*22*). Therefore, we hypothesize that porcine lactoferrin and its pepsin hydrolysate, LFcin P, may play an important role in antimicrobial action, immunomodulation, and iron-binding functions when piglets are raised in a poor growing environment.

In the present study, we identified the porcine lactoferricin region by sequence homologous BLAST among eight species including caprine, bovine, buffalo, equine, camel, human, mouse, and rat. We synthesized 20 and 9 residues of LFcin P, as well as LFcin B and human lactoferricin (LFcin H), to investigate the actual antimicrobial domain in the LFcin P core sequence and to compare the antimicrobial activity of porcine, bovine, and human lactoferricins.

MATERIALS AND METHODS

Bacterial and Fungal Strains. The strains used were Gram-negative bacterium *Escherichia coli* ATCC25922, Gram-positive bacterium *Staphylococcus aureus* ATCC25923, and the fungal strain *Candida albicans* ATCC14053. All strains were stored at −80 °C, and further grown in tryptic soy broth (TSB; Difco, France), pH 6.8, at 37 °C. All tests were performed with cells in the exponential growth phase, and the cell suspension was adjusted in 1% Bacto peptone water (BPW; Becton Dickinson Co., Sparks, MD) to give a final density of 1×10^6 colony-forming units (CFUs)/mL.

Synthesis of Peptides. The N-terminal 45-amino-acid sequences of porcine, bovine, buffalo, caprine, equine, camel, human, mouse, and rat lactoferrins with a putative antimicrobial domain were identified and compared by the MegAlign clustal method (DNASTAR Inc., Madison, WI). The 20-residue porcine lactoferricin derivatives (LFcin P-20) containing the two-amino-acid-deleted region and the corresponding fragments from bovine (LFcin B-20) and human (LFcin H-20) (see **Table 1** for detailed sequences) were synthesized for antimicrobial determination. The nine-residue core peptide of bovine lactoferricin (LFcin B-9) and its corresponding sequences from porcine (LFcin P-9) and human (LFcin H-9) were also tested in this study. All peptides were synthesized with a 9050 Plus PepSynthesizer (Milligen, Milford, MA) using solid-phase fluorenylmethoxycarbonyl (FMOC) chemistry. The peptides were analyzed and purified by reversed-phase HPLC on a Waters 600E chromatograph (Millipore, Billerica, MA) with UV detection at an optical density (OD) of 254 nm, according to previous reports (*23*, *24*). The peptides were dissolved in doubly distilled sterile water and stored at −80 °C until use.

Antimicrobial Activity Testing. The minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) of the various lactoferricins were determined according to previously described methods (*17*, *24*). Briefly, a standard microdilution technique with an inoculum of 1×10^6 CFUs/mL was used. The MIC of the various lactoferricins was determined in 1% BPW after incubation at 37 °C for 12 h. The concentration for the various lactoferricins ranged from 8 to 512 μg/mL. The MIC was defined as the lowest concentration at which bacterial growth was inhibited. For determination of the MBC, aliquots of 10 μL were transferred onto agar plates. The plates were incubated overnight at 37 °C, and the number of colony-forming units was determined. The MBC was set as the lowest concentration that reduced the number of CFUs by 99%. All assays were performed in triplicate.

Table 2. MICs (μg/mL) (Concentration (μM) in Parentheses) of the Various Lactoferricins against *E. coli*, *S. aureus*, and *C. albicans*[a]

	strain		
lactoferricin	*E. coli* ATCC25922	*S. aureus* ATCC25923	*C. albicans* ATCC14053
LFcin P-20	64 (25)	32 (12)	32 (12)
LFcin B-20	16 (7)	16 (7)	8 (3)
LFcin H-20	256 (105) $P = 0.009$[b]	128 (53) $P = 0.013$	256 (105) $P = 0.006$
LFcin P-9	512 (407)	256 (204)	512 (407)
LFcin B-9	32 (23)	16 (12)	32 (23)
LFcin H-9	512 (396) $P = 0.009$	256 (198) $P = 0.019$	256 (198) $P = 0.008$

[a] The MIC values of the six peptides from porcine, bovine, and human lactoferricin derivatives against *E. coli*, *S. aureus*, and *C. albicans* are the modes of three to six different experiments. [b] A P value of <0.05 was considered as statistically significant (Wilcoxon test).

Table 3. MBCs (μg/mL) (Concentration (μM) in Parentheses) of the Various Lactoferricins against *E. coli*, *S. aureus*, and *C. albicans*[a]

	strain		
lactoferricin	*E. coli* ATCC25922	*S. aureus* ATCC25923	*C. albicans* ATCC14053
LFcin P-20	64 (25)	64 (25)	32 (12)
LFcin B-20	32 (13)	32 (13)	16 (7)
LFcin H-20	512 (210) $P = 0.008$[b]	256 (105) $P = 0.014$	>512 (>210) $P = 0.004$
LFcin P-9	>512 (>407)	>512 (>407)	>512 (>407)
LFcin B-9	64 (47)	32 (23)	64 (47)
LFcin H-9	>512 (>396) $P = 0.019$	512 (396) $P = 0.014$	>512 (>396) $P = 0.017$

[a] The MBC values of the six peptides from porcine, bovine, and human lactoferricin derivatives against *E. coli*, *S. aureus*, and *C. albicans* are the modes of three to six different experiments. [b] A P value of <0.05 was considered as statistically significant (Wilcoxon test).

Scanning Electron Microscopy Observation. The test trains of *E. coli*, *S. aureus*, and *C. albicans* were individually grown to midlogarithmic phase in 2% BPW, and further diluted in 2% BPW to reach a final concentration of 2×10^6 CFUs/mL. Equal amounts of microbes and synthetic 20-residue porcine LFcin (dissolved in water) were mixed to give a total volume of 50 mL, yielding a final concentration of 100 μg/mL LFcin P-20 peptide. The solutions were placed in a water shaker (37 °C) for 2 h, and then centrifuged for 10 min at 1700*g*; the resulting pellet was kept for electron microscopy. The specimens were cut and washed in phosphate buffer prior to fixation under 0.7% glutaraldehyde and 1% OsO_4 and dehydration in 20%, 40%, 60%, 80%, 95%, and 100% ethanol sequentially; they were then further dried by a critical point dryer (Samdri-PVT-3B, Tousimis, Rockville, MD). The samples were observed under 10000−20000× magnification with a scanning electron microscope (JSM-6300, JEOL, Japan) as described (*17*).

Statistical Analysis. All values are expressed as modes (**Tables 2** and **3**). The MIC and MBC data for each strain were analyzed using the statistical software package SAS version 9.1 (SAS Institute, Cary, NC), and the significances of differences among groups were evaluated using the Wilcoxon test (*25*). A P value of <0.05 was considered as significant.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Porcine	A	P	K	K	G	V	R	W	C	V	I	S	T	A	E
Bovine	A	P	R	K	N	V	R	W	C	T	I	S	Q	P	E
Buffalo	A	P	R	K	N	V	R	W	C	T	I	S	Q	P	E
Caprine	A	P	R	K	N	V	R	W	C	A	I	S	P	P	E
Equine	A	P	R	K	S	V	R	W	C	T	I	S	P	A	E
Camel	A	S	K	K	S	V	R	W	C	T	T	S	P	A	E
Human	G	R	R	R	S	V	Q	W	C	A	V	S	Q	P	E
Mouse	A	K	A	T	T	V	R	W	C	A	V	S	N	S	E
Rat	R	I	D	T	V	V	R	W	C	T	I	S	R	N	E

	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Porcine	Y	S	K	C	R	Q	W	Q	S	K	I	R	R	T	N
Bovine	W	F	K	C	R	R	W	Q	W	R	M	K	K	L	G
Buffalo	W	L	K	C	H	R	W	Q	W	R	M	K	K	L	G
Caprine	G	S	K	C	Y	Q	W	Q	R	R	M	R	K	L	G
Equine	A	A	K	C	A	K	F	Q	R	N	M	K	K	V	R
Camel	S	S	K	C	A	Q	W	Q	R	R	M	K	K	V	R
Human	A	T	K	C	F	Q	W	Q	R	N	M	K	R	V	R
Mouse	E	E	K	C	L	R	W	Q	N	E	M	R	K	V	G
Rat	A	Q	K	C	F	M	W	Q	E	M	L	N	K	A	G

	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
Porcine	-	P	-	I	F	C	I	R	R	A	S	P	T	D	C
Bovine	A	P	S	I	T	C	V	R	R	A	F	A	L	E	C
Buffalo	A	P	S	I	T	C	V	R	R	A	F	V	L	E	C
Caprine	A	P	S	I	T	C	V	R	R	T	S	A	L	E	C
Equine	G	P	S	V	S	C	I	R	K	T	S	S	F	E	C
Camel	G	P	S	V	T	C	V	K	K	T	S	R	F	E	C
Human	G	P	P	V	S	C	I	K	R	D	S	P	I	Q	C
Mouse	G	P	P	L	S	C	V	K	K	S	S	T	R	Q	C
Rat	V	P	K	L	R	C	A	R	K	Y	F	M	P	H	C

Figure 1. Comparison of the N-terminal amino acid sequences of porcine, bovine, buffalo, caprine, equine, camel, human, mouse, and rat lactoferrins. The figure indicates the residue number of bovine lactoferrin. Solid boxed areas contain residues that are identical, and dashed boxed areas indicate residues that have the same property of amino acids in the side chain. A dash represents the amino acid deletion. Accession numbers of lactoferrin amino acid sequences of various species in NCBI GenBank are as follows: NP_999527 (porcine), P24627 (bovine), O77698 (buffalo), AAV92908 (caprine), O77811 (equine), Q9TUM0 (camel), AAW71443 (human), P08071 (mouse), and XP_236657 (rat).

RESULTS

Sequence Alignment of the N-Terminal Lactoferricin Fragment among Nine Species. The N-terminal amino acid sequences of lactoferrin exhibiting antimicrobial activity in various species have been identified and are referred to as lactoferricin fragments (*10*, *19*). An alignment analysis of putative lactoferricin fragments among nine species was done using the clustal method (MegAlign software) (**Figure 1**). After alignment of the first 45 N-terminal amino acids of porcine lactoferrin with the corresponding sequences of eight species, the sequence identity (%) was as follows: caprine (53.3%), bovine (48.9%), buffalo (46.7%), equine (44.4%), camel (44.4%), human (42.2%), mouse (35.6%), and rat (33.3%). The first five amino acids of the mature lactoferrins from porcine, bovine, buffalo, caprine, equine, and camel contain two basic amino acids (Arg or Lys), whereas the proteins from mouse and rat do not. This region is important for interaction and bactericidal ability (*14*, *15*). It has been well documented that LFcin B comprises residues 17−41 of the mature bovine lactoferrin and contains a disulfide bond between the cysteine residues in positions 19 and 36 (*8*, *10*).

LFcin sequences of eight species corresponding to LFcin B could readily be identified on the basis of the conserved residues Lys 18, Cys 19, Gln 23, Pro 32, and Cys 36 and the two basic residues (Arg and Lys) in positions 27 and 28 and 38 and 39 as shown in Figure 1. Only the porcine protein among the nine alignment sequences has two additional deletions within the lactoferricin fragment in positions 31 and 33 as shown in **Figure 1**.

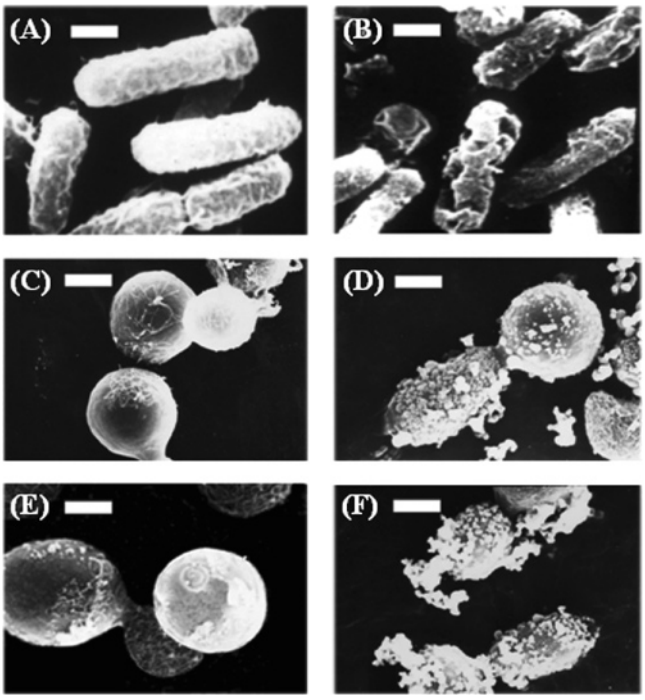

Figure 2. The antimicrobial activity of synthetic lactoferricin P-20 was directly observed by SEM. In the tested groups, *E. coli* (**B**), *S. aureus* (**D**), and *C. albicans* (**F**) were cultured to the logarithmic phase and resuspended in 2% PBW buffer for a further 2 h incubation with 100 μg/mL LFcin P-20 peptide. In the control groups, *E. coli* (**A**), *S. aurcus* (**C**), and *C. albicans* (**E**) were cultured in the same conditions but without LFcin P-20. The bars are equal to 500 nm.

Antimicrobial Activity of Lactoferricin Derivatives. In the 20-residue LFcin peptides, LFcin B-20 was the most active against Gram-negative and Gram-positive bacteria and fungi in the MIC range of 3−7 μM. In addition, LFcin P-20 also showed high growth-inhibitory activity against *E. coli*, *S. aureus*, and *C. albicans* in the MIC range of 12−25 μM when compared with LFcin H-20 (MIC = 53−105 μM) (**Table 2**, $P < 0.05$). However, in the nine-residue LFcin peptides, only LFcin B-9 maintained the same level of growth inhibition as LFcin B-20 against three tested bacteria in the MIC range of 12−23 μM. The MICs (198−407 μM) of LFcin P-9 and LFcin H-9 against *E. coli*, *S. aureus*, and *C. albicans* were 16 times less than the MIC of LFcin B-9, indicating that LFcin B-9 has much greater antimicrobial activity ($P < 0.05$).

The antimicrobial activity of various LFcin peptides of different sizes was further measured by determining the MBC in 1% BPW medium (**Table 3**). LFcin B-20 (7 μM MBC) and LFcin P-20 (12 μM MBC) exhibited good antimicrobial activity to the *C. albicans* yeast cells. According to the MBC, LFcin P-20 (25 μM) had a moderate antimicrobial effect on *E. coli* and *S. aureus*, but LFcin P-9 (>407 μM) did not. LFcin H-20 and LFcin H-9 exhibited the lowest antimicrobial activity against *E. coli*, *S. aureus*, and *C. albicans* (MBC values ranged from 105 to >396 μM).

Antimicrobial Effect of Porcine Lactoferricin P-20. Morphological changes were visualized by scanning electron microscopy (SEM) (**Figure 2**). In the *E. coli*-treated group (**Figure 2B**), a number of bacterium cells exhibited damage in their cell walls while others had membranes which had completely broken down. In the *S. aureus*-treated group (**Figure 2D**), an altered cell morphology consisting of membrane blisters was seen. In the *C. albicans*-treated group (**Figure 2F**), the yeast cells displayed severe morphological changes associated with

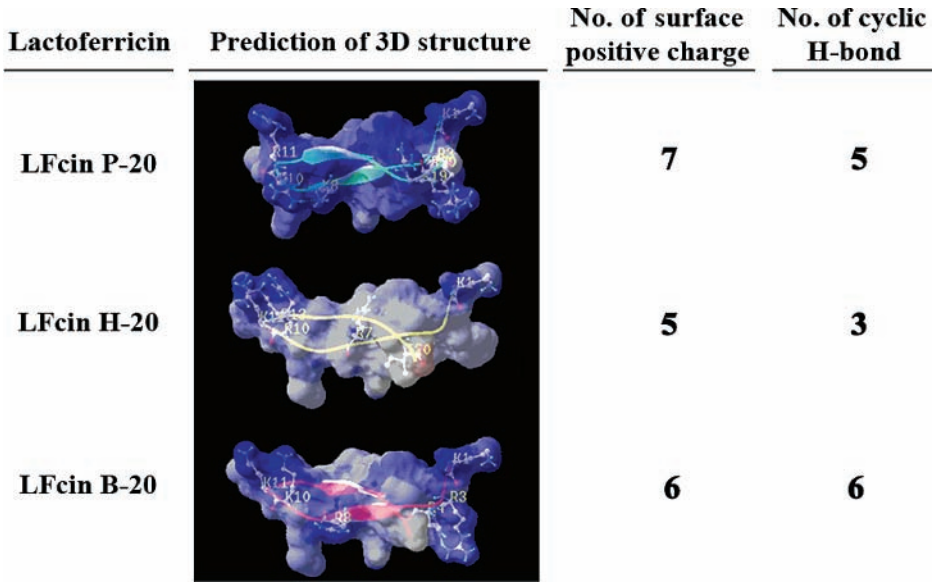

Figure 3. Stereoview of the predicted 3D structure of LFcin peptides. The backbones of the cyclic antiparallel structure of LFcin P-20, LFcin H-20, and LFcin B-20 are represented in green, yellow, and red colors, respectively. Regions of surface positive charge are indicated in gradient blue color (0–7 kT/e). Numbers of residues with surface positive charge and H-bonds presented in intrastrand cyclic formation are calculated by the program SWISS-MODEL (Deep View Swiss-PdbViewer 3.7). LFcin B-25 was used as a template for homology modeling (*9*).

a large amount of debris which had erupted through the cell membrane. SEM observations have revealed that LFcin P-20 treatment of *E. coli*, *S. aureus*, and *C. albicans* directly led to the disruption of the cell wall and breakdown of the outer membranes. This antimicrobial effect is similar to that of LFcin B (*6*, *26*), which made the cell permeable by disrupting the inner membrane, causing immediate development of electron-dense membrane blisters and subsequent depolarization and aggregation of cytoplasmic material.

DISCUSSION

In this work, we identified the potential porcine lactoferricin region by sequence homologous clustering and studied the antimicrobial activity of short derivatives of porcine, bovine, and human lactoferricins, corresponding to the cyclic fragment (residues 18−37) and the core sequence (residues 20−28) of mature bovine lactoferrin (**Table 1**). Of these peptide sequences, LFcin P was two amino acids shorter within the cyclic domain than lactoferricins obtained from the other eight species (**Figure 1**).

Current opinion on the mechanism of antimicrobial peptide action is focused on their interaction with the negatively charged elements in the membranes of susceptible bacteria (*6*, *14*, *27*). These elements are LPSs in Gram-negative bacteria, while it is assumed that these structures are lipoteichoic and/or teichoic acids in Gram-positive bacteria (*24*, *28*). The bactericidal and candidacidal effects of the antimicrobial peptides are thought to be caused by disrupting the cell membrane. In this study, most of the MBC values of lactoferricins (**Table 3**) were higher than the MIC values (**Table 2**). This might indicate that some of the peptides bind to the cell wall before gaining entry to the cell membrane, where they exert their lethal effect (*29*).

This is the first study to demonstrate the moderate antimicrobial ability in 20-residue LFcin P-20 against *E. coli*, *S. aureus*, and *C. albicans*, in contrast to earlier findings that only bovine lactoferricin showed significant bactericidal activity against bacteria tested (*30*, *31*). Strom et al. reported that the 15-residue porcine lactoferricin, a linear peptide, did not exhibit bactericidal activity (*31*). Cyclic bovine lactoferricin has been found to be more active in antimicrobial function than linear bovine lactoferricin (*29*). In our synthetic LFcin P-20 peptide, two cysteine residues at both ends might easily form a cyclic structure via a disulfide bridge, and numerous intrastrand hydrogen bonds (H-bonds) also contribute to stabilization of the speculated cyclic structure (**Figure 3**). Furthermore, this LFcin P-20 presented better amphiphilicity and more surface positive charge because of its additional six amino acids (IFCIRR; see **Table 1**) with hydrophobic, aromatic, and polar charged residues in the C-terminus when compared with the 15-residue porcine lactoferricin sequence reported by Strom et al. (*31*). Therefore, we proposed that the cyclic structure and the special residue properties of LFcin P-20 contributed to the antimicrobial activity. For comparison, we also studied the antimicrobial activity of short derivatives of nine-residue LFcin P-9, LFcin B-9, and LFcin H-9. We found that only LFcin B-9 had measurable growth-inhibitory and bactericidal activity against the three selected strains (**Tables 2** and **3**). In previous studies, it has been reported that tryptophans at positions 6 and 8 are crucial for the antimicrobial activity of lactoferricin derivatives and additional tryptophan residues introduced to a 15-residue LFcin B increase antimicrobial activity (*14*, *31*).

The development and persistence of multi-drug-resistant bacteria have created challenges to public health (*32*, *33*). Although the use of antibiotics in human medicine has influenced the emergence of antibiotic-resistant bacteria, the use of antibiotics in animal husbandry has markedly contributed to this critical problem as well (*34*, *35*). The swine industry alone uses an estimated 10.3 million pounds of antibiotics annually for nontherapeutic purposes, and the frequent use of antibiotics has been shown to select for resistance to high concentrations of antibiotics in both pathogenic and commensal bacteria in animals (*36*). Therefore, in the future, serious infections may be treated with systemically and topically active antibiotics developed from naturally occurring peptides. These peptides constitute an important part of the innate immunity of animals and insects, such as lactoferricins, cecropins, defensins, and magainins (*37*). This first line of defense is characterized by

amphipathic peptides consisting of less than 60 amino acids with a net positive charge (*38*).

In conclusion, the synthetic 20-residue porcine lactoferricin peptide exhibited antimicrobial activity against *E. coli*, *S. aureus*, and *C. albicans* according to MIC and MBC determinations and direct SEM observation. Short antimicrobial peptides are both easy to synthesize chemically and simple to purify. A limited amount of time and solvents are needed, which is advantageous from both economical and environmental points of view. Having carefully investigated the sequences of naturally occurring antimicrobial peptides, LFcin P-20 as well as bovine LFcin B-20 and LFcin B-9, we can conclude that the high antimicrobial activity of such lactoferricin derivates makes them potential novel agents for both topical and systemic treatment of bacterial infections. Further elucidation of the clinical efficacies and mechanisms of action of LFcin P-20 will increase the value of this natural antimicrobial peptide.

ABBREVIATIONS USED

BPW, Bacto peptone water; CFUs, colony-forming units; H-bond, hydrogen bond; LF, lactoferrin; LFcin B, bovine lactoferricin; LFcin H, human lactoferricin; LFcin P, porcine lactoferricin; LPSs, lipopolysaccharides; MIC, minimal inhibitory concentration; MBC, minimal bactericidal concentration; SEM, scanning electron microscopy.

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LITERATURE CITED

(1) Nuijens, J. H.; van Berkel, P. H. C.; Schanbacher, F. L. Structural and biological actions of lactoferrin. *J. Mammary Gland Biol. Neoplasia* **1996**, *1*, 285−295.

(2) Brock, J. Lactoferrin: a multifunctional immunoregulatory protein. *Immunol. Today* **1995**, *16*, 417−419.

(3) Tomita, M.; Wakabayashi, H.; Yamauchi, K.; Teraguchi, S.; Hayasawa, H. Bovine lactoferrin and lactoferricin derived from milk: production and applications. *Biochem. Cell Biol.* **2002**, *80*, 109−112.

(4) Appelmelk, B. J.; An, Y. Q.; Gerts, M.; Thijs, B. G.; De Boer, H. A.; Maclaren, D. M.; De Graaff, J.; Nuijens, J. H. Lactoferrin is a lipid A-binding protein. *Infect. Immun.* **1994**, *62*, 2628−2632.

(5) Emery, T. Iron deprivation as a biological defense mechanism. *Nature* **1980**, *287*, 776−777.

(6) Ulvatne, H.; Haukland, H. H.; Olsvik, O.; Vorland, L. H. Lactoferricin B causes depolarization of the cytoplasmic membrane of *Escherichia coli* ATCC 25922 and fusion of negatively charged liposomes. *FEBS Lett.* **2001**, *492*, 62−65.

(7) Crouch, S. P. M.; Slater, K. J.; Fletcher, J. Regulation of cytokine release from mononuclear cells by the iron-binding protein lactoferrin. *Blood* **1992**, *80*, 235−240.

(8) Tomita, M.; Bellamy, W.; Takase, M.; Yamauchi, K.; Wakabayashi, H.; Kawase, K. Potent antibacterial peptides generated by pepsin digestion of bovine lactoferrin. *J. Dairy Sci.* **1991**, *74*, 4137−4142.

(9) Hwang, P. M.; Zhou, N.; Shan, X.; Arrowsmith, C. H.; Vogel, H. J. Three-dimensional solution structure of lactoferricin B, an antimicrobial peptide derived from bovine lactoferrin. *Biochemistry* **1998**, *37*, 4288−4298.

(10) Bellamy, W.; Takase, M.; Wakabayashi, H.; Kawase, K.; Tomita, M. Antibacterial spectrum of lactoferricin B, a potent bactericidal peptide derived from the N-terminal region of bovine lactoferrin. *J. Appl. Bacteriol.* **1992**, *73*, 472−479.

(11) Isamida, T.; Tanaka, T.; Omata, Y.; Yamauchi, K.; Shimazaki, K.; Saito, A. Protective effect of lactoferricin against *Toxoplasma gondii* infection in mice. *J. Vet. Med. Sci.* **1998**, *60*, 241−244.

(12) Turchany, J. M.; Aley, S. B.; Gillin, F. D. Giardicidal activity of lactoferrin and N-terminal peptides. *Infect. Immun.* **1995**, *63*, 4550−4552.

(13) Yoo, Y. C.; Watanabe, S.; Watanabe, R.; Hata, K.; Shimazaki, K.; Azuma, I. Bovine lactoferrin and lactoferricin, a peptide derived from bovine lactoferrin, inhibit tumor metastasis in mice. *Jpn. J. Cancer Res.* **1997**, *88*, 184−190.

(14) Strom, M. B.; Haug, B. E.; Rekdal, O.; Skar, M. L.; Stensen, W.; Svendsen, J. S. Important structural features of 15-residue lactoferricin derivatives and methods for improvement of antimicrobial activity. *Biochem. Cell Biol.* **2002**, *80*, 65−74.

(15) Ulvatne, H.; Vorland, L. H. Bactericidal kinetics of 3 lactoferricins against *Staphylococcus aureus* and *Escherichia coli*. *Scand. J. Infect. Dis.* **2001**, *33*, 507−511.

(16) Schibli, D. J.; Hwang, P. M.; Vogel, H. J. The structure of the antimicrobial active center of lactoferricin B bound to sodium dodecyl sulfate micelles. *FEBS Lett.* **1999**, *446*, 213-217.

(17) Chen, H. L.; Lai, Y. W.; Yen, C. C.; Lin, Y. Y.; Lu, C. Y.; Yang, S. H.; Tsai, T. C.; Lin, Y. J.; Lin, C. W.; Chen, C. M. Production of recombinant porcine lactoferrin exhibiting antibacterial activity in methylotrophic yeast, *Pichia pastoris*. *J. Mol. Microbiol. Biotechnol.* **2004**, *8*, 141−149.

(18) Steijns, J. M.; van Hooijdonk, A. C. Occurrence, structure, biochemical properties and technological characteristics of lactoferrin. *Br. J. Nutr.* **2000**, *84*, S11−17.

(19) Nibbering, P. H.; Ravensbergen, E.; Welling, M. M.; van Berkel, L. A.; van Berkel, P. H.; Pauwels, E. K.; Nuijens, J. H. Human lactoferrin and peptides derived from its N terminus are highly effective against infections with antibiotic- resistant bacteria. *Infect. Immun.* **2001**, *69*, 1469−1476.

(20) Chu, R. M.; Wang, S. R.; Weng, C. N.; Pursel, V. G. Isolation and characterization of porcine milk lactoferrin. *Am. J. Vet. Res.* **1993**, *54*, 1154−1159.

(21) Hanson, L. A.; Korotkova, M.; Haversen, L.; Mattsby-Baltzer, I.; Hahn-Zoric, M.; Silfverdal, S. A.; Strandvik, B.; Telemo. E. Breast-feeding, a complex support system for the offspring. *Pediatr. Int.* **2002**, *44*, 347−352.

(22) Lee, W. J.; Farmer, J. L.; Hilty, M.; Kim, Y. B. The protective effects of lactoferrin feeding against endotoxin lethal shock in germfree piglets. *Infect. Immun.* **1998**, *66*, 1421−1426.

(23) Cheng, W. T. K.; Chen, B. C.; Chiou, S. T.; Chen, C. M. Use of nonautologous microencapsulated fibroblasts in growth hormone gene therapy to improve growth of midget swine. *Hum. Gene Ther.* **1998**, *9*, 1995−2003.

(24) Vorland, L. H.; Ulvatne, H.; Andersen, J.; Haukland, H. H.; Rekdal, O.; Svendsen, J. S.; Gutteberg, T. J. Antibacterial effects of lactoferricin B. *Scand. J. Infect. Dis.* **1999**, *31*, 179−184.

(25) van Winkelhoff, A. J.; Herrera, D.; Oteo, A.; Sanz, M. Antimicrobial profiles of periodontal pathogens isolated from periodontitis patients in the Netherlands and Spain. *J. Clin. Periodontol.* **2005**, *32*, 893−898.

(26) Yamauchi, K.; Tomita, M.; Giehl, T. J.; Ellison, R. T., III. Antibacterial activity of lactoferrin and a pepsin-derived lactoferrin peptide fragment. *Infect. Immun.* **1993**, *61*, 719−728.

(27) Hunter, H. N.; Demcoe, A. R.; Jenssen, H.; Gutteberg, T. J.; Vogel, H. J. Human lactoferricin is partially folded in aqueous solution and is better stabilized in a membrane mimetic solvent. *Antimicrob. Agents Chemother.* **2005**, *49*, 3387−3395.

(28) Schilling, O.; Vogel, A.; Kostelecky, B.; Natal da Luz, H.; Spemann, D.; Späth, B.; Marchfelder, A.; Tröger, W.; Meyer-Klaucke, W. Enhancement of endotoxin neutralization by coupling of a C12-alkyl chain to a lactoferricin-derived peptide. *Biochem. J.* **2005**, *385*, 135−143.

(29) Vorland, L. H.; Ulvatne, H.; Andersen, J.; Haukland, H.; Rekdal, O.; Svendsen, J. S.; Gutteberg, T. J. Lactoferricin of bovine origin

is more active than lactoferricins of human, murine and caprine origin. *Scand. J. Infect. Dis.* **1998**, *30*, 513−517.
(30) Chen, P. W.; Shyu, C. L.; Mao, F. C. Antibacterial activity of short hydrophobic and basic-rich peptides. *Am. J. Vet. Res.* **2003**, *64*, 1088−1092.
(31) Strom, M. B.; Rekdal, O.; Svendsen, J. S. Antibacterial activity of 15-residue lactoferricin derivatives. *J. Pept. Res.* **2000**, *56*, 265−274.
(32) Said-Salim, B.; Mathema, B.; Braughton, K.; Davis, S.; Sinsimer, D.; Eisner, W.; Likhoshvay, Y.; Deleo, F. R.; Kreiswirth, B. N. Differential distribution and expression of Panton-Valentine leucocidin among community-acquired methicillin-resistant *Staphylococcus aureus* strains. *J. Clin. Microbiol.* **2005**, *43*, 3373−3379.
(33) Murray, R. J.; Lim, T. T.; Pearson, J. C.; Grubb, W. B.; Lum, G. D. Community-onset methicillin-resistant *Staphylococcus aureus* bacteremia in Northern Australia. *Int. J. Infect. Dis.* **2004**, *8*, 275−283.
(34) Gorbach, S. L. Antimicrobial use in animal feed—time to stop. *N. Engl. J. Med.* **2001**, *345*, 1202−1203.
(35) Van den Boogard, A. E.; Stobberingh. E. E. Antibiotic usage in animals: impact on bacterial resistance and public health. *Drugs* **1999**, *58*, 589−607.
(36) Wegener, H. C. Antibiotics in animal feed and their role in resistance development. *Curr. Opin. Microbiol.* **2003**, *6*, 439−445.
(37) Boman, H. G. Gene-encoded peptide antibiotics and the concept of innate immunity: an update review. *Scand. J. Immunol.* **1998**, *48*, 15−25.
(38) Shai, Y.; Oren, Z. From "carpet" mechanism to *de-novo* designed diastereomeric cell-selective antimicrobial peptides. *Peptides* **2001**, *22*, 1629−1641.

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