

CHICKEN ANTIMICROBIAL PEPTIDES:
GENOME-WIDE IDENTIFICATION AND
FUNCTIONAL AND STRUCTURAL ANALYSIS

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ABSTRACT

With rapid emergence of antibiotic resistance, there is a pressing need for a new generation of anti-infective agents with a less likelihood of developing resistance. Antimicrobial peptides are regarded as promising therapeutic alternatives with desirable antimicrobial activities, favorable biological functions, and a low chance to develop resistance. Defensins and cathelicidins comprise two major families of cationic antimicrobial peptides in vertebrate animals. Recent availability of the chicken genome sequence provides an excellent opportunity to search for novel antimicrobial peptides with therapeutic potential. In this report, a genome-wide computational screen of the entire chicken genome led to identification of a gene cluster on chromosome 3q3.5-q3.7 containing thirteen different β -defensin genes as well as a cluster on chromosome 2p containing three cathelicidin genes. Among all novel chicken antimicrobial peptides identified, two cathelicidins, which we named fowlicidin-1 and -2, were chosen to study for their antibacterial properties. Both fowlicidins were found to display potent and salt-independent activities against a wide range of bacteria, including antibiotic resistant strains. Furthermore, both are capable of binding lipopolysaccharide (LPS) and neutralizing its inflammatory effects, suggesting that both peptides may be excellent candidates as novel antimicrobial and anti-sepsis agents. Based on the tertiary structure of fowlicidin-1, a series of truncation and substitution analogs were further synthesized and

tested for their antibacterial, cytolytic, and LPS-binding activities. A short peptide variant, namely fowlicidin-1(8-26), stands out with the highest therapeutic potential among all analogs, representing a safer and more attractive therapeutic candidate than the parent peptide.

CHAPTER I

INTRODUCTION

With rapid emergence of antibiotic-resistance pathogens in the last several decades, there is a pressing need for a new generation of antibiotics with a less likelihood of developing resistance for clinical use. One promising option to solve this problem is to develop cationic antimicrobial peptides as a new class of antimicrobial agents. Cationic antimicrobial peptides are a large group of gene-encoded molecules that have been discovered in all species of life, playing a critical role in innate host defense and disease resistance. Defensins and cathelicidins comprise two major families of antimicrobial peptides in vertebrate animals.

A number of defensin and cathelicidin peptides have potent and broad-spectrum activities toward microbes including antibiotic-resistant strains through their physical interaction with and disruption of microbial membranes. In addition to their ability to directly kill a wide range of bacteria, fungi, and enveloped viruses, defensins and cathelicidins plays an important regulatory role in innate immunity and other biological processes, including chemotaxis, dendritic cell activation, LPS-binding, and sperm maturation. Several antimicrobial peptides with therapeutic potential have been developed and are currently in clinical trials. The identification and functional and structural analysis of novel defensins and cathelicidins from different animal species will

help creating understanding the innate host defense mechanisms against microbial challenges and provide more potential therapeutic candidates against antibiotic-resistant infections.

The rest of this report is organized as follows: Chapter II is the literature review of cationic antimicrobial peptides, including their features, classification, antimicrobial activity, mechanisms of action, roles in innate immunity, other biological processes, and therapeutic potential. A genome-wide identification of a β -defensin gene cluster in the chicken is provided in Chapter III. Chapter IV describes the identification and functional characterization of three chicken cathelicidins with potent antimicrobial activity. Chapter V investigates the structure-activity relationship study of fowlicidin-1, a cathelicidin antimicrobial peptide in chicken. The summary, challenges and future prospects are discussed in Chapter VI.

CHAPTER II

LITERATURE REVIEW

The discovery of penicillin in 1929 by Alexander Fleming witnessed the beginning of a new era of antibiotics. Since then penicillin and other antibiotics, produced at industrial scale, have been widely used for the treatment of bacterial infections, as well as in many medical procedures including surgery and chemotherapy. The availability of antibiotics has revolutionized modern medicine and greatly improved human health by increasing the average human lifespan up to 10 years [1]. However, pathogens that are resistant to these antibiotics have emerged quickly in recent years due to the widespread availability and sometimes indiscriminate uses of antibiotics. For example, more than 95% of the strains of *Staphylococcus aureus* have been reported as resistant to penicillin [2]. Besides medical misuse, the application of antibiotics in animal feeds to prevent infections and promote growth is thought to be another major reason responsible for the emergence of resistance [3].

Conventional antibiotics kill bacteria by different mechanisms including inhibition of cell wall synthesis, interference with membrane function, inhibition of DNA replication and/or transcription, and inhibition of protein synthesis [4]. Different types of antibiotics have different cellular targets. For example, penicillin and cephalosporin bind to certain cell wall proteins and interfere with peptidoglycan cross-linking, and nalidixic acid

specifically binds to the enzymes necessary for DNA synthesis [4]. Mutations on the single or a limited number of specific targets could lead to emergence of new bacterial strains that are resistant to the actions of these antibiotics. Consequently, we are in an urgent need of novel drugs with different mechanism of actions than conventional antibiotics to combat bacterial infections in the near future.

Antimicrobial peptides, an important component of innate host defense, are currently being actively explored as promising antibiotic alternatives. Since a report on purothionin, a wheat peptide with antibacterial or antifungal activities in 1972 [5], hundreds of antimicrobial peptides have been discovered in virtually all species of life up to date. In addition to direct and broad-spectrum activity against bacteria, parasites, viruses, and fungi, antimicrobial peptides also play a critical regulatory role in innate and adaptive immune responses.

2.1 Features and classification of antimicrobial peptides

Antimicrobial peptides share several common features, although they have diverse sequences and structures [6-8]. These peptides are positively charged, normally with a net charge of +2 ~ +10, due to the presence of lysine and/or arginine molecules. Thus they are generally referred to as cationic peptides. Secondly, they are all small, ranging from 12 to 100 amino acids and are encoded by distinct genes. Thirdly, the peptides usually adopt amphipathic structures with hydrophilic segments being separated from

hydrophobic segments, which facilitate the interaction of the peptides with microbial membranes.

Based on the structure, antimicrobial peptides can be divided into four groups, namely peptides with α -helical, β -sheet, loops, and extended structures [6, 9]. The α -helical peptides are a class of the most widespread and best-studied cationic antimicrobial peptides. This class of peptides, such as SMAP29 [10], magainin [11], melittin [12], exhibit potent antimicrobial activity, but also display considerable cytotoxicity toward host cells. Another major class of cationic peptides is β -sheet peptides, which contain two or more β -sheets stabilized by 2 ~ 4 intramolecular disulfide bridges. Among all β -sheet peptides, there is one particular group that form the β -hairpin due to the presence of two disulfide bonds between the two antiparallel β -sheets, such as the horseshoe crab peptide, polyphemusin [13]. This group of peptides generally exhibit salt-independent antimicrobial activity against Gram-positive and Gram-negative bacteria [14]. The less commonly studied cationic peptides are loop and extended peptides. Loop peptides are cyclized with a single cysteine disulfide, such as cattle neutrophil bacterenecin [15]. Extended peptides are unstructured, but normally contain a high proportion of one or two amino acids, e.g. proline, tryptophan or histidine [9, 16]. Bovine neutrophil peptide indolicidin [17] (> 38% of tryptophan) and pig intestine peptide PR-39 [18] (> 49% of proline) are examples of this group of antimicrobial peptides with extended structures. Many of these extended peptides are unstructured in aqueous solution, but adopt their final conformations upon interacting with membranes.

2.2 *Defensins and cathelicidins*

In vertebrates, there are two major families of antimicrobial peptides, namely defensins and cathelicidins, based on their phylogenies. Defensins constitute a major family of cationic antimicrobial peptides that are widely distributed among living organisms [19-23]. Containing highly conserved 6 ~ 8 of cysteine residues, all defensins folded into 3 ~ 4 antiparallel β -sheet structures that are stabilized by 3 ~ 4 intramolecular disulfide bonds with or without an α -helix. Plant defensins are characterized by containing eight conserved cysteine residues forming four intramolecular disulfide bridges between Cys¹-Cys⁸, Cys²-Cys⁵, Cys³-Cys⁶, and Cys⁴-Cys⁷. The cysteine pairings in invertebrate defensins are Cys¹-Cys⁴, Cys²-Cys⁵, and Cys³-Cys⁶. In vertebrates, defensins contain a six-cysteine motif and are classified into three subfamilies, α -, β -, and θ -defensins, based on different disulfide bonding pattern (Figure 1). The cysteine pairings in α -defensins are Cys¹-Cys⁶, Cys²-Cys⁴, and Cys³-Cys⁵, while β -defensins forms the disulfide linkages between Cys¹-Cys⁵, Cys²-Cys⁴, and Cys³-Cys⁶. The θ -defensins are a class of circularly structured defensins with a cysteine spacing pattern of Cys¹-Cys⁶, Cys²-Cys⁵ and Cys³-Cys⁴.

The evolutionary relationships of defensins across plant, invertebrate and mammals are poorly understood, but highly conserved sequences, structures, and functions suggested that all defensins are evolutionarily related [24]. Especially in vertebrates, the defensin gene clusters on chromosomes are adjacent to each other, suggesting that all defensins are likely to arise from a common ancestor [25]. Hughes provided some

evidence indicating that vertebrate β -defensins are more like insect defensins rather than to vertebrate α -defensins [26]. Recent phylogenetic analyses from our research group revealed that α -defensins in glires and primates appeared after the divergence of mammalian species by a rapid duplication and positive selection [27], while θ -defensins are originated from α -defensins following the divergence of primates from other mammals [28].

β -defensins are the most ubiquitous defensins in vertebrate animals with a wide expression pattern including mucosal epithelial cells and phagocytes, while α -defensins are produced by leukocytes and Paneth cells of small intestine in the human, rhesus macaque, rabbit, guinea pig, mouse, and rat [27]. Till now, only three θ -defensins have been discovered in leukocytes [29] and bone marrow [30] of rhesus monkeys. Although the human has several θ -defensin genes, but no θ -defensin peptides are produced due to the presence of a premature stop codon in the upstream sequence that prevents translation [28]. Mature θ -defensin peptides contain only 18 amino acids and are cyclized by two α -defensin-like 9-amino acid sequences [31]. Generally defensins are stored as inactive precursors in cells. In response to microbial infections, active mature defensins will be released following proteolytic processing and target microbes locally [31]. All defensin precursors consist of a hydrophobic signal sequence, a pro-sequence, and a mature defensin peptide containing six-cysteine motif at the C-terminus [19]. To be biologically active, β -defensins need to be processed from the precursor by proteolytic enzymes, although enzymes for cleavage are yet to be identified. Recently, the processing enzymes

to release active α -defensin peptides, such as metalloproteinase Matrilysin (MMP-7) in mice [32] and trypsin in human [33], have been identified.

Cathelicidins are a family of highly diverse antimicrobial peptides that share a common ancestor [34], and they have been found in fish [35, 36], birds [37] and mammals [38-44]. The first member of the cathelicidin family was isolated in early 1990s from porcine neutrophils [34]. Similar to defensins, cathelicidins are also stored as inactive precursors in cells. All cathelicidin precursors share highly conserved pre- and pro-sequences (“cathelin”) at the N-terminus, followed by diversified, cationic mature sequences at the C-terminus [8, 45, 46] (Figure 2). Generally, cathelicidin genes contain four exons and three introns [47]. The first three exons encode the pre- and pro-sequences, whereas the fourth exon encodes the mature peptide sequence [47]. The presequences range from 29 ~ 30 amino acids that act as signal peptides for secretion. The length of the “cathelin” domain is around 94 ~ 114 amino acids that contain four invariant cysteines forming two disulfide bridges [48]. The highly diverse C-terminal mature peptides are around 12 ~ 100 amino acids [8].

Cathelicidins are mainly present in the granules of phagocytes, and also has been found in mucosal epithelial cells and keratinocytes as well [8, 45, 46]. The biologically active mature peptides are processed by proteolytic enzymes and released upon infections. Studies with bovine [49] and pig [50] cathelicidins indicated that elastase is the enzyme responsible for cleaving the pro-sequence and releasing the mature peptides to exert antimicrobial activity. The highly conserved “cathelin” domain of cathelicidins is

negatively charged and its biological function is still not clear. Recently, the “cathelin” domain of human cathelicidin precursor LL-37/hCAP-18 was found to kill bacterial strains that are resistant to mature peptide, suggesting a complementary antibacterial activity of the “cathelin” domain with mature peptides [51].

2.3 Antimicrobial activity

Antimicrobial peptides are so named because of their broad-spectrum antimicrobial activities [7, 16]. In addition to killing Gram-negative and Gram-positive bacteria [52, 53], antimicrobial peptides are active against enveloped viruses [54, 55], fungi [56-58], parasite [53, 59, 60], and even cancer cells [61, 62]. In general, antimicrobial peptides kill most bacteria in micromolar concentrations, including antibiotic-resistant strains. For example, a sheep cathelicidin, SMAP-29, kills *Pseudomonas aeruginosa* and a methicillin-resistant strain of *Staphylococcus aureus* with a minimum inhibitory concentration (MIC) of 0.25 μ M and 1 μ M, respectively [8]. The antibiotic-resistance mechanisms that limited the use of conventional antibiotics don't affect antimicrobial peptides due to their non-specific mode of action. But they are not perfect for all bacterial strains. For instance, the MICs of SMAP-29 and a cattle cathelicidin BMAP-29 against *Burkholderia cepacia* are ≥ 32 μ M [8]. Melittin had minimal effect on several strains of *Pseudomonas* and *Serratia* [63].

Rapid bactericidal action is one of the essential features of an effective therapeutic agent because bacteria can double every 20-30 min under appropriate conditions. A

number of antimicrobial peptides have been reported to kill microbes quickly within several minutes in vitro. At the concentration of 0.06 µg/ml, Factor C protein isolated from the horseshoe crab, *Carcinoscorpius rotundicauda*, achieved 90% reduction in viable counts against *Pseudomonas aeruginosa* within 7 min [64]. Similarly, pig cathelicidin, Protegrin-1, reduced the number of viable methicillin-resistant *S. aureus* (MRSA) or *P. aeruginosa* up to three log units in less than 15 minutes [65].

However, factors like salt and serum are known to alter the intensity of the microbial killing activity of antimicrobial peptides [66]. Kandasamy *et al.* [67] studied the effect of salt on the interactions of frog antimicrobial peptide, magainin, with zwitterionic lipid bilayers, indicating that the binding of peptide to the lipids is stronger at lower concentrations of salt. The peptide interacted quickly with the membrane and caused lipid disorder, but this effect was diminished as the salt concentration increased [67]. Nevertheless, a few peptides displayed salt-independent antimicrobial activities. For example, a sheep cathelicidin, SMAP-29, was more effective against bacteria in the presence of 100 mM NaCl [68], and LL-37/hCAP18 retained its killing efficacy against vancomycin-resistant enterococci in both low- and high- salt media [69, 70]. Human β -defensin 3 (hBD-3) exerted potent antimicrobial activity at physiologic concentrations of NaCl (150 mM), but in vitro assay showed that the activity was inhibited in the presence of 20% human serum [71]. It is likely that the proteases or certain proteins in the serum reduced the availability of the peptide to bacteria, and the high ionic strength or salt properties could also contribute to its inhibition [72].

Some antimicrobial peptides showed synergy with conventional antibiotics and other antimicrobial peptides in killing bacteria. For example, protegrin-1 is synergistic with cathelicidins from bovine (indolicidin) and human (LL-37) in killing antibiotic-resistant bacteria including *P. aeruginosa* and methicilin-resistant *S. aureus* efficiently [73]. Citropin 1.1, an antimicrobial peptide derived from the Australian tree frog, *Litoria citropa*, demonstrated synergy with conventional antibiotics like clarithromycin, doxycycline, and rifampicin against rifampicin-resistant *Rhodococcus equi* [74].

In addition to killing bacteria, many antimicrobial peptides exhibit direct antifungal and antiparasitic activities as well. For example, plant defensins, α -, β - and γ -thionins are selective against fungi [6, 75]. Insect defensins, heliomicin [76] and gallerimycin [77], also display similar antifungal activities [78]. In addition, the human cathelicidin LL-37 [79], sheep cathelicidin SMAP-29 [56], and mouse cathelicidin mCRAMP [79] are effective against *C. albicans*. Gomesin, purified from the hemocytes of the tarantula spider *Acanthoscurria gomesiana*, affected the viability of the parasite *Leishmania amazonensis* at 2.5 μ M [80]. Insect antimicrobial peptide, cecropin A, has a lethal effect on the parasite, *Trypanosoma cruzi*, at 150–500 μ M [81].

Moreover, certain antimicrobial peptides have antiviral activities. Horseshoe crab antimicrobial peptide, T22 ([Tyr^{5,12}, Lys⁷]-polyphemusin II), is of great interest because it specifically block T cell-line HIV-1 infection by recognizing and binding to a chemokine receptor (CXCR4) [82, 83]. Lactoferricin, an iron-binding protein that is present in milk, has been shown to effectively block the entry of herpes simplex virus

(HSV) into the host cells [84]. Human α -defensins 1-3 (HNPs 1-3) and human α -defensin 5 (HD-5) are potent against both cutaneous and mucosal papillomavirus types, which are the primary cause of cervical cancer [85].

2.4 Mechanisms of action

A common mechanism by which antimicrobial peptides kill bacteria appears to be mediated through physical interactions with negatively-charged bacterial membranes followed by membrane disruption [86, 87]. The interaction between antimicrobial peptides and bacterial membrane is regarded as the first and essential step for the antibacterial activity. Generally, antimicrobial peptides kill bacteria more efficiently than eukaryotic host cells because of the differences between prokaryotic and eukaryotic membrane characteristics [9]. Bacteria have highly negatively charged membranes due to the presence of a high percentage of negatively charged phospholipid, anionic LPS, lipoteichoic acid, and peptidoglycan. In contrast, eukaryotic membranes have much lower content of anionic lipids and high content of cholesterol [9]. In the case of Gram-negative bacteria, anionic LPS is stabilized by divalent cations such as Mg^{2+} and Ca^{2+} . Electrostatic interactions of cationic peptides with LPS can displace divalent cations leading to a local disturbance in the outer membrane [88]. Peptides then integrate into the outer membrane and cause membrane thinning as observed by X-ray diffraction [88].

However, the outer membrane interaction with peptides is believed not sufficient for cell death [89]. Three major models have been proposed to account for membrane

disruption [88, 90, 91]. The “barrel-stave” model suggests that peptide monomers bind to cytoplasmic membrane and insert into membrane by forming a pore. The pore size is increased by progressively recruiting more peptide monomers [87]. In the “toroidal pore” model, pores form differently due to the curvature strain produced by lipids bending when peptides accumulate on the surface of membrane [92]. The third “carpet” model suggests that peptides lay on the surface of cytoplasmic membrane and lead to disruption of membrane until the concentration of peptides reaches the threshold [87]. The physico-chemical properties of peptides and lipids dictate the mode of action [93]. Membrane damages caused by antimicrobial peptides are believed to be one of the major reasons responsible for microbial death.

Besides bacterial membranes, accumulating evidence has shown that antimicrobial peptides can attack intracellular targets after peptides translocate across the cytoplasmic membrane. Insect antimicrobial peptides such as pyrrhocoricin, apidaecin, and drosocin are capable of inhibiting the activity of heat shock protein, DnaK, leading to accumulation of misfolded protein within the cytoplasm causing cell death [94]. Buforin II, isolated from the stomach of Asian toad, can bind to DNA and RNA of cells and inhibit cellular functions [95]. Lactoferricin B, a cationic peptide derived from bovine lactoferrin, when applied to *E. coli* at concentrations insufficient to kill bacterial cells, can induce an initial increase in protein and RNA synthesis and a decrease in DNA synthesis [96].

The phenomenon that antimicrobial peptides have multiple intracellular targets is generally explained by a “multi-hit” model proposed by Hancock’s group [9, 95]. This model shows that, in addition to the physical disruption of the bacterial membrane, antimicrobial peptides can translocate across the membrane and bind to intracellular anionic molecules including DNA, RNA, and certain proteins, which collectively results in the inhibition of macromolecular synthesis.

The unique mechanism of action that differs from that of conventional antibiotics makes cationic antimicrobial peptides attractive to be developed as therapeutic agents. Because the peptides interact with membranes via electrostatic interactions, development of resistance is highly unlikely due to extreme difficulty in changing its membrane composition through mutations.

The mechanism of antiviral activity of antimicrobial peptides is not very clear. It is generally believed that peptides have a dual role in antiviral activity [97]. By a similar antibacterial mechanism, peptides can interact with viral envelopes and directly inactivate viral particles. In addition, peptides inhibit viral replication by interacting indirectly with host cells through the binding of cell-surface glycoproteins and/or interfering with cell-signaling pathways.

2.5 Roles in innate immunity and other biological processes

In addition to their direct antimicrobial activity, growing evidence has suggested that antimicrobial peptides play an important modulatory role in response to inflammation and infection [9, 98, 99]. For example, Human α -defensin -1 (HNP-1) was found to be capable of blocking NADPH oxidase activation in vitro and may act in vivo by inhibiting excessive activation of the oxidase thus preventing overproduction of oxygen radicals [100]. Human neutrophil peptides HNP-(1-3) are able to bind to endothelial cells and fibrin and subsequently inhibit tissue-type plasminogen activator (tPA) and plasminogen-mediated fibrinolysis thereby limiting the spread of infection [101].

A number of antimicrobial peptides display a direct chemotactic activity for neutrophils, monocytes, mast cells, immature dendritic cells, and T helper cells to the sites of infection. The peptides isolated from skin secretions of the North American dusky gopher frog, *Rana sevososa*, could attract neutrophils to the sites of infection and help to destroy bacteria [102].

Additionally, antimicrobial peptides can induce the production and release of chemokines and/or cytokines. Human neutrophil peptides HNP-(1-3) have been shown to significantly increase the IL-8 levels in airway epithelial cells [103], and defensin-induced IL-8 production enhanced the release of secretory leukocyte proteinase inhibitor (SLPI), which functions as an inhibitor of neutrophil elastase in addition to direct antibacterial and antifungal activities [104]. Similarly, human cathelicidin LL-37 induces IL-8, IL-18 and IL-20 production by human keratinocytes through activation of the MAP kinase pathway, and synergizes with HNP1-4 in increasing IL-18 production [105].

Paradoxically, antimicrobial peptides can also protect the host by suppressing the excessive inflammatory responses. Cationic peptides are able to bind anionic LPS and further neutralize LPS-induced production of cytokines/chemokines such as IL-1 β , TNF α , and IL-6 [106, 107]. The immuno-modulating effects of antimicrobial peptides probably involve multiple mechanisms, but remain elusive [98].

Some antimicrobial peptides have been reported to promote wound healing. The presence of pig cathelicidin PR-39 in wound fluid induces mesenchymal cells to express specific cell surface syndecan-1 and -4, which are able to bind heparin-binding growth factors such as fibroblast growth factor 2, heparin-binding epidermal growth factor-like growth factor and platelet-derive growth factor, leading to cell proliferation and migration [108]. The binding of syndecan-1 to fibronectin, tenascin and fibrillar collagens would contribute to wound repair [108]. Human cathelicidin LL-37 has been reported to associate with the wound repair process by stimulating keratinocyte proliferation and inducing angiogenesis [109, 110].

Besides a role in immunity and host defense, antimicrobial peptides are involved in other biological processes as well. For example, rat peptide Bin1b, an epididymis-specific β -defensin, is capable of binding sperm and initiating maturation process by inducing the uptake of Ca²⁺ and progressive mobility of immature sperm [111]. In fact, most β -defensins have been found to be expressed preferentially in epididymis [111-113].

2.6 *Therapeutic potential*

Although a few of antimicrobial peptides display potent antimicrobial activity and immunomodulatory effects, they also exhibit considerable cytotoxicity toward host cells. For example, melittin is a potent antimicrobial peptide found in European honey bee venom, but with significant cytotoxicity toward human intestinal epithelial cells, HT29 and Caco-2 [114]. Nevertheless, the concentrations of antimicrobial peptides that are required to exert a noticeable cytolytic effect are often much higher (10-1000 times) than microbicidal concentrations [8], indicating their therapeutic potential.

Structure-based rational mutagenesis of natural peptides is being employed to reduce cytotoxicity while maintaining or increasing the antimicrobial activity. Several physico-chemical properties like amphiphilicity, helicity, flexibility and charges are among the most critical factors that affect their biological activities and can be manipulated for a beneficial outcome [9]. For example, tritrpticin, a cathelicidin deduced from porcine myeloid, has a broad spectrum of antimicrobial activity with relatively strong hemolytic activity [115]. A perfect symmetric analogue of tritrpticin with C-terminal amidation, was shown to reduce hemolysis and permeabilization of the zwitterionic phosphatidylcholine membrane with 2~8 fold improvement in antimicrobial activity [116]. Novispirin G10, an antimicrobial peptide analogue derived from sheep cathelicidin SMAP-29, exhibited enhanced specificity and antimicrobial activity toward antibiotic-resistant *Pseudomonas* spp following rational changes of the native peptide [117].

Taken together, the characteristics of antimicrobial peptides including the broad-spectrum microbicidal activity, rapid action, potential low levels of resistance and immunomodulatory effects make them good candidates for therapeutic agents [118]. Antimicrobial peptides are being developed as novel therapeutics with several in various stages of clinical trials. For example, Pexiganan (MSI-78), a peptide derived from frog magainin 2, is the first cationic peptide to be developed as a commercial antibiotic to treat infected diabetic foot ulcers [9]. Omigana, an indolicidin-like peptide, is under the Phase III trial for prevention or treatment of catheter-associated infections [119]. Table 1 is a list of antimicrobial peptides that are being developed as therapeutic agents [9, 118, 120]. Detailed structural and functional studies about additional novel antimicrobial peptides will facilitate the development of new anti-infective therapeutic agents, particularly against antibiotic-resistant pathogens [121].

2.7 References

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Table 1. A list of cationic antimicrobial peptide drugs that are being developed for various disease treatments [9, 118, 120]

Compound	Company	Testing status	Disease drug designed to treat
Pexiganan	Magainin Pharmaceuticals	Phase III completed	Infection of diabetic foot ulcers
Iseganan	IntraBiotics Corporation	Phase III halted prematurely	Ventilator-associated pneumonia
Omiganan (MBI 226)	Migenix (formerly Micrologix)	Phase III completed	Infections at site of in-dwelling catheter insertion
MBI 594AN	Microbiologx	Phase II	Acute acne
IMXC001	Inimex Pharmaceuticals	Preclinical	Sepsis
P113 P113D	Demegen	Phase II completed	Oral candidiasis in HIV patients
RBPI21	Xoma Ltd.	Phase III completed	Severe bacterial meningitis
XMP.629	Xoma Ltd.	Phase III halted	Acute acne
Mycoprex (XMP366)	Xoma Ltd.	Preclinical development	Systemic fungal infections
Histatine variants	Periondotix	Phase II Phase I	Gingivitis and mouth infections oral candidiasis
Heliomocin variants (ETD151)	Entomed SA	Preclinical development	Systemic deep and invasive fungal infections, immunocompromised patients

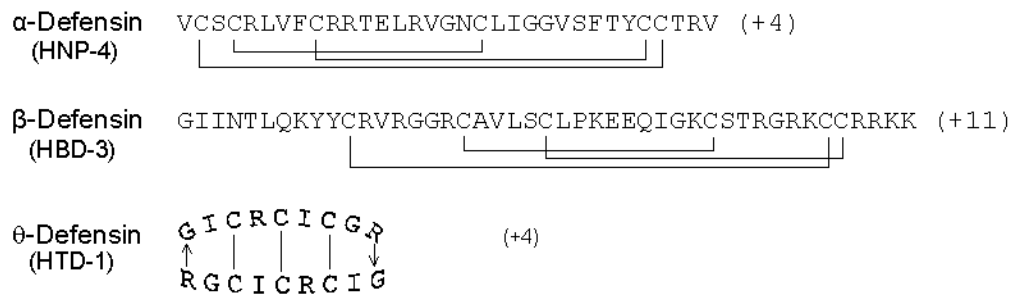


Figure 1. Representative peptide sequences of three classes of mammalian defensins.

Also shown are the net positive charges (in parenthesis) and intra-molecular disulfide bridges of each peptide. *Abbreviations:* HNP-4, human neutrophil α -defensin-4; hBD-3, human β -defensin-3; HTD-1, human θ -defensin-1.

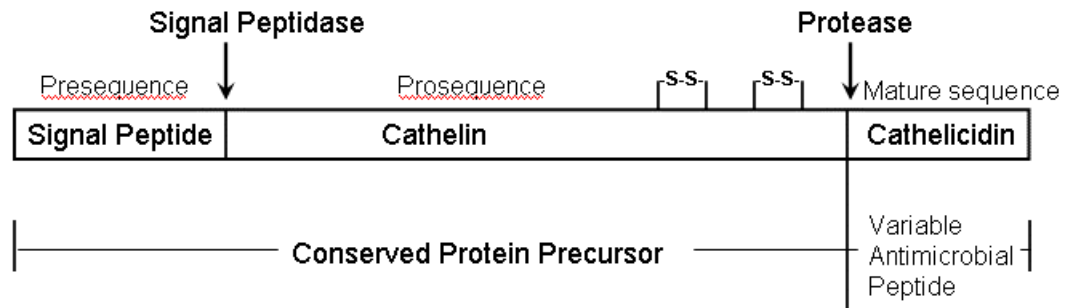


Figure 2. Schematic representation of a cathelicidin precursor. Cathelicidins consist of a highly conserved preprosequence followed by a diverse mature peptide that possesses antimicrobial activity. Four cysteines within the pro-sequence form two intracellular disulfide bridges.

CHAPTER III

**A GENOME-WIDE SCREEN IDENTIFIES A SINGLE β -DEFENSIN GENE
CLUSTER IN THE CHICKEN: IMPLICATIONS FOR THE ORIGIN AND
EVOLUTION OF MAMMLIAN DEFENSINS**

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3.1 Abstract

Background: Defensins comprise a large family of cationic antimicrobial peptides that are characterized by the presence of a conserved cysteine-rich defensin motif. Based on the spacing pattern of cysteines, these defensins are broadly divided into five groups, namely plant, invertebrate, α -, β -, and θ -defensins, with the last three groups being mostly found in mammalian species. However, the evolutionary relationships among these five groups of defensins remain controversial.

Results: Following a comprehensive screen, here we report that the chicken genome encodes a total of 13 different β -defensins but with no other groups of defensins being discovered. These chicken β -defensin genes, designated as *Gallinacin 1-13*, are clustered densely within a 86-Kb distance on the chromosome 3q3.5-q3.7. The deduced peptides vary from 63 to 104 amino acid residues in length sharing the characteristic defensin motif. Based on the tissue expression pattern, 13 β -defensin genes can be divided into two subgroups with *Gallinacin 1-7* being predominantly expressed in bone marrow and the respiratory tract and the remaining genes being restricted to liver and the urogenital tract. Comparative analysis of the defensin clusters among chicken, mouse, and human suggested that vertebrate defensins have evolved from a single β -defensin-like gene, which has undergone rapid duplication, diversification, and translocation in various vertebrate lineages during evolution.

Conclusions: We conclude that the chicken genome encodes only β -defensin sequences and that all mammalian defensins are evolved from a common β -defensin-like ancestor. The α -defensins arose from β -defensins by gene duplication, which may have occurred after the divergence of mammals from other vertebrates, and θ -defensins have

arisen from α -defensins specific to the primate lineage. Further analysis of these defensins in different vertebrate lineages will shed light on the mechanisms of host defense and evolution of innate immunity.

3.2 Background

Defensins constitute a large family of small, cysteine-rich, cationic peptides that are capable of killing a broad spectrum of pathogens, including various bacteria, fungi, and certain enveloped viruses [1-5]. These peptides play a critical role in host defense and disease resistance by protecting the hosts against infections. Transgenic mice expressing human enteric defensin *HD5* are fully protected against the doses of *Salmonella typhimurium* that are otherwise lethal to the wide-type mice [6]. Conversely, mice deficient in the matrilysin gene, which is responsible for activating enteric defensins, become more susceptible to oral infection with *S. typhimurium* [7].

Defensins have been identified in species ranging from plants, insects to animals and humans [1-5]. Characterized by the presence of 6-8 cysteine residues in relatively defined positions, all defensins are structurally related in that they form 3-4 intramolecular disulfide bonds and 2-3 antiparallel β -sheets with or without an α -helix. Based on the spacing pattern of cysteines, these peptides are broadly divided into five groups; namely plant, invertebrate, α -, β -, and θ -defensins [1-5]. Alignment of all known defensin sequences revealed the consensus defensin motif of each group as follows: plant defensin: C-X₈₋₁₁-C-X₃₋₅-C-X₃-C-X₉₋₁₂-C-X₄₋₁₁-C-X₁-C-X₃-C; invertebrate defensin: C-X₅₋₁₆-C-X₃-

C-X₉₋₁₀-C-X₄₋₇-C-X₁-C; α -defensin: C-X₁-C-X₃₋₄-C-X₉-C-X₆₋₁₀-C-C; and β -defensin: C-X₄₋₈-C-X₃₋₅-C-X₉₋₁₃-C-X₄₋₇-C-C. The α - and β -defensins are unique to vertebrate animals with α -defensins only being found in rodents and primates, while β -defensins are present in all mammalian species investigated [1-3]. On the other hand, θ -defensins have only been found in certain primates as a result of posttranslational ligation of two α -defensin-like sequences [8-10]. A pseudogene for θ -defensin is also present in humans [11].

Analysis of human and mouse genomes indicated that β -defensins form 4-5 distinct clusters on different chromosomes with each cluster consisting of multiple defensin genes [12]. Interestingly, the single mammalian α -defensin locus is located on a β -defensin cluster with θ -defensins residing in the center of α -defensins [12]. Studies with mammalian defensins suggested a rapid duplication followed by positive selection and diversification within each group [13-18]. However, the evolutionary relationships among three groups of mammalian defensins and among plant, invertebrate, and mammalian defensins remain controversial. Similarity in spatial structure and biological functions favors the notion that all mammalian defensins are evolutionarily related [19], although a phylogenetic analysis suggested a closer relationship between β - and insect defensins than between α - and β -defensins [16].

Existence of a large number of expressed sequence tag (EST) sequences and recent completion of chicken genome sequencing at a 6.6 \times coverage [20] provided a timely opportunity to discover a complete repertoire of defensin-related sequences in birds for studying the evolutionary relationship between invertebrate and mammalian defensins.

Here we report identification of a single β -defensin cluster that is composed of 13 genes located on the chicken chromosome 3q3.5-q3.7. Evolutionary and comparative analyses of these chicken β -defensins with mammalian homologues strongly suggested that all mammalian defensins have evolved from a common β -defensin-like ancestor, which has undergone rapid duplication, positive diversifying selection, and chromosomal translocations, thereby giving rise to multiple gene clusters on different chromosomal regions.

3.3 Results and discussion

Discovery of novel chicken defensins

To identify novel defensin genes in the chicken, all five groups of known defensin-like peptide sequences from plants, invertebrates, and vertebrates were first queried individually against the translated chicken nonredundant (NR), EST, high throughput genomic sequence (HTGS), and whole-genome shotgun sequence (WGS) databases in the GenBank by using the TBLASTN program[21]. All potential hits were then examined manually for the presence of the characteristic cysteine motifs. For every novel defensin identified, additional iterative BLAST searches were performed until no more novel sequences could be found. In addition to three known chicken β -defensins (*Gal 1-3*) [22, 23], nine novel putative sequences, namely *Gal 4-12*, have been found in the EST database with at least two hits for each, and such sequences have also been confirmed in genomic sequences (Table 1). Because of the fact that mammalian defensins tend to form clusters [12, 14, 15, 18], all chicken HTGS and WGS sequences containing defensin

sequences were also retrieved from GenBank, translated into six open reading frames, and manually curated. As a result, an additional putative β -defensin, *Gal13*, was identified in several genomic clones (Table 1). The open reading frame of *Gal13* was predicted by GENSCAN [24] and confirmed by directly sequencing of RT-PCR product amplified from chicken kidney.

No other sequence containing β -defensin-like six-cysteine motif has been found in NR, EST or genomic databases, suggesting that 13 *Gal* genes constitute the entire repertoire of the β -defensin family encoded in the chicken genome. Although it is highly unlikely, we could not rule out the possibility that additional defensin-related genes with distant homology might be uncovered in the chicken by different computational search methods such as the use of Hidden Markov models [12, 15]. It is noted that none of other groups of defensins have been discovered in the chicken, indicating that plant, invertebrate, α -, and θ -defensins are absent in the chicken lineage.

Similar to Gal 1-3, 10 novel β -defensins, deduced from either EST or genomic sequences, vary from 63 to 104 amino acid residues in length. Alignment of these peptides revealed a conservation of the signal sequence at the N-terminus and the characteristic six-cysteine defensin motif at the C-terminus (Figure 1). Consistent with the fact that all β -defensins are a group of secreted molecules in response to infections, the signal sequences of all chicken defensins are hydrophobic and rich in leucines. In addition, the mature C-terminal sequences are all positively charged due to the presence of excess arginines and lysines. Interestingly, Gal11 contains two tandem, but highly

divergent, copies of the six-cysteine motif at the C-terminus, and is the only defensin having such sequences. Functional significance for existence of such two defensin motifs remains to be studied.

Evolutionary analysis of vertebrate β -defensins

Phylogenetic analysis of vertebrate β -defensins showed that chicken defensins clustered with various different groups of mammalian β -defensins (Figure 2). However, the bootstrap support for these patterns was very weak (less than 50% in all cases). The clustering of certain chicken β -defensins with mammalian homologues suggests that major subfamilies of β -defensins arose before the last common ancestor of birds and mammals, estimated to have occurred about 310 million years ago [25]. This in turn implies that some duplication of β -defensin genes must have taken place before the divergence of birds and mammals. The apparent lack of α -defensins in the chicken and other non-mammalian species (G. Zhang, unpublished data) suggests that α -defensins may have evolved after mammals diverged from other vertebrates.

Comparison of the numbers of synonymous and nonsynonymous nucleotide substitutions provides a powerful test of the hypothesis that positive Darwinian selection has acted to favor changes at the amino acid level [26]. This approach has previously been applied to both α - and β -defensins of mammals and has revealed positive selection acting on the mature defensin but not on other regions of the gene [16, 17]. In the comparison of the chicken β -defensin sequences, synonymous sites were saturated with changes or nearly so, making it impossible to test the hypothesis of positive selection in

every case. In pairwise comparisons among all sequences, mean p_S in the propeptide region was 0.551 ± 0.036 (S.E.), while mean p_N was 0.369 ± 0.040 . In the mature defensin region, mean p_S was 0.673 ± 0.027 , while mean p_N was 0.534 ± 0.051 . Mean p_N in the mature defensin was significantly greater than that in the propeptide (z-test; $P < 0.05$), indicating lesser functional constraint on the amino acid sequence of the former. The high mean p_S shows that chicken β -defensin genes have not duplicated recently, unlike β -defensin genes of the bovine [16]. In the comparison between the most closely related pair of sequences (*Gal6* and *Gal7*), mean p_S in the mature defensin was 0.221 ± 0.082 , while mean p_N was 0.331 ± 0.076 . While these values are not significantly different at the 5% level, the fact that p_N was higher than p_S suggested that positive selection may have acted to diversify the mature defensin region between these two genes.

Genomic organization and chromosomal localization of the chicken β -defensin gene cluster

Searching through HTGS database led to identification of two overlapping bacterial artificial chromosome (BAC) sequences, TAM31-54I5 (accession no. AC110874) and CH261-162O9 (accession no. AC146292), both of which were sequenced and deposited earlier by one of us (J.F. Chen). Alignment of these two sequences allowed to re-order three DNA fragments in AC110874 and to construct a continuous, gap-free genomic contig that includes 11 *Gal* genes except for *Gal4* and *Gal5*. Later search of chicken WGS sequences released on February 29, 2004 confirmed the order of the genomic contig that we assembled and also revealed the locations of two remaining genes, *Gal4* and *Gal5*, both of which reside on a WGS (accession no. AADN01058096) that overlaps

with AC110874 (Figure 3). The position and orientation of each *Gal* gene were obtained by comparing its cDNA with the assembled DNA sequence. As shown in Figure 3, all 13 *Gal* genes were clustered densely within a distance of 86.0 Kb on the genome. It was also confirmed by aligning such a contig with the chicken genome assembly, in which 13 *Gal* genes are located on six WGS contigs (Table 1) of chromosome 3 that are only ~3.3 Mb from the distal end. Consistent with this, the *Gal* gene cluster was physically mapped to the tip of chicken chromosome 3 at the region of q3.5-q3.7 by fluorescence in situ hybridization (FISH) using the TAM31-54I5 BAC DNA as probe (Figure 4).

Comparing the cDNA with genomic sequences also revealed the structure of each *Gal* gene. Unlike most mammalian β -defensin genes, which primarily consist of two exons and one intron, the *Gal* genes were found to be composed of four short exons separated by three introns with variable lengths ranging from 117 bp to 3,322 bp (Table 1). *Gal12* is an exception, in which the last two exons have been fused together. While the first exon of the *Gal* genes encodes 5'-untranslated region (UTR) and the majority of the last exon encodes 3'-UTR as well as a few C-terminal amino acids, two internal exons resemble mammalian β -defensin genes in that one exon encodes the signal and pro-sequence and the other encodes the mature sequence with six-cysteine motif [19, 27-29]. Apparently, the first two and the last two exons of the *Gal* genes have joined together during the evolution as a result of exon shuffling, which occurred in many other evolutionarily conserved gene families [30], including invertebrate defensins [5]. The fusion of defensin exons in mammals is presumably adaptive because it allows a faster mobilization of such host defense molecules to better cope with invading microbes.

Tissue expression patterns of chicken β -defensins

It has been shown that *Gal1* and *Gal2* are expressed in bone marrow and lung, while *Gal3* is more preferentially expressed in bone marrow, tongue, trachea, and bursa of Fabricius [23]. To study the tissue expression patterns of novel *Gal* genes that we identified, RT-PCR was performed with a panel of 32 different chicken tissues. Similar to *Gal 1-3*, *Gal 4-7* are highly restricted to bone marrow cells with *Gal5* also expressed in tongue, trachea, lung, and brain at lower levels (Figure 5). By contrast, the six remaining genes, *Gal 8-13*, were not found in bone marrow, but instead in liver, kidney, testicle, ovary, and male and female reproductive tracts (Figure 5). These results clearly suggested that all chicken β -defensin genes can be divided into two subgroups. Seven genes (*Gal 1-7*) are predominantly expressed in bone marrow and the respiratory tract, whereas the other six genes (*Gal 8-13*) are more restricted to liver and the urogenital tract. However, the functional significance and transcriptional regulatory mechanisms of these genes during inflammation and infection remain to be investigated.

Comparative analysis of chicken and mammalian β -defensin gene clusters

To study the origin and evolution of mammalian defensins, a comparative analysis of β -defensin gene clusters in the chicken, mouse, and human was performed by employing additional, more phylogenetically conserved gene markers surrounding the defensin clusters. As shown in Figure 6, two genes, *CTSB* (Cathepsin B, accession no. NP_680093) and a human EST sequence (accession no. BE072524) immediately located centromeric to chicken defensins, were also found to be conserved in the defensin gene clusters on

human chromosome 8p22 and mouse chromosome 14C3. Similarly, another gene, *HARL2754* (accession no. XP_372011) that is 6-Kb telemetric to *Gal4* is also conserved in another defensin cluster in human (8p23) or mouse (8A1.3) (Figure 6).

These results strongly suggested that all vertebrate β -defensins are evolved from a single gene. This conclusion is further supported by the fact that there are three highly similar β -defensin-like sequences present in the largely finished zebrafish genome (G. Zhang, unpublished data). In addition, a group of homologous β -defensin-like sequences, namely crotamine and myotoxins, have been found in several *Crotalus* snakes [31], which are presumably derived from a single ancestral gene. The appearance of multiple β -defensin gene clusters on different chromosomal regions in mammalian species [12] is apparently a result of rapid gene duplication, positive diversifying selection, and chromosomal translocation following divergence of mammals from other vertebrate lineages.

In addition to the structural conservation between β -defensin-like sequences in the rattlesnake and mammals [32], a growing body of evidence suggests that their functions appear to be largely conserved in that both are capable of interacting negatively-charged lipid membranes followed by formation of ion channels or pores [32-34]. It is noteworthy that the conservation of Cathepsin B (CTSB) adjacent to β -defensins is perhaps not surprising, given the recent finding that cathepsins are involved in the cleavage and inactivation of β -defensins [35].

3.4 Conclusions

We have showed that chicken genome encodes a total of 13 different β -defensin genes clustered densely within a 86-Kb distance on the chromosome 3q3.5-q3.7, but with no α -defensin genes. These peptides exhibit homology to different subgroups of mammalian β -defensins, consistent with the hypothesis that α -defensins and β -defensins arose by gene duplication after the divergence of birds and mammals. The θ -defensins are specific to primates; and thus appear to have arisen from α -defensins by gene duplication specific to the primate lineage. Apparently, the evolution of defensins is rapid and driven by duplication and positive diversifying selection. Collectively, this study represents the first large-scale detailed investigation of defensins in non-mammalian vertebrates. There is no doubt that further analysis of these defensin genes will lead to a better understanding of host defense mechanisms and evolution of innate immunity.

3.5 Methods

Computational search for novel chicken defensins

To identify novel defensins in the chicken, all known cysteine-containing defensin-like peptide sequences discovered in plants, invertebrates, birds, and mammals were individually queried against the translated chicken NR, EST, HTGS, and WGS databases in the GenBank by using the TBLASTN program [21] with default settings on the NCBI web site [36]. All potential hits were then examined for the presence of the characteristic defensin motif. For every novel defensin identified, additional iterative BLAST searches were performed until no more novel sequences could be revealed. Because mammalian

defensins tend to form clusters [12, 14, 15, 18], all chicken genomic sequences containing defensin sequences were also retrieved from the GenBank and translated into six open reading frames and curated manually for the presence of the defensin motif in order to discover potential sequences with distant homology.

Alignment and phylogenetic analysis of chicken β -defensins

Multiple sequence alignment was constructed by using the ClustalW program (version 1.82) [37]. A phylogenetic tree of amino acid sequences of mature β -defensins was constructed by the neighbor-joining method [38]. So that a comparable data set would be used for all pairwise comparisons, any site at which the alignment postulated a gap in any sequence was excluded from the analysis. To maximize the number of sites available for analysis, certain sequences with large deletions were excluded from the analysis. Because the sequences were very short (25 aligned sites), no correction for multiple hits was applied. The reliability of clustering patterns within the tree was assessed by bootstrapping; 1000 bootstrap pseudo-samples were used. The proportion of synonymous nucleotide differences per synonymous site (p_S) and the proportion of nonsynonymous nucleotide differences per nonsynonymous site (p_N) were estimated by the method of Nei and Gojobori [26]. Again, no correction for multiple hits was applied because a small number of sites were examined.

Assembly of the chicken β -defensin gene cluster

To generate a continuous defensin gene cluster, the HTGS and WGS sequences containing the putative defensin genes were retrieved from the GenBank, aligned to

generate a longer contig, which was confirmed later by searching through the assembled chicken genome released on February 29, 2004, by using the BLAT program [39] under the UCSC Genome Browser web site [40]. The relative positions, orientations, and structural organizations of individual genes were determined by comparing its cDNA sequence to the continuous genomic contig that we assembled.

Chromosome localization of the chicken β -defensin gene cluster

Fluorescence in situ hybridization (FISH) was used for chromosomal assignment of the chicken β -defensin gene cluster by using the BAC clone TAM31-54I4 as probe, which harbors 11 *Gal* genes. Metaphase chromosome spreads were prepared from mitogen-stimulated chicken splenocyte culture as we described [41, 42]. The BAC clone was labeled by nick translation with biotin 16-dUTP (Roche Diagnostics), hybridized to metaphase chromosome DNA, followed by detection with FITC-labeled avidin (Roche Diagnostics) and staining with propidium iodide to simultaneously induce the R-banding.

RT-PCR analysis of the tissue expression patterns of chicken β -defensins

Total RNA was extracted with Trizol (Invitrogen) from a total of 32 different tissues from healthy, 2-month-old chickens (see Figure 5). A total of 4 μ g RNA from each tissue were reverse transcribed with random hexamers and Superscript II reverse transcriptase by using a first-strand cDNA synthesis kit (Invitrogen) according to the instructions. The subsequent PCR was carried out with 1/40 of the first-strand cDNA and gene-specific primers for each β -defensin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as described [28, 43]. Every pair of primers were designed to locate on different exons to

aid in distinguishing PCR products amplified from cDNA vs. genomic DNA (Table 2). The PCR program used was: 94°C denaturation for 2 min, followed by different cycles of 94°C denaturation for 20 sec, 55°C annealing for 20 sec, and 72°C extension for 40 sec, followed by a final extension at 72°C for 5 min. The number of PCR cycle was optimized for each gene to ensure linear amplification (Table 2). A half of the PCR products were analyzed by electrophoresis on 1.2% agarose gels containing 0.5 µg/ml ethidium bromide. The specificity of each PCR product was confirmed by cloning of the PCR product into T/A cloning vector, followed by sequencing of the recombinant plasmid.

3.6 Note added in proof

Following submission of this manuscript, Lynn *et al.* reported independently discovery of seven novel chicken β -defensins in the chicken EST database by using homology search strategies [44]. Consistent with our conclusion, they also revealed occurrence of positive selection particularly in the mature region of chicken β -defensins following evolutionary analysis. Moreover, albeit the use of a different nomenclature, they confirmed that the expressions of *Gal 4-7* are primarily in bone marrow, while other genes are more restricted to liver and the genitourinary tract.

3.7 List of abbreviations

Abbreviations: Gal, Gallinacin; NR, nonredundant; EST, expressed sequence tag; HTGS, high throughput genomic sequence; WGS, whole-genome shotgun sequence; BAC, bacterial artificial chromosome; FISH, fluorescence in situ hybridization; UTR, untranslated region; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

AUTHOR'S CONTRIBUTIONS

YX carried out the tissue collection, RT-PCR analysis of tissue expression patterns, and drafted the manuscript. ALH carried out the phylogenetic and molecular evolutionary analyses. JA and YM carried out the fluorescence in situ hybridization. JFC carried out the sequencing of two chicken defensin-containing BAC clones. DSN participated in tissue collection and preparation. GZ conceived of the study, carried out all computational analyses and annotation, drafted the manuscript, and participated in its design and coordination. All authors read and approved the final manuscript.

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	Signal Peptide	Propiece	Mature Peptide
Gal1	MRIVYLLLPFILLL A QGAAG	--SSQALGRKSD	CFRKSGFCAF L-KP S L T L I-SGKSR-FYLCKR
Gal2	MRILYLLFSLFLALQVSPG	--LSSPRRDMLECK	--CGSCHFG-GCP S H L I K-VGSCFG-FRSCCKW
Gal3	MRIVYLLLPFLLFLQGAAG	-----T--ATQCR	IRGGFORVG-SRFP H I A-IGK S A T-FISCKR
Gal4	MRILCFFIVLLFVA VEGAVG	--FSSSPRYHMO	CGYRGTFC T P G-KCP Y G N A Y-LGLCRP-KYSCCKW
Gal5	MRILPILLFAVLLMLRAEPG	--L S L A R G L P Q D	C E R R G G F C S H K-S C P P G I G R-I G L C S K-E D F C C S
Gal6	MRILYLLLSVLFVVLQGVAG	--QPYPSSPIHAC	RYQRGVCI P G-PORW P Y Y R-VGSCG S G L K S C C V R
Gal7	MRILYLLLSVLFVVLQGVAG	--QPFI PRP I D T C R	L R N G I C P P G-I C R R P Y Y W-I G T C N N G I G S C C A R
Gal8	MRILYLLLAVLLTVLQSSLG	--FMRVFNNEA	QCEQAGGICSKD-HCFHLHTRAFGH C Q R G-V P C C R T
Gal9	MRILEFLVAVLFFLFQAAPA	--YSQEDADTLA	C R Q S E G S C S F V-A C R A P S V D-I G T C R G G K L K C C K W
Gal10	MRILCLLFAVLLFLFQAAPGSADPLFP	-DTVACR	TQGNFCRAG-AC P P T F T I-SGQ C H G G L L N C C A K
Gal11	MRILFSCLLMALLFL LQAVPG	---LGLPRDTSR	OVGYHGVCIRSKVCPK P F A A-FGTCSWRQKTCQVD
Gal12	MRNLCFVFI F I S L L A H G S T H	-----G-PDS	C N H D R G L C R V G-N O N P G E Y L-A K Y C F E P V I L C C K P
Gal13	MRILQLLFAIVVILLLQDAP	---ARGFSDS	QLCRNNEGECRRL--CFHME S W-A G S C M N G E L R C C K F
Gal1	IWG	-----	65
Gal2	PWNA	-----	64
Gal3	AYEVDALNSVRTSPWLLAPGNNPH	-----	80
Gal4	L	-----	63
Gal5	RWYS	-----	66
Gal6	NRWA	-----	67
Gal7	GWRS	-----	67
Gal8	VYD	-----	66
Gal9	APSS	-----	67
Gal10	IPAQ	-----	68
Gal11	T T S D F H T C Q D R G G H C V S P K I E C L E E Q L G L C P L K R W T C C K E I	-----	104
Gal12	L S P T P T K T	-----	65
Gal13	S T R Q P F S N P K H S V L H T A E Q D P S P S L G G T	-----	89

Figure 1. Multiple sequence alignment of chicken β -defensins: The intervening region between signal and mature peptide sequence is the short propiece. The conserved residues are shaded. Also shown is the length of each peptide. Notice the six-cysteine defensin motif is highly conserved. The six cysteines in the second tandem copy of the defensin motif in Gal11 are boxed.

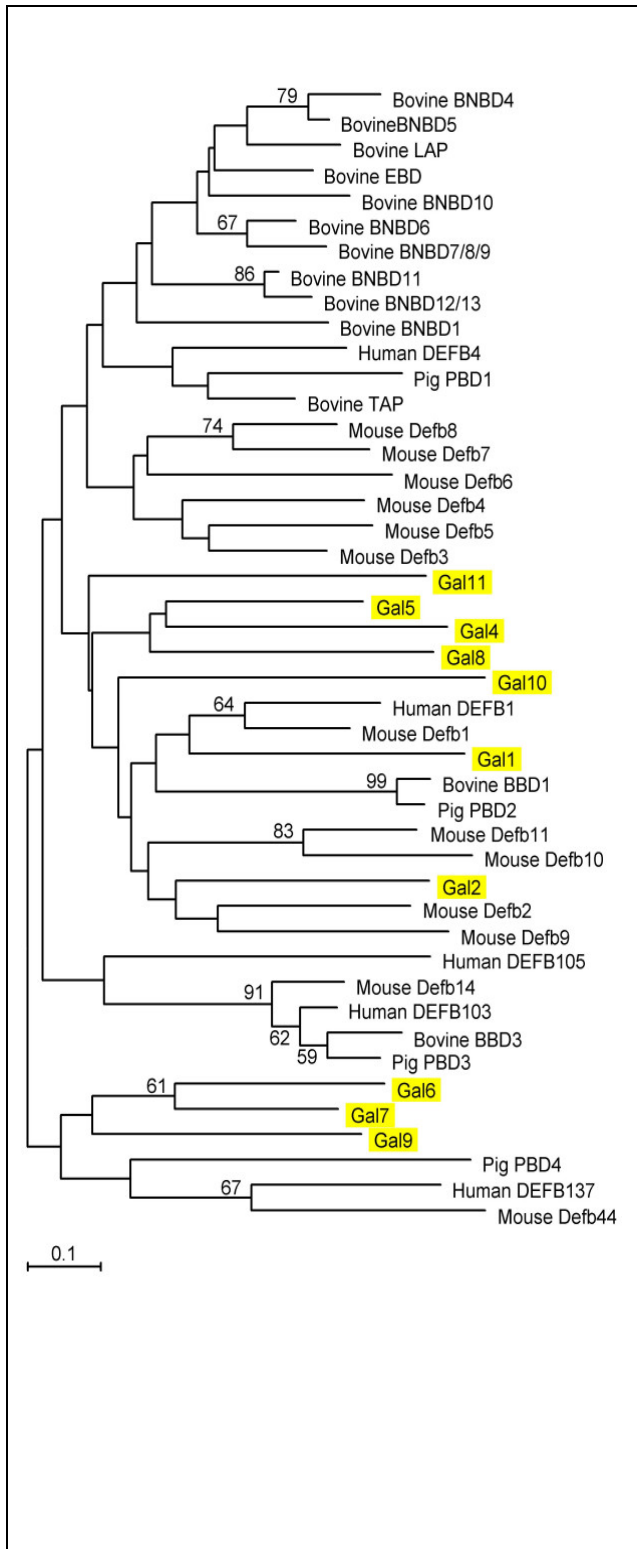


Figure 2. Phylogenetic relationship of vertebrate β -defensins: The tree was constructed by the neighbor-joining method and the reliability of each branch was assessed by using 1000 bootstrap replications. Numbers on the branches indicate the percentage of 1000 bootstrap samples supporting the branch. Only branches supported by a bootstrap value of at least 50% are indicated. Chicken β -defensins are highlighted in yellow. Abbreviations: *BNBD*, bovine neutrophil β -defensin; *LAP*, lingual antimicrobial peptide; *EBD*, enteric β -defensin; *TAP*, tracheal antimicrobial peptide; *PBD*, porcine β -defensin; *DEFB/Defb*, β -defensin; *Gal*, Gallinacin; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

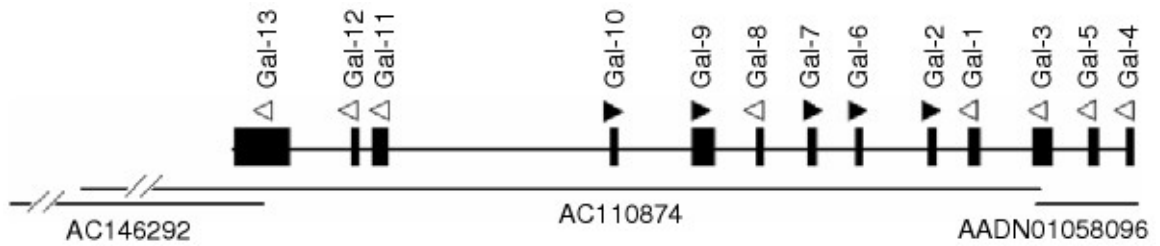


Figure 3. Genomic organization of the chicken β -defensin gene cluster: The horizontal lines at the bottom represent the three overlapping genomic clones that were used to assemble the continuous, gap-free contig. The position of each gene is represented by a solid vertical bar and the width of each bar is proportional to the size of each gene. The direction of transcription is indicated by the triangle above each gene. The genes with solid triangles are transcribed in the direction opposite to the ones with open triangles. Slanted lines refer to the sequences omitted. Note that the three fragments of AC110874 sequence have been re-ordered and the gaps have been filled following alignment with AC146292.

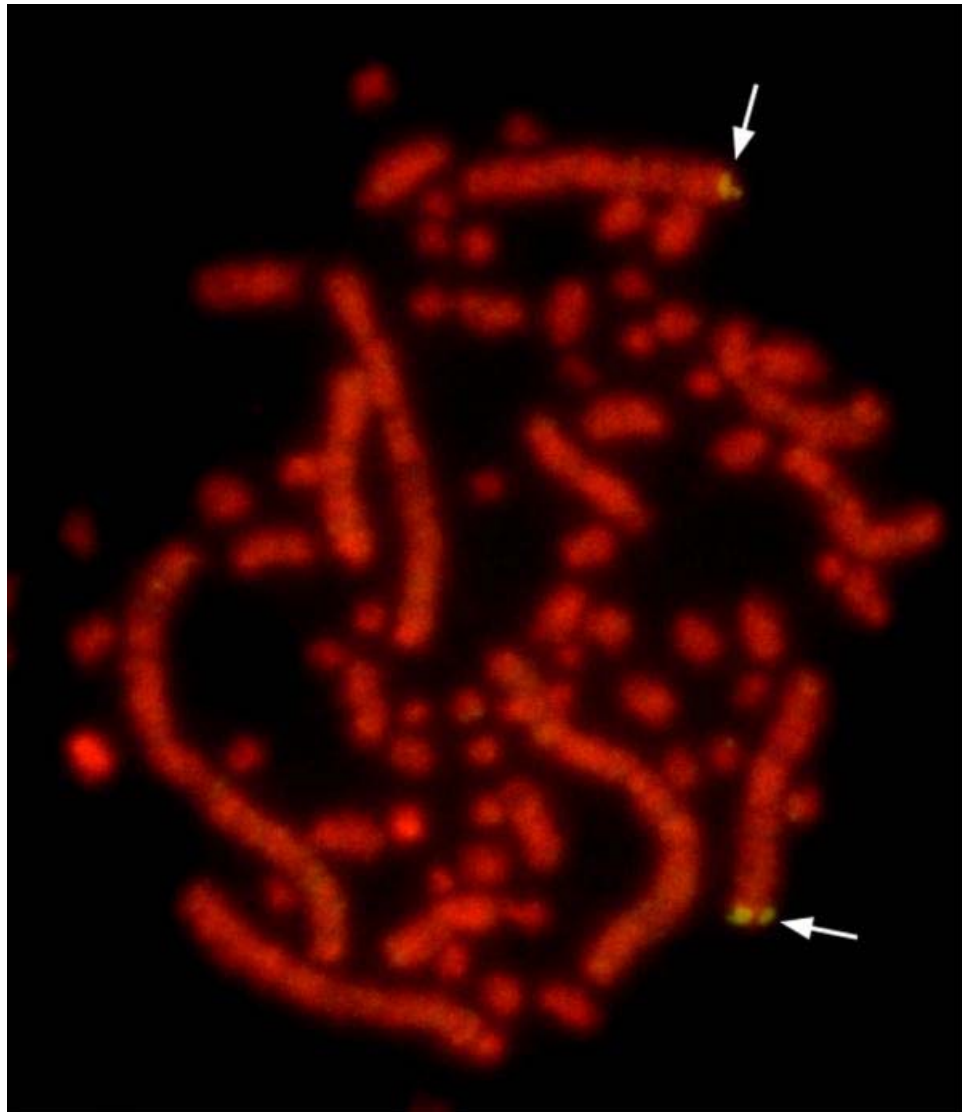


Figure 4. Chromosomal localization of the chicken β -defensin gene cluster by fluorescence in situ hybridization: The BAC clone TAM31-54I4, which harbors 11 *Gal* genes, was mapped to chicken chromosome 3q3.5-q3.7. Arrows indicate the hybridization signals.

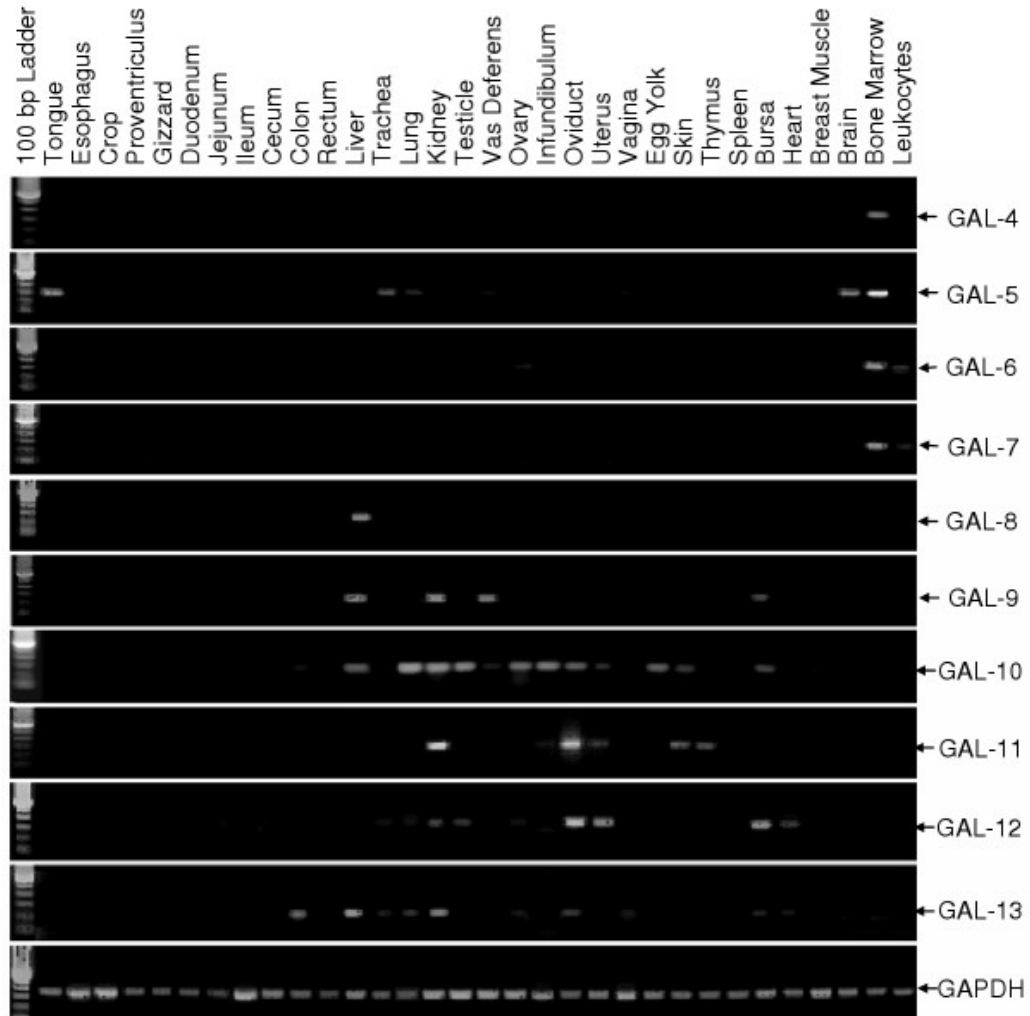


Figure 5. Tissue expression patterns of 10 novel chicken β -defensins by RT-PCR: See Materials and Methods for details. The number of PCR cycles was optimized for each gene, and the specificity of each PCR product was confirmed by sequencing. The house-keeping gene, GAPDH, was used for normalization of the template input.

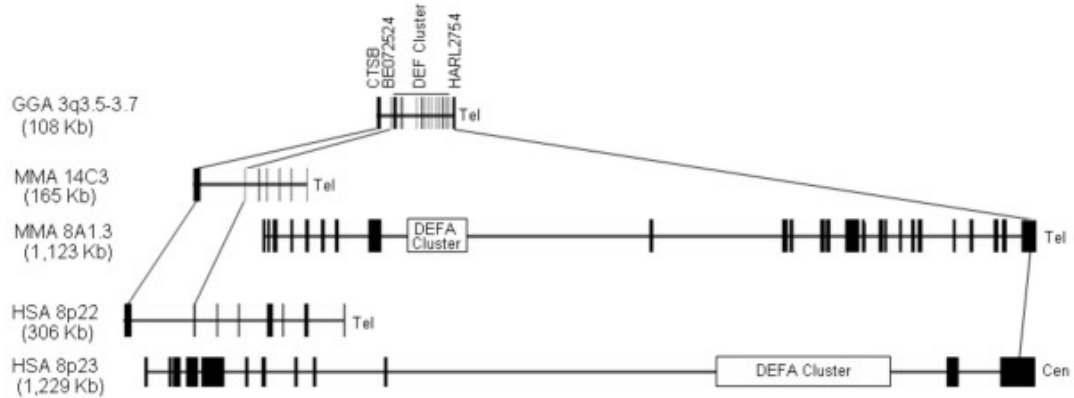


Figure 6. Comparative analysis of defensin clusters among the chicken, mouse, and human: The gene clusters were drawn proportionally according to their sizes. Each vertical line/bar represents the position of a gene, and the width of each line/bar is proportional to the size of each gene. Three highly conserved genes (*CTSB*, *BE072524*, and *HARL2754*) surrounding the defensin clusters in the chicken, mouse, and human were connected by solid lines. The position of the α -defensin locus (*DEFA*) was indicated as an open square. Note that the human θ -defensin pseudogene resides in the *DEFA* locus. The positions and orders of defensin genes in human and mouse were drawn based on the genome assemblies released in July 2003 and October 2003, respectively. Abbreviations: *GGA*, chicken chromosome; *MMA*, mouse chromosome; *HSA*, human chromosome; *Tel*, telomere; *Cen*, centromere.

Table 1. Identification of chicken β -defensins

Gene	EST ^{1,2}	HTGS	WGS	Gene Size (bp) ³						
				E 1	I 1	E 2	I 2	E 3	I 3	E 4
Gal1	BX260462	AC110874	AADN01058097	70	972	88	482	127	496	217
Gal2	BX540940	AC110874	AADN01058097	66	1113	143	183	121	674	204
Gal3		AC110874	AADN01058097 AADN01058096			53	980	109	1180	215
Gal4	BU451960		AADN01058096			136	461	127	117	141
Gal5	BU389548		AADN01058096			290	445	127	355	187
Gal6	CF251501	AC110874	AADN01058097 AADN01058098	52	704	86	705	130	249	234
Gal7	CF251115	AC110874	AADN01058098	50	656	86	201	130	234	248
Gal8	BU242665	AC110874	AADN01058098	71	915	91	259	134	706	494
Gal9	BX270804	AC110874	AADN01058098			220	1592	130	781	343
Gal10	AW198592	AC110874	AADN01058099			118	268	133	1719	381
Gal11	BM440069	AC110874	AADN01058101			63	966	129	1001	460
Gal12	BX257296	AC110874	AADN01058102			84	396	420		
Gal13		AC110874	AADN01058102			61	1016	118	3322	91

¹ Abbreviations: *EST*, expressed sequence tag; *HTGS*, high throughput genomic sequence; *WGS*, whole-genome shotgun sequence; *E*, exon; *I*, intron.

² One EST sequence entry is given only for the exemplary purpose. In each case, more than two independent EST sequences have been found, except for *Gal3* and *Gal13*, both of which have no EST sequences. *Gal3* was found through homology cloning [23], and *Gal13* was predicted by us from the genomic sequence.

³ All *Gal* genes are predicted to consist of four exons separated by three introns, except for *Gal12*, whose last two exons are fused together. The absence of additional sequence information at the 5'-untranslated regions of the cDNA sequences prevented prediction of the sizes of first exon and intron for *Gal 3-5* and *Gal 9-13* genes.

Table 2. Primer sequences used for RT-PCR analysis of novel chicken β -defensins

Gene	Primer Sequence		Product Size (bp)		Cycles Used
	Sense	Antisense	cDNA	Genomic	
Gal4	CATCTCAGTGTCTGTTTCTCTGC	ACAATGGTTCCCCAAATCCAAC	321	899	36
Gal5	CTGCCAGCAAGAAAGGAACCTG	TGAACGTGAAGGGACATCAGAG	300	1100	36
Gal6	AGGATTTACATCCCAGCCGTG	CAGGAGAAGCCAGTGAGTCATC	249	1203	36
Gal7	CTGCTGTCTGTCTCTTTGTGG	CATTTGGTAGATGCAGGAAGGA	230	665	35
Gal8	ACAGTGTGAGCAGGCAGGAGGGA	CTCTTCTGTTTCAGCCTTTGGTG	261	967	35
Gal9	GCAAAGGCTATTCCACAGCAG	AGCATTTTCAGCTTCCCACCAC	211	1802	33
Gal10	TGGGGCACGCAGTCCACAAC	ATCAGCTCCTCAAGGCAGTG	298	2285	33
Gal11	ACTGCATCCGTTCCAAAAGTCTG	TCGGGCAGCTTCTCTACAAC	301	1299	33
Gal12	CCCAGCAGGACCAAAGCAATG	GTGAATCCACAGCCAATGAGAG	335	731	36
Gal13	CATCGTTGTCATTCTCCTCCTC	ACTTGCAGCGTGTGGGAGTTG	175	4514	50
GAPDH	GCACGCCATCACTATCTTCC	CATCCACCGTCTTCTGTGTG	356	876	30

CHAPTER IV
IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF THREE
CHICKEN CATHELICIDINS WITH POTENT ANTIMICROBIAL ACTIVITY

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4.1 Abstract

Cathelicidins comprise a family of antimicrobial peptides sharing a highly conserved cathelin domain. Here we report that the entire chicken genome encodes three cathelicidins, namely fowlicidins 1-3, which are densely clustered within a 7.5-kb distance at the proximal end of chromosome 2p. Each fowlicidin gene adopts a four-exon, three-intron structure, typical for a mammalian cathelicidin. Phylogenetic analysis revealed that fowlicidins and a group of distantly related mammalian cathelicidins known as neutrophilic granule proteins are likely to originate from a common ancestral gene prior to the separation of birds from mammals, whereas other classic mammalian cathelicidins may have been duplicated from the primordial gene for neutrophilic granule proteins after mammals and birds are diverged. Similar to ovine cathelicidin SMAP-29, putatively mature fowlicidins displayed potent and salt-independent activities against a range of Gram-negative and Gram-positive bacteria, including antibiotic-resistant strains, with minimum inhibitory concentrations in the range of 0.4-2.0 μM for most strains. Fowlicidin-1 and -2 also showed cytotoxicity, with 50% killing of mammalian erythrocytes or epithelial cells in the range of 6-40 μM . In addition, two fowlicidins demonstrated a strong positive cooperativity in binding lipopolysaccharide (LPS), resulting in nearly complete blockage of LPS-mediated proinflammatory gene expression in RAW264.7 cells. Taken together, fowlicidin-1 and -2 are clearly among the most potent cathelicidins that have been reported. Their broad-spectrum and salt-insensitive antibacterial activities, coupled with their potent LPS-neutralizing activity, make fowlicidins excellent candidates for novel antimicrobial and anti-sepsis agents.

4.2 Introduction

Cationic antimicrobial peptides comprise a large group of gene-encoded molecules that have been discovered in virtually all species of life, playing a critical role in innate host defense and disease resistance [1-4]. Two major families of antimicrobial peptides exist in mammals, namely defensins and cathelicidins. Whereas defensins are characterized by the presence of six cysteines at well-defined positions [5, 6], all cathelicidins share a highly conserved “cathelin” pro-sequence at the N-terminus, followed by diversified, cationic mature sequences at the C-terminus [7-9]. Cathelicidins are most abundantly present in the granules of phagocytic cells and also to a lesser extent in many other cell types such as mucosal epithelial cells and skin keratinocytes [7-9].

Upon activation, most cathelicidin precursors are proteolytically cleaved to release the cathelin domain and the C-terminal mature peptides with antimicrobial activities, although the unprocessed or differentially processed forms are often found in the biological fluids where cathelicidins are expressed [8, 9]. The physiological role of the cathelin domain or uncleaved precursors remains elusive, but is more likely to be involved in immune modulation other than just bacterial killing [10, 11].

In addition to their ability to directly kill a wide range of bacteria, fungi, and enveloped viruses, mature cathelicidins are actively involved in various phases of host defense. Certain cathelicidins are found to chemoattract and activate a variety of immune cells, inhibit NADPH oxidase, kill activated lymphocytes, and promote angiogenesis and wound healing [1, 8, 9]. Consistent with their critical role in host defense and disease resistance, aberrant expression of cathelicidins are often associated with various disease processes. For example, LL-37/hCAP-18 deficiency correlates with recurrent skin

infections in the atopic dermatitis patients [12] and chronic periodontal disease in the morbus Kostmann patients [13]. Similarly, deletion of the cathelicidin gene (*CRAMP*) in mice resulted in a loss of protection against skin infection by Group A *Streptococcus* [14] or oral infection with murine enteric pathogen *Citrobacter rodentium* [15]. Conversely, local or systemic administration of cathelicidins conferred enhanced protection against experimental infections [16-20].

A common mechanism by which cathelicidins kill bacteria appears to be mediated through physical interactions with negatively charged microbial membrane phospholipids, followed by membrane disruption [3, 21]. Many cathelicidins exhibit LPS-binding activity, and the binding affinity is often positively correlated with their antibacterial activity [7]. Because of this physical mechanism, these peptide antibiotics are equally effective in killing both drug-resistant and susceptible strains with little possibility of developing resistance [3, 7]. One side-effect commonly associated with cathelicidins is their cytotoxicity to mammalian cells; however, the concentrations that are required to exert an appreciable degree of cytolytic effect are often much higher than the microbicidal concentrations [7].

To date, cathelicidins have been discovered in a range of mammalian species [8, 9]. In contrast to the vast majority of “classic” cathelicidins, P15 in rabbits [22] and neutrophilic granule protein (NGP)² in mice [23] are distantly related to classic cathelicidins with less homology in the cathelin domain. Hagfish was also found recently to contain two cathelicidin-like sequences [24]. However, the evolutionary relationship of these cathelicidins remains uncertain.

Following our genome-wide computational screening and molecular cloning, here we report identification and functional analysis of the complete repertoire of the cathelicidin gene family in the chicken. Discovery of these non-mammalian cathelicidins helped reveal for the first time the origin and evolution of mammalian cathelicidins. Our data clearly suggested that fowlicidins and mammalian NGPs are likely to originate from a common ancestral gene prior to the separation of birds from mammals and that other classic mammalian cathelicidins may have been duplicated from the NGP gene after the split of mammals and birds. Moreover, a series of functional analyses indicated that these chicken cathelicidins are among the most efficacious cathelicidins that have been identified to date with potent antibacterial and LPS-neutralizing activities, making them attractive candidates as novel antimicrobial and anti-sepsis agents.

4.3 Experimental procedures

Computational Search for Novel Chicken Cathelicidins - To identify potential novel cathelicidins in the chicken, all known cathelicidin peptide sequences discovered in the hagfish and mammals were individually queried against the translated chicken expressed sequence tags (EST), nonredundant sequences (NR), unfinished high throughput genomic sequences (HTGS), and whole genome shotgun sequences (WGS) in GenBank by using the TBLASTN program [25] as we described [26-28]. All potential hits were then examined for presence of the characteristic cathelin domain, including the highly conserved four cysteines. If necessary, the genomic sequences containing chicken

cathelicidin genes were retrieved from GenBank to predict the exon sequence and genomic structure by using GenomeScan [29].

Cloning of the Chicken Fowlicidin Genes - Because no complete genomic sequence is available in GenBank for any of the three fowlicidin genes, the missing sequence of each gene was cloned separately from chicken genomic DNA that was isolated from liver using a genomic DNA isolation kit (Zymo Research, Orange, CA). The first exon/ intron sequence of the fowlicidin-2 gene that is missing in the WGS sequence (AADN01005055) was cloned by using a genome walking approach, namely vectorette PCR, as previously described [30, 31]. Briefly, chicken genomic DNA was digested separately with the blunt-end restriction enzymes (*Dra* I, *EcoR* V, *Puv* I, *Rsa* I, and *Stu* I), followed by ligation with annealed, bubble oligonucleotides (5'-CAAGGGAGGACGCTGTCTGTCGAAGGTAAGGAACGGACGAGAGAAGGGAGAG-3'), and 5'-CTCTCCCTTCTCGAATCGTAACCGTTCTACGAGAATCGCTGTCCTCTCCTTG-3'). Subsequent PCR was performed by using the forward primer indicated in the underlined region of the bubble oligonucleotide and a gene-specific reverse primer. Nested PCR was further performed with the same forward primer and a second gene-specific reverse primer. Two rounds of vectorette PCR were performed to obtain a total of 1.8 kb upstream sequence of the fowlicidin-2 gene.

Because only partial sequence of the last exon of the fowlicidin-1 gene is present in AADN01081708, the entire fowlicidin-1 gene sequence was directly cloned from genomic DNA by PCR using the primers (Forward: 5'-GTTTCCGCATTGCCCAACTTCAG-3'; Reverse: 5'-

GGAACAGTGCTAACAGTGGCTC-3'), which are located in the first and last exons flanking the open reading frame, according to the EST Sequence. The missing first intron sequence of the fowlicidin-3 gene, i.e., the gap between AADN01005055 and AADN01005056, was cloned from chicken genomic DNA by PCR using primers (Forward: 5'-GCTGTGGACTCCTACA ACCAAC-3'; Reverse: 5'-TTGAGGTTGTGCA GGGAGCTGA-3') located in two flanking exons. All PCR products were recovered from agarose gel, ligated into pGEM T-Easy Vector (Promega, USA), and sequenced from both directions.

Assembly of the Chicken Cathelicidin Gene Cluster - To confirm the orientation of three fowlicidin genes on the chromosome, additional PCR reactions were performed to clone the intergenic sequences with chicken genomic DNA and combinations of gene-specific primers located in the first and last exons of each fowlicidin gene. The DNA sequence between fowlicidin-1 and fowlicidin-2 was obtained by using the primers (Forward: 5'-CGCTGGTCATCAGGACTGTGA T-3'; Reverse: 5'-CCATCGTGTCTCCATTCTA TC-3'), while the sequence between fowlicidin-2 and fowlicidin-3 was obtained by using the primers (Forward: 5'-CACCGTGTTGATGGCC ACTGG-3'; Reverse: 5'-TGAGGCCACCGAGTG TCACCT-3'). PCR products were subsequently cloned into pGEM T-Easy Vector and sequenced from both directions. No other primer combinations yielded any PCR products.

To generate a continuous, gap-free cathelicidin gene cluster, three WGS sequences containing fowlicidin genes (AADN01005055, AADN01005056, and AADN01081708) were retrieved from GenBank and annotated together with our newly cloned intra- and

intergenic sequences. Structures of fowlicidin genes were determined by comparing their cDNA sequences with the genomic contig that we assembled. Chromosomal location of the chicken cathelicidin gene cluster was revealed by using the Map Viewer Program (<http://www.ncbi.nlm.nih.gov/mapview/>) in the most current chicken genome assembly (Build 1.1) released on July 1, 2004.

Alignment and Phylogenetic Analysis of Chicken Cathelicidins - Multiple sequence alignment was constructed by using the ClustalW program (version 1.82) [32]. The phylogenetic tree was constructed using the neighbor-joining method [33] by calculating the proportion of amino acid differences (p-distance) among all known cathelicidin precursors with and without the last exon sequence. The reliability of each branch was tested by 1000 bootstrap replications.

Peptide Synthesis - Given that valine is the preferred cleavage site for elastase in the processing and maturation of bovine and porcine cathelicidins [34, 35], we reasoned that the first valine in the fourth exon of fowlicidin-1 and -2 (Fig. 1) is likely to be cleaved by chicken elastase. Therefore, putative mature fowlicidin-1 (RVKRVWPLVIRTVIAGYNLYRAIKKK) and -2 (LVQRGRFGRFLRKIRRFKVTITIQGSARF) were chemically synthesized by SynPep (Dublin, CA), and a sheep cathelicidin, SMAP-29 (RGLRRLGRKIAHGPKKYGPTVLRRIIRIA-NH₂) was synthesized by Bio-Synthesis (Lewisville, TX) by the standard solid-phase synthesis method. All peptides were purified to >95% purity through reverse phase-HPLC. The mass and purity of each peptide was confirmed

by the matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry using the Voyager DE-PRO instrument (Applied Biosystems, Foster City, CA) housed in the Recombinant DNA/Protein Core Facility of Oklahoma State University. The molecular masses of three peptides are as follows: fowlicidin-1 (calculated: 3141.9 and observed: 3141.6), fowlicidin-2 (calculated: 3760.6 and observed: 3760.1), and SMAP-29 (calculated: 3199.0 and observed: 3198.7).

Bacterial Culture - Gram-negative bacteria (*Escherichia coli* ATCC 25922, *E. coli* O157:H7 ATCC 700728, *Salmonella typhimurium* ATCC 14028, *Klebsiella pneumoniae* ATCC 13883, and *Pseudomonas aeruginosa* ATCC 27853), and Gram-positive bacteria (*L. monocytogenes* ATCC 19115 and *Staphylococcus aureus* ATCC 25923) were purchased from either ATCC (Manassas, VA) or MicroBiologics (St. Cloud, MN) and tested individually against fowlicidins and SMAP-29. Three multidrug-resistant bacterial strains (*S. typhimurium* DT104 ATCC 700408, *S. aureus* ATCC BAA-39, and *S. aureus* ATCC 43300) were also purchased from ATCC and used in the antibacterial testing. All bacteria were maintained on trypticase soy agar (TSA) plates. Fresh colonies were cultured and subcultured in trypticase soy broth (TSB) with shaking at 250 rpm at 37°C in a shaking incubator.

Antibacterial Assays - Standard colony counting assay was used to determine the antibacterial activity of fowlicidins as previously described [36]. Briefly, overnight cultures of bacteria were subcultured for additional 3-5 h at 37°C in TSB to the mid-logarithmic phase, washed once with 10 mM sodium phosphate buffer, pH 7.4, and

suspended to 4×10^5 colony forming units (CFU)/ml in the same buffer. Bacteria (90 μ l) were dispensed into 96-well microtiter plates, followed by addition of 10 μ l of serial twofold dilutions of peptides in duplicate. After 2-h incubation at 37°C, surviving bacteria were counted (CFU/ml) after serial plating onto TSA plates and overnight incubation. Minimum inhibitory concentration (MIC_{90}) of individual peptides against each bacterial strain was determined as the lowest concentration that reduced bacterial growth by 90%.

For the kinetics of bacterial killing, fowlicidin-1 (0.1 μ M), fowlicidin-2 (0.16 μ M), and SMAP-29 (0.1 μ M) at MIC_{90} concentrations were incubated separately with *E. coli* ATCC 25922 at 37°C in 10 mM sodium phosphate buffer, pH 7.4. The reaction was stopped by addition of ice-cold PBS at 0, 5, 10, 15, 20, 30, and 60 min and plated immediately for counting viable bacteria. To study the effect of salinity on the antimicrobial activity, fowlicidin-1 (0.1 μ M) and fowlicidin-2 (0.16 μ M) were incubated separately with *E. coli* ATCC 25922 for 2 h with different concentration of NaCl (0, 25, 50, 100, and 150 mM) in 10 mM sodium phosphate buffer, pH 7.4. Surviving bacteria were counted, following overnight incubation on TSA plates.

To examine the antibacterial spectrum of each peptide, a modified broth microdilution assay was used essentially as described [37]. Briefly, bacteria were subcultured to the mid-log phase, washed with 10 mM sodium phosphate buffer and suspended to 5×10^5 CFU/ml in 1% cation-adjusted Mueller Hinton broth (MHB) (BBL, Cockeysville, MD) with and without 100 mM of NaCl. Bacteria (90 μ l) were then dispensed into 96-well plates, followed by addition in duplicate of 10 μ l of serially diluted peptides in 0.01% acetic acid. Because of poor growth of *P. aeruginosa* ATCC

27853 in 1% MHB, this strain was grown in 10% cation-adjusted MHB with peptides in the presence and absence of 100 mM NaCl. After overnight incubation at 37°C, the MIC value of each peptide will be determined as the lowest concentration that gave no visible bacterial growth.

Hemolysis Assay - The hemolytic activities of fowlicidins were determined essentially as described [38, 39]. Briefly, fresh human and chicken blood were collected, washed twice with PBS, and diluted to 0.5% in PBS with and without addition of 10% FBS, followed by dispensing 90 µl into 96-well plates. Different concentrations of peptides (10 µl) dissolved in 0.01% acetic acid were added in duplicate to cells and incubated at 37°C for 2 h. Following centrifugation at $800 \times g$ for 10 min, the supernatants were transferred to new 96-well plates and monitored by measuring the absorbance at 405 nm for released hemoglobin. Controls for 0 and 100% hemolysis consisted of cells suspended in PBS only and in 1% Triton X-100, respectively. Percent hemolysis (%) was calculated as $[(A_{405\text{nm, peptide}} - A_{405\text{nm, PBS}}) / (A_{405\text{nm, 1\% Triton X-100}} - A_{405\text{nm, PBS}})] \times 100$. The effective concentration (EC_{50}) was defined as the peptide concentration that caused 50% lysis of erythrocytes.

Cytotoxicity Assay - The cytotoxic effect of fowlicidins on mammalian cells was measured by using the alamarBlue dye (Biosource), which has been shown to be equivalent to the classic MTT-based assay [40]. Madin-Darby canine kidney (MDCK) epithelial cells were purchased from ATCC and maintained in DMEM with 10% FBS. MDCK cells were seeded into 96-well plates at 1.5×10^5 /well. Following overnight growth, the cells were washed once with DMEM, followed by addition of 90 µl of fresh

DMEM with or without 10% FBS, together with 10 μ l of serially diluted peptides in 0.01% acetic acid in duplicate. After incubation for 18 h, 10 μ l of alamarBlue dye was added to cells for 6 h at 37°C in a humidified 5% CO₂ incubator. The fluorescence of dye was read with excitation at 545 nm and emission at 590 nm. Percent cell death (%) was calculated as $[1 - (F_{\text{peptide}} - F_{\text{background}})/(F_{\text{acetic acid}} - F_{\text{background}})] \times 100$, where F_{peptide} is the fluorescence of cells exposed to different concentrations of peptides, $F_{\text{acetic acid}}$ is the fluorescence of cells exposed to 0.01% acetic acid only, $F_{\text{background}}$ is the background fluorescence of 10% alamarBlue dye in cell culture medium without cells. Cytotoxicity (EC₅₀) of individual peptides was defined as the peptide concentration that caused 50% cell death.

LPS Binding Assay - The binding of LPS to fowlicidins was measured by the kinetic chromogenic *Limulus* amoebocyte lysate assay (Kinetic-QCL 1000 kit; BioWhittaker, Walkersville, MD) as previously described [37, 41]. Briefly, 25 μ l of serially diluted peptide were added in duplicate into 25 μ l of *E. coli* O111:B4 LPS containing 0.5 endotoxin units/ml and incubated for 30 min at 37°C, followed by incubation with 50 μ l of the amoebocyte lysate reagent for 10 min. The absorbance at 405 nm was measured at 10 and 16 min after addition of 100 μ l of chromogenic substrate, Ac-Ile-Glu-Ala-Arg-*p*-nitroanilide. Percent LPS binding was calculated as $[(\Delta D1 - \Delta D2 + \Delta D3)/ \Delta D1] \times 100$, where $\Delta D1$ represents the difference in the absorbance between 10 and 16 min for the sample containing LPS only, $\Delta D2$ represents the difference in the absorbance between 10 and 16 min for the samples containing LPS and different concentrations of peptides, and $\Delta D3$ represents the difference in the absorbance between 10 and 16 min for the samples

containing different concentrations of peptides with no LPS. Hill plot was graphed as described [37, 41] by plotting \log_{10} fowlicidin concentrations against $\log_{10} [F_1/(1.0 - F_1)]$, where F_1 was the fractional inhibition of LPS binding activity.

Modulation of LPS-induced Proinflammatory Gene Expression by Fowlicidins -

Mouse macrophage RAW 264.7 cells were plated in 12-well plates at 5×10^5 cells/well in DMEM with 10% FBS and allowed to grow overnight. The cells were pretreated for 30 min with 1, 5, and 20 μM of fowlicidin-1, fowlicidin-2, and SMAP-29 in duplicate, followed by stimulation with 100 ng/ml LPS from *E. coli* O114:B4 (Sigma) for 4 h. The supernatant was removed and total RNA was isolated from cells using TRIzol (Invitrogen) according to the manufacturer's instructions. The first strand cDNA from 1.5 μg of each RNA sample was synthesized at 42°C for 30 min by using QuantiTect® Reverse Transcription Kit (Qiagen), which includes removal of genomic DNA contamination prior to cDNA synthesis.

The first-strand cDNA of each sample was then used as a template for subsequent real-time PCR amplification by using QuantiTect® SYBR Green qRT-PCR Kit (Qiagen) and MyiQ® Real-Time PCR Detection System (Bio-Rad). Three common proinflammatory cytokines and chemokines, namely interleukin (IL)-1 β , CC chemokine ligand 2 (CCL2)/MCP-1, and CCL3/MIP-1 α , were selected. All primers were designed to expand at least an intron sequence (Table I). The PCR reaction was set up in a total volume of 15 μl containing 0.4 μM of each primer and 0.2 μg of the first-strand cDNA. PCR cycling conditions were as follows: 95°C for 10 min, followed by 50 cycles of 95°C for 15 sec, 55°C for 30 sec, and 72°C for 30 sec.

Gene expression levels were quantified by the comparative $\Delta\Delta C_T$ method as described [42] by using β -actin as an internal standard for normalization. The ΔC_T value was determined by subtracting the C_T value of each sample from that of β -actin in the corresponding sample. The $\Delta\Delta C_T$ values were further calculated by subtracting the highest mean ΔC_T value as an arbitrary constant from all other ΔC_T values. Relative gene expression levels were calculated using the formula $2^{-\Delta\Delta C_T}$. The presence of contaminating genomic DNA was determined by including a no-reverse transcriptase control and signal generated by primer dimers was determined through no-template controls. Melting curve analysis (55-95°C) was performed and confirmed no visible nonspecific amplification of any PCR products from genomic DNA or primer dimers.

4.4 Results

Identification of Three Novel Chicken Cathelicidin Genes - To identify potential cathelicidins in the chicken, all known cathelicidin peptide sequences were queried individually against the translated genomic and EST sequences in GenBank by using the TBLASTN program [25] as we previously described [26-28]. As a result, seven chicken EST sequences (GenBank accession numbers BX936022, BU106516, AJ393748, CB018183, BU420865, CR389785, and BQ484540) were identified (Table II). Three putative cathelicidin peptide sequences were subsequently deduced and termed fowlicidins 1-3. Because the N-terminal sequence including the start codon of fowlicidin-2 was missing in GenBank, a genome walking approach known as vectorette PCR was performed by using chicken genomic DNA as previously described [30, 31]. As a result,

a 1.8-kb upstream sequence of the fowlicidin-2 gene was obtained following two rounds of vectorette PCR (data not shown). The missing N-terminal peptide sequence of fowlicidin-2 was predicted by GenomeScan [29] based on its homology with the other two fowlicidins.

Alignment of three fowlicidin peptide sequences revealed that they are highly homologous to each other (Fig. 1). Among all three peptides, fowlicidin-1 and -3 are more closely related with >90% identity throughout the entire sequence. Chicken cathelicidins also share a high degree of similarity with all known mammalian cathelicidins, particularly in the prosequence region (Fig. 1). Noticeably, four cysteines that are conserved in the cathelin domain of all mammalian cathelicidins are also invariantly spaced in three fowlicidins. These results clearly suggest that three chicken fowlicidins are *bona fide* non-mammalian cathelicidins.

Despite of sequence conservation at the N-terminus, fowlicidins and classic cathelicidins are drastically diverged at the C-terminus (Fig. 1). Similar to classic cathelicidins, fowlicidins 1-3 are positively charged at the C-terminus due to the presence of an excess number of cationic residues (R and K). The preferred cleavage site for elastase in the processing and maturation of bovine and porcine cathelicidins [34, 35] also appears to be conserved in the chicken. Therefore, mature fowlicidins 1-3 are predicted to be devoid of cysteines and composed of 26, 32, and 29 amino acid residues with a net charge of +8, +10, and +7, respectively (Fig. 1).

In addition to fowlicidins, we also identified the orthologs of rabbit P15 [22] and mouse NGP [23] in the rat, pig, and cow, which we named rNGP, pNGP, and bNGP, respectively (Fig. 1). However, no NGP-like genes were found in dogs or primates.

Surprisingly, fowlicidins share a higher degree of similarity particularly in the signal sequence region with NGPs than to classic cathelicidins (Fig. 1), implying that chicken cathelicidins and NPGs may be more closely related. It is noted that all NGPs are highly conserved throughout the entire sequence among rodents and ungulates with a net negative charge at the C-terminus. The functional significance of such anionic sequences remains to be studied.

Genomic Organization of the Chicken Fowlicidin Gene Cluster - A screening through genomic sequences in the NR, HTGS and WGS databases in GenBank identified three WGS sequences. AADN01081708 contains a part of the last exon sequence of the fowlicidin-1 gene, and AADN01005055 and AADN01005056 encode the majority of the fowlicidin-2 and -3 genes (Fig. 2A). The fowlicidin-1 gene was cloned from chicken genomic DNA by PCR using the primers located in the first and last exons, whereas the missing first intron sequence of the fowlicidin-3 gene, i.e., the gap between AADN01005055 and AADN01005056, was cloned directly by PCR with primers located in two flanking exons (Fig. 2A). The 5'-end of the fowlicidin-2 gene was cloned by two rounds of vectorette PCR as described in the previous section. Structural organizations of three fowlicidin genes were obtained by comparing their cDNA with genomic DNA sequences. As shown in Table I, all three genes are organized similarly with four exons separated by three introns. The first three exons encode the signal and cathelin pro-sequences, whereas the last exon primarily encodes the mature sequences. Such structures are surprisingly identical to the mammalian cathelicidin genes, a clear indication of significant conservation during evolution.

Annotation of AADN01005055, AADN01005056, AADN01081708, and our newly cloned intra- and intergenic sequences resulted in the formation a continuous, gap-free contig that harbors all three fowlicidin genes, which has been deposited to GenBank under accession number DQ092350. As shown in Fig. 2A, three fowlicidin genes are packed tightly in a 7.5-kb distance on the chromosome with fowlicidin-2 and -3 in a head-to-head orientation that is separated only by 736 bp from the stop codons of both genes. However, fowlicidin-1 and -2 are separated 2.4 kb from each other by a gene homologous to the C-terminal end of vesicle-associated, calmodulin kinase-like kinase (CamKV) (GenBank accession no. NP_076951). Chromosomal location of the chicken cathelicidin gene cluster was further revealed by using the Map Viewer Program. AADN01005055 and AADN01005056 were found to locate on the p arm of chromosome 2 that are less than 3.5 Mb from the proximal end in the current chicken genome assembly (Build 1.1) released on July 1, 2004, but AADN01081708 remains unmapped.

Comparative and Evolutionary Analyses of Vertebrate Cathelicidins - Identification of three chicken fowlicidins provides an excellent opportunity to study the evolutionary relationship of mammalian cathelicidins. We first examined physical locations of the cathelicidin gene clusters across several phylogenetically distant vertebrate species. As shown in Fig. 2B, the cathelicidin genes are located in the syntenic regions flanking an evolutionarily conserved gene, Kelch-like 18 (KLHL18) (NP_071737) across rodents, dogs, and humans, clearly indicating that cathelicidins in mammals and birds share a common ancestor. It is noteworthy that the chicken CamKV gene is absent in syntenic regions in mammals (Fig. 2B).

We then performed the phylogenetic analysis of fowlicidins together with all known mammalian cathelicidins and two recently identified hagfish cathelicidins using the neighbor-joining method [33] by calculating the proportion of amino acid differences. All vertebrate cathelicidins clearly formed three distinct clusters with two hagfish peptides located in a separate clade from others (Fig. 3). Supported by a bootstrap value of 56%, fowlicidins clustered with NGPs, suggesting that fowlicidins and NGPs are likely to originate from a common ancestor prior to the separation of birds from mammals. This is further supported by the close proximity of fowlicidins and NGPs with KLHL18 on chromosomes (Fig. 2B). All classic mammalian cathelicidins comprised a separate cluster supported by a bootstrap value of 99% (Fig. 3) and are located more than 500 kb away from KLHL18 and NGP, implying that classic cathelicidins are likely to be duplicated from NGPs after the mammal-bird split. Apparent missing of NGPs in the dog, chimpanzee, and human genomes (data not shown) suggested that the NGP lineage was lost after canines and primates diverged from other mammals.

However, it is also possible that two different primordial genes for NGPs/fowlicidins and classic cathelicidins were present in the common ancestor of aves and mammals. Both gene lineages are preserved in most mammals, but the classic cathelicidin lineage was lost in aves after they split from mammals. Because two hagfish cathelicidins are too divergent, it is impossible to point out the evolutionary relationship of fish cathelicidins with their avian and mammalian homologs. Availability of genomic sequences of additional phylo-genetically distant lower vertebrate species is expected to help bridge the gap.

Antibacterial Properties of Fowlicidins - To test antibacterial properties of chicken cathelicidins, putatively mature fowlicidin-1 and -2 were synthesized commercially by the standard solid phase synthesis method and purified to >95% purity. A reference strain of *E. coli* ATCC 25922 was tested by using the colony counting assay as previously described [36]. As shown in Fig. 4A, fowlicidin-1 and -2 displayed a MIC₉₀ of 65 nM and 180 nM, respectively, against *E. coli*. In fact, when compared directly with SMAP-29, which is the most potent cathelicidin that has been reported thus far [7], both fowlicidin-1 and -2 showed comparable antibacterial potency, implying the promising therapeutic potential of these two chicken cathelicidins. Furthermore, similar to SMAP-29, both fowlicidins showed a rapid killing of *E. coli* with the maximum killing occurring at 30 min at MIC₉₀ concentrations (Fig. 4B), reinforcing the notion that both fowlicidins kill bacteria most likely through physical membrane disruption. However, unlike many antimicrobial peptides whose antimicrobial activities are inhibited by salt at physiological concentrations [36-38, 43, 44], fowlicidin-1 and -2 maintained their activities up to 150 mM NaCl (Fig. 4C), implying their potential for systemic therapeutic applications. It is noted that we did not observe any obvious synergistic effect of two fowlicidin peptides in killing *E. coli* when applied together (data not shown).

To test the antibacterial spectrum of fowlicidins, six Gram-negative and four Gram-positive bacterial strains were used in a modified broth microdilution assay [37]. Both chicken cathelicidins were broadly effective against all bacteria tested in a salt-independent manner, with most MIC values in the range of 0.4-2.0 μ M (Table III). *P. aeruginosa* appeared to be the only exception, being slightly more resistant to fowlicidins with the MIC of 3-6 μ M for both peptides. Strikingly, both peptides displayed

comparable antibacterial potency with SMAP-29 against all bacteria, although there was a tendency that fowlicidin-1 is slightly more efficacious than fowlicidin-2 in most cases (Table III). More desirably, both peptides were equally effective against antibiotic-resistant bacterial strains, such as multidrug-resistant *S. typhimurium* DT104 and methicilin-resistant *S. aureus* (MRSA) (Table III). Fowlicidin-3 also showed similar antibacterial activities against Gram-positive and Gram-negative bacteria to fowlicidin-1 (data not shown), but was omitted for further functional analyses because of its high homology with fowlicidin-1 (Fig. 1).

Cytotoxicity of Fowlicidins - To evaluate the hemolytic activity of fowlicidins against red blood cells, freshly isolated human and chicken erythrocytes were incubated with fowlicidins, together with SMAP29 as a positive reference. Hemolysis was monitored by measuring the absorbance at 405 nm for released hemoglobin as described [38, 39]. Hemolytic activities of both chicken cathelicidins were similar toward human and chicken erythrocytes with EC_{50} occurring at approximately 6-10 μ M and 15-20 μ M for fowlicidin-1 and -2, respectively (Fig. 5A and 5B). Hemolytic activities of both fowlicidins and SMAP-29 were reduced by 2-4 fold in the presence of 10% FBS (data not shown).

To further examine the cytotoxicities of fowlicidins toward mammalian epithelial cells, the viability of MDCK cells was measured by an alamarBlue-based, colorimetric method [40], following exposure to either peptide for 24 h. As shown in Fig. 5C, in the absence of 10% FBS, EC_{50} occurred in the range of 10-20 μ M toward MDCK cells for both fowlicidins, with SMAP-29 being the most toxic. A similar trend also occurred with

RAW264.7 macrophage cells (data not shown). A 2- to 5-fold reduction in toxicity was observed in the presence of 10% FBS for all three peptides. It is noted that the cytotoxicities of fowlicidins toward mammalian host cells are at least several fold higher than their MIC values toward bacteria (compare Table III and Fig. 5), suggestive of the therapeutic potential of two fowlicidins. Nevertheless, a further reduction of their toxicity through rational mutagenesis will be more desirable.

LPS Binding and Host Gene Modulatory Activities of Fowlicidins - To test the potential of fowlicidins as anti-sepsis agents, a chromogenic *Limulus* amoebocyte lysate assay was used to measure the binding of LPS to fowlicidins by the competitive inhibition of LPS-induced procoagulant activation as described [37, 41]. As shown in Fig. 6A, typical sigmoidal curves of LPS binding activity were observed for both peptides, which exhibited a similar LPS binding efficiency with 50% binding occurring at 7.5 μ M and 8.6 μ M for fowlicidin-1 and -2, respectively. Both peptides completely inhibited the LPS procoagulant activity at 10-15 μ M concentrations. Because the sigmoidal shapes imply cooperativity, we also graphed the data on a Hill Plot (Fig. 6B). The Hill plot coefficients (slopes) were calculated to be 2.44 and 3.22 for fowlicidin-1 and -2, respectively, suggesting the presence of cooperative LPS binding sites possibly on each peptide molecule for both peptides. These results are reminiscent of SMAP-29 and LL-37 with multiple intramolecular LPS binding sites that function cooperatively to allow peptides to bind to LPS with high affinity [37, 41].

To further evaluate whether binding of fowlicidins to LPS can neutralize LPS-induced proinflammatory responses, RAW264.7 macrophage cells were stimulated with

LPS in the presence of different concentrations of peptides, followed by evaluation of proinflammatory cytokine/chemokine gene expression by real time reverse transcriptase-PCR. Fowlicidin-1 and -2, when applied up to 20 μ M in the absence of LPS, did not alter gene expression. However, they blocked LPS-induced expression of IL-1 β and CCL-2/MCP-1 in a dose-dependent fashion (Fig. 6C and 6D). The same trend also occurred with CCL-3/MIP-1 α for both peptides (data not shown). In fact, fowlicidins inhibited the expression of all three genes by >90% at 20 μ M (Fig. 6C and 6D). To our surprise, albeit with in vitro LPS-binding activity [41], SMAP-29, even at 20 μ M, failed to suppress the expression of any of the three proinflammatory genes that we examined. Collectively, these data strongly suggested the potential of fowlicidin-1 and -2 as both antibacterial and anti-sepsis agents. It is noted that all three peptides, when applied at 20 μ M, caused only minimal, < 5% cell death to RAW264.7 cells in the presence of 10% FBS (data not shown).

4.5 Discussion

Three chicken cathelicidins consist of linear cationic sequences at the C-termini, which are expected to be freed from the cathelin domain to become biologically active. Indeed, putatively mature fowlicidins possess potent antibacterial activities (Fig. 4 and Table III). Although we suspected that valine on the fourth exon of fowlicidins (Fig. 1) is likely to be the processing site for chicken elastase-like protease as in the case of bovine and porcine cathelicidins [34, 35], the protease and exact cleavage site for fowlicidins need to be experimentally confirmed.

In the course of screening for chicken cathelicidins, we also identified NGPs in rats, pigs and cows that are highly homologous to P15s in rabbits [22] and NGP in mice [23] (Fig. 1). These NGP-like proteins appear to be evolutionarily conserved only in glires and ungulates, but not in dogs and primates. In spite of relatively low homology in the cathelin domain with the majority of other mammalian cathelicidins, NGPs share similar tissue expression pattern [22, 23], chromosomal location (Fig. 2B), gene structure (data not shown), and antimicrobial activities [22] to classic mammalian cathelicidins, and therefore, clearly belong to the cathelicidin family. Identification of three fowlicidins, which are more closely related to NGPs (Fig. 1 and 3), suggested that the ancestral gene for fowlicidins/NGPs arose in the common ancestor of mammals and birds, which may have further given rise to classic mammalian cathelicidins as a result of gene duplication after the mammal-bird split. Classic cathelicidins must have been duplicated from NGPs prior to the divergence of mammals from each other, because of a high degree of homology within classic cathelicidins particularly in the cathelin domain. Apparently, independent duplications have occurred after mammals were separated from each other, which is supported by species-specific clustering and presence of a varied number of classic cathelicidins in most cases (Fig. 3). For example, ungulates have multiple cathelicidins, whereas most other mammals have one or very few members.

As for the origin of three chicken cathelicidins, fowlicidin-1 and -3 are apparently a result of gene duplication, because of significant homology across the entire open reading frame (Fig. 1). Furthermore, the intron sequences of these two cathelicidin genes are highly similar (data not shown). Fowlicidin-2 also shares significant homology in the first three exons with fowlicidin-1 and -3, but diverged greatly in the last exon encoding the

mature sequence (Fig. 1). However, alignment of the last intron and exon nucleotide sequences of three fowlicidin genes revealed approximately 45% identity (data not shown). This suggested that, the entire fowlicidin-2 gene may have been duplicated directly from fowlicidin-1 or -3, but not a result of exon shuffling [45], which probably gave rise to multiple cathelicidins with drastic sequence divergence in the last exon in ungulates.

Although P15s is unique in that it does not undergo proteolytic processing when released [22], it will be interesting to see whether it is also true with other mammalian NGPs. The presence of valines in the fourth exons of NGPs in the pig and rat at equivalent elastase cleavage site raises the possibility that at least some NGPs may be enzymatically processed upon activation (Fig. 1). However, the C-terminal peptides of NGPs are all negatively charged, as opposed to classic cathelicidins with cationic sequences. Therefore, it will be interesting to study the processing and biological roles of these NGPs. Because of the existence of two cathelicidins (NGP and CRAMP) in mice as opposed to a single cathelicidin (LL-37/hCAP-18) in humans, extrapolation of the data from CRAMP-deficient mouse to the human system needs to be more prudent.

During the preparation of our manuscript, the sequences identical to fowlicidin-1 and -2 have been reported independently [46, 47]. Similar to most mammalian cathelicidins, fowlicidin-1 and -2 were shown to be expressed primarily in bone marrow, but none of the functional information has been provided [46, 47]. Our data clearly indicated that putatively mature fowlicidins are clearly among the most potent cathelicidins discovered to date, killing a variety of bacteria at $<2 \mu\text{M}$ (Table III). This is perhaps not surprising, given the fact that chicken heterophils, which are equivalent to mammalian neutrophils

and likely the store sites of fowlicidins, lack myeloperoxidase and depends primarily on non-oxidative mechanisms for antimicrobial activity [48]. Unlike many cationic antimicrobial peptides that are inactivated at physiological concentrations of salt, fowlicidins maintained antibacterial activity in the presence of high NaCl (Fig. 4C and Table III). Salt-independent killing of bacteria of fowlicidins may offer an attractive therapeutic option for cystic fibrosis and Crohn's disease, both of which are associated with aberrant local expression or inactivation of antimicrobial peptides [49-51].

In summary, discovery and functional characterization of these chicken cathelicidins offer new insights on the evolution of mammalian cathelicidins. Potent, broad-spectrum, salt-independent antibacterial activities with strong LPS-neutralizing activity make fowlicidins excellent candidates as antimicrobial and anti-sepsis agents. Structure-activity relationship studies of these peptides aiming at potentiating their antimicrobial and LPS-neutralizing activities while further reducing their toxicities are currently ongoing.

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4.7 References

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4.8 Footnotes

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The nucleotide sequences reported in this paper have been submitted to the GenBankTM/EMBL Data Bank with accession numbers DQ092350, DQ092351, DQ092352, and DQ092353.

² The abbreviations used are: NGP, neutrophilic granule protein; EST, expressed sequence tag; HTGS, high throughput genomic sequence; WGS, whole-genome shotgun sequence; CFU, colony forming units; TSB, trypticase soy agar; MHB, Mueller Hinton broth; MIC₉₀, minimum inhibitory concentration of an antimicrobial that reduces bacterial growth by 90%; EC₅₀, 50% effective concentration; LPS, lipopolysaccharide; MDCK, Madin-Darby canine kidney cells; SEM, standard error of the mean.

TABLE I. Primer sequences used for real-time RT-PCR analysis of murine cytokines and chemokines

Gene	Primer Sequence		Product Size (bp)	
	Forward	Reverse	cDNA	Genomic
IL-1 β	AGAATCTATACCTGTCCTGTGT	TGTGCTCTGCTTGTGAGGTG	916	195
CCL2	ACAAGAGGATCACCAGCAGC	CTGAAGACCTTAGGGCAGATG	511	186
CCL-3	CACGCCAATTCATCGTTGAC	CATTCAGTTCCAGGTCAGTG	372	147
β -actin	GGAGATTACTGCTCTGGCTC	CTCCTGCTTGCTGATCCACA	264	139

TABLE II. Identification of chicken fowlicidins

The chicken genome contains three cathelicidin genes, namely fowlicidins 1-3, encoded by expressed sequence tags (EST) and whole genome shotgun sequences (WGS) as indicated. Each fowlicidin gene consists of four exons (E) separated by three introns (I). The sizes of the first and last exons are given according to the coding sequences without the 5'- and 3'-untranslated regions being counted. Note that the first three exons are 100% identical between fowlicidin-1 and fowlicidin-3, except for exon 4, which encodes different mature sequences.

Gene	EST	WGS	Gene Size (bp)						
			E 1	I 1	E 2	I 2	E 3	I 3	E 4
fowlicidin-1	BX936022	AADN01081708	168	537	108	84	84	99	87
	BU106516								
	AY534900								
fowlicidin-2	AJ393748	AADN01005055	168	901	108	70	84	293	105
	CB018183								
	BU420865								
fowlicidin-3	CR389785	AADN01005055	168	535	108	84	84	99	96
		AADN01005056							

TABLE III. Antibacterial spectrum of fowlicidins against Gram-negative and Gram-positive bacteria

Mid-log phase bacteria (5×10^5 CFU/ml) in 1% cation-adjusted Muller Hinton broth (MHB) were incubated overnight with serial twofold dilutions of peptides in the presence or absence of 100 mM NaCl. The exception was *P. aeruginosa* ATCC 27853, which was incubated with peptides in 10% cation-adjusted MHB with or without addition of 100 mM NaCl. The minimum inhibitory concentrations (MIC) of individual peptides against each bacterial strain were determined as the peptide concentration that gave no visible bacterial growth after overnight incubation. The experiments were repeated at least twice with essentially the same results.

Bacteria	ATCC #	MIC (μ M)					
		Fowlicidin-1 (NaCl, mM)		Fowlicidin-2 (NaCl, mM)		SMAP-29 (NaCl, mM)	
		0	100	0	100	0	100
Gram-negative:							
<i>E. coli</i>	25922	1.59	1.59	2.66	2.66	1.56	1.56
<i>E. coli</i> O157:H7	700728	0.80	0.80	0.66	1.33	0.78	0.78
<i>S. typhimurium</i>	14028	0.80	1.59	1.33	1.33	0.39	0.78
<i>S. Typhimurium</i> DT104	700408	0.40	1.59	0.66	1.33	0.39	0.78
<i>K. pneumoniae</i>	13883	0.40	0.80	0.66	1.33	0.39	0.39
<i>P. aeruginosa</i>	27853	3.18	3.18	5.32	2.66	3.12	3.12
Gram-positive:							
<i>L. monocytogenes</i>	19115	0.80	1.59	1.33	1.33	0.78	0.78
<i>S. aureus</i>	25923	0.80	0.80	0.66	1.33	0.78	0.78
<i>S. aureus</i> (MRSA) ¹	BAA-39	0.40	0.80	0.66	1.33	0.39	0.39
<i>S. aureus</i> (MRSA)	43300	0.40	0.40	0.33	2.66	0.39	0.39

¹ MRSA, methicilin-resistant *S. aureus*.

	Signal Peptide	Prosequence (Cathelin)	
Fowlicidin-1	-----MLSCWVLL LALLGGACALPAP----	LGYSQALAQAVDSYNQRPEVQNAFRLLSAD-PEP	
Fowlicidin-3	-----MLSCWVLL LALLGGACALPAP----	LGYSQALAQAVDSYNQRPEVQNAFRLLSAD-PEP	
Fowlicidin-2	-----MLSCWVLL LALLGGV CALPAP----	LGYSQALAQAVDSYNQRPEVQNAFRLLSAD-PEP	
pNGP	-----MAGAWRALVLVAGLAAVVSVA-QRSLSYEEIINKALWFFNQGRPGQRLFRLLVAM-PPP		
bNGP	-----MAGAWKALVLVAGLAAVACVA-QRGLSYEEIVTQALKFFNQGRRGQRIFGLLESTPPPP		
rNGP	-----MARLWKT FMLVV ALAVVACEA-HRRLRYEDIVNRAIEAYNRGQRGRPLFRLLSAT-PPP		
mNGP	-----MAGLWKT FVLVV ALAVV SCEA-LRQLRYEEIVDRAIEAYNQGRQGRPLFRLLSAT-PPS		
P15	-----MAGVWVKV L VLVVGLAVVACAI PHRRRLRYEEVVAQALQFYNEGQQGQPLFRLLSAT-PPP		
LL37	MKTQRNGHSLGRWSLV LLLLGLVMP LAII-AQVLSYKEAVLRAIDGINQRS SDANLYRLLDLD-PRP		
CRAMP	MQFQRDVP SLWLWRSLS LLLLGLGF---SQTPSYRDAVLRVDDFNQQLDNTNLYRLLDLD-PEP		
Protegrin-1	METQRASLCLGRWSLW LLLLALVVP SAS--AQALS YREAVLRAVDRLNEQS SEANLYRLELD-QPP		
Indolicidin	MQQRASLSLGRWSLW LLLLGLVVP SAS--AQALS YREAVLRAVDQLNELS SEANLYRLELD-PPP		
Fowlicidin-1	GNVQLSSLHNLNFTIMETRCQARS GA---QLDSCFEKEDGLVKDCAAPVVLQGGRAVLDVT CVDSM		
Fowlicidin-3	GNVQLSSLHNLNFTIMETRCQARS GA---QLDSCFEKEDGLVKDCAAPVVLQGGRAVLDVT CVDSM		
Fowlicidin-2	GPVLDLSTLRALNFTIMETECTPSARL---PVDDCDFKENGVI RDCSGPVS VLQDTP E INLRCRDAS		
pNGP	NI---NFTTNIPLNFR IKETVCFSTR FHLHRQPRKCAFREGGEERNCTGTFMFLPQFRLLSFQCTEDP		
bNGP	DI---NSTT-IPLNFR IKETVCFLLWYR---RRRQCPFRREGGEERNCTGSFFMLRQLRLLSLNCVPDR		
rNGP	GC---NPTSNVPLEFR IKETVCI STTER---RLENCDFREGGEERNCTGEFSRRQWSTSLTLTCDRDC		
mNGP	SC---NPATNIPLQFRIKETECTS TQER---QPKDCDFLENGEERNCTGKFFRRRQSTSLTLTCDRDC		
P15	SI---NSKSRIPLNFR IKETVICIFTLDR---QPGNCAFREGGEERI CRGAFVRRRRVRALTLCRDRDQ		
LL37	TMDGDPDTPKPV SFTVKETVCPRTTQQ---SPEDCDFKDKGLVKRCMGTVTLNQARGSFDISCDK---		
CRAMP	QDEDPDTPKSVRFRVVKETVCGK AERQ---LPEQCAFKEQGVVKQCMGAVT LNP AADSFDIS CNE---		
Protegrin-1	KADEDPTPKPV SFTVKETVCPRP TRQ---PELCDFKENG RVKQCVGTVTLDQ IKDPLDITCNE---		
Indolicidin	KNEDLDGTRKPV SFTVKETVCPRT IQQ---PAEQCDFKDKGLVKRCMGTVTLDPSNDQFDLNCNE---		
Fowlicidin-1	AD-----PVRV KR-VWPLV---IRTVIAGYNLYRAIKKK-----	(+ 8) 26	
Fowlicidin-3	AD-----PVRV KR-FWPLVPVAINTVAAGINLYKAIRK-----	(+ 7) 29	
Fowlicidin-2	SD-----PVLVQRGRFGRFLRKIRRFKPKVTITIQGSARFG-----	(+10) 32	
pNGP	DRERE---LNRQIPRVRRSAVSSDVAPPETD-ISKLPAAARDLYERTKYDIINNILRNF		
bNGP	ELEPE---PRRREERSAGSAGEDPPELD-SSNLPPAVRDMYERAKYDIISNILRNF		
rNGP	RREV---SQWATFSDNKSDDSEKDKLEGLPPHAKNIYENAKYDIISNILHNF		
mNGP	SRED---TQETSFND-KQDVSEKEKFEDVPPHIRNIYEDAKYDIIGNILKNF		
P15	RRQP---EFPRVTRPAGPTA-----		
LL37	-----DNKRFALLGDFFRK SKEKIGKEFKRIVORIKDFLRNLVPRTES-----	(+ 6) 37	
CRAMP	-----PGAQPF RFKKISRLAGLLRKGGEKIGEKLLKIGOKIKNFFOKLVPQPEQ-----	(+ 6) 33	
Protegrin-1	-----VQGVRRGRLCYCRRRFCVCGRG-----	(+ 6) 19	
Indolicidin	-----LQSVILPWKWPWWPWRRG-----	(+ 3) 14	

FIG. 1. Multiple sequence alignment of chicken fowlicidins with representative mammalian cathelicidins. Fowlicidins are aligned with classic cathelicidins (human LL37, mouse CRAMP, porcine Protegrin-1, and bovine Indolicidin) and a group of distantly related neutrophilic granule proteins (NGP) in the pig (pNGP), cow (bNGP), rat (rNGP), and rabbit (P15). Dashes are inserted to optimize the alignment and conserved residues are shaded. The positions of four exon boundaries are indicated by vertical lines. Two intramolecular disulfide bonds in the cathelin pro-sequence are shown. Also indicated are the net positive charge (in parenthesis) and length of each mature cathelicidin as underlined. Because NGP proteins may not be cleaved following activation, no mature sequences are postulated. Note that chicken fowlicidins share higher sequence homology with NGP proteins in the first three exons than with classic mammalian cathelicidins.

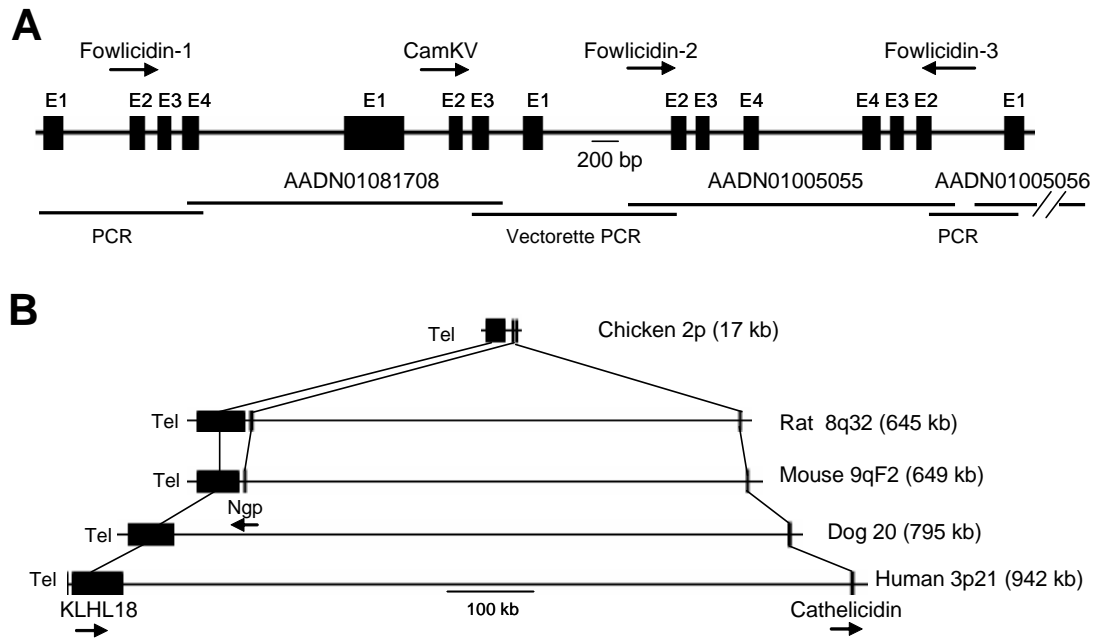


FIG. 2. Genomic organization of the chicken fowlicidin cluster (A) and comparative analysis of the mammalian cathelicidin gene clusters (B). The continuous genomic contig containing chicken fowlicidins was obtained by annotation of three whole-genome shotgun sequences (AADN01005055, AADN01005056, and AADN01081708) and additional genomic sequences obtained by PCR and vectorette PCR (see “Experimental Procedures”). The direction of transcription of each gene is indicated by the arrow. In Panel A, the chicken cathelicidin cluster consists of three fowlicidin genes each containing four exons (*E*) shown as solid rectangles. Located between fowlicidin-1 and fowlicidin-2 genes is chicken CamKV gene, which is homologous to vesicle-associated, calmodulin kinase-like kinase (NP_076951). In Panel B, relative position of each gene is indicated by a solid rectangle or vertical line. Note that chicken fowlicidins, similar to neutrophilic granule proteins (*NGP*), are located closely adjacent to an evolutionarily conserved gene (*KLHL18*), but a *NGP*-like protein is missing in the dog or human genome.

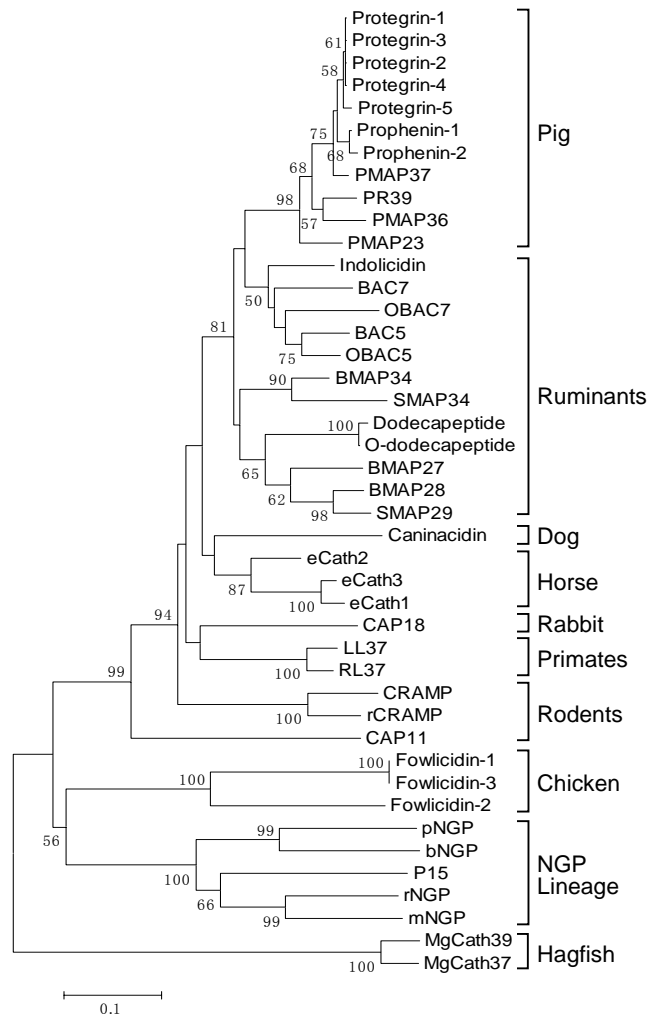


FIG. 3. **Phylogenetic analysis of cathelicidins.** The tree was constructed by the neighbor-joining method based on the proportion difference (p-distance) of aligned amino acid sites of the full-length peptide sequences. A total of 1000 bootstrap replicates were used to test the reliability of each branch. Numbers on the branches indicate the percentage of 1000 bootstrap samples supporting the branch. Only branches supported by a bootstrap value of at least 50% are shown. The tree constructed with the peptide sequences from the first three conserved exons is essentially the same as the one constructed with the full-length peptides, and therefore, is not shown.

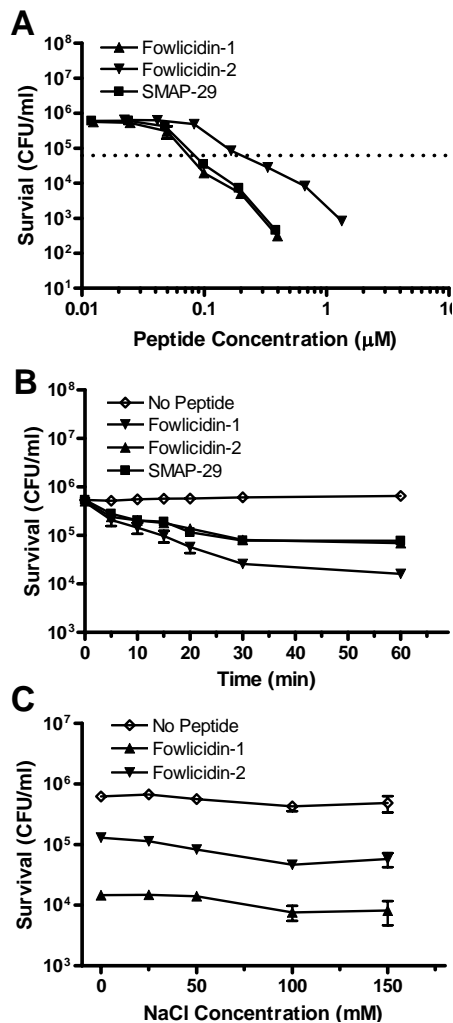


FIG. 4. **Antibacterial properties of fowlicidins.** *E. coli* ATCC 25922 was incubated with peptides in 10 mM phosphate buffer, pH7.4 at 37°C and surviving bacteria were plated onto trypticase soy agar plates and quantitated as CFU/ml following overnight incubation. *A*, dose-dependent killing of *E. coli* by fowlicidins and an ovine cathelicidin (SMAP-29). Bacteria were exposed to indicated peptide concentrations for 2 h followed by quantitative CFU assays. The MIC₉₀ value is indicated as a dotted line. *B*, Time-dependent killing of *E. coli* by peptides at MIC₉₀ concentrations. Following exposure to peptides (0.1 μM fowlicidin-1, 0.16 μM fowlicidin-2, and 0.1 μM SMAP-29) or an equal volume of 0.01% acetic acid (control) for 0, 5, 10, 15, 20, 30, and 60 min, surviving bacteria were plated and counted. *C*, Effect of Salinity on the antibacterial activity of fowlicidins. *E. coli* were exposed to peptides (0.1 μM fowlicidin-1 and 0.16 μM fowlicidin-2) or an equal volume of solvent (control) in 10 mM phosphate buffer with addition of different concentrations of NaCl (0, 25, 50, 100, and 150 mM). Data shown are means ± SEM of 2-4 independent experiments.

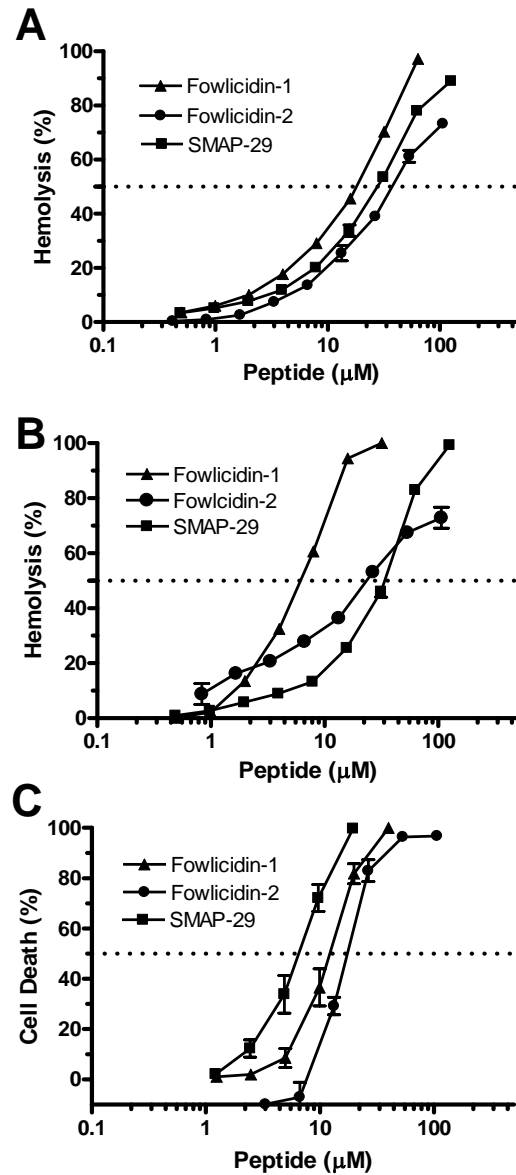


FIG. 5. **Cytolytic activities of fowlicidins.** *A*, hemolytic activity of fowlicidins and SMAP-29 to chicken erythrocytes. *B*, hemolytic activity of fowlicidins and SMAP-29 to human erythrocytes. In panels *A* and *B*, freshly isolated red blood cells were incubated with different concentrations of peptides in PBS for 2 h before measuring the absorbance at 405 nm for the released hemoglobin. *C*, cytotoxicity of fowlicidins and SMAP-29 to MDCK cells. Cells were incubated with serially diluted peptides for 24 h in serum-free medium, followed by measurement of the viability of cells by an alamarBlue dye-based, colorimetric method. The EC_{50} values are indicated as dotted lines. Data shown are means \pm SEM of 2-3 independent experiments.

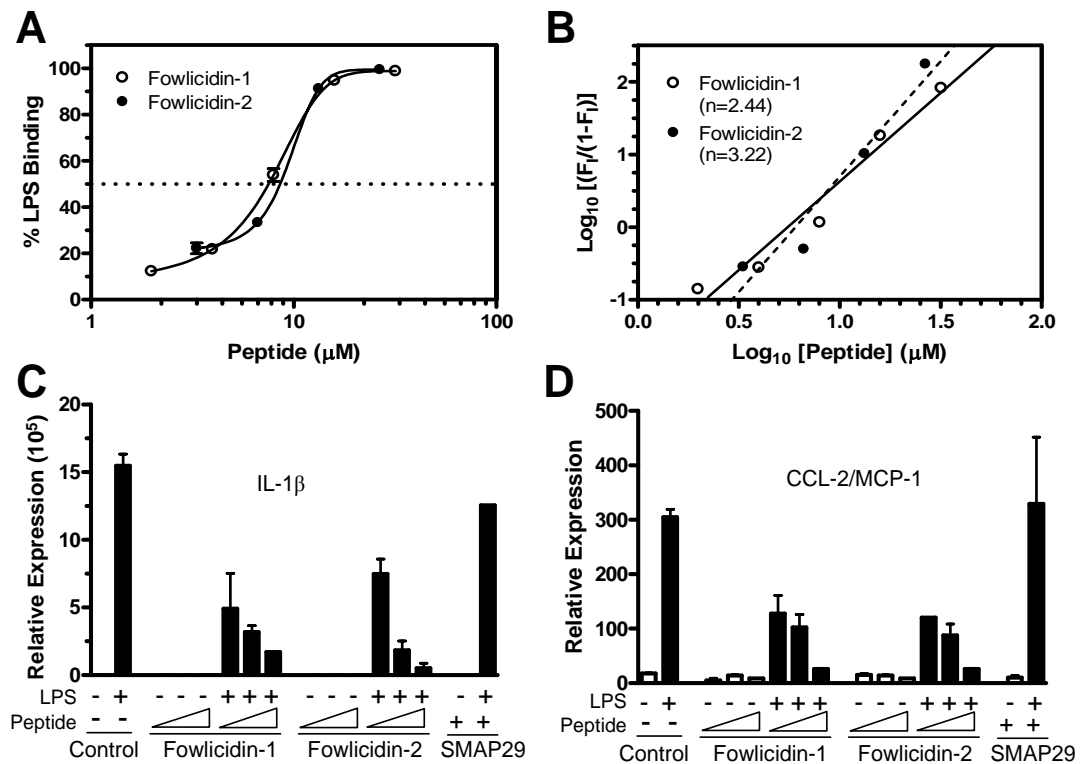


FIG. 6. Neutralization of LPS by fowlicidins. *A*, LPS binding by fowlicidins. Chromogenic *Limulus* amebocyte lysate assay was used to evaluate the binding of fowlicidins to LPS from *E. coli* O111:B4. The EC_{50} value is indicated as a dotted line. Data shown are means \pm SEM of three independent experiments. *B*, Hill plot of LPS binding by fowlicidins showing the binding affinity. The plot was graphed from the means in panel *A*. The Hill's coefficient was derived from the slope of the linear regression. *C*, Blockage of LPS-induced IL-1 β gene expression by fowlicidins. *D*, Blockage of LPS-induced CCL-2/MCP-1 gene expression by fowlicidins. In panels *C* and *D*, RAW264.7 cells were pretreated for 30 min with increasing concentrations (1, 5, and 20 μ M) of peptides, and then stimulated with 100 ng/ml LPS or left untreated for 4 h. Total RNA was isolated from cells and subjected to real time RT-PCR. Data shown are a representative of two independent experiments with similar results.

CHAPTER V
STRUCTURE-ACTIVITY RELATIONSHIP OF FOWLICIDIN-1, A
CATHELICIDIN ANTIMICROBIAL PEPTIDE IN CHICKEN

(Published in *FEBS J.* 2006, 273:2581-2593)

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Abbreviations: MIC, minimum inhibitory concentration; EC₅₀, 50% effective concentration; SAR, structure-activity relationship; LPS, lipopolysaccharide; TFE, trifluoroethanol; CFU, colony forming units; MDCK, Madin-Darby canine kidney cells.

5.1 Summary

Cationic antimicrobial peptides are naturally occurring antibiotics that are actively being explored as a new class of anti-infective agents. We recently identified three cathelicidin antimicrobial peptides from chicken with potent and broad-spectrum antibacterial activities in vitro (Xiao Y, Cai Y, Bommineni YR, Fernando SC, Prakash O, Gilliland SE & Zhang G. 2006. *J Biol Chem* 281, 2858-2867). Here we report that fowlicidin-1 mainly adopts an α -helical conformation with a slight kink induced by glycine close to the center, in addition to a short flexible unstructured region near the N-terminus. To further gain insight into the structural requirements for function, a series of truncation and substitution mutants of fowlicidin-1 were synthesized and tested separately for their antibacterial, cytolytic, and lipopolysaccharide (LPS)-binding activities. The short C-terminal helical segment after the kink, consisting of a stretch of eight amino acids (residues 16 to 23), was shown to be critically involved in all three functions, suggesting that this region may be required for the peptide to interact with LPS and lipid membranes and to permeabilize both prokaryotic and eukaryotic cells. We also identified a second segment comprised of three amino acids (residues 5 to 7) in the N-terminal flexible region that participate in LPS binding and cytotoxicity, but are less important in bacterial killing. The fowlicidin-1 analog with deletion of the second N-terminal segment (residues 5 to 7) was found to retain substantial antibacterial potency with a significant reduction in cytotoxicity. Such a peptide analog may have considerable potential for development as an anti-infective agent.

5.2 *Introduction*

Cathelicidins are a major family of animal antimicrobial peptides with hallmarks of a highly conserved prosequence (cathelin domain) and an extremely variable, antibacterially active sequence at the C-terminus [1-3]. The exact microbicidal mechanism for this family of antimicrobial peptides is not clearly understood. However, it is generally believed that the electrostatic interaction between the C-terminal cationic peptides with anionic lipids followed by membrane permeabilization is mainly responsible for killing prokaryotic cells. Because of such a non-specific membrane-lytic mechanism, many cathelicidins kill a variety of bacteria at low micromolar concentrations with much less chance of developing resistance [4-6]. More importantly, they are equally active against antibiotic-resistant bacterial strains with some demonstrating synergism in killing bacteria with conventional antibiotics or structurally different antimicrobial peptides [7-9]. One side-effect that is commonly associated with cathelicidins as potential therapeutic agents is their cytotoxicity toward mammalian host cells [4-6]. However, the concentrations that are required for cathelicidins to exert an appreciable cytolytic effect are often higher than the bactericidal concentrations.

Structure-activity relationship (SAR) studies of cathelicidins revealed that cationicity, amphipathicity, hydrophobicity, and helicity (helical content) are among the most important determinants of their microbicidal and cytolytic activities [10, 11]. However, in general there is no simple correlation between any of these physicochemical properties and peptide functions. A delicate balance of these parameters often dictates the antimicrobial potency and target selectivity [10, 11]. Moreover, the domain that is responsible for cytotoxicity can sometimes be separated from that for antimicrobial

activity [12, 13]. Therefore, it is possible that strategic manipulation of structural and physicochemical parameters of cathelicidins may maximize their antimicrobial activity while reducing their cytotoxicity.

We and others have recently identified three novel chicken cathelicidins [14-16], which are called fowlicidins 1-3 in this report. All three fowlicidins share little similarity with mammalian cathelicidins in the C-terminal sequence [16]. Putatively mature fowlicidin-1, a linear peptide of 26 amino acid residues, was found to be broadly active against a range of Gram-negative and Gram-positive bacteria with a similar potency to SMAP-29 [16]. However, fowlicidin-1 also displayed considerable cytotoxicity toward human erythrocytes and mammalian epithelial cells with 50% lysis in the range of 6-40 μM [16].

To better understand its mechanism of action, we determined its tertiary structure by NMR spectroscopy in this study. Fowlicidin-1 was shown to be composed of an α -helical segment with a slight kink near the center and a flexible unstructured region at the N-terminal end. A series of deletion and substitution mutants of fowlicidin-1 were further synthesized and tested separately for their antibacterial, LPS-binding, and cytolytic activities. The regions that are responsible for each of these functions have been revealed. In addition, we identified a fowlicidin-1 analog with deletion of the N-terminal flexible region that retains the antibacterial potency but with substantially reduced cytotoxicity. Such a peptide analog may represent an excellent candidate as a novel antimicrobial agent against bacteria that are resistant to conventional antibiotics.

5.3 Results

Solution structure of fowlicidin-1

To first determine the secondary structure of fowlicidin-1, CD spectroscopy was performed in the increasing concentrations of structure-promoting agents, trifluoroethanol (TFE) and SDS. As shown in Fig. 1A, fowlicidin-1 was largely unstructured in the aqueous solution, but underwent a dramatic transition to a typical α -helical conformation with addition of TFE. The α -helical content of fowlicidin-1 increased dose-dependently from 10% in 50 mM phosphate buffer to 81% in 60% TFE, with a concomitant reduction of the random coiled structure. Significant α -helical content (81%) was similarly observed in the presence of 0.25% or 0.5% SDS (Fig. 1B).

Because of adoption of a well-defined structure in the presence of TFE or SDS, subsequent NMR experiments were done in 50% deuterated TFE. The spectra acquired at 35°C gave good chemical shift dispersion with limited spectral overlap, enabling the assignment of most spin systems for fowlicidin-1 (Table S1, Figs. S1 and S2). The complete proton resonance assignments were obtained for the peptide using spin system identification and sequential assignments [17] from 2D NMR spectra recorded at 35°C. Some ambiguities due to overlapping signals were also solved by the comparative use of spectra recorded at 10°C and 35°C. In these assignments, $H_{\alpha}(i)$ - $H_{\delta}(i+1:\text{Pro})$ ($d_{\alpha\delta}$) or $H_{\alpha}(i)$ - $H_{\alpha}(i+1:\text{Pro})$ ($d_{\alpha\alpha}$) NOEs instead of $d_{\alpha N}$ were used for Pro⁷, which showed strong $d_{\alpha\delta}$ NOEs, indicating that Pro⁷ in fowlicidin-1 has trans configuration.

Stereo-specific assignments of β -methylene protons were obtained by using information on $^3J_{H_{\alpha}H_{\beta}}$ coupling constants estimated qualitatively from short-mixing time

TOCSY spectra combined with intra-residue NH-H β and H α -H β NOEs. Qualitative analysis of short- and medium-range NOEs, $^3J_{\text{HNH}\alpha}$ coupling constants, and slowly-exchanging amide proton patterns was used to characterize the secondary structure of fowlicidin-1. The sequential and medium distance NOEs connectivities as well as C α -proton chemical shift index ($\Delta C_{\alpha\text{H}}$) [18] are illustrated in Fig. 2. A number of nonsequential $d_{\alpha\text{N}}(i, i+3)$ and $d_{\alpha\beta}(i, i+3)$ NOEs that are clearly characteristics of α -helical conformation were observed for fowlicidin-1 from Leu⁸ to Lys²⁵. A continuous stretch of $d_{\text{NN}}(i, i+1)$ also extended from Leu⁸ to Lys²⁵, except for Gly¹⁶. The helicity of fowlicidin-1 was further supported by the chemical shift index (Fig. 2).

To determine the tertiary structure of fowlicidin-1, a total of 247 NOE distance constraints involving 90 interresidue, 81 sequential, and 76 medium range constraints were used in structural calculations (Table 1). Of 100 conformers calculated, 20 structures with the lowest energy were retained for further analysis. All 20 structures were in good agreement with the experimental data, with no distance violations $> 0.3 \text{ \AA}$ and no angle violations $> 5^\circ$. A Ramachandran plot was also produced by PROCHECK-NMR [19] showing that 76.1% of the residues are in the most favored region and 21.8 and 1.1% in additional and generously allowed regions, respectively (Table 1).

The minimized average structure is shown in Fig. 3A, indicating that fowlicidin-1 is primarily an α -helical peptide consisting of a helical segment from Leu⁸ to Lys²⁵ and a disordered region near the N-terminus from Arg¹ to Pro⁷. No unambiguous long range NOEs for the first four N-terminal residues were observed (Fig. 2), indicative of their extremely flexible nature. A closer examination revealed that the long helix of fowlicidin-1 is further composed of two short, but perfect, α -helical segments (Leu⁸-Ala¹⁵

and Arg²¹-Lys²⁵) with a slight bend between Gly¹⁶ and Tyr²⁰, due to the presence of Gly¹⁶ (Fig. 3A). A superimposition of the backbones of 20 lowest energy structures best fitted to residues 8-16 or residues 17-25 indicated that the two short helices are highly rigid, but with some degree of flexibility in between (Fig. 3B and 3C). The superimposition of the two short helical segments of the 20 final structures against an averaged structure resulted in a RMSD value of backbone $< 0.5 \text{ \AA}$ (Table 1). Greater flexibility between the helices was revealed when only one segment of the helix was superimposed (Table 1). It is noteworthy that the angle between the two helical axes could not be measured because of a lack of NOEs in the Gly¹⁶ region and fluidity between the two segments. However, flexibility of the “hinge” is somewhat restricted by the side chains of nearby residues, such as Tyr¹⁷ (Fig. 3A).

Design and physicochemical properties of fowlicidin-1 analogs

In contrast with most cathelicidins containing a highly cationic, amphipathic α -helix [10], the central helical region (residues 6-23) of fowlicidin-1 is highly hydrophobic, containing only two cationic residues (Arg¹¹ and Arg²¹) and two uncharged polar residues (Thr¹² and Gln¹⁸) (Fig. 4A). Positively charged residues are instead highly concentrated at both ends. To probe the impact of N- and C-terminal cationic regions and two short helical segments on antibacterial, LPS-binding, and cytolytic activities of fowlicidin-1, several N- and C-terminal deletion mutants were designed (Table 2). All mutants have fewer net positive charges than the parent peptide, in addition to missing one or two structural components.

To further investigate the influence of helicity on the functional properties, Gly¹⁶ of fowlicidin-1 was replaced with a helix-stabilizing residue, leucine, to give rise to fowlicidin-1-L¹⁶. Such a variant minimized the bend and flexibility between two short helices as modeled by Modeller [20] (data not shown), without significantly altering any other structural or physicochemical characteristics. Another substitution variant, fowlicidin-1-K⁷L¹²K¹⁴L¹⁶K¹⁸, was designed mainly for significant augmentation of its amphipathicity. This mutant now has cationic residues clearly aligned along one side and hydrophobic residues aligned along the opposite side of the helix (compare Fig. 4A with 4B). The net charge of this mutant has increased from +8 to +11, as compared with the parent peptide. Replacement of two helix-breaking residues, Pro⁷ and Gly¹⁶, with helix-stabilizing residues, lysine and leucine, respectively, also enhanced the helical content of fowlicidin-1-K⁷L¹²K¹⁴L¹⁶K¹⁸ by concomitant reduction of the kink in the center and extension of the helix at the N-terminus. Along with simultaneous enhancement of amphipathicity, cationicity, and helicity, it is understandable that such a peptide variant also has reduced hydrophobicity in the helical region as a result of incorporation of several positively charged residues. Consistent with the modeling results, two substitution mutants showed increased α -helical contents in the presence of 50% TFE by CD spectroscopy, relative to the parent peptide (data not shown).

All peptides were synthesized commercially by the standard solid-phase method and ordered at >95% purity. The molecular mass and purity of each synthetic peptide were further confirmed by mass spectrometry (Table 2).

Antibacterial activities of fowlicidin-1 and its analogs

Two representative Gram-negative bacteria (*Escherichia coli* ATCC 25922 and *Salmonella enterica* serovar Typhimurium ATCC 14028) and two Gram-positive bacteria (*Listeria monocytogenes* ATCC 19115 and *Staphylococcus aureus* ATCC 25923) were used to test antibacterial potency of fowlicidin-1 and its analogs by a modified broth microdilution assay as described [16, 21]. As compared with the parent peptide, the analog with deletion of three C-terminal lysines [fowlicidin-1(1-23)] or four N-terminal residues [fowlicidin-1(5-26)] or seven N-terminal residues [fowlicidin-1(8-26)] retained much of the bactericidal activity (Table 3), suggesting that the cationic residues at both ends are dispensable for its antibacterial activity, but all or part of the central hydrophobic α -helical region between residues 8-23 plays a major role in killing bacteria. However, the peptide analog that is composed of entirely the central hydrophobic α -helix (residues 8-23) with a net charge of +2 became insoluble in 0.01% acetic acid and, therefore, was excluded from antibacterial assays.

To further examine the differential role of the N- and C-terminal short helical segments in antibacterial potency, fowlicidin-1(1-15) with omission of the C-terminal helical region after the kink at Gly¹⁶, was tested against the four bacterial strains and found to have only < 2-fold reduction in minimum inhibitory concentration (MIC) toward Gram-negative bacteria, but 7- to 18-fold reduction toward Gram-positive bacteria (Table 3), suggesting that the C-terminal short helix (residues 16-23) is critical in maintaining its antibacterial potency against Gram-positive but not Gram-negative bacteria. This is consistent with earlier observations that activity of cationic antimicrobial peptides against Gram-negative bacteria is generally more tolerant to structural changes [10].

In contrast to our expectations, two substitution mutants (fowlicidin-1-L¹⁶ and fowlicidin-1-K⁷L¹²K¹⁴L¹⁶K¹⁸) with significant improvement in helicity, amphipathicity, and/or cationicity were found to have reduced antibacterial activity, relative to the wild-type peptide (Table 3), reinforcing the notion that an intricate balance, rather than a simple enhancement in those structural parameters, dictates the antibacterial potency of the α -helical antimicrobial peptides [10, 11]. It is noteworthy that all peptide analogs showed similar kinetics in killing bacteria as the full-length peptide, with maximal activities being reached 30 min after incubation with bacteria in the presence or absence of 100 mM NaCl (data not shown). It is not clear why fowlicidin-1-K⁷L¹²K¹⁴L¹⁶K¹⁸ largely maintained its potency against *S. aureus* and *Salmonella enterica* serovar Typhimurium, but failed to completely inhibit the growth of *E. coli* and *L. monocytogenes* even at the highest concentration (7.6 μ M = 25 μ g/ml) that we tested.

Cytotoxicity of fowlicidin-1 and its analogs

To map the region that is responsible for lysis of eukaryotic cells and to identify a peptide analog with reduced cytolytic activity, all deletion and substitution mutants of fowlicidin-1 were tested individually against human erythrocyte and Madin-Darby canine kidney cells (MDCK) for their toxicity as previously described [13, 16, 22]. As summarized in Table 3, Fowlicidin-1 exhibited considerable toxicity toward erythrocytes and epithelial cells with 50% effective concentrations (EC₅₀) in the range of 6-15 μ M. Deletion of the last three lysines [fowlicidin-1(1-23)] resulted in a modest < 4-fold reduction in toxicity, while truncation of the entire C-terminal short helix [fowlicidin-1(1-

15)] caused a nearly complete loss of lytic activity toward both erythrocytes and epithelial cells, indicating that the C-terminal helix (residues 16-23), but not the last three lysines, is a critical determinant of cytotoxicity.

Relative to the full-length peptide, fowlicidin-1(5-26) maintained a similar lytic activity, whereas fowlicidin-1(8-26) only caused minimal 20% lysis of human red blood cells at 360 μ M, the highest concentration tested (data not shown), suggesting possible presence of another cytotoxicity determinant in the N-terminal unstructured segment between residues 5-7. Consistent with these results, a significant >10-fold reduction in killing MDCK cells was also observed with fowlicidin-1(8-26) (Table 3). Because of the fact that two peptide analogs, fowlicidin-1(1-15) and fowlicidin-1(8-26) each containing one cytolytic determinant, had substantially reduced toxicity, it is likely that the two lytic sites (residues 5-7 and 16-23) act in a synergistic manner in lysis eukaryotic cells, i.e., the presence of one determinant facilitates the action of the other.

Single substitution of Gly¹⁶ for leucine (fowlicidin-1-L¹⁶) did not lead to any obvious alterations in killing eukaryotic cells (Table 3). In contrast, fowlicidin-1-K⁷L¹²K¹⁴L¹⁶K¹⁸ with a nearly perfect amphipathic helix in the center showed a 6-fold increase in lysis of red blood cells, but only slightly higher lytic activity against mammalian epithelial cells (Table 3). This suggested that the amphipathic helix has a stronger binding affinity and permeability toward erythrocyte membranes than to epithelial membranes, perhaps due to the difference in the lipid composition of the two host cell types.

LPS-binding activity of fowlicidin-1 and its analogs

Binding and disrupting anionic LPS, the major outer membrane component of Gram-negative bacteria, is often the first step for antimicrobial peptides to interact with bacteria and permeabilize membranes [10]. Several cathelicidins, including human LL-37/hCAP-18 [21, 23], rabbit CAP-18 [24], and sheep SMAP-29 [25], have been shown to bind and neutralize LPS with EC_{50} at low micromolar concentrations. We have also demonstrated that fowlicidin-1 has at least two LPS binding sites [16]. To map the regions involved in the binding of fowlicidin-1 to LPS, the N- and C-terminal deletion mutants were mixed with LPS, and their ability to bind LPS and inhibit LPS-mediated procoagulant activation was measured by a chromogenic *Limulus* amoebocyte assay [21, 25]. As shown in Fig. 5A, fowlicidin-1(1-23) and fowlicidin-1(5-26) had similar affinities for LPS to the full-length peptide, with EC_{50} in the range of 10-39 μ M (Table 3), suggesting that LPS-binding sites are likely to be located in the central helical region between residues 5-23.

Residues 5-7 is clearly involved in LPS binding and may constitute the core region of one LPS-binding site, because fowlicidin-1(8-26) showed a >15-fold reduction in binding to LPS relative to fowlicidin-1(5-26), which had a similar affinity for LPS to the full-length peptide. The other LPS-binding site is likely located in the C-terminal short helix between residues 16-23, because deletion of that region [fowlicidin-1(1-15)] resulted in a >25-fold reduction in LPS binding, as compared to fowlicidin-1(1-23) (Fig. 5A, Table 3). It is important to note that two LPS-binding sites of fowlicidin-1 are located in the same regions where the two cytotoxicity determinants reside. This is perhaps not surprising, given that sequences that interact with anionic LPS or phospholipids on bacterial membranes are likely involved in interactions with eukaryotic cell membranes, which is a

prerequisite for cytotoxicity. In fact, the hemolytic domain of SMAP-29 was also shown to overlap with a LPS-binding site at the C-terminal end [25].

To determine whether the two LPS-binding sites act in a synergistic manner, an equimolar mixture of fowlicidin-1(1-15) and fowlicidin-1(8-26) with each containing one LPS-binding site was incubated with LPS and measured for their ability to bind to LPS. As shown in Fig. 5A, the mixture displayed an enhanced affinity for LPS, approaching the full-length peptide, indicative of the synergistic nature of two LPS-binding sites. Both substitution mutants, fowlicidin-1-L¹⁶ and fowlicidin-1-K⁷L¹²K¹⁴L¹⁶K¹⁸, had minimal changes in LPS-binding affinity, relative to the native peptide (Fig. 5B), suggesting that a simultaneous enhancement in helicity, cationicity, and amphipathicity has little impact on interactions of peptides with LPS and possibly with bacterial membranes as well, which may explain why the antibacterial activities of both mutants remained largely unchanged (Table 3).

5.4 Discussion

Cathelicidins are highly conserved from birds to mammals in the prosequence, but are extremely divergent in the C-terminal mature sequence [1-3]. Cathelicidin-like molecules have also been found in the hagfish, the most ancient extant jawless fish with no adaptive immune system [26]. With the finding that fowlicidin-1 adopts an α -helix (Fig. 3), it is now evident that at least one cathelicidin in α -helical conformation is present in each of the fish, bird, and mammalian species examined. This suggests that, in addition to the prosequence, cathelicidins appear to be conserved in the mature region structurally and

presumably functionally as well. It is plausible that presence of additional structurally different cathelicidins in certain animal species may help the hosts better cope with unique microbial insults in the ecological niche where each species inhabits, given the fact that different cathelicidins appear to possess non-overlapping antimicrobial spectrum [6] and that some act synergistically in combinations in killing microbes [7]. On the other hand, innate host defense of animal species like primates and rodents containing a single cathelicidin may be compensated by the presence of a large number of other antimicrobial peptides such as α - and β -defensins [27, 28]. Conversely, pig and cattle have multiple cathelicidins but no alpha-defensins reported.

Our NMR studies revealed that, in addition to a short flexible unstructured region at the N-terminus, fowlicidin-1 is primarily composed of two short α -helical segments connected by a slight kink caused by Gly¹⁶ near the center (Fig. 3). Interestingly, such a helix-hinge-helix structural motif is not uncommon for cathelicidins. Mouse cathelicidin CRAMP [22], bovine BMAP-34 [29], and porcine PAMP-37 [30] all adopt a helix-hinge-helix structure with the hinge occurring at the central glycine (Fig. 6). In fact, none of the linear, naturally occurring cathelicidins are strictly α -helical. Besides peptides with helix-hinge-helix structures, a few other linear cathelicidins consist of a N-terminal helix followed by non-helical and mostly hydrophobic tails, such as rabbit CAP-18 [31], sheep SMAP-29 [25], and bovine BMAP-27 and BMAP-28 [12] (Fig. 6).

In addition to cathelicidins, a scan of over 150 helical antimicrobial peptides revealed that glycine is frequently found near the center and acts as a hinge to increase flexibility in many other protein families [10] (Fig. 6). Presence or insertion of such a hinge in the helix has been shown in many cases to be desirable, attenuating the toxicity of peptides to

host cells while maintaining comparable antimicrobial potency with the peptides having no hinge sequences [10, 11]. Mutation of the hinge sequence with a helix-stabilizing residue, like leucine, will generally result in an increase in cytotoxicity and in several cases antimicrobial potency as well. However, substituting Gly¹⁶ of fowlicidin-1-L¹⁶ for leucine did not enhance antibacterial or cytolytic activity (Table 3), which is likely due to the relatively low flexibility of the wild-type peptide.

A careful comparison of fowlicidin-1 with other α -helical cathelicidins indicated that the α -helix (residues 8-23) of fowlicidin-1 is much more hydrophobic and much less amphipathic than most of the mammalian cathelicidins (Fig. 6). The positive charges of fowlicidin-1 are more concentrated in the non-helical regions at both ends. Because high hydrophobicity is often associated with strong cytotoxicity [10, 11], it is perhaps not surprising to see that fowlicidin-1 is relatively more toxic than many other cathelicidins. Interestingly, fowlicidin-1 structurally more resembles melittin, a helical peptide found in honey bee venom that has a curved hydrophobic helix with positively charged residues located primarily at the C-terminal end [32] (Fig. 6). Like fowlicidin-1, melittin displays considerable antibacterial and hemolytic activities. An attempt to reduce hydrophobicity and enhance amphipathicity of the helical region of fowlicidin-1 to make fowlicidin-1-K⁷L¹²K¹⁴L¹⁶K¹⁸ led to a dramatically increased toxicity particularly toward erythrocytes with a minimum change in the antibacterial activity against certain bacteria (Table 3). This is consistent with an earlier conclusion that an amphipathic helix is more essential for interactions with zwitterionic lipid membranes on eukaryotic cells than for anionic lipids on prokaryotic cells [33].

Our SAR data revealed the regions that are responsible for each of the antibacterial, LPS-binding, and cytolytic activities of fowlicidin-1 (Fig. 7). The C-terminal α -helix after the kink (residues 16-23), consisting of a stretch of eight amino acids, is required for all three functions, suggesting that this region is likely a major site for the peptide to interact with LPS and lipid membranes and to permeabilize both bacterial and eukaryotic cells. It is not surprising to see the presence of two lipophilic tyrosines (Tyr¹⁷ and Tyr²⁰) that might be critical in mediating membrane interactions for fowlicidin-1. However, the α -helix before the kink at Gly¹⁶ is likely to be involved in membrane penetration as well, because the minimum length required for a helical peptide to traverse membranes and exert antimicrobial and lytic activities is about 11-14 residues [34].

Another region, comprised of three amino acids in the N-terminal flexible region (residues 5-7), is also involved in both LPS binding and cytotoxicity, but not so important in bacterial killing (Fig. 7). It is interesting to note that among the three residues in this region is Trp⁶, which is known to have a preference to be inserted into lipid bilayers at the membrane-water interface [35, 36]. Because of such membrane-seeking ability, inclusion of tryptophan often renders peptides with higher affinity for membranes and more potency against bacteria [37, 38]. It is not known why tryptophan is not significantly involved in the antibacterial activity of fowlicidin-1.

It is noteworthy that the N-terminal helix of many cathelicidins plays a major role in LPS binding and bacterial killing, while the C-terminal segment is either dispensable for antimicrobial activity or more involved in cytotoxicity [12, 25, 39, 40]. However, the C-terminal helix after the kink of fowlicidin-1 is more important in killing bacteria than the N-terminal helix. Such a sharp difference in the distribution of functional domains along

the peptide chain between fowlicidin-1 and other cathelicidins is probably because of a more pronounced hydrophobic nature of the helix and the presence of an additional highly flexible segment at the N-terminus of fowlicidin-1.

One aim of our study was to identify peptide analog(s) with better therapeutic potential. Fowlicidin-1(1-23) and fowlicidin-1(5-26) had only marginal effect on either antibacterial potency or cytotoxicity, whereas fowlicidin-1(1-15) exhibited minimal toxicity up to 443 μM , but with an obvious decrease in antibacterial activity particularly against Gram-positive bacteria, implying less desirable therapeutic relevance of these peptide analogs as a broad-spectrum antibiotic. Fowlicidin-1-L¹⁶ and fowlicidin-1-K⁷L¹²K¹⁴L¹⁶K¹⁸ also had a more pronounced reduction in antibacterial activity than in toxicity, therefore with reduced clinical potential. In contrast, fowlicidin-1(8-26) with the N-terminal toxicity determinant (residues 5-7) deleted and the C-terminal antibacterial domain (residues 16-23) left unaltered, had a slight reduction in MIC against bacteria, but with >10-fold reduction in toxicity toward mammalian epithelial cells and negligible toxicity toward erythrocytes (Table 3). Coupled with its smaller size, this peptide analog may represent a safer and more attractive therapeutic candidate than the parent peptide. Given the fact that fowlicidin-1 is broadly effective against several common bacterial strains implicated in cystic fibrosis, including *S. aureus*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* in a salt-independent manner [16], its analog, fowlicidin-1(8-26), might prove useful in controlling chronic respiratory infections of cystic fibrosis patients. These results also suggested the usefulness of systematic SAR studies in improving the safety and target specificity of antimicrobial peptides.

5.5 *Materials and methods*

Peptide synthesis

Fowlicidin-1 was synthesized using the standard solid-phase method by SynPep (Dublin, CA) and its analogs were synthesized by either Sigma Genosys (Woodlands, TX) or Bio-Synthesis (Lewisville, TX) (Table 1). The peptides were purified through RP-HPLC and purchased at >95% purity. The mass and purity of each peptide were further confirmed by 15% Tris-Tricine polyacrylamide gel electrophoresis (data not shown) and by MALDI-TOF mass spectrometry (Table 1) using the Voyager DE-PRO instrument (Applied Biosystems, Foster City, CA) housed in the Recombinant DNA/Protein Resource Facility of Oklahoma State University.

CD spectroscopy

To determine the secondary structure of fowlicidin-1, CD spectroscopy was performed with a Jasco-715 spectropolarimeter using a 0.1-cm path length cell over the 180-260 nm range as we previously described [41]. The spectra were acquired at 25°C every 1 nm with a 2-s averaging time per point and a 1-nm band pass. Fowlicidin-1 (10 μ M) was measured in 50 mM potassium phosphate buffer, pH 7.4, with or without different concentrations of TFE (0%, 10%, 20%, 40%, and 60%) or SDS micelles (0.25% and 0.5%). Mean residue ellipticity (MRE) was expressed as $[\theta]_{\text{MRE}}$ ($\text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$). The contents of six types of the secondary structural elements, including regular and

distorted α -helix, regular and distorted β -sheet, turns, and unordered structures, were analyzed with the program SELCON3 [42].

NMR spectroscopy

2D [^1H - ^1H] NMR experiments for fowlicidin-1 were performed as previously described [43, 44]. Briefly, NMR data were acquired on a 11.75T Varian UNITYplus spectrometer (Varian, Palo Alto, CA), operating at 500 MHz for ^1H , with a 3-mm triple-resonance inverse detection probe. The NMR sample of fowlicidin-1, consisting of 4 mM in water containing 50% deuterated TFE (TFE- d_3 , Cambridge Isotope Laboratories) and 10% D_2O , was used to record spectra at 10, 20, 30, and 35°C. The spectra acquired at 35°C were determined to provide the optimal resolution of overlapping NMR resonances. These spectra were processed and analyzed using Varian software VNMR Version 6.1C on a Silicon Graphics Octane workstation. The invariant nature of the NMR chemical shifts and line widths upon 10-fold dilution indicated that fowlicidin-1 was monomeric in solution at the concentration used for 2D NMR analysis. A total of 512 increments of 4K data points were collected for these 2D NMR experiments. The high digital resolution DQF-COSY spectra were recorded using 512 increments and 8K data points in t_1 and t_2 dimensions. Sequential assignments were carried out by comparison of cross-peaks in a NOESY spectrum with those in a TOCSY spectrum acquired under similar experimental conditions. NOESY experiments were performed with 200, 300, 400 and 500 ms mixing times. A mixing time of 200 ms was used for distance constraints measurements. The NOE cross-peaks were classified as strong, medium, weak and very weak based on an

observed relative number of contour lines. TOCSY spectra were recorded by using MLEV-17 for isotropic mixing for 35 and 100 ms at a B1 field strength of 7 KHz.

Water peak suppression was obtained by low-power irradiation of the water peak during relaxation delay. The residual TFE methylene peak was considered as reference for the chemical shift values. The temperature dependencies of amide proton chemical shifts were measured by collecting data from 10° to 35°C in steps of 5°C by using a variable temperature probe. All experiments were zero-filled to 4K data points in t1 dimension and when necessary, spectral resolution was enhanced by Lorentzian-Gaussian apodization.

Structure calculations

For structure calculations, NOE-derived distance restraints were classified into four ranges: 1.8-2.7, 1.8-3.5, 1.8-4.0 and 1.8-5.0 Å, according to the strong, medium, weak and very weak NOE intensities. Upper distance limits for NOEs involving methyl protons and non-stereospecifically assigned methylene protons were corrected appropriately for center averaging [45]. In addition, a distance of 0.5 Å was added to the upper distance limits only for NOEs involving the methyl proton after correction for center averaging [46]. The distance restraints were then used to create initial peptide structures starting from extended structures using the program CNS (version 1.1) [47]. CNS uses both a simulated annealing protocol and molecular dynamics to produce low energy structures with the minimum distance and geometry violations. In general, default parameters supplied with the program were used with 100 structures for each CNS run. The final

round of calculations began with 100 initial structures and 20 best structures with the lowest energy were selected and analyzed with MOLMOL [48] and PROCHECK-NMR [19]. Structure figures were generated by using MOLMOL. The structures of fowlicidin-1 analogs were further modeled by using Modeller [20], based on the parent peptide.

Antibacterial assay

Two representative Gram-negative bacteria (*E. coli* ATCC 25922 and *S. enterica* serovar Typhimurium ATCC 14028) and two Gram-positive bacteria (*L. monocytogenes* ATCC 19115 and *S. aureus* ATCC 25923) were purchased from ATCC (Manassas, VA) and tested separately against fowlicidin-1 and its analogs by using a modified broth microdilution assay as described [16, 21]. Briefly, overnight cultures of bacteria were subcultured for additional 3-5 h at 37°C in trypticase soy broth to the mid-log phase, washed with 10 mM sodium phosphate buffer, pH 7.4, and suspended to 5×10^5 CFU/ml in 1% cation-adjusted Mueller Hinton broth (BBL, Cockeysville, MD), which was prepared by a 1:100 dilution of conventional strength Mueller Hinton broth in 10 mM phosphate buffer. If necessary, 100 mM of NaCl were added to test the influence of salinity on antibacterial activity. Bacteria (90 μ l) were then dispensed into 96-well plates, followed by addition in duplicate of 10 μ l of serially diluted peptides in 0.01% acetic acid. After overnight incubation at 37°C, the MIC value of each peptide was determined as the lowest concentration that gave no visible bacterial growth. The antibacterial assays were repeated at least 3-4 times for each bacterial strain with <2-fold difference in MIC values in all cases, and therefore, representative MIC values were tabulated in Table 3.

Hemolysis assay

The hemolytic activity of fowlicidin-1 and its mutants were determined essentially as described [13, 22]. Briefly, fresh anti-coagulated human blood was collected, washed twice with PBS, diluted to 0.5% in PBS, and 90 μ l were dispensed into 96-well plates. Serial 2-fold dilutions of peptides were added in duplicate to erythrocytes and incubated at 37°C for 2 h. Following centrifugation at 800 \times g for 10 min, the supernatants were transferred to new 96-well plates and monitored by measuring the absorbance at 405 nm for released hemoglobin. Controls for 0 and 100% hemolysis consisted of cells suspended in PBS only and in 1% Triton X-100, respectively. Percent hemolysis (%) was calculated as $[(A_{405\text{nm, peptide}} - A_{405\text{nm, PBS}}) / (A_{405\text{nm, 1\% Triton X-100}} - A_{405\text{nm, PBS}})] \times 100$. EC₅₀ of the hemolytic activity was defined as the peptide concentration that caused 50% lysis of erythrocytes.

Cytotoxicity assay

The toxic effect of fowlicidin-1 and its analogs on mammalian epithelial cells was evaluated with MDCK cells by using alamarBlue dye (Biosource) as previously described [16]. Briefly, cells were seeded into 96-well plates at 1.5×10^5 /well and allowed to grow overnight in DMEM containing 10% FBS. Cells were then washed once with DMEM, followed by addition of 90 μ l of fresh DMEM, together with 10 μ l of serially diluted peptides in 0.01% acetic acid in triplicate. After incubation for 18 h, 10 μ l

of alamarBlue dye were added to cells for 6 h at 37°C in a humidified 5% CO₂ incubator before the fluorescence was read with excitation at 545 nm and emission at 590 nm. Percent cell death was calculated as $[1 - (F_{\text{peptide}} - F_{\text{background}})/(F_{\text{acetic acid}} - F_{\text{background}})] \times 100$, where F_{peptide} is the fluorescence of cells exposed to different concentrations of peptides, $F_{\text{acetic acid}}$ is the fluorescence of cells exposed to 0.01% acetic acid only, and $F_{\text{background}}$ is the background fluorescence of 10% alamarBlue dye in cell culture medium without cells. Cytotoxicity (EC₅₀) of individual peptides was defined as the peptide concentration that caused 50% cell death.

LPS binding assay

The binding of LPS to fowlicidin-1 and its analogs was measured by a kinetic chromogenic *Limulus* amoebocyte lysate assay (Kinetic-QCL 1000 kit; BioWhittaker, Walkersville, MD) as previously described [21, 25]. Briefly, 25 µl of serially diluted peptide were added in duplicate into 25 µl of *E. coli* O111:B4 LPS containing 0.5 endotoxin units/ml and incubated for 30 min at 37°C, followed by incubation with 50 µl of the amoebocyte lysate reagent for 10 min. The absorbance at 405 nm was measured at 10 and 16 min after addition of 100 µl of chromogenic substrate, Ac-Ile-Glu-Ala-Arg-*p*-nitroanilide. Percent LPS binding was calculated as $[(\Delta D1 - \Delta D2 + \Delta D3) / \Delta D1] \times 100$, where $\Delta D1$ represents the difference in the optical density between 10 and 16 min for the sample containing LPS only, $\Delta D2$ represents the difference in the optical density between 10 and 16 min for the samples containing LPS and different concentrations of peptides, and $\Delta D3$ represents the difference in the optical density between 10 and 16 min for the

samples containing different concentrations of peptides with no LPS. EC₅₀ of the LPS-binding activity was defined as the peptide concentration that inhibited LPS-mediated procoagulant activation by 50%.

Protein Data Bank accession code

The atomic coordinates and structure factors of putatively mature fowlicidin-1 have been deposited under accession code 2AMN in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (<http://www.rcsb.org/>).

5.6 Acknowledgments

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Table 1. Structural statistics of the 20 lowest energy structures of fowlicidin-1

Total NOE constraints	247
Intraresidue ($ i-j = 0$)	90
Sequential ($ i-j = 1$)	81
Medium Range ($ i-j \leq 4$)	76
Constraints/residue	9.5
Energies (kcal.mol ⁻¹)	
Overall	31.76 ± 1.24
Bonds	1.46 ± 0.12
Angles	18.61 ± 0.39
Improper	1.09 ± 0.13
van der Waals	5.30 ± 0.96
NOE	5.30 ± 0.63
Pairwise RMSDs for residues 1-26 (Å)	
Backbone	2.98 ± 0.98
Heavy atoms	4.48 ± 0.96
RMSDs to mean structure (backbone/heavy atoms) (Å)	
Residues 1-26	1.76/2.50
Residues 8-16	0.28/0.98
Residues 17-25	0.48/1.96
Percentage of residues in regions of ϕ - ψ space	
Core	76.1%
Allowed	21.8%
Generously allowed	1.1%
Disallowed	0.9%

Table 2. Fowlicidin-1 and its analogs

Peptide	Sequence	Charge	Length	Mass	
				Calculated	Observed
Fowlicidin-1(1-26)	RVKRVWPLVIRTVIAGYNLYRAIKKK	+8	26	3141.9	3141.6
Fowl-1(1-15)	RVKRVWPLVIRTVIA	+4	15	1807.3	1807.6
Fowl-1(1-23)	RVKRVWPLVIRTVIAGYNLYRAI	+5	23	2758.4	2757.2
Fowl-1(8-26)	LVIRTVIAGYNLYRAIKKK	+5	19	2220.8	2220.9
Fowl-1(5-26)	VWPLVIRTVIAGYNLYRAIKKK	+5	22	2603.2	2600.3
Fowl-1-L ¹⁶	RVKRVWPLVIRTVIALYNLYRAIKKK	+8	26	3199.0	3197.3
Fowl1-K ⁷ L ¹² K ¹⁴ L ¹⁶ K ¹⁸	RVKRVWKLVIRLVKALYKLYRAIKKK	+11	26	3271.2	3271.1

Table 3. Functional properties of fowlicidin-1 and its analogs

Peptide	Antibacterial Activity (MIC, μ M)				Cytolytic Activity (EC ₅₀ , μ M)		LPS Binding Activity (EC ₅₀ , μ M)
	<i>S. aureus</i>	<i>Listeria</i>	<i>Salmonella</i>	<i>E. coli</i>	Hemolytic	Cytotoxic	
Fowlicidin-1(1-26)	0.5	2.0	2.0	4.0	6	15	11
Fowl-1(1-15)	13.8	13.8	3.5	6.9	>443	>443	>443
Fowl-1(1-23)	1.1	2.3	2.3	4.5	38	40	39
Fowl-1(8-26)	2.8	5.6	2.8	5.6	>360	159	>260
Fowl-1(5-26)	0.6	2.4	2.4	4.8	11	9	10
Fowl-1-L ¹⁶	2.0	3.9	2.0	7.8	3	15	9
Fowl-1-KLKLK	1.9	>7.6	1.9	>7.6	1	11	6

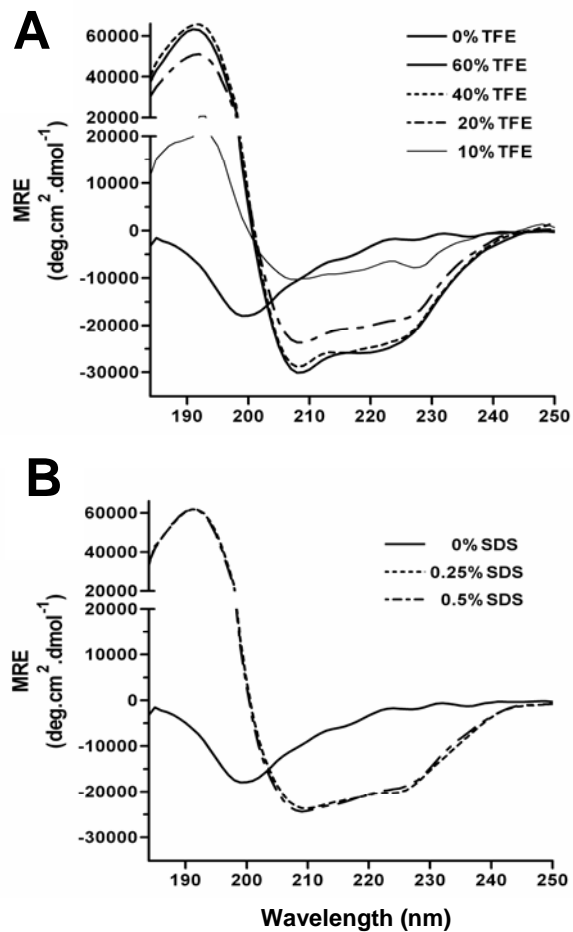


Fig. 1. CD spectra of fowlicidin-1in different concentrations of TFE (A) and SDS micelles (B). The CD spectra of the peptides were acquired at 10 μ M in 50 mM potassium phosphate buffer, pH 7.4, with or without different concentrations of TFE or SDS micelles.

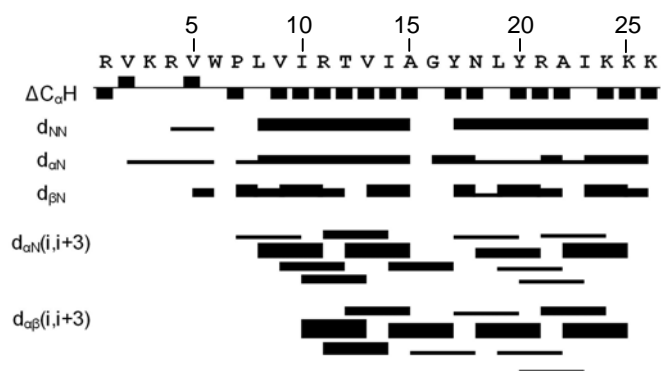


Fig. 2. Schematic diagram of sequential and medium distance NOE connectivities and $C_{\alpha H}$ chemical shift index for fowlicidin 1. The thickness of the bar reflects the strength of the NOE connectivities.

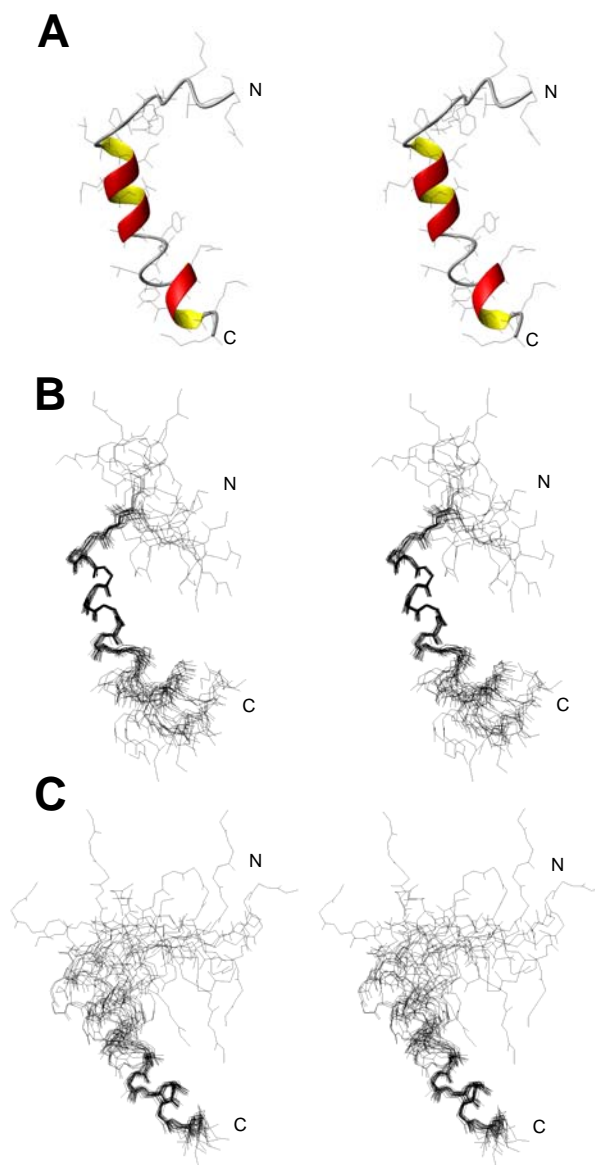


Fig. 3. Solution structure of fowlicidin-1. *A*, ribbon stereo-diagram of the restrained minimized average structure of fowlicidin-1. *B*, stereo-diagrams of the backbone trace of 20 lowest energy structures of fowlicidin-1 with residues 8-16 overlaid. *C*, stereo-diagrams of the backbone trace of 20 lowest energy structures of fowlicidin-1 with residues 17-25 overlaid. This figure was generated with MOLMOL.

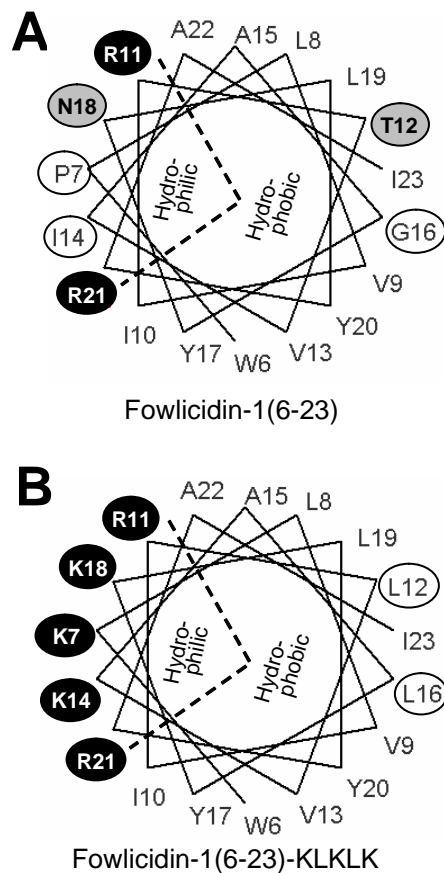


Fig. 4. Helical wheel projections of the central helical regions (residues 6-23) of fowlicidin-1 (A) and its substitution mutant, fowlicidin-1-K⁷L¹²K¹⁴L¹⁶K¹⁸ (B). The representation shows the amphipathic structure of the helical region. Charged residues are indicated in black background, and polar uncharged residues are in gray background. The mutated residues are circled. Notice a significant enhancement in amphipathicity of the mutant peptide relative to the native peptide.

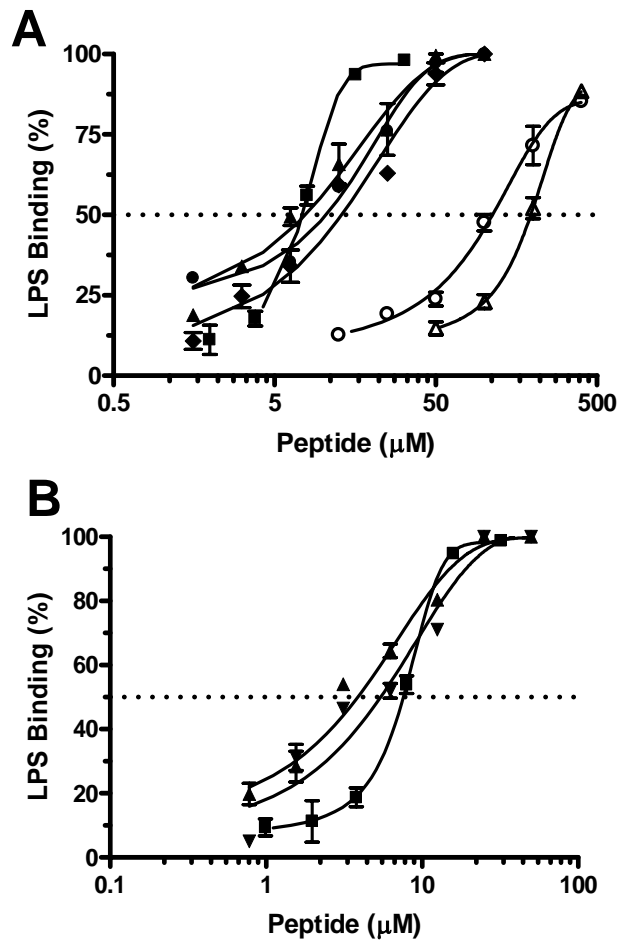


Fig. 5. LPS-binding isotherms of the deletion (A) and substitution mutants (B) of fowlicidin-1. The EC_{50} value, indicated by a dotted line in each panel, was defined as the peptide concentration that inhibited LPS-mediated procoagulant activation by 50%. In panel A, ■, fowlicidin-1(1-26); ○, fowlicidin-1(8-26); △, fowlicidin-1(1-15); ▲, fowlicidin-1(5-26); ◆, fowlicidin-1(1-23); ●, fowlicidin-1(8-26) + fowlicidin-1(1-15). In panel B, ■, fowlicidin-1(1-26); ▲, fowlicidin-1- L^{16} ; and ▼, fowlicidin-1-KLKLK. Data shown are means \pm SEM of three independent experiments.

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Fowlicidin-1  - - - - - PVRVKRVWPLVIRTVIAG-YNL-YRAIKKK-
Melittin      - - - - - GIGAVLKVLTTGLPAL-ISWIKRKR
Cecropin-A1   GWLKKIGKKIERVGHTRD-ATIQGLGIAQQAANVAATA
Cecropin-P1   - - - - SWLSKTAKKLENSAKKRISEGIAIA-IQGGPR-
CRAMP         - - - - - GLLRKGGEKIGEKLKKIGQKIKNFFQKLVPQI
CAP18        - - - - GLRKRLRKFRNKIKEKLEKIGQKIQGFVPKLAPR
BMAP34       GLFRRLRDSIRRGQQKILEKARRIGERIKDI--FRG--
SMAP34       GLFGRRLRDSLQRGGQKILEKAERIGDRIKDI--FRG--
PMAP37       GLLSRLRDFLSDRGRRLGEKIERIGQKIKDLSSEFFQS--
BMAP28       - - - - - GGLRSLGRKILRAWKKYGPPIIVPIIR-IG--
SMAP29       - - - - - RGLRRLGRKIAHG VKKYGPTVLRIR- IAG--

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Fig. 6. Alignment of representative linear α -helical antimicrobial peptides demonstrating the conservation of a kink induced by glycine near the center. Putatively mature fowlicidin-1 sequence is aligned with representative cathelicidins (mouse CRAMP, rabbit CAP18, bovine BMAP34 and BMAP28, sheep SMAP34 and SMAP29, and porcine PMAP37) as well as three insect peptides (fruit fly cecropin A1, a putative porcine cecropin P1, and honey bee melittin). Dashes are inserted to optimize the alignment and conserved residues are shaded. Note that each peptide aligned has an α -helix N-terminal to the conserved glycine (boxed) near the center, followed by either a helical or unstructured tail. The only exception is CRAMP, which has a kink at Gly¹¹ instead of Gly¹⁸ [22].

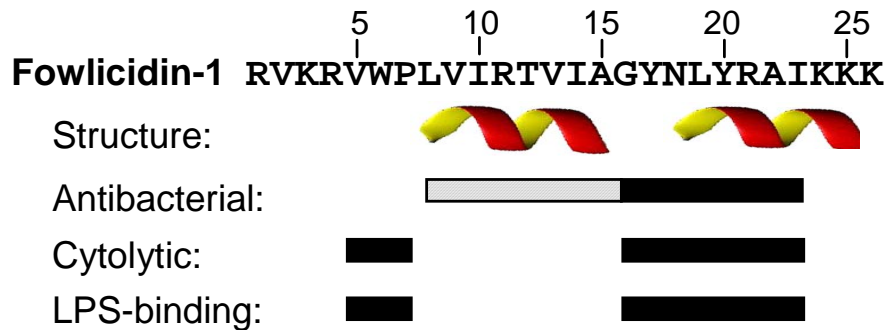


Fig. 7. Schematic drawing of the distribution of functional determinants of fowlicidin-1. Note that the C-terminal helix from Gly¹⁶ to Ile²³ is indispensable for antibacterial, cytolytic, and LPS-binding activities, whereas the three residues (Val⁵-Pro⁷) in the N-terminal unstructured region constitute the core of the second determinant that is critically involved in cytotoxicity and LPS binding, but less significant in the bactericidal activity. The N-terminal helix (Leu⁸-Ala¹⁵) also presumably facilitates the interactions of the C-terminal helix (Gly¹⁶-Ile²³) with lipid membranes.

Supplementary material

Table S1. Proton chemical shift assignments of fowlicidin 1 in deuterated TFE: H₂O (1:1) and 35°C.

Residue	HN	H α	H β	Other
Arg ¹		4.11	1.96	C γ H 1.71; C δ H 3.23; NH 7.22
Val ²	8.34	4.18	2.08	C γ H 0.98
Lys ³	8.11	4.83	1.82, 1.71	C γ H 1.49; C δ H 1.39; C ϵ H 2.99
Arg ⁴	8.01	4.31	1.62	C γ H 1.41; C δ H 3.09; NH 7.08
Val ⁵	7.60	4.25	2.20	C γ H 0.93
Trp ⁶	7.74	4.75	3.37	C δ_1 H 7.26; C ϵ H 3 7.52; C η_2 H 7.2; C ζ_2 H 7.45; C ζ_3 H 7.09; NH 9.75
Pro ⁷		4.19	2.30, 1.67	C γ 1.98; C δ 3.59
Leu ⁸	7.23	4.15	1.74	C γ H 1.64; C δ H 0.94, 0.86
Val ⁹	7.67	3.62	2.25	C γ H 0.99, 0.93
Ile ¹⁰	7.86	3.73	1.82	C γ H 1.50, 1.21, 0.88; C δ H 0.88
Arg ¹¹	7.72	3.96	1.96	C γ H 1.89; C δ H 3.16; NH 7.07
Thr ¹²	7.74	3.99	4.44	C γ H 1.29
Val ¹³	8.18	3.72	2.27	C γ H 1.06
Ile ¹⁴	8.28	3.78	1.94	C γ H 1.57, 1.09, 0.96; C δ H 0.81
Ala ¹⁵	8.27	4.20	1.57	
Gly ¹⁶	8.27	3.92, 3.90		
Tyr ¹⁷	8.40	4.38	3.19	C δ H 7.11; C ϵ H 6.78
Asn ¹⁸	8.30	4.39	2.99, 2.74	NH 7.44, 6.54
Leu ¹⁹	8.18	4.18	1.79	C γ H 1.69; C δ H 0.92
Tyr ²⁰	8.16	4.14	3.15	C δ H 7.01; C ϵ H 6.77
Arg ²¹	7.96	3.82	1.81, 1.66	C γ H 1.51; C δ H 3.1; NH 7.07
Ala ²²	7.76	4.15	1.53	
Ile ²³	7.94	3.95	1.91	C γ H 1.61, 1.24, 0.9; C δ H 0.83
Lys ²⁴	7.87	4.16	1.70	C γ H 1.59; C δ H 1.27; C ϵ H 2.95
Lys ²⁵	7.76	4.24	1.88	C γ H 1.70; C δ H 1.50; C ϵ H 2.99
Lys ²⁶	7.75	4.26	1.91	C γ H 1.73; C δ H 1.50; C ϵ H 3.02

Fig. S1. Fingerprint region of a 500-MHz 2D [¹H, ¹H]-TOCSY NMR spectrum of fowlicidin-1 in deuterated TFE: H₂O (1:1) and 35°C.

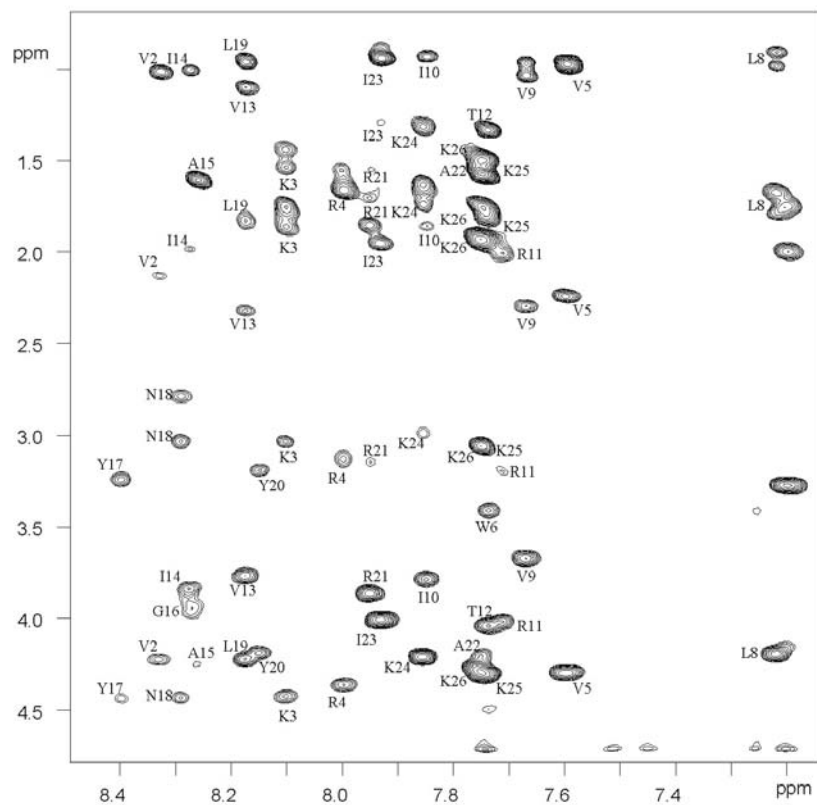
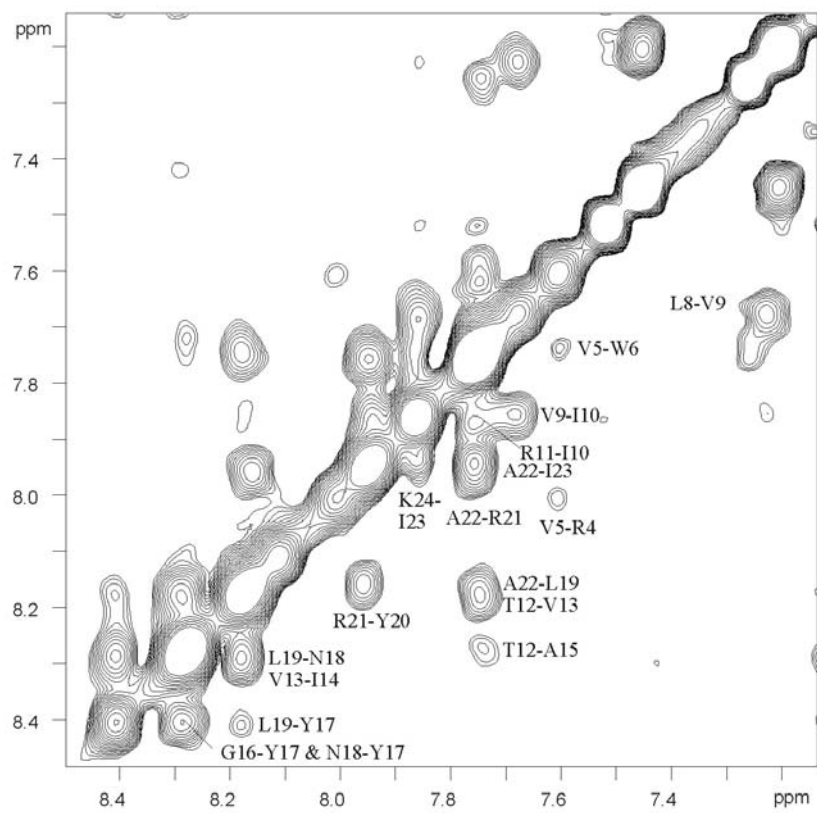


Fig. S2. Fingerprint (NH-NH) region of a 500-MHz 2D [^1H , ^1H]-NOESY NMR spectrum of fowlicidin-1 in deuterated TFE: H_2O (1:1) and 35°C .



CHAPTER VI

SUMMARY AND FUTURE PROSPECTS

6.1 Summary

In this dissertation, a brief review of antimicrobial peptides including their features, classifications, roles in immunity, mechanisms of action and therapeutic potential were discussed. This study focused on the chicken antimicrobial peptides. Chicken β -defensin gene cluster was identified and studied by comparative analyses with mammalian homologues. Chicken cathelicidins were identified and analyzed for their structures and functions. At last one therapeutic candidate was suggested based on the structure-activity relationship study of fowlicidin-1.

Following a comprehensive screen, here we report that the chicken genome encodes a total of 13 different β -defensins but with no other groups of defensins being discovered. These chicken β -defensin genes, designated as *Gallinacin 1-13*, are clustered densely within a 86-Kb distance on the chromosome 3q3.5-q3.7. The deduced peptides varied from 63 to 104 amino acid residues in length sharing the characteristic defensin motif. Based on the tissue expression pattern, 13 β -defensin genes can be divided into two subgroups with *Gallinacin 1-7* being predominantly expressed in bone marrow and the respiratory tract and the remaining genes being restricted to liver and the urogenital tract.

Comparative analysis of the defensin clusters among chicken, mouse, and human suggested that vertebrate defensins have evolved from a single β -defensin-like gene or gene cluster, which has undergone rapid duplication, diversification, and translocation in various vertebrate lineages during evolution. We conclude that the chicken genome encodes only β -defensin sequences and that all mammalian defensins are evolved from a common β -defensin-like ancestor. The α -defensins arose from β -defensins by gene duplication, which may have occurred after the divergence of mammals from other vertebrates, and θ -defensins have arisen from α -defensins specific to the primate lineage. Further analysis of these defensins in different vertebrate lineages will shed light on the mechanisms of host defense and evolution of innate immunity.

A genome-wide computational screen of the entire chicken genome led to identification of three novel cathelicidins, namely fowlicidins 1-3. Three fowlicidin genes are densely clustered within a 7.5-kb distance on chromosome 2 with each gene adopting a four-exon, three-intron structure. Synthetic mature fowlicidin-1 and -2 displayed potent and salt-independent activities against a broad range of Gram-negative and Gram-positive bacteria including antibiotic-resistant strains, mostly in the range of 0.2-0.6 μ M. In addition, two cathelicidins demonstrated a strong positive cooperativity in binding LPS. More desirably, fowlicidin-1 and -2 showed a low cytotoxicity with 50% lysis of erythrocytes and epithelial cells occurring at the concentrations of 10-20 μ M. Taken together, fowlicidin-1 and -2 are clearly among the most potent cathelicidins that have been reported. Broad-spectrum and salt-insensitive antibacterial activities, coupled with

potent LPS-neutralizing activity and low cytotoxicity, make fowlicidins excellent candidates for novel antimicrobial and anti-sepsis agents.

To further explore the potential of fowlicidin-1 as a therapeutic drug, we studied the structure-activity relationship of this peptide. The solution structure of fowlicidin-1 was solved by nuclear magnetic resonance (NMR) spectroscopy. Apart from a short flexible unstructured region near the N-terminus, fowlicidin-1 mainly adopts a α -helical conformation in 50% trifluoroethanol with a slight kink induced by glycine close to the center. The long helix of fowlicidin-1 is further composed of two shorter, but perfect, α -helical segments (Leu⁸-Ala¹⁵ and Arg²¹-Lys²⁵) with a slight bend between Gly¹⁶ and Tyr²⁰, due to the presence of Gly¹⁶.

To gain insight into the structural requirements for function, a series of truncation and substitution mutants were synthesized and tested separately for their antibacterial, cytolytic, and lipopolysaccharide (LPS)-binding activities. The short C-terminal helical segment after the kink (residues 16-23), consisting of a stretch of eight amino acids, was shown to be critically involved in all three functions, suggesting that this region is a major site for the peptide to interact with LPS and lipid membranes and to permeabilize both prokaryotic and eukaryotic cells.

We also identified a second segment comprised of three amino acid residues (residues 5-7) in the N-terminal flexible region that participate in LPS binding and cytotoxicity, but are less important in bacterial killing. The fowlicidin-1 analog with deletion of the second

N-terminal segment was found to retain substantial antibacterial potency with drastic reduction in cytotoxicity. The substitution mutant fowlicidin-1-L¹⁶ and fowlicidin-1-K⁷L¹²K¹⁴L¹⁶K¹⁸, which showed the significant enhancement of the helicity and amphipathicity, respectively, led to a dramatically increased toxicity particularly toward erythrocytes with a minimum change in the antibacterial activity, indicating that an amphipathic helix is more essential for interactions with lipid membranes on eukaryotic cells than for anionic lipids on prokaryotic cells. A short peptide variant, namely fowlicidin-1(8-26), stands out with the highest therapeutic index among all analogs, which represents a safer and more attractive therapeutic candidate than the parent peptide. Such a peptide analog may hold great promise as a novel antimicrobial agent against bacteria that are resistant to conventional antibiotics.

6.2 Challenges and future prospects

Human and animal health has been improved dramatically since the development and application of antibiotics. However, the efficacies of antibiotics have declined dramatically due to rapid emergence of antibiotic-resistant microbes. The serious public health crisis drives the search for novel antibiotic drugs with a less possibility of gaining resistance. A growing body of evidence has suggested that cationic antimicrobial peptides represent excellent therapeutic candidates against antibiotic-resistant microbes.

Although with potent in-vitro activities, the potency of some antimicrobial peptides diminished when they were applied systemically. For example, Gordon et al. reported

that a potent antimicrobial peptide tested in rabbit ocular infection models failed to exhibit significant activities [1]. Polyphemusin I, a horseshoe crab antimicrobial peptide, displayed excellent in-vitro antimicrobial activity with a MIC of $< 0.2 \mu\text{M}$, but no activity was observed in animal models [2]. It is likely that these peptides were cleared by host proteases before exerting antibacterial activities when applied in vivo. Stability is one of the major barriers to in vivo application of antimicrobial peptides [3]. Consequently, modified peptides have been developed to overcome this stability issue. For instance, a structure variant of polyphemusin I, with an additional arginine residue inserted in the loop region, showed reasonable activity in vivo [2]. A cecropin B analog with one amino acid substituted reduced the degradation rate by 5.7-fold than the parent peptide [4].

Toxicity is another barrier that may limit the systemic use of antimicrobial peptides [1, 3]. The optimizations of the physico-chemical parameters such as positive charges, amphipathicity, hydrophobicity, and helicity can reduce their cytotoxicity while enhancing or remaining the bactericidal activity [5]. Based on the structure information, rational designs of antimicrobial peptide analogs offer a valuable tool to overcome the toxicity problem. Our approaches to reduce the cytotoxicity of fowlicidin-1 by structure-based rational design have clearly supported this strategy.

To avoid proteolytic degradation and toxicity of peptides in systemic applications, limiting the use of peptides for tropic therapy is an alternative option. For instance, MBI

594AN, a modified bovine indolicidin peptide, is developed as a tropic treatment for acute acne, which is currently in a phase II clinical trial [1].

Because of the high cost associated with the use of chemically synthesized peptides, cost-effective strategies including recombinant protein techniques, transgenic approaches make the application of antimicrobial peptides practical. A number of antimicrobial peptides have been expressed as fusion proteins in bacteria or adenoviruses. In several cases, transgenic plants expressing antimicrobial peptides displayed broad-spectrum antimicrobial activities against phytopathogens [6]. Clearly, producing fowlicidins on a large scale with the strategies mentioned above will make these potent peptide antibiotic possible for medical or agricultural use.

Undoubtedly, antimicrobial peptides with desirable microbicidal activities, favorable immunomodulatory functions, and a less possibility to develop resistance make them promising therapeutic candidates in controlling infections. Although there are a few barriers that are currently limiting their potential as antibiotic agents, a better understanding of their mechanisms of action and structure-activity relationships will help address questions about safety and efficacy and bring a bright future to them as a new generation of antibiotics.

6.3 References

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Scope and Method of Study: The purpose of the study is to identify novel antimicrobial peptides in chickens and characterize the functions and structures of a few selected peptides for their therapeutic potential. A genome-wide computational screen of the entire chicken genome has been used to identify novel chicken defensins and cathelicidins. Putatively mature cathelicidins, fowlicidin-1 and -2, were synthesized and evaluated for their antibacterial, cytolytic, and LPS-binding activities. The tertiary structure of fowlicidin-1 was determined by CD and NMR. Based on the tertiary structure of fowlicidin-1, a series of truncation and substitution analogs were synthesized and tested separately for their antibacterial, cytolytic, and LPS-binding activities.

Findings and Conclusions: A gene cluster containing thirteen different β -defensin genes and a cluster containing three cathelicidin genes have been identified. The β -defensin gene cluster is found to localize on the chromosome 3q3.5-q3.7 as well as a cluster on chromosome 2p containing three cathelicidin genes. Fowlicidin-1 and -2 were found to display potent and salt-independent activities against a wide range of bacteria, including resistant strains. Furthermore, both fowlicidins are capable of binding lipopolysaccharide (LPS) and neutralize its inflammatory effects. Fowlicidin-1 was revealed to be largely an α -helical peptide with a slight kink close to the center. A short peptide variant, namely fowlicidin-1(8-26), stands out with the highest therapeutic potential among all peptide analogs, and represents a safer and more attractive therapeutic candidate than the parent peptide.

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