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 fowlicidin-1 to -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.

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 chemotract 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 is 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 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) in mice (23) are distantly related to classic cathelicidins with less homology in the cathelin domain. Hagfish was also found recently to contain two cathe-
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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 nonmammalian cathelicidins helped reveal for the first time the origin and evolution of mammalian cathelicidins. Our data clearly suggested that fowlidins 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.

**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, unfinished high throughput genomic sequences, 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 the 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 Fowlidin Genes**—Because no complete genomic sequence is available in GenBank™ for any of the three fowlidin 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 fowlidin-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 (DraI, EcoRV, PvuII, Rsal, and StuI), followed by ligation with annealed, bubble oligonucleotides (5¢-CAAGGAGGACCGCTGGTCATCAGGACTGTGAT-3¢ and 5¢-CCTCCCTTCTGCAGAATCGGTTGTACGGAATACCGCTGCTTCTCCTCGGATGGA-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 fowlidin-2 gene.

Because only partial sequence of the last exon of the fowlidin-1 gene is present in AADN01081708, the entire fowlidin-1 gene sequence was directly cloned from genomic DNA by PCR using the primers (forward: 5¢-GTTTCCCGATGCCCACACTTGCAG-3¢; reverse: 5¢-GGAACAGTGGCTAAAGGCGG-3¢) that 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 fowlidin-3 gene (i.e. the gap between AADN01005055 and AADN01005056) was cloned from chicken genomic DNA by PCR using primers (forward: 5¢-GCTGGTGGACTCCTACAAACCAAC-3¢; reverse: 5¢-TTGAGGTTGTGCGAGGAGCTGA-3¢) located in two flanking exons. All PCR products were recovered from agarose gel, ligated into pGEM T-Easy Vector (Promega), and sequenced from both directions.

**Assembly of the Chicken Cathelicidin Gene Cluster**—To confirm the orientation of three fowlidin genes on the chromosome, additional PCRs 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 fowlidin gene. The DNA sequence between fowlidin-1 and fowlidin-2 was obtained by using the primers (forward: 5¢-CGCTGTGGTCATCAGGACTGTGAT-3¢; reverse: 5¢-CCATCGTGGTCCTCCACCTCATTAC-3¢), whereas the sequence between fowlidin-2 and fowlidin-3 was obtained by using the primers (forward: 5¢-CAGGATTGTGAGCCACTTG-3¢; reverse: 5¢-TGAGGAGGACCGAGGCACCGGACACCAACCGGCGGATGACAG-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 fowlidin genes (AADN01005055, AADN01005056, and AADN01081708) were retrieved from GenBank™ and annotated together with our newly cloned intra- and intergenic sequences. Structures of fowlidin 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 (available on the World Wide Web at 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 fowlidin-1 and -2 (Fig. 1) is likely to be cleaved by chicken elastase. Therefore, putative mature fowlidin-1 (RKVRVPLVITRVYANGLYRAIKKK) and fowlidin-2 (LQVRQGRFGLRKRFPKTVI-TIQGSARF) were chemically synthesized by SynPep (Dublin, CA), and a sheep cathelicidin, SMAP-29 (RGLRLRGLKIAHGVKYGPTVLIRI-LIR-IH-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 high pressure liquid chromatography. The mass and purity of each peptide was confirmed by matrix-assisted laser desorption ionization-time-of-flight 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: fowlidin-1 (calculated: 3141.9; observed: 3141.6), fowlidin-2 (calculated: 3760.6; observed: 3760.1), and SMAP-29 (calculated: 3199.0; 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 (Listeria monocytogenes ATCC 19115 and Staphylococcus aureus ATCC 25923) were purchased from either ATCC (Manassas, VA) or MicroBiotests (St. Cloud, MN) and tested individually against fowlidins and SMAP-29. Three multidrug-resistant bacterial strains (S. typhimurium DT104 ATCC 700408, ...
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*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 plates. Fresh colonies were cultured and subcultured in trypticase soy broth 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 an additional 3–5 h at 37 °C in trypticase soy broth to the midlogarithmic 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 μl) were dispensed into 96-well microtiter plates, followed by the addition of 10 μl of serial 2-fold dilutions of peptides in duplicate. After a 2-h incubation at 37 °C, surviving bacteria were counted (CFU/ml) after serial plating onto trypticase soy agar 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 μM), fowlicidin-2 (0.16 μM), and SMAP-29 (0.1 μM) 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 the 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 μM) and fowlicidin-2 (0.16 μM) were incubated separately with *E. coli* ATCC 25922 for 2 h with different concentrations 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 trypticase soy agar 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 midlog phase, washed with 10 mM sodium phosphate buffer, and suspended to 5 × 10^5 CFU/ml in 1% cation-adjusted Mueller Hinton broth (BBL, Cockeysville, MD) with and without 100 mM of NaCl. Bacteria (90 μl) were then dispensed into 96-well plates, followed by the addition in duplicate of 10 μl of serially diluted peptides in 0.01% acetic acid. Because of poor growth of *P. aeruginosa* ATCC 27853 in 1% Mueller Hinton broth, this strain was grown in 10% cation-adjusted Mueller Hinton broth 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 the 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 × 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. The percentage of hemolysis was calculated as (((A_{405 nm, peptide} − A_{405 nm, PBS})/A_{405 nm, 1% Triton X-100} − A_{405 nm, PBS}) × 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 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-based assay (40). Madin-Darby canine kidney (MDCK) epithelial cells were purchased from ATCC and maintained in Dulbecco’s modified Eagle’s medium 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 Dulbecco’s modified Eagle’s medium, followed by the addition of 90 μl of fresh Dulbecco’s modified Eagle’s medium with or without 10% FBS, together with 10 μl of serially diluted peptides in 0.01% acetic acid in duplicate. After incubation for 18 h, 10 μl of alamarBlue dye was added to cells for 6 h at 37 °C in a humidified 5% CO_2_ incubator. The fluorescence of dye was read with excitation at 545 nm and emission at 590 nm. The percentage of cell death was calculated as (1 − (F_{peptide} − F_{background})/ (F_{acetic acid} − F_{background})) × 100, where F_{peptide} is the fluorescence of cells exposed to different concentrations of peptides, F_{acetic acid} is the fluorescence of cells exposed to 0.01% acetic acid only, F_{background} is the background fluorescence of 10% alamarBlue dye in cell culture medium without cells. Cytotoxicity (EC_{50}) 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 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 amebocyte lysate reagent for 10 min. The absorbance at 405 nm was measured at 10 and 16 min after the addition of 100 μl of chromogenic substrate, Ac-Ile-Glu- Ala-Arg-p-nitroanilide. Percentage LPS binding was calculated as ((ΔD1−ΔD2 + ΔD3)/ΔD1) × 100, where ΔD1 represents the difference in the absorbance between 10 and 16 min for the sample containing LPS only, ΔD2 represents the difference in the absorbance between 10 and 16 min for the samples containing LPS and different concentrations of peptides, and ΔD3 represents the difference in the absorbance between 10 and 16 min for the samples containing different concentrations of peptides with no LPS. The Hill plot was graphed as described (37, 41) by plotting log_{10} (f/(100 − f)), where f is 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 Dulbecco’s modified Eagle’s medium 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* O111: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 the QuantiTect® SYBR Green qRT-PCR kit (Qiagen) and MyiQ® real-time PCR detection system (Bio-Rad). Three common proinflammatory cytokines and chemokines (namely interleukin-1β, CC chemokine ligand 2/MCP-1, and CCL3/MIP-1α) were selected. All primers were designed to expand at least an intron sequence (Table 1). The PCR was set up in a total volume of 15 μl containing a 0.4 μM concentration of each primer and 0.2 μl 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 s, 55 °C for 30 s, and 72 °C for 30 s.
Gene expression levels were quantified by the comparative ΔΔC_{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 ΔΔC_{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 non-specific amplification of any PCR products from genomic DNA or primer dimers.

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 2). 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 nonmammalian cathelicidins.

Despite 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 (Arg and Lys). 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 NGPs 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 nonredundant sequence, high throughput genomic sequences, 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.

| Gene     | Primer sequences used for real time reverse transcription-PCR analysis of murine cytokines and chemokines |
|----------|----------------------------------------------------------------------------------------------------------|
| Gene     | Primer sequence                                                                                         | Product size |
|          | Forward                                                                                                 | Reverse      | cDNA | Genomic |
|          |                                                                                                          |              |      |         |
| Interleukin-1β | AGAATCTATACCTGTCCTGTGT                                                                                   | TGGCTCTGCTGTTGAGGTG | 195  | 916     |
| CCL-2    | ACAGAGAATACACACAGACC                                                                                      | CTGAACGTTTTAGGCAAGAG | 186  | 511     |
| CCL-3    | ACACCCCAAGTTCTAGTTQQC                                                                                     | CATTGATCCAGGTCAAGTG | 147  | 372     |
| β-Actin  | GAGAATCTACCTGCTGCTGCT                                                                                     | CTGTTGGCTGATCCACA | 139  | 264     |

| Gene     | Identification of chicken fowlicidins                                                                 |
|----------|--------------------------------------------------------------------------------------------------------|
| Gene     | EST                                                                                                    | WGS          |
|          |                                                                                                        |              |
| Fowlidin-1| BX936022                                                                                               | AADN01081708 |
| Fowlidin-2| AY534900                                                                                               | AADN01005055 |
| Fowlidin-3| CR389785                                                                                               | AADN01005055 |

| Gene     | Gene size |
|----------|------------|
|          | E 1 | I 1 | E 2 | I 2 | E 3 | I 3 | E 4 | bp  |
| Fowlidin-1| 168 | 537 | 108 | 84  | 84  | 99  | 87  |
| Fowlidin-2| 168 | 901 | 108 | 70  | 84  | 293 | 105 |
| Fowlidin-3| 168 | 535 | 108 | 84  | 84  | 99  | 96  |
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 1, 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.

As shown in Fig. 2A, 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 1, 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.
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 (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_077137) across rodents, dogs, and humans, clearly indicating that cathelicidins in mammals and birds share a common ancestor. It is noteworthy that the chicken calmodulin kinase-like kinase gene is absent in syntenic regions in mammals (Fig. 2B).
**Identification and Characterization of Chicken Cathelicidins**

**Table 3**

Antibacterial spectrum of fowlicidins against Gram-negative and Gram-positive bacteria

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

**Table notes:**

- MRSA, methicillin-resistant S. aureus.

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 phylogenetically 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 MIC<sub>50</sub> of 65 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 MIC<sub>50</sub> 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 µM (Table 3). *P. aeruginosa* appeared to be the only exception, being slightly more resistant to fowlicidins with the MIC of 3–6 µM 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 3). More desirably, both peptides were equally effective against antibiotic-resistant bacterial strains, such as multidrug-resistant *S. typhimurium* DT104 and methicillin-resistant *S. aureus* (Table 3). 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 SMAP-29 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<sub>50</sub> occurring at ~6–10 µM and 15–20 µM for fowlicidin-1 and -2, respectively (Fig. 5, A and B). 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<sub>50</sub> occurred in the range of 10–20 µM toward MDCK cells for both fowlicidins, with SMAP-29 being the most toxic. A similar trend also...
Identification and Characterization of Chicken Cathelicidins

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 3). Although we suspected that valine on the fourth exon of fowlicidins (Fig. 1) is likely to become biologically active. Indeed, putatively mature fowlicidins possess potent antibacterial activities (Fig. 4 and Table 3). 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. Despite 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) as classic mammalian cathelicidins and therefore clearly belong to the cathelicidin family. Identification of three fowlicidins, which are more closely related to NGPs (Figs. 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 occurring at 7.5 and 8.6 µM for fowlicidin-1 and -2, respectively. Both peptides completely inhibited the LPS procoagulant activity at 10–15 µM 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 µM in the absence of LPS, did not alter gene expression. However, they blocked LPS-induced expression of interleukin-1β and CCL-2/MCP-1 in a dose-dependent fashion (Fig. 6, C and D). The same trend also occurred with CCL-3/MIP-1α for both peptides (data not shown). In fact, fowlicidins inhibited the expression of all three genes by >90% at 20 µM (Fig. 6, C and D). To our surprise, albeit with in vitro LPS binding activity (41), SMAP-29, even at 20 µM, 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 µM, caused only minimal, <5% cell death to RAW264.7 cells in the presence of 10% FBS (data not shown).

DISCUSSION

To test the potential of fowlicidins as anti-sepsis agents, a chromogenic *Limulus* amebocyte 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 values are indicated as dotted lines. Data shown are means ± S.E. of 2–3 independent experiments.

LPS Binding and Host Gene Modulatory Activities of Fowlicidins—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 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 ± S.E. of 2–3 independent experiments.
Identification and Characterization of Chicken Cathelicidins

As for the origin of three chicken cathelicidins, fowlcicidin-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). Fowlcicidin-2 also shares significant homology in the first three exons with fowlcicidin-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 fowlcidin genes revealed ~45% identity (data not shown). This suggested that the entire fowlcicidin-2 gene may have been duplicated directly from fowlcicidin-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 its 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 sites 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 mice to the human system needs to be more prudent.

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