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Distinctive Structural Hallmarks and Biological Activities of the Multiple Cathelicidin Antimicrobial Peptides in a Primitive Teleost Fish

Xu-Jie Zhang,*† Xiang-Yang Zhang,*† Nu Zhang,*† Xia Guo,*† Kai-Song Peng,*‡ Han Wu,* Long-Feng Lu,*† Nan Wu,* Dan-Dan Chen,*‡ Shun Li,* Pin Nie,* and Yong-An Zhang*

Cathelicidin antimicrobial peptides (CAMPs) represent a crucial component of the innate immune system in vertebrates. Although widely studied in mammals, little is known about the structure and function of fish CAMPs. Further to the previous findings, two more cathelicidin genes and multiple transcripts from rainbow trout were identified in the present study. Interestingly, we found that trout have evolved energy-saving forms of cathelicidins with the total deletion of the characteristic cathelin-like domain. Sequence analysis revealed that salmonid CAMPs have formed a special class of antimicrobial peptides in vertebrates with three distinctive hallmarks: the N terminus is intensified by positive charges, the central region consists of repetitive motifs based on RPGGGS, and the C terminus is lowly charged. Immunofluorescence localization of trout CAMPs demonstrated that these peptides expressed mainly at the mucosal layer of gut. Meanwhile, signals around sinusoids were also detected in head kidney. Moreover, the biological activities of trout CAMPs were proved to be mediated by the N terminus. Additionally, the repetitive motifs characteristically existing in Salmonidae increased the structural flexibilities of peptides and further increased the antibacterial and IL-8-stimulating activities. Unlike most α helical and cytokytic mammalian CAMPs, trout CAMPs, mainly consisting of β-sheet and random coil, exhibited no cytoxic activities. The distinctive structural features of trout CAMPs provide new insights into the understanding of the evolution of CAMPs in vertebrates. Moreover, the high bacterial membrane selectivity of trout CAMPs will help to design excellent peptide antibiotics. The Journal of Immunology, 2015, 194: 4974–4987.

Antimicrobial peptides represent an evolutionarily old component of the innate immune system in all organisms (1, 2). They form the first line of host defense against infectious microorganisms prior to stimulating the adaptive immune system of animals (1–3). The increasing resistance of bacteria to conventional antibiotics stimulates the exploitation of antimicrobial peptides as candidates for new antibiotics (3, 4). Among them, cathelicidins and defensins represent the major and well-studied antimicrobial peptide families in animals (2, 5).

Antimicrobial peptides are present in all vertebrates (2, 6, 7). The discovery of three cathelicidin-like members in the ancient vertebrate hagfish (Myxine glutinosa) implies that cathelicidins already existed ∼450 million years ago (8). Until now, only one cathelicidin antimicrobial peptide (CAMP) has been found in primates (2). Nevertheless, at least six CAMPs have been discovered in other mammals belonging to Euungulata, such as cattle, goat, sheep, and pig, whereas four have been discovered in birds, three in reptiles, and two in amphibians (2, 9, 10). As in fish, three CAMPs have been identified in Atlantic cod (Gadus morhua), whereas two or one has been discovered in several other species (5, 7, 11). In vivo studies conducted in mice have suggested that the duplication and divergence of cathelicidins are beneficial to host defense against infections (12, 13).

In mammals, CAMPs are produced by epithelial cells and lymphocytes (14, 15) and are mainly expressed at bone marrow and the epithelial surfaces of skin, gastrointestinal tract, and lung (15–17). CAMPs are first produced as inactive preproteins, which consist of a signal peptide followed by a cathelin-like domain (CLD), as well as a C-terminal mature peptide containing 12–80 residues (2, 18). The CLD shows high interspecies sequence homology, whereas the mature peptide shows high diversity both inter- and intraspecies (2, 6, 19). The active mature peptide is released via proteolytic processing with specific proteases (2, 18). The released CAMPs are small, cationic, and amphipathic peptides (2, 5, 6). Although varying in amino acid sequence and size, most mammalian CAMPs are linear peptides consisting of 23–37 residues and adopting amphipathic α helical structures in the environments mimicking biological membranes (6). These CAMPs exhibit a broad spectrum of antimicrobial activities against bacteria, viruses, and...
fungi (2, 6, 18). In addition to their direct antimicrobial effects, mammalian CAMPs possess other modulatory activities, such as LPS neutralizing (20, 21) and cytokine stimulating (22, 23).

Although CAMPs have been widely studied in mammals, research on fish CAMPs has been predominantly limited to gene identification and antibacterial activity test. No information is available about the structure/activity relationships of fish CAMPs. However, the special living environment and evolutionary status make fish an interesting host to study the functions of CAMPs. Thus, the aim of the present study was two-fold: first, to unearth more cathelicidin genes and transcripts in rainbow trout (Oncorhynchus mykiss), a commercially farmed fish throughout the world; and second, to further investigate the structure and multifunction of these CAMPs. We found that trout possess at least four genes and seven transcripts of CAMPs. Sequence analysis revealed that trout CAMPs, together with other salmonid CAMPs, form a special class of antimicrobial peptides with distinctive hallmarks in vertebrates. Moreover, the structure/activity relationships of trout CAMPs have been established.

Materials and Methods

Fish

Rainbow trout (50–70 g) obtained from Zhanhe Reservoir Rainbow Trout Farm (Jingmen, China) were maintained and acclimated to the laboratory conditions as previously described (24). All animal experiments were approved by the Committee on the Ethics of Animal Experiments of the Institute of Hydrobiology, Chinese Academy of Sciences.

Isolation of cDNAs for trout cathelicidins

Healthy trout were euthanized and total RNA was isolated from the head kidney by using the TRizol reagent (Invitrogen). First-strand cDNA was synthesized from the total RNA by using a reverse transcription system (Promega) with oligo(dT)18 primer and stored at -20°C for use.

Using the previously reported cDNA sequence of rtCATH-1 (accession no. AY382478, termed rtCATH-1a in this study) to search trout in the Expressed Sequence Tags database (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov), we obtained a putative splicing isoform (GE834401, termed rtCATH-1b). Primer sets rtCATH1-F1/rtCATH1-R1 and rtCATH1-F1/rtCATH1-1R (Table I) matching the 5’ and 3’ ends of the cDNA and open reading frame sequences, respectively, were applied to amplify other potential splicing isoforms of rtCATH-1a. Alternatively, we have previously sequenced a genomic clone carrying a homolog gene (AY542962, termed rtCATH2b) of rtCATH-2 (AY542963, termed rtCATH-2a). Primer set rtCATH2-F1/rtCATH2-R1 (Table I) matching the 5’ and 3’ ends of the genomic sequences was applied to amplify the transcripts from trout cDNA.

All PCRs were performed in 20-µl reaction volumes containing 14 µl water, 2 µl 10× buffer, 2 µl 2°-dioxymucose 5°-triphosphate (2.5 mM each), 0.25 µl Ex Taq HS DNA Polymerase (TaKaRa), 1 µl primer set (10 µM each), and 0.75 µl cDNA. The amplification program was as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 20 s, 60°C for 20 s, and 72°C for 30 s, and then 72°C for 5 min. Amplified DNA was analyzed using 1.8% (w/v) agarose gel electrophoresis stained with ethidium bromide and visualized with a UV transilluminator (Bio-Rad).

Isolation of genomic sequences of trout cathelicidins

Genomic DNA was extracted from the liver of trout with a genomic DNA extraction kit (Tiangen Biotech) and was used as a PCR template. The homolog genes of trout rtCATH-1a and rtCATH-2a were PCR amplified with the primer sets and program as described above. Amplified DNA was analyzed on a 1% agarose gel. Owing to the high sequence similarity, the gene fragments of rtCATH-2a and rtCATH-2b were amplified with the same primer set rtCATH-2F1/rtCATH-2R (Table I) and further cloned and sequenced to detect the coexistence of both genes in individual trout.

DNA cloning and sequence analysis

All PCR products were cloned into pMD18-T vector (TaKaRa). Plasmid DNA was isolated from the positive colonies and sequenced with an ABI 3730XL sequencer (Applied Biosystems) by Tsingke Company (Wuhan, China). Predictions of the open reading frame and signal peptide were performed with the Translate and SignalP programs, respectively, at the ExPaSy proteomics server (http://www.expasy.org/). Multiple sequence alignment was generated using ClustaW2 at the European Molecular Biology Laboratory–European Bioinformatics Institute Web site (http://www.ebi.ac.uk/).

Expression patterns of trout cathelicidin genes

Three healthy trout (50–70 g) were euthanized and total RNA was isolated from the blood, head kidney, spleen, thymus, gut, skin, gill, liver, heart, muscle, and brain. Meanwhile, two groups of healthy trout were injected i.p. with LPS (from Escherichia coli 0111:B4; Sigma-Aldrich; 1 mg/ml in PBS) or PBS (Invasive, 100 µl per fish, and total RNA was isolated from the head kidney and gut of three fish at 0 h, 12 h, 24 h, 3 d, and 7 d postinjection. The first-strand cDNA was synthesized as described above.

The transcripts of rtCATH-2a and rtCATH-2b were amplified with the primer set rtCATH-2F1/rtCATH-2R (Table I) by 38 cycles and then cloned and sequenced as described above to detect the coexpression of both genes in healthy trout tissues. The expression levels of trout cathelicidin genes in the tissues under normal and challenged situations were further analyzed by quantitative real-time PCR (qPCR) using primer sets specific for rtCATH-1a/1b/1c (qCATH1aF/qCATH1aR), rtCATH-2a/2b (qCATH2F/qCATH2R), and the reference gene elongation factor 1a (EF-1a; qEF-1aF/qEF-1aR) (Table I). The reactions were conducted in duplicate and each 10-µl reaction volume contained 1 µl cDNA template, 5 µl SYBR Green PCR master mix (2× Bio-Rad), and 1 µl of each forward and reverse primer (10 µM). The amplification profile consisted of an initial denaturation step at 95°C for 3 min, and then 45 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 20 s, followed by melting (dissociation) stage from 65°C to 95°C in a CFX real-time PCR detection system (Bio-Rad). Amplification efficiencies of the primer sets were 96.2% for qCATH1aF/qCATH1aR (R² = 0.995), 100% for qCATH2F/qCATH2R (R² = 0.999), and 96.7% for qEF-1aF/qEF-1aR (R² = 0.995), calculated by using 2-fold series dilution of cDNA in qPCR. Specificities of the primer sets were verified by the dissociation curves and sequencing the qPCR products. The relative expression levels of trout cathelicidin genes were normalized by using the expression of EF-1a.

Peptide synthesis

Trout CAMPs and the truncated variants were synthesized using a peptide synthesizer from Attepeptide (Nanjing, China) and analyzed by HPLC and MALDI-TOF mass spectroscopy to confirm that the purity was >95%. Human CAMPs LL-37 was synthesized as a reference. All peptides were lyophilized and stored at -80°C for use.

Expression and purification of GST-tagged trout CAMPs in E. coli

The DNA fragments encoding trout CAMPs 1a and 2a were amplified using the primer sets Ex-Cath1aF/Ex-Cath1aR and Ex-Cath2aF/Ex-Cath2aR (Table I, respectively), and then digested with BanHI and XhoI (New England Biolabs) and ligated to the pGEX-4T-1 expression vector (GE Healthcare). The expression plasmids were subsequently transformed into E. coli BL21 (DE3)–CodonPlus-RIL competent cells, which were cultured in Luria–Bertani broth and induced with 0.5 mM isopropyl β-D-thiogalactoside at 12°C for 12 h. Then cells were harvested and resuspended in 25 ml lysis buffer (500 mM NaCl, 20 mM Tris, 1 mM EDTA, 0.1% Triton X-100, 1 mM PMSF, 1 mM aprotinin [pH 7.5]). Cell suspensions were mechanically disrupted using a JN-24C high-pressure cell press (JNBIO, Guangzhou, China) and then centrifuged at 16,000 g for 30 min at 4°C. The supernatants were dialyzed against 500 mM NaCl, 20 mM Tris, 1 mM EDTA, 0.1% Triton X-100, 1 mM PMSF, and 1 mM aprotinin (pH 7.5). The supernatants were incubated with GST resin (Clontech, Palo Alto, CA) for 4 h at 4°C. The GST column was then washed with 500 ml washing buffer (500 mM NaCl, 20 mM Tris, 1 mM EDTA, 0.1% Triton X-100, 1 mM PMSF, 1 mM aprotinin [pH 7.5]). Then the GST fusion protein was eluted with lysis buffer (150 mM NaCl, 20 mM Tris, 20 mM reduced glutathione [pH 7.2]). The purified protein was then analyzed by SDS-PAGE (12%) and subjected to Western blot analysis with a polyclonal antibody raised against recombinant recombinant trout cathelicidin. The expression and purification of GST-tagged trout CAMPs in E. coli were performed with BacMam (Invitrogen) and the expression products were analyzed by 12% SDS-PAGE (12%) and subjected to Western blot analysis with a polyclonal antibody raised against recombinant trout cathelicidin.

Production, purification, and detection of rabbit anti-trout CAMP polyclonal Abs

GST-tagged trout CAMPs 1a and 2a were used to raise polyclonal Abs (pAbs) in rabbits (Yangtze Biotech, Shanghai, China). The pAbs in rabbit antiserum were purified by affinity chromatography using the GST-tagged trout CAMPs coupled to SulfoLink coupling resin (Thermo Scientific).
Thereafter, the GST-specific pAbs were removed by affinity chromatography using the GST-coupled SulfoLink coupling resin. Thus, the remaining pAbs were trout CAMP-specific. Western blot was performed to analyze the reactivity of the pAbs with trout CAMPs as previously described (25).

**Immunofluorescence localization of trout CAMPs**

Immunofluorescence was performed as previously described (26) to analyze the tissue localization of trout CAMPs in head kidney and gut. Briefly, cryosections of tissue samples were incubated with rabbit anti-trout CAMP pAbs (0.5 μg/ml), followed by FITC-conjugated goat anti-rabbit IgG Ab (2.5 μg/ml, Thermo Scientific). Rabbit anti-GST pAb (0.5 μg/ml) was used as the negative control. After staining with DAPI (Beyotime), the images were acquired using a confocal microscope (Zeiss).

**Antibacterial activity assay of trout CAMPs and their truncated variants**

Five species of Gram-negative bacteria, including *E. coli* ATCC 25922, *Edwardsiella ictaluri*, *Vibrio flavidus*, *Aeromonas hydrophila*, and *Yersinia ruckeri*, and one species of Gram-positive bacteria, *Streptococcus dysgalactiae*, were used to test the antibacterial activities of trout CAMPs and their truncated variants. All of these bacteria, except *E. coli* ATCC 25922, are fish pathogenic bacteria. Human CAMP LL-37 was used as a reference. Antibacterial activity assay was performed by 2-fold microtiter broth dilution method as previously described with minor modifications (27, 28). Briefly, 80 μl serial 2-fold dilutions of peptides prepared in water were added to each corresponding well of a 96-well microtiter plate (Thermo Scientific). Then, 20 μl bacteria diluted in Mueller–Hinton broth (MHB; Oxoid) or brain heart infusion broth (BD Biosciences) (for *S. dysgalactiae*) to a concentration of 5 × 10^6 CFU/ml were added. The wells without peptide were used as controls. Initial OD_{955} was measured using an ELX800 microplate reader (BioTek). Then these plates were incubated at 20 °C, except *S. dysgalactiae* (at 28 °C), for 18 h. OD_{955} was measured and corrected by initial OD_{955} values. Bacterial grow rates were calculated as the bacterial densities in the presence of peptides to the bacterial densities of controls.

**Bacterial membrane permeability analysis of trout CAMPs and their truncated variants**

The bacterial membrane permeability of trout CAMPs and their truncated variants was assessed by flow cytometry as previously described (29) with minor modifications. Briefly, to 20 μl *E. ictaluri* cells (10^6 CFU/ml in MHB), 80 μl peptides diluted in water was added to give a final concentration of 8 μM. Meanwhile, a bacterial suspension without peptides was included as a control. After incubated at 20 °C for 1.5 h, propidium iodide (PI, Sigma-Aldrich) was added to give a final concentration of 9 μM. The influx of PI into bacterial cells was investigated by using an InFlux C6 flow cytometer (BD Biosciences) at an accelerating voltage of 3 kV.

**LPS-binding activities**

The LPS-binding activities of trout CAMPs were measured through ELISA as previously described (20, 36). All reactions were done in duplicate and the experiment was repeated. Binding of peptides to LPS was expressed as a percentage of absorbance developed by 0.1 μg peptide. Additionally, the LPS-binding isotherms of trout CAMPs and their truncated variants were quantified by the kinetic chromogenic *Limulus* amebocyte lysate assay (QCL-1000 kit; Lonza) as previously described (21, 37).

**Induction of chemokine IL-8 expression in trout peripheral blood leukocytes**

Trout peripheral blood leukocytes (PBLs) were isolated and stimulated with CAMPs at the concentration of 10 μM as previously described (28). Total RNA was isolated from the PBLs at 0, 4, 8, 16, and 24 h postincubation. Then, cDNA synthesis and qPCR were performed as above described to quantify the expression of the two reference genes. The relative expression of the two reference genes was subsequently measured at 490 nm. The relative cell viability was determined by comparison with 0 and 100% lysis using the following formula: [(A_{Abetasilicon} – A_{100% lysis}/(A_{100% lysis} – A_{0% lysis})) × 100, where A is the absorbance at 490 nm.

**Synteny analyses of cathelicidin genes in vertebrates**

Gene synteny of cathelicidin family members in representative vertebrates, including human, mouse, cattle, goat, chicken, frog, and trout, were analyzed. Cathelicidin members were located to chromosomes or scaffolds by using BLAST algorithm.

**Statistical analysis**

The statistic p values were calculated by one-way ANOVA with a Dunnett post hoc test (SPSS Statistics, version 19, IBM). A p value <0.05 was considered significant.

**Results**

**Genomic, cDNA, and protein sequences of trout cathelicidins**

Gene-specific primers (Table I) were used to amplify the transcripts of *rCATH-1* interestingly, among the PCR products amplified by the primer set rCATH1-F1/rCATH1-R1, besides *rCATH-1b*, an additional splicing isoform (termed as *rCATH-1c* of *rCATH-1a* was obtained. While among the PCR products amplified by the primer set rCATH1-F1/rCATH1-1R, another isoform (termed as *rCATH-1am*) of *rCATH-1a* and a transcript of the second *rCATH-1* gene (termed as *rCATH-1d*) were obtained. Then, the genomic sequences of *rCATH-1a* and *rCATH-1d*...
were amplified with the primer set rtCATH1-F1/rtCATH-1rtR.
The deduced amino acid sequences were aligned and schematic
diagrams were created to show the organizations of the genes,
transcripts, and preproproteins (Fig. 1A, 1B). The preproprotein of
rtCATH-1a consists of a signal peptide and a CLD followed by
a mature peptide. The central region of the mature peptide is
classified by five tandem repeats based on the (R/P)(P/L)
GGGS motif, and it is flanked by 16 residues at the N-terminal
and C-terminal, respectively. The splicing isoforms
rtCATH-1b
and
rtCATH-1c are characterized by the loss of small portions

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** Schematic diagrams of the gene, mRNA, and protein organizations of trout CAMPs and multiple alignment of the deduced amino acid sequences. (A) Schematic diagrams showing the gene, mRNA, and protein organizations of rtCATH-1a and rtCATH-1d. (B) Multiple alignment of the deduced amino acid sequences of the preproteins rtCATH-1a and rtCATH-1d. (C) Schematic diagrams showing the gene, mRNA, and protein organizations of rtCATH-2a and rtCATH-2b. (D) Multiple alignment of the deduced amino acid sequences of the preproteins rtCATH-2a and rtCATH-2b. The signal peptide, CLD, and mature peptide are denoted above the alignment. The repetitive motifs [(R/P)(P/L)GGGS] in mature peptides are in boldface. The cysteine residues are shaded black.
of exon 4, resulting in reduction of the copy numbers of the repetitive motif in the mature peptide, whereas rtCATH-1am is characterized by the loss of a large portion ranging from exon 1 to exon 3, resulting in complete deletion of the CLD of the preproprotein. Interestingly, the rtCATH-1d gene has no introns, and the preproprotein only consists of a signal peptide and a mature peptide, which is similar to that of rtCATH-1am, but without the repetitive motif.

The genomic and cDNA sequences of rtCATH-2a and rtCATH-2b were amplified. The deduced amino acid sequences were compared and schematic diagrams were created to show the organizations of the genes, transcripts, and preproproteins (Fig. 1C, 1D). Similar to rtCATH-1a, the preproprotein of rtCATH-2a and rtCATH-2b also consist of a signal peptide and a CLD followed by a mature peptide. The central regions of the mature peptides possess two tandem repeats of the RPG(S/F)GS motif. Interestingly, these two genes share a surprisingly high degree of sequence identity (99%) at the nucleotide level. However, owing to the earlier occurring stop codon, the preproprotein of rtCATH-2a is shorter than that of rtCATH-2b, with lack of the C-terminal 16 residues (Fig. 1C, 1D). The present study revealed that both genes coexisted in each of the 24 tested trout (data not shown), which means that rtCATH-2a and rtCATH-2b are two different genes and are not segregated in trout.

Tissue distribution and stimulation of trout cathelicidins

The expressions of rtCATH-2a and rtCATH-2b in a range of healthy trout tissues were analyzed by RT-PCR. The sequenced PCR products showed that both genes were coexpressed in all of the tested tissues, although the expression level of rtCATH-2a was higher than that of rtCATH-2b (data not shown). Then, the expression levels of rtCATH-1a/1b/1c and rtCATH-2a/2b were further analyzed by qPCR. As shown in Fig. 2A, in normal conditions, rtCATH-1a/1b/1c and rtCATH-2a/2b are constitutively expressed. The expression level of rtCATH-1a/1b/1c was higher than that of rtCATH-2a/2b in thymus and gill but lower in heart and muscle. Interestingly, both types of CAMPs were highly expressed in thymus and mucosal tissues, including skin and gill. In LPS-stimulated conditions, the expressions of both types of CAMPs in head kidney (Fig. 2B) and gut (Fig. 2C) were increased dramatically.

Immunofluorescence localization of trout CAMPs in head kidney and gut

Anti-trout CAMP pAbs were used to detect the expression and localization of CAMPs in head kidney and gut of trout. Western blot showed that anti–rtCATH-1a pAb could specifically recognize rtCATH-1a, -1b, -1c, and -1d; however, anti–rtCATH-2a pAb could only recognize rtCATH-2a, but not rtCATH-2b (Supplemental Fig. 1). In head kidney, the immunoreactive signals of trout CAMPs were observed in lymphoid cells around the sinusoids (Fig. 3A, 3B). However, in gut, the signals were observed mainly at the mucosal layer (Fig. 3D, 3E). Thus, trout CAMPs were mainly expressed by the columnar epithelial cells of the gut villus. Higher magnification further revealed that some lymphoid cells in the lamina propria of gut also expressed CAMPs. Negative controls showed no immunoreactive signal (Fig. 3C, 3F).
Antibacterial activities of trout CAMPs and their truncated variants

Trout CAMPs derived from rtCATH-1a, rtCATH-1d, rtCATH-2a, and rtCATH-2b genes displayed various net charges (Fig. 4) and antibacterial activities (Fig. 5). All of the trout CAMPs, including 1a, 1b, 1c, 1d, 2a, and 2b, were antibacterial to all strains tested.

As for cathelicidin 1 peptides, with the gradual deletion of the repetitive motif in the central region, the antibacterial activities of 1a, 1b, 1c, and 1d were gradually decreased. The antibacterial activities of 1a and 1b were almost equal, whereas those of 1c and 1d were 3- to 4-fold lower. Among the series of truncated variants of 1a, only the negatively charged C-terminal variant 1Ct had no antibacterial activity. The deletion of the highly cationic N-terminal of 1a (1ΔN) resulted in a significant decrease of the antibacterial activity, whereas the deletion of the lowly cationic central region (1ΔIn) or C terminus (1ΔC) almost did not lead to a decrease of the activity. The N-terminal variant 1Nt independently had antibacterial activity that was slightly lower than that of 1ΔC. The order of the antibacterial activities of trout cathelicidin 1 peptides and their truncated variants was 1a > 1b > 1ΔC > 1c > 1d > 1Nt > 1In > 1ΔN > 1Ct.

As for cathelicidin 2 peptides (2a and 2b), with lack of the C-terminal 16 residues, the antibacterial activities of 2b were slightly lower than those of 2a. Among the series of truncated variants of 2a, the lowly cationic central variant 2In and C-terminal variant 2Ct had no antibacterial activities. The deletion of the highly cationic N-terminal of 2a (2ΔN) resulted in a significant decrease of the antibacterial activity, whereas the deletion of the lowly cationic central region (2ΔIn) or C terminus (2ΔC) almost did not lead to a decrease of the activity. The N-terminal variant 2Nt independently had antibacterial activity that was slightly lower than that of 2ΔIn and 2ΔC. The order of the antibacterial activities of trout cathelicidin 2 peptides and their truncated variants was 2a > 2b > 2ΔIn > 2ΔC > 2Nt > 2ΔC > 2Nt > 2ΔN > 2In = 2Ct.

Bacterial membrane permeability of trout CAMPs and their truncated variants

The penetrating efficiencies to bacteria, according to the relative fluorescence intensities, of trout CAMPs and their truncated variants are shown in Fig. 6. The results indicated that trout CAMPs killed...
bacteria through membrane-permeabilizing action. Compared to LL-37, trout CAMPs showed weaker bacterial membrane permeability. The penetrating efficiency of 1a was higher than that of 2a. Among the truncated variants of 1a and 2a, 1Nt, 1In, and 2Nt could independently penetrate the bacterial membrane, whereas 1Ct, 2In, and 2Ct could not. The results precisely accorded with the antibacterial activities of trout CAMPs and their truncated variants.

SEM observation of the bacteria treated with trout CAMPs
Bacterial morphological changes after treatment with trout CAMPs were directly observed by SEM. As shown in Fig. 7, the untreated E. ictaluri cells possessed normal shape and smooth surfaces. In contrast, E. ictaluri cells treated with trout CAMPs had obvious morphological alterations. After treatment with 1a, 1b, 1c, 1d, and 2b, the cell walls of bacteria were severely perturbed; however, with complete deletion of the repetitive motif in the central region, the cell wall perturbation ability of 1d was significantly decreased.

Secondary structural elements of trout CAMPs measured by CD
The structural features of trout CAMPs and their truncated variants in different solvent environments were measured by CD spectroscopy (Fig. 8, Supplemental Fig. 2). In phosphate buffer, the CD spectrum of all peptides, except human LL-37, showed a strong negative band at 200 nm, indicative of a mostly random-coiled conformation. Interestingly, in SDS micelles or TFE solvent, mimic of bacterial cell membrane environment, the CD spectra of cathelicidin 1 peptides (1a, 1b, 1c, and 1d) changed dramatically, with double minimal signals at 208 and 222 nm, indicating an amphipathic α helical conformation. Especially in

FIGURE 5. Antibacterial activities of trout CAMPs and their truncated variants against E. ictaluri, V. fluvialis, E. coli, A. hydrophila, Y. ruckeri, and S. dysgalactiae. (A) Antibacterial activities of cathelicidin 1 peptides and their truncated variants. (B) Antibacterial activities of cathelicidin 2 peptides and their truncated variants. LL-37 was used as a control. The bacteria in mid-logarithmic phase were diluted in medium and incubated with serially diluted peptides at a concentration of 10^5 CFU/ml for 18 h. OD_595 was measured and corrected by initial OD_595 values. Bacterial grow rates were calculated as the bacterial density in the presence of peptides to the bacterial density of controls. Data shown are the means of duplicate reactions in a representative of three independent experiments.
TFE, an organic solvent whose dielectric constant resembles that of biological membrane, the signals were intensified. However, the calculated helical content of peptides 1a, 1b, 1c, and 1d in TFE was only 10.4, 12.8, 18.1, and 13.3%, respectively (Table II). The results demonstrated that the peptides were mainly composed of β-sheet and random coil. For the truncated variants of 1a, 1Ct showed the most significant signals of α helical conformation, followed by 1AN. However, 1ΔC, 1Nt, and 1In showed no signal of α helical conformation. The results indicated that the α helical conformation locates at the C-terminal of 1a. All of these peptides, except 1Ct, showed β-sheet conformation. Unlike cathelicidin 1 peptides, cathelicidin 2 peptides and the truncated variants showed no obvious signal of α helical conformation, but partial signals of β-sheet conformation. The calculated secondary structural elements of 2a and 2b demonstrated that they mostly consisted of random coil, with 62.0 and 52.4% content, respectively. In contrast with trout CAMPs, human LL-37 showed strong signal of α helical conformation, with 98.3% α helix in TFE.

**Simulated tertiary structures of trout CAMPs**

The simulated tertiary structures of trout CAMPs are shown in Fig. 9. As for 1a, the N-terminal 16 aa formed an antiparallel β-sheet, the central repetitive motifs formed another antiparallel β-sheet and a flexible random coiled region, whereas the C-terminal 16 aa formed a one-turn helix flanked by an antiparallel β-sheet on the C-terminal side. From peptide 1a to 1d, with gradual deletion of the repetitive motifs in the central region, the second antiparallel β-sheet and the flexible region were gradually diminished, resulting in a decrease of the structural flexibility of peptides. As for 2a and 2b, except for an obvious antiparallel β-sheet located in the central region, the entire molecule consisted of random coil.

**Hemolytic and cytotoxic activities of trout CAMPs**

As shown in Fig. 10, trout CAMPs did not exhibit apparent hemolytic and cytotoxic activities. In contrast, human LL-37 exhibited strong hemolytic and cytotoxic activities against all cells tested.

**LPS-binding activities of trout CAMPs**

The LPS-binding activities of trout CAMPs were investigated by ELISA. As shown in Fig. 11A, trout CAMPs could bind to LPS in a dose-dependent manner. To investigate the LPS binding sites of trout CAMPs, the LPS-binding isotherms of 1a and its truncated variants were evaluated by the kinetic chromogenic Limulus amebocyte lysate assay (Fig. 11B). The EC50 is indicated as a dotted line. The LPS-binding activity of 1ΔC was almost equal to that of 1a. However, the activities of 1AN and 1In were significantly lower. The results suggested that there are at least two LPS binding sites in 1a, one at the N terminus and the other at the central region. The 6-fold higher affinity of 1ΔC (EC50 ≈ 9 μM)
for LPS than 1In (EC50 ≈ 64 μM) suggested that the N-terminal binding site has higher affinity for LPS than does the central one. The LPS-binding isotherm of 1a showed a sigmoidal shape, indicating positive cooperativity of the two LPS-binding sites.

Induction of IL-8 expression in PBLs by trout CAMPs

In human and Atlantic salmon (Salmo salar), CAMPs have been associated with the ability to stimulate IL-8 expression (22, 23, 28, 38, 39). As shown in Fig. 11C, trout CAMPs 1a and 2a could also significantly stimulate the expression of IL-8 in PBLs at 4 h after incubation. Interestingly, with gradual deletion of the repetitive motif in the central region, the IL-8 stimulating ability of 1a, 1b, 1c, and 1d was gradually decreased. Compared to 2a, the IL-8 stimulation by 2b was weaker, and the time point with the greatest stimulation effect (16 h after incubation) was much later than that by 2a.

Synteny analyses of cathelicidin genes in vertebrates

As shown in Supplemental Fig. 3, only a single cathelicidin gene exists in humans and mice. However, in cattle, goats, chickens, and frogs, multiple cathelicidin genes form a gene cluster in one chromosome, although the two genes in frogs are separated by other genes. Unlike higher vertebrates, the cathelicidin genes discovered so far in trout locate in three different chromosomes, which may result from the salmonid-specific fourth whole-genome duplication dated from 25 to 100 million y ago (40). Among trout cathelicidin genes, rCATH-1a and rCATH-2a genes from trout (24, 27), two more cathelicidin genes, rCATH-1d and rCATH-2b, have been identified in this study. Interestingly, rCATH-1d contains no intron and its preproprotein lacks the whole CLD. Despite absence of the CLD and (R/P)(P/L)GGGS motif, the protein sequence of rCATH-1d is

![CD spectra of trout CAMPs. The spectra were taken in 20 mM phosphate buffer (pH 6.0), 90 mM SDS (pH 6.0), and 60% TFE (pH 6.0), respectively, and measured between 190 and 250 nm. Three consecutive scans per sample were performed and averaged followed by subtraction of the solvent signals.](http://www.jimmunol.org/)

<table>
<thead>
<tr>
<th>Peptide</th>
<th>α-Helix (%)</th>
<th>β-Sheet (%)</th>
<th>β-Turn (%)</th>
<th>Random Coil (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>10.4</td>
<td>37.7</td>
<td>21.0</td>
<td>30.9</td>
</tr>
<tr>
<td>1b</td>
<td>12.8</td>
<td>32.5</td>
<td>21.8</td>
<td>33.0</td>
</tr>
<tr>
<td>1c</td>
<td>18.1</td>
<td>27.5</td>
<td>22.5</td>
<td>32.0</td>
</tr>
<tr>
<td>1d</td>
<td>13.3</td>
<td>31.7</td>
<td>22.3</td>
<td>32.6</td>
</tr>
<tr>
<td>2a</td>
<td>5.5</td>
<td>21.1</td>
<td>11.4</td>
<td>62.0</td>
</tr>
<tr>
<td>2b</td>
<td>6.6</td>
<td>27.1</td>
<td>13.9</td>
<td>52.4</td>
</tr>
<tr>
<td>LL-37</td>
<td>98.3</td>
<td>1.1</td>
<td>0.4</td>
<td>0.2</td>
</tr>
</tbody>
</table>
identical to that of rrtCATH-1a. Moreover, three splicing isoforms of rrtCATH-1 were cloned in this study. When compared with rrtCATH-1a, rrtCATH-1am lacks the whole CLD, whereas rrtCATH-1b and rrtCATH-1c lack several of the repetitive motifs in the mature peptide. Coincidentally, a combination of these splicing sites can almost lead to the production of rrtCATH-1d. These results indicated that a splicing mechanism exists in trout resulting in the production of rrtCATH-1d from rrtCATH-1a, which might occur very recently, as the sequence variation did not happen in such a short evolutionary time. To our knowledge, this is the first report of a cathelicidin gene and a splicing isoform with total deletion of the whole characteristic CLD in the cathelicidin family. This indicated that trout have evolved energy-saving forms of cathelicidins. Thus, the discovery of more cathelicidins in primitive teleost fish may help to reveal the trajectory of the evolution of cathelicidins in vertebrates.

Cathelicidins are produced as inactive forms and processed extracellularly to release the active peptides in mammals (42, 43). Most mammalian CAMPs exhibit high cytotoxicity in vitro (44). Thus, the function of the CLD has long been considered to neutralize the potential intracellular cytotoxicity of CAMPs (45). However, limited studies are concerned with the function of the CLD, and the biological role of this domain is still a matter for debate (46, 47). As shown in this study, trout CAMPs possessed no obvious hemolytic and cytotoxic activities. Hence, discovery of the cathelicidin preproproteins with complete (in rainbow trout) or partial (in Arctic char and brook trout) (7) deletion of the CLD provides ideas for revealing the function of the domain. In future studies, the structure and function of the CLDs in Salmonidae fish species are worth examination, which will provide implications for understanding the possible roles of CLDs in vertebrates.

A homologous gene of rrtCATH-2a was cloned in this study. Interestingly, both genes almost have identical nucleotide sequences (99% identity). The present study revealed that they are indeed two different genes coexisting and coexpressing in trout. However, owing to the stop codons locating at different positions, the preproprotein of rrtCATH-2b lacks the C-terminal 16 residues of rrtCATH-2a. The discovery of rrtCATH-2a and rrtCATH-2b in trout strongly supported the viewpoint that cathelicidin family members expanded in vertebrates by gene duplication and divergence (6, 48). The high nucleotide sequence identity of rrtCATH-2a and rrtCATH-2b demonstrated that the duplication occurred very recently. Based on sequence alignments (data not shown), rrtCATH-2b is more similar to the CATH-2 in other Salmonidae fish species. Besides, the rrtCATH-2b gene shows the highest synteny with other cathelicidin genes in higher vertebrates. The results indicated that rrtCATH-2b is a more conserved gene from which rrtCATH-2a might be duplicated and diverged. In vivo studies conducted in mice have demonstrated that the duplication and divergence of cathelicidins are beneficial to host defense against bacterial infections (12, 13). Thus, it is certain that the duplication events that occurred recently in trout can promote host disease resistance.

In mammals, CAMPs are produced by epithelial cells and lymphocytes and are mainly expressed at bone marrow and epithelial surfaces of skin, gastrointestinal tract, and lung (2, 15–17). In this study, at the mRNA level, trout CAMPs were constitutively expressed in different tissues, especially in mucosal tissues, including skin and gill. In LPS-stimulated conditions, the expressions of trout CAMPs were increased dramatically. Furthermore, specific Abs against trout CAMPs were raised and the expression sites of these peptides were detected by immunofluorescence. In head kidney, the equivalent of bone marrow in higher vertebrates (49), the immunoreactive signals of trout CAMPs were observed in lymphoid cells around sinusoids, which would circulate to the entire body. In gut, trout CAMPs were largely expressed at the mucosal layer, especially in the columnar epithelial cells of the villus, and in some lymphoid cells in the lamina propria, which indicated that, similar to their mammalian orthologs, CAMPs also form the first line of host defense in fish.

In higher vertebrates, all discovered CAMPs belong to three structural classes: the α helical class consisting of 23–37 residues, the β-hairpin class consisting of 12–18 residues, and linear class enriched with repetitive Pro-Arg motifs and consisting of 39–94 residues (6, 50). Among these CAMPs, the α helical class is...
subsequently measured at 490 nm. Data shown are the means ± SD of duplicate reactions in a representative of three independent experiments.

Copy numbers, which suggested that it is a genetically unstable

The hemolytic and cytotoxic activities of trout CAMPs at a concentration of 60 μM. (A and B) Hemolytic activities of CAMPs to trout (A) and human (B) erythrocytes. The CAMPs were added to the erythrocytes and incubated at 20°C for 2 h and then OD₄₀₅ of the supernatant was measured. (C) Cytotoxic activities of CAMPs to trout RTG-2 cells. The CAMPs were added to RTG-2 cells and incubated at 20°C for 48 h; MTS solution was then added and incubated at 20°C for an additional 4 h. The absorbance was subsequently measured at 490 nm. Data shown are the means ± SD of duplicate reactions in a representative of three independent experiments.

present in every vertebrate thus far investigated, even in primates with a sole cathelicidin. Thus, the α helical class is considered to be the ancestral molecule from which this family differentially expanded (6). In recent years, the mysterious veil of fish CAMPs has been uncovered. Sequence analysis revealed that teleost CAMPs form a novel class of peptides enriched with Arg, Gly, and Ser (7). In this class, salmonid CAMPs are more special and have three distinctive hallmarks: the N terminus is intensified by positive charges, the central region consists of (R/P)(P/L)GGGS-based repetitive motifs, and the C terminus is lowly positive charged (Supplemental Table I). In this study, two isoforms of trout cathelicidin 1 peptides also contained cysteines and formed antiparallel β-sheets. The antibacterial activities of these peptides were revealed to be mediated by this region. Previous studies have demonstrated that the bacterial membrane is the key target for CAMPs (6, 19). Thus, the highly cationic N terminus can facilitate the binding of trout cathelicidin 1 peptides to the negatively charged bacterial membranes. The cell permeability assay revealed that this region can independently disrupt the bacterial membranes. The repetitive (R/P)(P/L)GGGS motifs in trout cathelicidin 1 peptides formed an antiparallel β-sheet and a flexible random coil region. From 1a to 1d, with gradual deletion of the repetitive motifs, the structural flexibilities of these peptides were gradually decreased, accompanied by the reduction of the antibacterial activities. These findings are particularly intriguing in view of the conformational effects of Pro and Gly residues on peptide secondary structures. Many studies have shown that the Pro residue could act as a flexible element in determining peptide structures, and it was a potent helix breaker (51–53). It has been reported that a Pro residue insertion in the central region of antimicrobial peptides could induce a hinge structure and reduce the hemolytic activities of peptides (51, 52). This is consistent with the high bacterial membrane selectivity of trout CAMPs, which possessed no hemolytic and cytotoxic activities. Additionally, Gly is frequently found together with Pro in the antiparallel β-sheets or flexible coiled structures of peptides (53, 54). These previous findings precisely accord with the simulated tertiary structures of trout cathelicidin 1 peptides. Furthermore, the Pro-Gly motif was found in the transmembrane domain of several ion channel-forming peptides (53). Consistent with this, the cell permeability assay revealed that the repetitive motifs in 1a could independently disrupt the bacterial membranes, although the activity was low. Unlike human LL-37, only the C termini of trout cathelicidin 1 peptides formed partial α helical conformations, which retained little effect on the antibacterial activities. In summary, trout cathelicidin 1 peptides possess all the structural elements of mammalian CAMPs, including α helix, β-sheet, and random coil, which are more likely to represent the ancestral molecules from which this family differentially expanded.

Unlike trout cathelicidin 1 peptides, the entire molecule of trout cathelicidin 2 peptides mainly consists of random coil, except a short piece of antiparallel β-sheet. In mammals, a linear class of CAMPs consisting of random coil also has been discovered in cattle, sheep, goats, and pigs, and these peptides are enriched with residues Pro and Arg (19, 50). In trout CAMPs 2a and 2b, Pro, Arg,
and Gly residues are distributed throughout the entire molecular, accounting for 40.9 and 46%, respectively. This precisely accords with their simulated tertiary structures. Similar to cathelicidin 1 peptides, cathelicidin 2 peptides also exhibited no cytotoxic and hemolytic activities, and the antibacterial activities were also mediated by the highly cationic N terminus. In contrast, both of the central region (consisting of only two copies of the repetitive motif) and the C terminus (with low charges) had minimal impact on the antibacterial activities of these peptides. Taken together, our results suggest that positive charges and structural flexibilities are the key factors determining the antibacterial activities of trout CAMPs. Substitution studies have proved the critical role of cationic Arg residues for the activity of Bac5 (55). In future studies, the importance of different residues in trout CAMPs needs to be clarified by site-specific amino acid substitutions.

In addition to the direct antimicrobial effects, mammalian CAMPs possess other modulatory activities, such as LPS-neutrophilizing (20, 21) and IL-8-stimulating activities (22, 23, 28, 38, 39). Furthermore, the LPS-binding activities are often positively correlated with their antibacterial activities (21). Similar to their mammalian orthologs, trout CAMPs also possessed these activities. The LPS binding was mainly mediated by the N terminus, whereas the repetitive motifs showed lower affinity, and the C terminus did not show any. Thus, the LPS-binding activities of fish CAMPs are also positively correlated with their antibacterial activities, which demonstrates that the activity is a primordial function that has been conserved throughout 350 million years of evolution. Moreover, trout CAMPs exhibited different abilities to stimulate the expression of IL-8 in PBLs. However, the mechanism of IL-8 stimulation by CAMPs is unclear. A study conducted by Braff et al. (39) suggested that LL-37 may directly interact with cell membrane to cause conformational changes and further activate the surface receptors linked to intracellular signaling pathways indirectly. In trout CAMPs, from 1a to 1d, with deletion of the repetitive motifs, the structural flexibilities were gradually reduced. Thus, the cell membrane conformational changes caused by these peptides should have been gradually diminished, resulting in the decreased IL-8–stimulating capacities. These results indicated that, besides antibacterial activities, the emergence of the repetitive motifs in salmonid CAMPs has also increased the IL-8–stimulating activities. Compared to 2a, the IL-8–stimulating ability of 2b was weaker and the time point with greatest stimulation effect was significantly latter. As discussed above, 2a may be duplicated and diverged from 2b, and this evolution has led to the acquisition of more potent IL-8–stimulating activities. Thus, in fish, CAMPs may recruit immune cells to the site of infection via stimulating chemotactic IL-8.

Taken together, similar to their mammalian orthologs, trout CAMPs also formed the first line of host defense. Interestingly, trout have evolved energy-saving forms of cathelicidins. The activities of trout CAMPs were mainly mediated by the highly cationic N terminus, whereas the emergence of (R/P)(P/L)G(G/S/F)GS motifs increased the antibacterial and IL-8–stimulating activities. The distinctive structural hallmarks and biological activities of trout CAMPs offered new insights into the understanding of the evolution of this family in vertebrates. Moreover, the high bacterial membrane selectivity of trout CAMPs provided new ideas for the design of excellent peptide antibiotics based on CAMPs in vertebrates.

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