

Identification of a Novel Cathelicidin Gene in the Rainbow Trout, *Oncorhynchus mykiss*

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Received 24 September 2004/Returned for modification 15 December 2004/Accepted 10 March 2005

We report the cloning of a novel antimicrobial peptide gene, termed *rtCATH_1*, found in the rainbow trout, *Oncorhynchus mykiss*. The predicted 216-residue *rtCATH_1* prepropeptide consists of three domains: a 22-residue signal peptide, a 128-residue cathelin-like region containing two identifiable cathelicidin family signatures, and a predicted 66-residue C-terminal cationic antimicrobial peptide. This predicted mature peptide was unique in possessing features of different known (mammalian) cathelicidin subgroups, such as the cysteine-bridged family and the specific amino-acid-rich family. The *rtCATH_1* gene comprises four exons, as seen in all known mammalian cathelicidin genes, and several transcription factor binding sites known to be of relevance to host defenses were identified in the 5' flanking region. By Northern blot analysis, the expression of *rtCATH_1* was detected in gill, head kidney, and spleen of bacterially challenged fish. Primary cultures of head kidney leukocytes from rainbow trout stimulated with lipopolysaccharide or poly(I · C) also expressed *rtCATH_1*. A 36-residue peptide corresponding to the core part of the fish cathelicidin was chemically synthesized and shown to exhibit potent antimicrobial activity and a low hemolytic effect. Thus, *rtCATH_1* represents a novel antimicrobial peptide gene belonging to the cathelicidin family and may play an important role in the innate immunity of rainbow trout.

Rapid and effective defense against pathogens is essential for the survival of all living organisms that coexist with microbes. Increasing evidence suggests that endogenous peptides with nonspecific antimicrobial properties play a critical role in the host first line of defense (1, 15, 19). More than 700 antimicrobial peptides have been isolated from diverse species. Most of them share characteristics, such as small size, cationic charge, and an amphipathic conformation. They tend to display broad-spectrum antimicrobial activity and have many potential roles in innate immunity (15). These peptides are found on epithelial surfaces (7, 16), where they act as a local defense mechanism, and in circulating myeloid-derived cells (25, 38), which gather at the site of microbial invasion. Based on their common features, these peptides can be divided into several families (5) that include well-known molecules, such as the cecropins from (primarily) insects, magainins from amphibians, and in mammals, defensins and cathelicidins.

The cathelicidin family was first recognized in the early 1990s (46). They are a numerous group of proteins that share a highly conserved preproregion that contains the cathelin-like domain, a 12-kDa protein from porcine leukocytes (36), at the N terminus and carry diverse antimicrobial peptides at the C terminus. In most species, cathelicidins are expressed in myeloid precursor cells (34) and stored in neutrophil granules as

propeptides without antimicrobial activities. After neutrophil activation leading to elastase-mediated endoproteolytic cleavage, the highly variable C-terminal antimicrobial peptide is released (19). The mature cathelicidin peptides are relatively small, most <100 amino acids, with an amphipathic molecular structure and a net positive charge. These two characteristics are related to the interaction of such peptides with bacterial membranes as part of their bactericidal mechanism. Cathelicidins are typically found in neutrophils and at mucosal surfaces, suggesting they contribute to systemic and local defense (13). Thus far, the cathelicidins have been described only in mammals, including human, monkey, horse, cattle, sheep, goat, pig, rabbit, mouse, and guinea pig (34), except for two sequences from hagfish published recently and cited as representing an "ancient ancestor of the cathelicidin gene family" (43).

Relatively few studies of antimicrobial peptides from fish have been performed, despite the problem of infectious diseases and the limitation of using antibiotics in aquaculture worldwide. Attempts to find antimicrobial peptides have had a number of successes in fish but have so far focused mainly on the epithelial surfaces. For example, paradaxin, a 33-amino-acid toxic polypeptide with bactericidal activity, was discovered in the mucous glands of Moses sole fish (30); pleurocidin, a 25-residue linear peptide active against a broad spectrum of microorganisms, was found in skin mucous secretions of winter flounder (7) and immunolocalized to mucin granules of goblet cells in the intestine; misgurin, a 21-amino-acid pore-forming antimicrobial peptide without significant hemolytic activity, was isolated from the loach (31); piscidin, a 22-amino-acid peptide rich in histidine and phenylalanine, was first isolated from skin of hybrid striped bass and immunolocalized to eo-

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TABLE 1. Primers used for cloning and sequencing of rainbow trout *rtCATH_1*

Name	Sequence (5' → 3')	Position
Trout cathelicidin primers		
<i>rtCATH_1</i> -F1	CATCCTGCTCGCTGTGGCTGTCCCT	+34 to +57
<i>rtCATH_1</i> -F2	GATTCGGACAAGAAGAAGCAAAG	+1056 to +1078
<i>rtCATH_1</i> -F3	GAAGATGAAGGTCCAGGTGAGA	+7 to +28
<i>rtCATH_1</i> -F4	CTGACCTGTCTACATTTGTTG	+728 to +749
<i>rtCATH_1</i> -R1	GCGATTTCCATCACTGTGATCTCT	+1249 to +1272
<i>rtCATH_1</i> -R2	CACAATTTTGCCTCTGGAGCATATTCT	+1081 to +1108
<i>rtCATH_1</i> -R3	CACAAACAAATGTAGACAGGTCAGTGTT	+724 to +751
Other primers		
RT-BT-F	ATGGAAGATGAAATCGCC	
RT-BT-R	TGCCAGATCTTCTCCATG	
M13F	CGTTGTA AAAACGACGGCCAG	
T7	TGTAATACGACTCACTATAGGG	
GR5P	CGACUGGAGCACGAGGACACUGA	
GR5NP	GGACACUGACAUGGACUGAAGGAGUAGAAA	
AP1	GTAATACGACTCACTATAGGGC	
AP2	ACTATAGGGCACGCGTGTT	

sinophilic granular cells in gill, skin, and gut and those lining blood vessels in the viscera (40); and moronecidin, an α -helical antimicrobial peptide with 22 residues, was found in the skin and gills of hybrid striped bass (23). Neither systemic antimicrobial peptides produced by myeloid cells nor members belonging to the cathelicidin family have yet been found in jawed fish.

Through routine screening of rainbow trout *Oncorhynchus mykiss* clones for immunologically important genes, a partial sequence was obtained with apparent homology to the mammalian cathelicidin gene family. This allowed the design of gene-specific primers to confirm and extend the sequence. In this paper, we report the cloning of this novel antimicrobial peptide gene, termed the *rtCATH_1* gene (from rainbow trout cathelicidin_1). The gene organization and expression were studied, and a peptide based on the mature peptide sequence of the *rtCATH_1* gene was synthesized chemically and found to have potent antimicrobial activities against fish-pathogenic bacteria.

MATERIALS AND METHODS

Fish, cell culture, and microorganisms. Rainbow trout *O. mykiss* weighing 300 to 350 g, obtained from a local fish farm (Almond Bank, Perthshire, United Kingdom), were maintained and acclimatized in a recirculating freshwater system at 14°C with regular feeding.

The reference strain *Escherichia coli* NCIMB12210 (ATCC 25922) was obtained from NCIMB Ltd., Aberdeen, United Kingdom. Nine field isolates of fish pathogens were provided by FRS Marine Laboratory, Aberdeen, United Kingdom. *Aeromonas salmonicida* A-layer-negative avirulent strain MT004 and the A-layer-positive virulent strain MT423 (17) were isolated from rainbow trout. *Photobacterium damsela* subsp. *piscicida* MT1415 was a virulent capsulated strain isolated from sea bass *Dicentrarchus labrax*, and EPOY8803II was an avirulent noncapsulated strain isolated from red grouper *Epinephelus akaara* (26). *Vibrio anguillarum* serogroup O1 MT1741 (3) and serogroup O2 MT1742 (4) and *Yersinia ruckeri* MT252 were isolated from rainbow trout. *Lactococcus garvieae* noncapsulated strain MT2055 and capsulated strain MT2291 were originally isolated from yellowtail and rainbow trout, respectively (2). *E. coli* was maintained in Luria-Bertani (LB) medium at 37°C. The aquatic strains were maintained in tryptic soy broth medium at 22°C. All the strains were transferred to Mueller-Hinton broth (Sigma) and cultured to optimal concentrations before the antimicrobial assays.

Isolation of the full-length cDNA. Wax-mediated hot-start PCR was performed using 0.5 μ l of an enzyme mixture of *Taq* (5 U/ μ l; Bioline, United

Kingdom) DNA polymerase and *Pfu* (3 U/ μ l; Promega, United Kingdom) DNA polymerase (50:1) in a 50- μ l reaction volume containing the following components: 5 μ l of 10 \times NH₄ buffer, 2 μ l of 10 mM deoxynucleoside triphosphate mixture, 10 μ l of 10 mM MgCl₂, 2 μ l of 10 μ M forward primer, 2 μ l of 10 μ M reverse primer, 2 μ l of DNA template, and 26.5 μ l of sterile H₂O. The cycling protocol was 1 cycle of 94°C for 5 min and 28 to 32 cycles of 94°C for 45 s, 58 to 62°C for 45 s, and 72°C for 1 min, followed by 1 cycle of 72°C for 10 min. Initial PCR using *rtCATH_1*-F1 forward (Table 1) and *rtCATH_1*-R1 reverse primers was performed on a rainbow trout phytohemagglutinin-stimulated leukocyte cDNA library (inserted into the λ ZAP express vector using the ZAP Express cDNA synthesis kit [Stratagene]) (9) and gave a product of 613 bp, confirming the presence of the gene (Fig. 1). Anchored PCR using the gene-specific primer *rtCATH_1*-F1 with the vector-specific primer M13F, followed by a nested PCR using *rtCATH_1*-F2 with the vector-specific primer T7, was used to obtain the 3' end of the gene and gave a product of 541 bp. However, attempts to obtain the 5' end of the gene by a similar procedure failed. Therefore, the cloning strategy to obtain the 5' end was switched to random amplification of cDNA ends PCR.

The transcription initiation start was determined using a GeneRACER kit (Invitrogen, United Kingdom) that allowed amplification of full-length capped mRNA. Total mRNA was extracted from stimulated rainbow trout head kidney using an Oligotex mRNA Mini Kit (QIAGEN, United Kingdom). The full-length, ligase-mediated rapid amplification of 5' end RNA was conducted according to the manufacturer's instructions. The treated RNA was reverse transcribed by treatment with Moloney murine leukemia virus reverse transcriptase (RNase H low; Bioline, United Kingdom) at 60°C for 20 min, followed by 65°C for 50 min. The full-length 5' end of the transcript was then obtained by PCR with primers GR5P (supplied with the kit) and *rtCATH_1*-R2, followed by a nested PCR using primers GR5NP (supplied with the kit) and *rtCATH_1*-R3, and gave a product of 425 bp.

Isolation of genomic DNA. Genomic DNA was isolated from rainbow trout liver using a genomic DNA isolation kit (QIAGEN, United Kingdom). The resultant DNA was dissolved in distilled H₂O to a final concentration of 0.25 mg/ml. The genomic sequence was obtained by PCR using the primers *rtCATH_1*-F3 and *rtCATH_1*-R1 and gave a product of 1,266 bp. The 5' flanking region was obtained using a gene-walking approach. Gene-walking libraries were constructed with a Universal GenomeWalker kit (Clontech) according to the manufacturer's instructions. Amplification of the 5' end of the gene was performed using primers AP1 (supplied with the kit) and *rtCATH_1*-R2, which gave a product of 1,118 bp. Nested PCRs were carried out on the DraI-restricted DNA using primers AP2 (supplied with the kit) and *rtCATH_1*-R3. The cycling protocol for the PCR was 1 cycle of 94°C for 5 min, 5 cycles of 94°C for 45 s and 72°C for 2 min, 5 cycles of 94°C for 45 s and 70°C for 2 min, and 25 cycles of 94°C for 45 s, 64°C for 45 s, and 70°C for 2 min, followed by 1 cycle of 72°C for 10 min.

Cloning and sequencing. All products obtained by PCR were directly cloned into the pGEM-T Easy vector (Promega, United Kingdom). Plasmid DNA was isolated from bacterial colonies that had an appropriately sized insert using a Qiaprep spin miniprep kit (QIAGEN, United Kingdom). Three randomly selected clones representing each product were sequenced on an ABI 377 Auto-

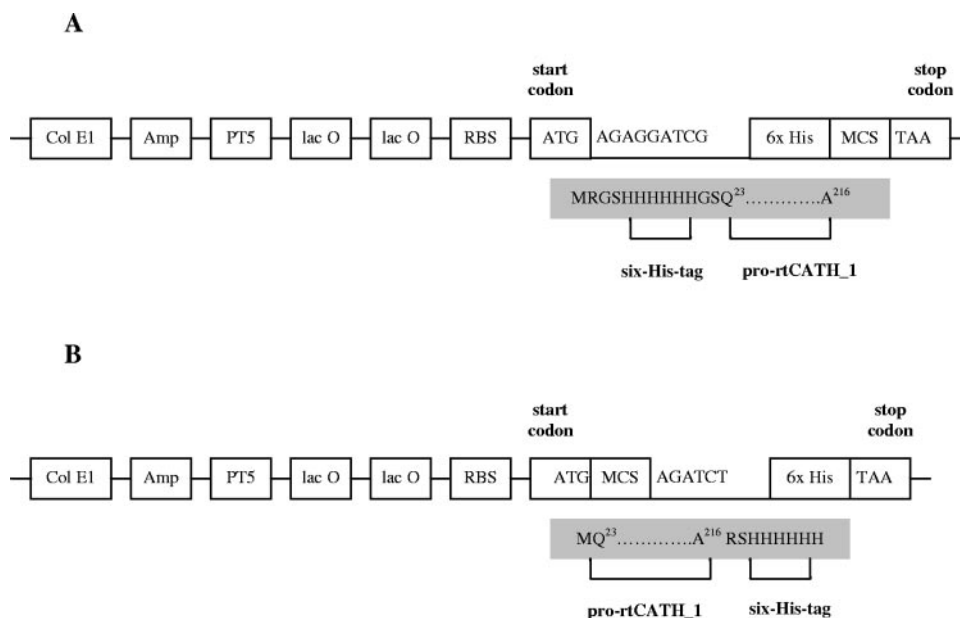


FIG. 2. Diagram showing the *prrCATH_1* expression vectors: pQE30-*prrCATH_1* (A) and pQE70-*prrCATH_1* (B). Col E1, Col E1 origin of replication; Amp, ampicillin resistance gene; PT5, T5 promoter; lac O, lac operator; RBS, ribosome-binding site; six-His, six-His tag sequence; MCS, multiple cloning site. The shaded areas represent the amino acid sequences translated from the vector.

RNA at 42°C for 50 min with the oligo(dT) primer (Invitrogen, United Kingdom). PCR was performed on the resulting cDNAs using the specific primers *prrCATH_1*-F4 and R1, which span intron 3 of the trout genomic sequence. β -Actin was amplified as a positive control for RT-PCR, using the RT-BT-F and RT-BT-R primers listed in Table 1. PCR reagents were as described above, and the cycling protocol was 1 cycle of 94°C for 4 min and 33 cycles of 94°C for 45 s, 62°C for 45 s, and 70°C for 1 min, with a final extension step of 10 min at 72°C. Selections of PCR products were sequenced to confirm correct amplification of the *prrCATH_1* gene in each tissue/cell type with primers F4 and R1.

Construction of *prrCATH_1* expression vector. The cDNA of *prrCATH_1* was amplified by PCR, sequenced, and cloned into pGEM-T easy vector (Promega). Using the sequenced plasmid DNA as a template, the cDNA fragment encoding the proregion of *prrCATH_1* (*prrCATH_1*), from Q²³ to the C terminus, was amplified again by PCR, and a translation start codon ATG was added. Two expression vectors named pQE30 and pQE70 (QIAGEN) containing a six-His tag at the N terminus and C terminus, respectively, were used to produce the recombinant proteins. To ligate into the pQE30 vector, restriction enzyme sites for BamHI and for HindIII were added by PCR at the 5' and 3' ends, respectively. To ligate into the pQE70 vector, restriction enzyme sites for SphI and BglII were added by PCR at the 5' and 3' ends, respectively. The resultant expression plasmids were named pQE30-*prrCATH_1* and pQE70-*prrCATH_1* (Fig. 2).

Production and purification of *prrCATH_1*. To express the recombinant *prrCATH_1* protein, the plasmids pQE30-*prrCATH_1* and pQE70-*prrCATH_1* were transformed into *E. coli* JM109 cells (Promega). A single colony containing target plasmid was cultured in 5 ml LB medium (Sigma) containing ampicillin (100 μ g/ml) and cultured at 37°C with shaking (300 rpm) overnight prior to culture in 250 ml LB medium containing ampicillin for 4 h and then incubated for 2 h with the addition of isopropyl β -D-1-thiogalactopyranoside (IPTG; 0.5 mM final concentration). The *E. coli* cells were then harvested by centrifugation at 4°C, lysed in phosphate buffer (100 mM NaH₂PO₄, 10 mM Tris-Cl, pH 8.0) containing 8 M urea, and loaded onto a Ni-nitrilotriacetic acid column (QIAGEN). Purification was conducted according to the QIAGEN protocols. Briefly, the column containing bacterial lysate was washed several times with the above-mentioned buffer at pH 6.3 to remove proteins without a six-His tag. The recombinant protein was then eluted using the phosphate buffer at pH 5.9 and 4.5, respectively. The protein size, purification, and concentrations of flowthrough fractions were determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and UVP image analysis. The resultant proteins generated from plasmids pQE30-*prrCATH_1* and pQE70-*prrCATH_1* were named 6His-*prrCATH_1* and *prrCATH_1*-6His, respectively.

Enzymatic cleavage of *prrCATH_1* and Western blot analysis. Human neutrophil elastase (ELAStin Products Company, Inc., Owensville, Mo.) was applied to analyze the cleavage site of the *prrCATH_1* protein. Fifty nanograms of elastase in Tris buffer (2 M Tris, 0.1 M NaOAc, 0.01% bovine serum albumin, pH 7.4) was incubated with 6His-*prrCATH_1* or *prrCATH_1*-6His protein (5 μ g) at 20°C for different times (see the legend to Fig. 4). The reaction was stopped by adding an equal volume of 5% acetic acid.

Western blotting was performed using the Westernbreeze Chemiluminescent Immunodetection System (Invitrogen) according to the manufacturer's instructions. Briefly, samples were loaded onto a 4 to 12% polyacrylamide gel and electroblotted to a nitrocellulose membrane. The membrane was blocked with the Westernbreeze blocking solution for 30 min and incubated with a monoclonal anti-six-His antibody (Invitrogen) to detect the six-His tag at the N terminus and C terminus of 6His-*prrCATH_1* and *prrCATH_1*-6His, respectively, for 1 h. The membranes were rinsed with antibody washing solution and water, covered with detection buffer, and exposed to X-ray film.

Peptide synthesis. Since the yield of recombinant protein cleaved by elastase was low and could be detected only by Western blotting, it was decided to test the antimicrobial activity using synthesized peptides. However, the full length of the predicted mature peptide (66 amino acids) was difficult to make chemically, and thus, a 36-amino-acid peptide, *prrCATH_1*(R151-V186), with a net charge of +6 and containing key features of the known cathelicidins, was synthesized commercially by the Proteome Facility at the University of Aberdeen (United Kingdom) using solid-phase 9-fluorenylmethoxy carbonyl chemistry and purified by reverse-phase high-performance liquid chromatography. The peptide mass of 3,464.49 Da was verified by mass spectroscopy using an ABI Voyager DE-STR matrix-assisted laser desorption ionization-COF (AME Bioscience, United Kingdom) and was not C-terminally amidated. To compare the *prrCATH_1* peptide activity directly against that of a mammalian cathelicidin, the bovine cyclic dodecapeptide (35) was also synthesized, since it is similar to the N-terminal region of the *prrCATH_1* mature peptide in molecular structure and charge.

Antimicrobial assays. The antimicrobial activities were determined as MICs using the modified twofold microtiter broth dilution method (45). Briefly, serial twofold dilutions of peptides were prepared in 0.2% bovine serum albumin-0.01% acetic acid buffer in tubes. Eighty microliters of each concentration was added to each corresponding well of a 96-well microtiter plate. Bacteria were incubated to mid-logarithmic phase and diluted in Mueller-Hinton broth to give a final inoculum concentration of 10⁵ CFU/ml. A suspension of 20 μ l of bacteria was added to each well of a 96-well plate and incubated for 24 to 48 h at 22°C. The growth was determined by the optical density at 600 nm. Inhibition was

defined as growth less than or equal to one-half of the growth observed in control wells, to which no peptide was added.

Kinetic analysis. A total of 10^3 *A. salmonicida* CFU were incubated at 22°C for increasing times (1 min to 8 h) with one of three concentrations (0.5×, 1×, and 2× MIC) of *rtCATH_1*(R151-V186). The reactions were terminated by plating on Mueller-Hinton agar (Sigma), and plates were incubated at 22°C for 48 h. Viable colonies were counted and shown as percentage of control *rtCATH_1*(R151-V186), plated at time zero.

Hemolytic assay. The hemolytic activity was assayed as described by Skerlavaj et al. (41) with a slight modification described by Park et al. (31). Freshly bled rainbow trout erythrocytes (3 ml), from blood collected in lithium-heparin-coated vacutainers, were washed with PBS, pH 7.4, until the supernatant was colorless. The washed erythrocytes were resuspended in 30 ml PBS supplemented with glucose (0.2% [wt/vol]). To 190 μ l of the cell suspension in microcentrifuge tubes, peptide samples (10 μ l, serially diluted in PBS) or mellitin (a hemolytic peptide) were added. Following gentle mixing, the samples were incubated for 1 h at 22°C and centrifuged for 10 min at $1,100 \times g$ at room temperature. One hundred microliters of supernatants was transferred to a microtiter plate, and the optical density was determined at 405 nm. The percentage of hemolysis was defined relative to the hemolysis obtained with the erythrocyte suspension treated with 0.2% Triton X-100 (100% hemolysis).

Nucleotide sequence accession number. The genomic and cDNA sequences reported in this paper have been submitted to the GenBank/EMBL Data Bank with accession numbers AY594646 and AY382478, respectively.

RESULTS

Cloning and characterization of rainbow trout antimicrobial peptide gene. PCR with primers F1 and R1, using a rainbow trout phytohemagglutinin-stimulated leukocyte cDNA library as a template, generated a 613-bp product that corresponded to the core sequence of *rtCATH_1*. Anchored PCR was used to isolate the 3' end of this transcript. Using a nested approach, PCR with primers F1 and M13F was followed by reamplification with primers F2 and T7 and resulted in a 541-bp product that contained the 3' end of the gene through to the poly(A) tail. The 3' untranslated region of the transcript contained 183 nucleotides, with a polyadenylation signal (AATAAA) 17 bp upstream from the poly(A) tail. The transcription initiation start and the 5' end of the mature peptide were determined by a GeneRacer kit using RNA from *A. salmonicida*-stimulated rainbow trout head kidney. A 425-bp product was obtained by nested PCR with primers GR5P and R2, followed by reamplification with primers GR5NP and R3, and contained 4 nucleotides of 5' untranslated region and the 5' end of the mature peptide. Analysis of the three sequenced overlapping products enabled the contiguous open reading frame of 651 nucleotides to be compiled (Fig. 1), which translated into a 216-residue peptide representing the first bony-fish homolog of the cathelicidin gene family.

Primers designed from the *rtCATH_1* cDNA sequence were used to isolate the full genomic sequence from rainbow trout. PCR with primers F3 and R1 gave a 1,266-bp product that was composed of four exons—the same as the gene structures of all mammalian cathelicidins—that were 180, 123, 117, and 231 bp, respectively. The three introns, 239, 117, and 270 bp, respectively, all possessed a classical “GT-AG” intron splicing motif (Fig. 1). By gene walking with primers AP1 and R2, followed by primers AP2 and R3, a 1,118-bp PCR product was obtained. An overlapping region of 700 bp allowed the assembly of a contiguous sequence. The genomic sequence contained 1,464 nucleotides from the start to the end of the transcript described above.

Scanning of the 5' flanking region of the *rtCATH_1* se-

quence for eukaryotic promoter elements indicated the presence of a typical TATA box (TATAAA) at -31 to -26 (Fig. 1). Two putative CCAAT boxes were found in a sense orientation at -209 and -180 . Clusters of potential recognition sites for nuclear factors involved in the transcription of immune-related molecules were found in the region. Within 331 bp upstream of the transcriptional start site there was one nuclear factor kappa B (NF- κ B) recognition site, one alpha interferon recognition site, one binding site for CCAAT/enhancer-binding protein β (C/EBP β), one nuclear factor 1, and two nuclear factor interleukin 6 motifs, two gamma interferon response elements, and five GAAA motifs.

The coding region of trout *rtCATH_1* was compared with the sequences of four different mammalian cathelicidins (LL-37 of human, BCT1 of cow, SC52 of sheep, and PR39 of pig), using the GAP program in the Genetics Computer Group package. Comparing the full cDNA sequence, *rtCATH_1* was found to share 41 to 44% nucleotide identity with mammalian cathelicidins. The highest homology (44 to 48%) was seen in exon 2, which encoded the cathelin-like domain. The lowest homology (31 to 36%) was seen in exon 4, which encoded the last few residues of the proregion and all of the predicted mature antimicrobial peptide.

The full-length cDNA sequence of the novel cathelicidin had an open reading frame of 216 amino acid residues, corresponding to a 23.6-kDa polypeptide with a calculated pI of 9.4. The peptide showed the characteristic features of cathelicidins, including a conserved N-terminal preproregion and a unique C-terminal sequence. Multiple alignment using the CLUSTAL program indicated conservation of both the mammalian cathelicidin family signature I, Y-X-[ED]-X-V-X-[RQ]-A-[LIVMA]-[DQG]-X-[LIVMFY]-N-[EQ], and signature II, F-X-[LIVM]-K-E-T-X-C-X₁₀-C-X-F-[KR]-[KE], in the rainbow trout sequence (12), with a few exceptions, e.g., V to I and N to L in signature I and K to Q and F to L in signature II (Fig. 3). Analysis of the N-terminal region predicted a cleavage site for signal peptidase at residues S²²-Q²³. The putative signal peptide is followed by a proregion that conserves the four cysteine residues present in all the congeners characterized thus far. This proregion is predicted to end at residues V¹⁵⁰-R¹⁵¹, which is a common elastase cleavage site for many mammalian cathelicidins (13). Thus, the predicted mature peptide would be 66 residues in size, of which 14 are positively charged. The peptide as a whole is basic (calculated pI, 11.71), with four residues being acidic.

Elastase cleavage of *prtCATH_1*. Enzymatic cleavage and Western blot analysis were performed to confirm that the *prtCATH_1* molecule is indeed processed by elastase and to determine the size of the mature *rtCATH_1* after processing. Elastase (50 ng) cleaved *prtCATH_1* (5 μ g) in a time-dependent manner into two fragments (Fig. 4). The molecular masses of the generated fragments detected after cleavage of the 6His-*prtCATH_1* and *prtCATH_1*-6His proteins were 15 kDa and 8 kDa, respectively, and matched the sizes of the predicted precursor fragment (Fig. 4A) and mature (Fig. 4B) *rtCATH_1* peptide. In both cases, the second fragment was not visualized, as it did not contain a six-His tag. The uncleaved precursor decreased as digestion time increased.

Antimicrobial and hemolytic assays. The effects of synthesized peptides against a panel of bacteria were evaluated as the

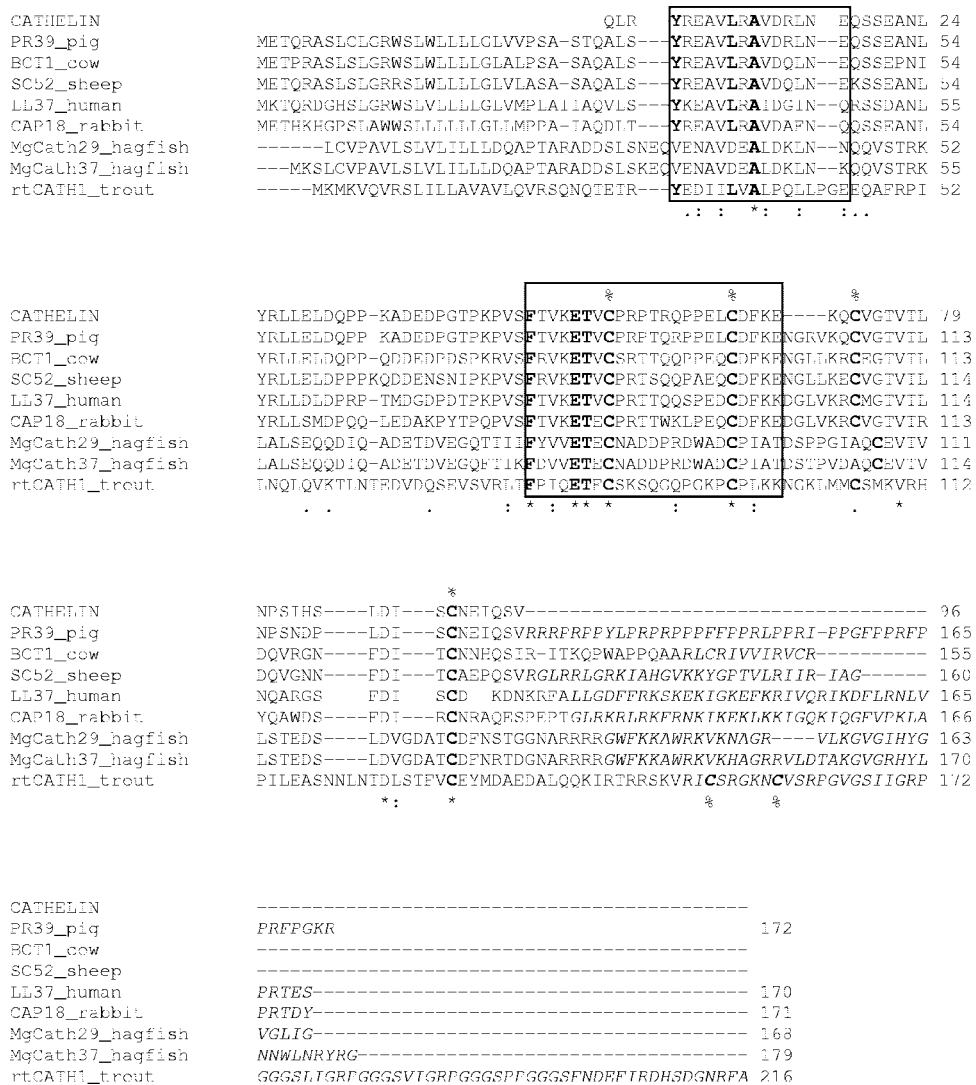


FIG. 3. Multiple alignment of the predicted translation of *rtCATH_1* propeptide with cathelin and other known cathelicidins from human, bovine, sheep, pig, rabbit, and hagfish. Identical (*) and similar (· or :) residues identified by the CLUSTAL program are indicated. The cathelicidin family signature is boxed, and the critical residues conforming to the signature are in boldface. Cysteines involved in disulfide bond formation are in boldface text and indicated with a %. The mature peptides are in italics.

minimal concentration capable of inhibiting visible microbial growth. The *rtCATH_1*(R151-V186) peptide displayed various antibacterial activities against all 10 different microorganisms studied, including gram-negative and gram-positive bacteria, with MICs of 2 to 40 μM (Table 2). The mammalian cathelicidin, bovine cyclic dodecapeptide, also demonstrated antimicrobial activities against a broad spectrum of the fish bacterial pathogens tested but was noticeably less effective than the *rtCATH_1*(R151-V186) peptide against *A. salmonicida* MT423, *P. damsela* MT1415, and *V. anguillarum* MT1741.

To determine the rate of bactericidal activity of *rtCATH_1*, a kinetic study was performed using *A. salmonicida* MT004 (Fig. 5). A time-dependent mechanism of action was demonstrated, with increased bacterium-*rtCATH_1*(R151-V186) incubation time leading to higher bactericidal activity (i.e., lower numbers of CFU). At 2× MIC (16.0 μM), antibacterial action increased dramatically after 30 min, and at 120 min, less than

20% of the CFU remained. At 1× MIC (8.0 μM), antibacterial action was noted after 60 min, with few CFU at 240 min. At 0.5× MIC (4.0 μM), antibacterial action started after 240 min, and more than 30% of the bacteria were still viable at 480 min. The overall mean activity, as represented by the average percentage of control CFU, increased with decreasing *rtCATH_1* (R151-V186) concentration.

Sodium, calcium, and magnesium were added to determine their effects on the MICs of *rtCATH_1*(R151-V186). An increased calcium or magnesium concentration resulted in decreased antimicrobial activity against *A. salmonicida* MT004 (Table 3). NaCl, added at concentrations 10-fold greater than those of either CaCl₂ or MgCl₂, did not affect the antimicrobial activity of *rtCATH_1*(R151-V186).

Very modest hemolytic activity (<12% hemolysis) was observed with the *rtCATH_1*(R151-V186) peptide and bovine cyclic dodecapeptide (up to 2.56 mM, 40 to 1,000 times the

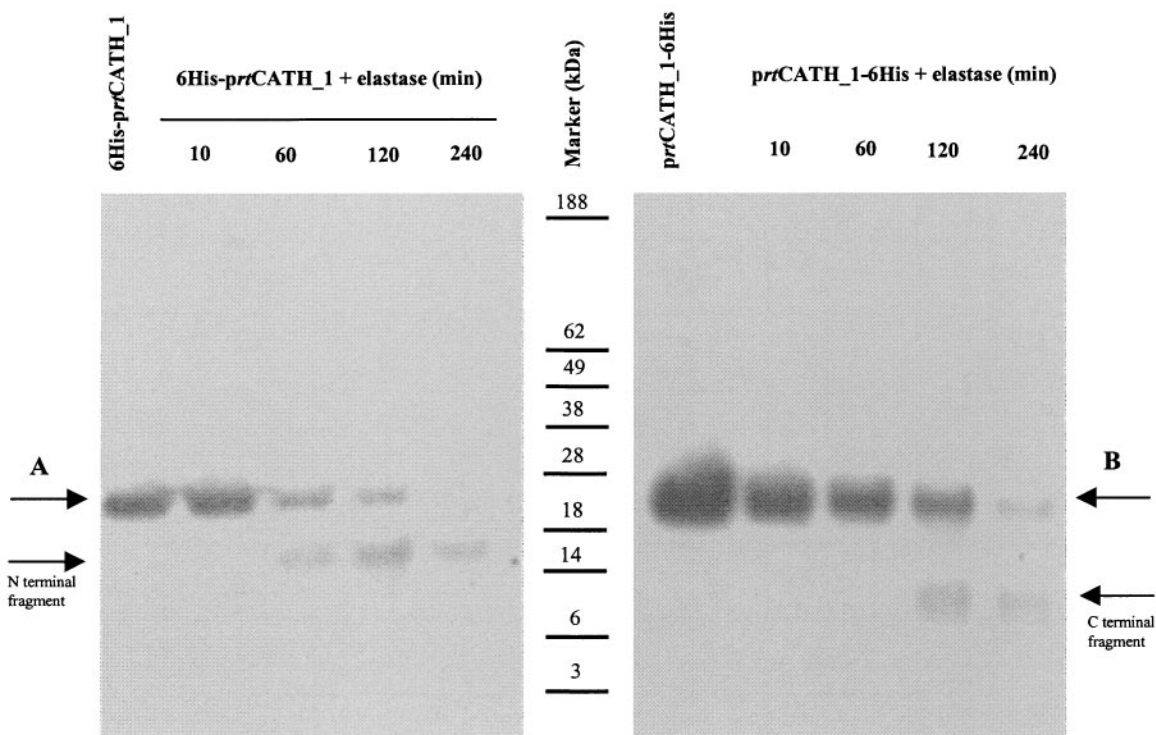


FIG. 4. Time-dependent cleavage of *prrCATH_1* by elastase. Shown is a Western blot of a 4 to 12% polyacrylamide gel after reaction with an anti-six-His tag antibody. Five micrograms of 6His-*prrCATH_1* (A) and *prrCATH_1*-6His (B) was incubated with elastase (50 ng) separately for the times indicated, and the reactions were stopped by acidification with equal volumes of 5% acetic acid. Note the appearance of the N-terminal and C-terminal fragments of 15 kDa and 8 kDa, respectively, with time.

MIC levels) when tested against rainbow trout erythrocytes (Fig. 6). In contrast, melittin, a hemolytic peptide, induced complete lysis of trout erythrocytes, with the exception of the lowest concentration used, where >80% lysis still occurred.

Expression studies. Northern blot analysis showed that no significant transcript level of *rtCATH_1* was apparent in samples from normal fish. However, after stimulation with bacteria, the spleen samples showed a significant increase in expression level, with samples from gill and head kidney also showing detectable levels of the *rtCATH_1* transcript (Fig. 7). By RT-PCR, clear dose-dependent effects of both LPS and poly(I · C)

stimulation were apparent using the head kidney leukocytes. Stimulation of the head kidney cells with increasing concentrations of LPS or poly(I · C) resulted in an increased *rtCATH_1* transcript level (Fig. 8).

DISCUSSION

Thus far, all cathelicidin family genes show similar structural organizations, with the coding sequence distributed over four exons. Exon 1 encodes the 29- to 30-residue signal peptide and some initial residues of the proregion, exons 2 and 3 encode 98 to 114 residues of the cathelicidin-like proregion, and exon 4 encodes several final residues of the proregion and the mature peptide, which varies in length from 12 to 100 residues (13). Rainbow trout cathelicidin has some interesting differences from known mammalian molecules. It has a shorter (22 residues) signal peptide and a longer (128 residues) cathelin-like domain. Homology analysis indicated that the percentage nucleotide identity in trout and mammalian cathelicidin genes drops in exon 4, which encodes the mature peptide and is the most diverse part of all the cathelicidin molecules. The next lowest homology was seen in exon 1, suggesting that the signal peptide of the fish cathelicidin was markedly different from those in mammals.

Phylogenetic analysis (Fig. 9) shows rainbow trout cathelicidin has branched away from the mammalian cathelicidins, especially the molecules belonging to species of the grandorder Ungulata. The hagfish cathelicidin family (MgCATH29 and

TABLE 2. Antimicrobial activities of *rtCATH_1*(R151-V186) and bovine cyclic dodecapeptide (Bovdode)

Organism and strain	Relevant phenotype	MIC (µM)	
		Bovdode	<i>rtCATH_1</i> (R151-V186)
Gram negative			
<i>Escherichia coli</i> NCIMB 12210	ATCC25922	4	5
<i>Aeromonas salmonicida</i> MT004	A-layer negative	16	8
<i>A. salmonicida</i> MT423	A-layer positive	>64	20
<i>Photobacterium damsela</i> MT1415	Capsulated	40	10
<i>P. damsela</i> EPOY 8803 II	Noncapsulated	2	16
<i>Vibrio anguillarum</i> MT1741	Serogroup O1	10	2
<i>V. anguillarum</i> MT1742	Serogroup O2	1	2
<i>Yersinia ruckeri</i> MT252		16	10
Gram positive			
<i>Lactococcus garvieae</i> MT2055	Noncapsulated	2.5	40
<i>L. garvieae</i> MT2291	Capsulated	1.25	2

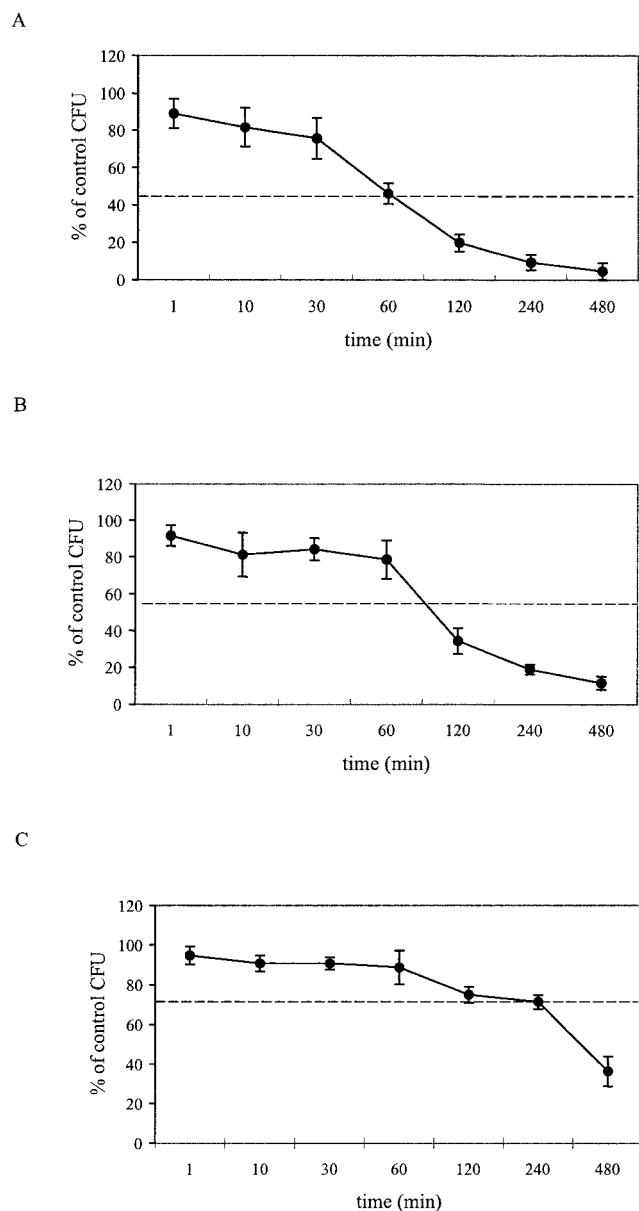


FIG. 5. Kinetics of *rCATH_1*(R151-V186) bactericidal activity against *A. salmonicida* MT004. The results represent viable colonies (CFU) shown as percent of control (without *rCATH_1*, plated at time zero). (A) 16.0 μ M ($2\times$ MIC); (B) 8.0 μ M ($1\times$ MIC); (C) 4.0 μ M ($0.5\times$ MIC). The dotted horizontal lines indicate the overall mean bactericidal activity for each concentration. The error bars indicate standard deviations.

MgCATH37) branches with *rCATH_1* but has a very distant evolutionary relationship.

Four invariant cysteines are present in mammalian cathelicidins, within the motif C-X₁₀-C-X₅-G-X₄-C-X₁₆-C, clustered in the conserved region of the cathelin-like propeptide. The two pairs of intramolecular disulfide bonds formed by the four cysteines may impose structural constraints on the molecule (34). In the *rCATH_1* sequence, four cysteines (C⁸⁴, C⁹⁵, C¹⁰⁶, and C¹³⁰) were also found to be coded for in exon 2 and exon 3. The disulfide bridge motif was C-X₁₀-C-X₅-G-X₄-C-X₂₃-C,

TABLE 3. Effects of increasing concentrations of common cations on MICs of *rCATH_1*(R151-V186) against *A. salmonicida* MT004

Salt	Concn (mM)	MIC (μ M)
NaCl	0	8
	10	8
	50	8
	100	8
CaCl ₂	0	8
	1	10
	5	32
	10	>64
MgCl ₂	0	8
	1	8
	5	16
	10	>64

which conformed very closely to the mammalian motif. In hagfish cathelicidin (43), the four cysteines were present but there was no glycine between cysteines 2 and 3, giving a motif of C-X₁₀-C-X₁₂-C-X₁₇-C. The fact that two pairs of cysteines would be needed within the proregion of cathelicidins, together with an analysis of possible elastase cut sites and the likelihood that the mature peptide would be encoded entirely within the final exon (exon 4), allowed the putative antimicrobial peptide to be deduced in the C-terminal region of *rCATH_1*. Probably the most interesting feature of the *rCATH_1* molecule is that the C-terminal 66-amino-acid sequence has features present in multiple cathelicidin subgroups, including the cysteine-bridged peptide family and the specific amino-acid-rich family (13), where, unusually, a high content of glycine is present in the fish sequence.

The *rCATH_1*(R151-V186) peptide, tested for antimicrobial activity in this study, represented the first 36 amino acids of the predicted mature peptide. It is highly basic (net charge, +6), due to the presence of multiple arginine residues and a lysine residue, and had potent antimicrobial activity. Bovine cyclic dodecapeptide was also tested in these studies, and in some cases it was more active than the *rCATH_1*(R151-V186)

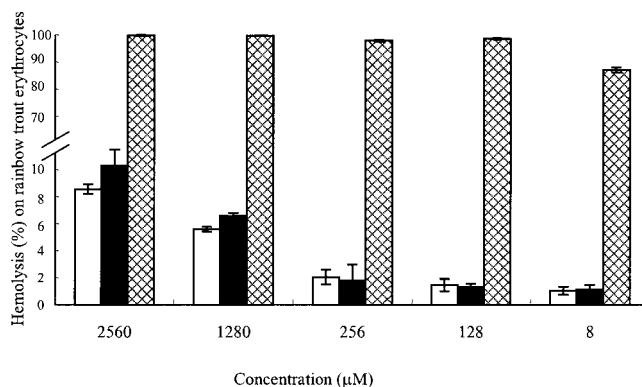
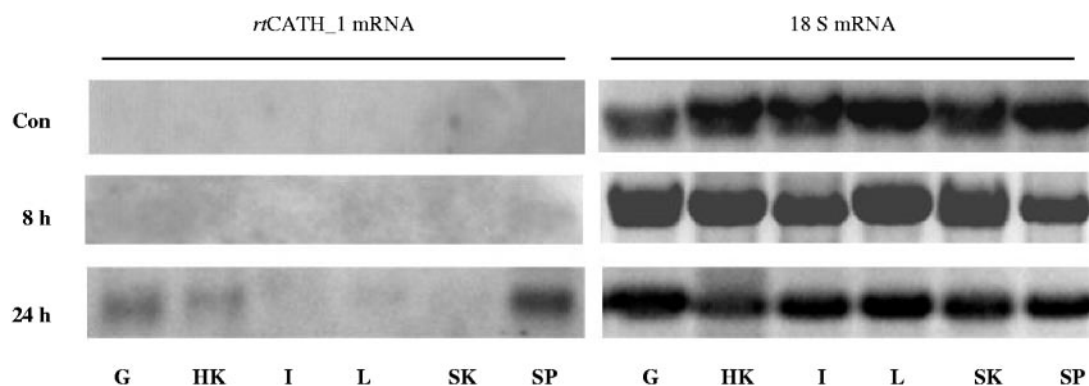


FIG. 6. Hemolysis assays of *rCATH_1*(R151-V186) peptide (open bars), bovine cyclic dodecapeptide (solid bars), and melittin (hatched bars) on rainbow trout erythrocytes. The values are expressed relative to total hemolysis, obtained by adding 0.2% Triton X-100 to the cell suspension. The results are the mean of three independent experiments with standard error values ranging from ± 0.1 to ± 1.2 .

A



B

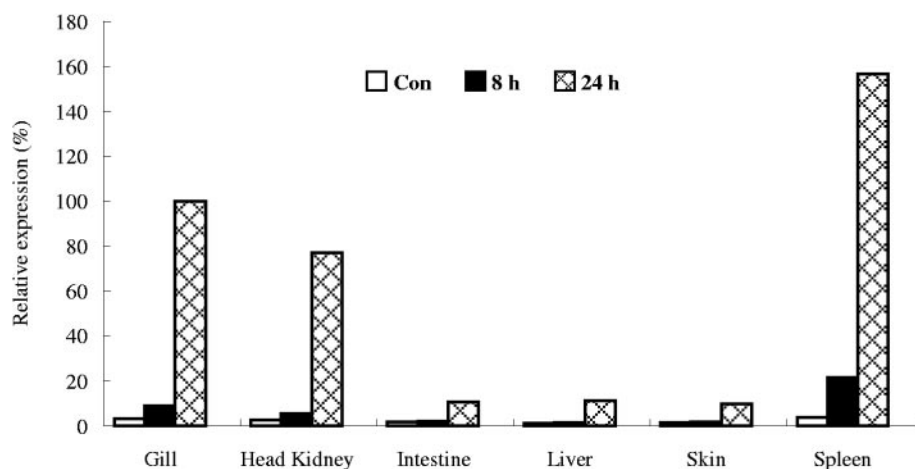


FIG. 7. Northern blot analysis of *rtCATH_1* mRNA expression. Exposed film (A) and densitometric readings of the film (B) are shown. The mRNA levels were normalized to the 18S RNA level and expressed as percentages of the mRNA level in the gills of 24-h-challenged fish (100%). Con, control fish; 8 h, fish injected intraperitoneally (i.p.) with 2×10^6 CFU of *Aeromonas salmonicida* MT423 for 8 h; 24 h, fish injected i.p. with *A. salmonicida* for 24 h; G, gill; HK, head kidney; I, intestine, L, liver; SK, skin; SP, spleen.

peptide, as seen with *P. damsela* EPOY 8803 II and the non-capsulated strain of *L. garvieae*. However, with *A. salmonicida*, which causes the well-known salmonid bacterial disease furunculosis, the *rtCATH_1*(R151-V186) peptide had more killing activity than bovine cyclic dodecapeptide. Our kinetic study (Fig. 5) indicates a direct relationship between the concentration of peptide, incubation time, and bactericidal activity.

Bacteria can evolve different strategies to resist bactericidal agents. For example, producing β -lactamase to destroy some antibiotics (6), secreting an outer capsule to prevent phagocytosis by host leukocytes (27), or producing an A layer to aggregate bacteria and decrease susceptibility to antimicrobial peptides (17). The results in this study showed that production of a capsule did not affect susceptibility to the *rtCATH_1*

(R151-V186) peptide, since the MICs for the two capsulated strains of *P. damsela* MT1415 and *L. garvieae* MT2055 were lower than those for the noncapsulated strains. In the case of A-layer production, *A. salmonicida* strains with an A layer were shown previously to be less susceptible to cecropins B and P1 (17), and a similar conclusion was made regarding resistance to the *rtCATH_1*(R151-V186) peptide, where the MIC against the A-layer-positive strain MT423 was higher than that to the A-layer-negative strain MT004.

Divalent cations can increase bacterial membrane stabilization (8), thus affecting the microbicidal activities of some antimicrobial peptides. Calcium and magnesium have been reported to inhibit the activities of mammalian cathelicidins (34) and some fish antimicrobial peptides (7, 23), where increasing

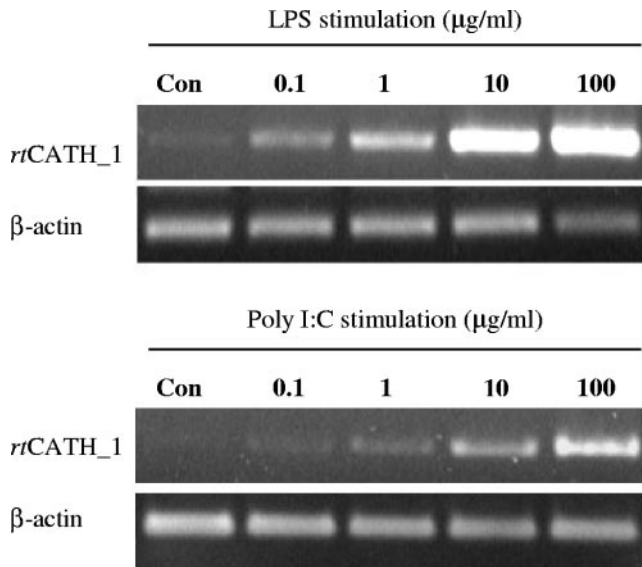


FIG. 8. RT-PCR analysis of *rtCATH_1* and β -actin expression in head kidney cells after stimulation without (Con) or with various concentrations of LPS (top) and poly(I · C) (bottom) for 5 h.

calcium or magnesium concentration resulted in decreased antimicrobial activity against the strains tested. A similar trend was seen with the rainbow trout antimicrobial peptide *rtCATH_1*(R151-V186) against *A. salmonicida* MT004. The divalent calcium and magnesium, but not monovalent sodium, affected the interaction between the antimicrobial peptide and the bacterial membrane. The functional relevance of such find-

ings relates to the aquatic habitat in which fish reside. Although normally found in freshwater, rainbow trout can live in seawater, in which both the calcium and magnesium concentrations (52.3 and 10.0 mM, respectively) are much higher than the levels tested here and would thus presumably decrease the activity of *rtCATH_1* at external surfaces. The reported concentrations of calcium and magnesium in trout body fluids, though, are on the order of 1 mM (22), which is within the active range, and thus, *rtCATH_1* would be expected to be active within physiologic fluids under all environmental circumstances.

Expression studies revealed an inducible expression of *rtCATH_1* in rainbow trout. The levels of *rtCATH_1* expression in rainbow trout were markedly increased after bacterial challenge, when the *rtCATH_1* mRNA was appreciable in gill, head kidney, and spleen. Induction at the systemic sites was correlated with the detection of bacteria internally, suggesting that pathways to upregulate *rtCATH_1* expression exist, possibly within myeloid cells at these sites. In mammals, cathelicidins are also expressed at various mucosal and systemic sites. The human cathelicidin, FALL-39/LL-37, is present in testis, airway epithelia, thymus, and liver (34). The porcine cathelicidin, PR-39, appears in bone marrow and lymphoid organs, including the thymus, spleen, and mesenteric lymph nodes (44). A murine cathelicidin, CRAMP, was found in the testis, spleen, stomach, and intestine (11). In the present study, it is possible that the challenge route (injection), which was chosen because rainbow trout do not typically succumb to an immersion challenge with *A. salmonicida*, affected the sites of expression detected. Studies with other routes of exposure and other pathogens, which are now possible, will confirm if other mu-

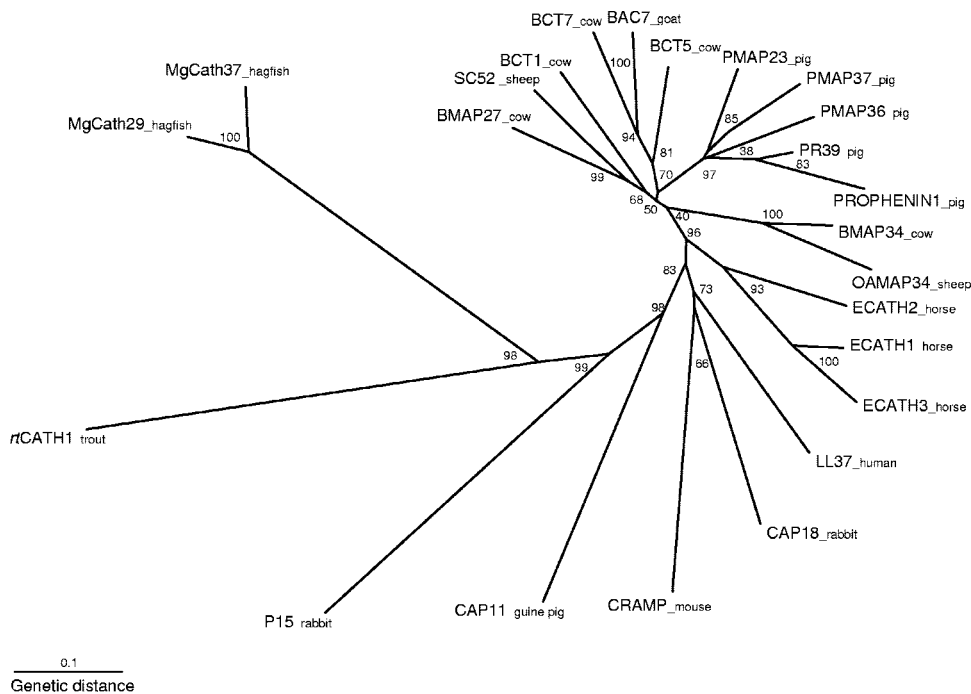


FIG. 9. Unrooted phylogenetic tree showing the relationship between *rtCATH_1* amino acid sequence of the precursor molecule and other known cathelicidin precursor sequences. The tree was constructed by the neighbor-joining method using the CLUSTAL W and PHYLIP packages and was bootstrapped 1,000 times.

cosal tissues express *rtCATH_1* in trout. Mammalian cathelicidins were originally isolated in neutrophils, as seen in humans, cattle, pigs, rabbits, horses, and mice (34). While this still remains to be determined for fish, the reverse transcription-PCR studies showed that *rtCATH_1* can be expressed in head kidney cells after stimulation with LPS or poly(I · C) (Fig. 7) and is thus present in one or more of the cell types present, which are predominantly leukocytes. Additional experiments are needed to investigate further the cellular source(s) of trout cathelicidin.

Analysis of the 5' flanking region of the *rtCATH_1* gene revealed a number of consensus binding sequences present in the promoter regions of other immune-relevant genes, including those for C/EBP β , nuclear factor 1, nuclear factor interleukin 6, and NF- κ B. Such transcription factors are frequently present adjacent to others, and the factors interact to effect transcription of antimicrobial peptide genes in different species, including the inducible antimicrobial peptides in invertebrates (20), the bovine β -defensin produced by respiratory tract epithelial cells (10), and other mammalian cathelicidin gene family members (14, 37, 47). In fish, such regulatory elements are known to be present in the promoters of the antimicrobial peptide gene in flounder (7), as well as the promoters of the transferrin gene in salmon (21), the interferon-inducible Mx genes (24) and the insulin-like growth factor gene in trout (39), and the antifreeze protein gene in flounder (28). Thus, in agreement with expression results from the bacterially challenged fish presented above, *rtCATH_1* gene expression is likely to be affected by transcription factors released in response to infection and inflammation, in a manner similar to the modulation of other immune-relevant genes in insects, fish, amphibians, and mammals.

Binding sites for various transcription factors known to be involved in viral defense were also found in the 5' flanking region of the *rtCATH_1* gene, including multiple GAAA motifs commonly found upstream of interferon-induced genes, two gamma interferon response elements, and one alpha interferon recognition site. Indeed, poly(I · C), a synthetic double-stranded RNA that is used as a viral mimic, enhanced the mRNA expression level of *rtCATH_1* in head kidney cells (Fig. 8). Thus, it is possible that the *rtCATH_1* gene has some role in host clearance of viral infections, and this requires further study.

Overall, the above-mentioned results confirmed that cathelicidin genes evolved at an early stage of vertebrate evolution and are present in bony fish. The *rtCATH_1* molecule was shown to be novel, in terms of possessing combined features of known mammalian subgroups, and the *rtCATH_1*(R151-V186) peptide was shown to have potent antimicrobial activity against a broad spectrum of fish bacterial pathogens while showing low hemolysis to host red blood cells. Thus, it appears that cathelicidins represent an important first line of defense at mucosal sites in fish as in mammals and that *rtCATH_1*, as the first bony-fish cathelicidin discovered, may be a good candidate antibiotic for preventing disease outbreaks in aquaculture and a starting point for the development of novel synthetic antimicrobial agents for fish.

ACKNOWLEDGMENTS

This work was supported by a Marie Curie Research Training grant from the EC to O.P. (FAIR-GT-1997-3355) and by EWOS Innovation.

Thanks also go the Taiwanese government for financial assistance to C.-I.C.

We thank Tony Ellis (FRS Marine Laboratory, United Kingdom) for kindly providing the field isolates of fish pathogens and Ian Davidson (Proteome Facility, University of Aberdeen, Aberdeen, United Kingdom) for synthesizing the *rtCATH_1* peptide.

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Editor: J. F. Urban, Jr.