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Antimicrobial and host cell -directed activities of Gly/Ser-rich peptides from salmonid cathelicidins

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Running title: Functional characterization of salmonid cathelicidins

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Abbreviations

AMP: antimicrobial peptide; BSA: bovine serum albumin; CD: circular dichroism; CFU: colony forming units; DCM: dichloromethane; DIPEA: diisopropylethylamine; DMF: dimethylformamide; DODT: 1,8-octanedithiol; dPG: diphosphatidylglycerol/cardiolipin; EDTA: ethylenediaminetetraacetic acid; ESI-MS: electron spray ionization mass spectrometry; FBS: fetal bovine serum; Fmoc: fluorenylmethyloxycarbonyl; HBSS: Hanks' Balanced Salt Solution; HKL: head kidney leukocytes; L-15: Leibovitz-15; LCIS: Live Cell Imaging Solution; LDH: lactate dehydrogenase; LPS: lipopolysaccharide; LUV: large unilamellar vesicles; MBC: minimum bactericidal concentration; MFI: mean fluorescence intensity; MIC: minimum inhibitory concentration; MH: Mueller-Hinton; PBS: phosphate-buffered saline; PI: propidium iodide; PG: L-α-phosphatidylglycerol; PMA: phorbol 12-myristate 13-acetate; PyBOP: benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate; RLU: relative luminescence units; ROS: reactive oxygen species; SPB: sodium phosphate buffer; SPPS: solid-phase peptide synthesis; TFA: trifluoroacetic acid; TFE: trifluoroethanol; TSA: tryptic soy agar; TSB: tryptic soy broth.

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Highlights

- Salmonid cathelicidin-derived peptides show medium-sensitive antimicrobial activity
- They are not cytotoxic to fish cells and promote proliferation of trout fibroblasts
- Peptide STF(1-37) potentiates phagocytosis and respiratory burst in trout leukocytes
- STF(1-37) and β-glucan act synergistically to immunostimulate trout leukocytes

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Abstract

Cathelicidins, a major family of vertebrate antimicrobial peptides (AMPs), have a recognized role in the first line of defense against infections. They have been identified in several salmonid species, where the putative mature peptides are unusually long and rich in serine and glycine residues, often arranged in short multiple repeats (RLGGGS/RPGGGS) intercalated by hydrophobic motifs. Fragments of 24 to 40 residues, spanning specific motifs and conserved sequences in grayling or brown, rainbow and brook trout, were chemically synthesized and examined for antimicrobial activity against relevant Gram-positive and Gram-negative salmonid pathogens, as well as laboratory reference strains. They were not active in complete medium, but showed varying potency and activity spectra in diluted media. Bacterial membrane permeabilization also occurred only under these conditions and was indicated by rapid propidium iodide uptake in peptide-treated bacteria. However, circular dichroism analyses indicated that they did not significantly adopt ordered conformations in membrane-like environments. The peptides were not hemolytic or cytotoxic to trout cells, including freshly purified head kidney leukocytes (HKL) and the fibroblastic RTG-2 cell line. Notably, when exposed to them, HKL showed increased metabolic activity, while a growth-promoting effect was observed on RTG-2 cells, suggesting a functional interaction of salmonid cathelicidins with host cells similar to that shown by mammalian ones. The three most active peptides produced a dose-dependent increase in phagocytic uptake by HKL simultaneously stimulated with bacterial particles. The peptide STF(1-37), selected for further analyses, also enhanced phagocytic uptake in the presence of autologous serum, and increased intracellular killing of live *E. coli*. Furthermore, when tested on HKL in combination with the immunostimulant β-glucan, it synergistically potentiated both phagocytic uptake and the respiratory burst response, activities that play a key role in fish immunity. Collectively, these data point to a role of salmonid cathelicidins as modulators of fish microbicidal mechanisms beyond a salt-sensitive antimicrobial activity, and encourage further studies also in view of potential applications in aquaculture.

Introduction

Antimicrobial peptides (AMPs) are an important component of the innate immune system of vertebrates, contributing to the first line of defense against microbial pathogens. Several different families of AMPs have been described, and their members often display broad-spectrum antimicrobial activities and in many cases also immunomodulatory functions [1-3]. Families of AMPs isolated in fish include some that are also present in other classes of vertebrates, such as defensins, cathelicidins, and hepcidins, as well as others that are peculiar to fish [4].

Cathelicidins are characterized by a conserved N-terminal proregion that contains a cathelin-like motif, with the antimicrobial activity being located in the variable C-terminal region [5]. This region is generally quite cationic and, when released from the proregion, generally adopts an amphipathic active conformation on interaction with bacterial membranes. These two features promote the initial interaction and subsequent insertion into bacterial membranes, whose integrity is eventually compromised.

To date, cathelicidins have been identified in several salmonid species [6-10], the smelt *Osmerus mordax* [8], the ayu *Plecoglossus altivelis* [11], and the Atlantic cod *Gadus morhua* [7, 12]. An ancient ancestor cathelicidin has also been found in the jawless hagfish *Myxine glutinosa* [13]. Two different cathelicidins, rtCATH1 and rtCATH2 are present in the rainbow trout *Oncorhynchus mykiss* [6] as well as in most other Salmonidae [8], and two additional cathelicidin genes have been recently identified in rainbow trout [14]. The C-terminal antimicrobial domains in the paralogous CATH1 and CATH2 peptides have rather different sequences, while there is a convincing homology between orthologous peptides from different salmonid species. Both CATH1 and CATH2 C-terminal regions are unusually long with respect to most other vertebrate cathelicidins, and rich in serine/glycine residues. They show an initial QKIRTRR sequence that is highly conserved in Salmonidae, Osmeridae and Gadidae families [8], while the downstream sequences are quite divergent and characterized by the presence of short multiple repeats, such as RLGGGS or RPGGGS, sometimes intercalated by hydrophobic motifs (LIG, IAGA, AGFI).

Peptides derived from the long C-terminal cathelicidin regions of *O. mykiss* [6, 14, 15] and other fish [9, 11, 16, 17] were shown to be antibacterial against several species of Gram-positive and Gram-negative bacteria, including fish pathogens. However, in these studies antibacterial assays were either carried out in diluted media or with unspecified conditions, so that the often discordant data does not definitely demonstrate that the principal biological function of the C-terminal peptides is a direct antibacterial activity. In this respect, atlantic salmon cathelicidins have been shown to be

ineffective *in vitro* against a pathogen which upregulated their expression *in vivo* during bacterial infection, thus suggesting an immunomodulatory role of these molecules [16]. Their ability to stimulate cytokine gene expression in salmon leukocytes [16], and similar effects displayed by trout cathelicidins on leukocytes [14] and epithelial cells [18], support a role for salmonid cathelicidins in fish immunity beyond that simply being endogenous antibiotics.

In this study we have investigated the functions of selected fragments from the C-terminal antimicrobial domain of salmonid cathelicidins, in terms of both the direct antimicrobial and immunomodulatory activities. Peptide fragments from brown trout, grayling, rainbow trout and brook trout cathelicidins were evaluated against a bacterial panel including both reference strains and trout pathogens. The more active peptides were further characterized for their effects on host cell viability as well as for the ability to modulate phagocyte functions, such as phagocytosis and respiratory burst activity. In the case of a brown trout peptide, these effects have also been examined in combination with the fish immunostimulant β-glucan. Results obtained extend and enhance previous findings on the biological functions of these important immune effectors beyond direct microbial killing, and support potential applications of these compounds in aquaculture.

Materials and methods

Reagents

L-α-phosphatidylglycerol (PG, egg yolk) and cardiolipin (dPG, bovine heart) were from Avanti polar lipids (Alabaster, AL, USA), lipopolysaccharide (LPS, *Salmonella minnesota*) and propidium iodide (PI) were from Sigma-Aldrich (St. Louis, MO, USA). Dehydrated media for microbiological assays (Mueller-Hinton broth, tryptic soy broth and agar technical) were obtained from Difco laboratories (Detroit, MI, USA). Solutions, media and supplements used for cell culture and leukocyte purification were purchased from Sigma-Aldrich with the exception of fetal bovine serum (FBS) (EuroClone, Pero, Italy). FBS was inactivated at 56°C for 30 min prior to use. Gentamicin was obtained from Gibco/Thermo Fisher Scientific (Waltham, MA, USA). Phorbol 12-myristate 13-acetate (PMA), β-glucan from baker's yeast (*Saccharomyces cerevisiae*), colchicine and all other reagents, unless otherwise specified, were purchased from Sigma-Aldrich.

Peptide synthesis

All peptides were synthesized by Fmoc-SPPS on a CEM Liberty automated microwave peptide synthesizer (CEM, Matthews, NC, USA). By default, a 5-fold excess of Fmoc-amino

acid/PyBOP/DIPEA (1:1:1.7) and a solvent mixture of DMF/DCM (80:20 v/v) was used for each coupling step. Potentially difficult sequences were predicted using the Peptide Companion software (Coshi-Soft/PeptiSearch, Tucson, AZ, USA) and subjected to double coupling cycles. The coupling temperature was 75°C except for Fmoc-Cys(Trt)-OH (45°C). The Fmoc(Hmb)-Gly-OH was used to minimize the deamidation and aspartimide formation at N-G sequence [19]. Peptides were cleaved from the resin using a cocktail of trifluoroacetic acid, thioanisole, water, DODT, triisopropylsilane (85:3:2:8:2 v/v). The crude peptides were analyzed by ESI-MS (Esquire 4000 Bruker Daltonics, Billerica, MA, USA) to confirm the correct structure.

The crude STF(1-37) peptide was directly folded in oxidative condition in the presence of the cysteine-cystine pair (peptide/cystine/cysteine 1:10:100) in N_2 saturated aqueous buffer (0.1 M ammonium acetate, 2 mM EDTA and 0.5 M guanidinium chloride, pH 7.5) as described in [20]. Peptides were purified on a preparative RP-HPLC column (Waters X-Terra^{TM} C_{18} 7 µm 19 x 300 mm; Waters, Milford, MA, USA). Peptide concentrations in water were calculated from the absorbance at 215 nm and 225 nm [21].

Sequence analysis

Sequences of peptides listed in Table 1 were analyzed and physico-chemical parameters acquired using tools present on the *ExPASy* Server. The HeliQuest program *(http://heliquest.ipmc.cnrs.fr/)* was used to estimate hydrophobicity per residue (H), hydrophobic moment (µH) and amphiphilicity of the peptides.

Circular dichroism analysis

CD spectra were taken on a Jasco 720 spectropolarimeter (Jasco, Tokyo, Japan) in the 190 - 240 nm range, under different conditions: a) in sodium phosphate buffer (SPB 10 mM pH 7.4), b) in the presence of increasing proportions of trifluoroethanol (TFE, up to 50% v/v in SPB), c) in the presence of anionic large unilamellar vesicles (LUV, PG:dPG 95:5 in SPB to a final lipid concentration of 0.4 mM), or d) LPS micelles in SPB. Liposomes and micelles were prepared as described previously [22, 23]. Three consecutive scans were accumulated per sample (40 µM peptide) in a 1-mm cell, at room temperature. The helicity of the peptides was determined from the mean residue helicity at 222 nm. All data are the mean of three independent measurements, which did not deviate more than 5%.

Bacterial strains and growth conditions

The reference strains used were *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Aeromonas salmonicida* ATCC 33658, *Aeromonas hydrophila* ATCC 7966, *Yersinia ruckeri* NCIMB 1315, *Vibrio anguillarum* ATCC 43305 and *Lactococcus garvieae* ATCC 49156. Fish pathogens were kindly provided by the Istituto Zooprofilattico Sperimentale delle Venezie (Legnaro, Italy). Bacteria were grown on tryptic soy agar (TSA) at 30°C with the exception of *E. coli* and *S. aureus* that were grown at 37°C. All the strains were transferred to tryptic soy broth (TSB) and cultured overnight to optimal density before use in antimicrobial assays.

Antimicrobial assays

Bacterial susceptibility test was used [24] with the following modifications: serial twofold dilutions of peptides were prepared in 96-well microtiter plates in 5% TSB in 10 mM SPB pH 7.4 to a final volume of 50 µl. Bacteria were grown to the mid-logarithmic phase and diluted in 5% TSB to give a final inoculum of 5 x 10^5 CFU/mL. A suspension of 50 µl of bacteria was added to each well, and the plate incubated for 24 h at 37°C for *E. coli* and *S. aureus* strains, and at 22°C for fish pathogen strains. *V. anguillarum* was assayed in the same medium with the addition of 2% NaCl. Assays were replicated at least three times. Some experiments were also performed in complete TSB or Mueller-Hinton (MH) broth, or in 20% MH diluted in SPB, or in M9 minimal medium. To determine the minimum bactericidal concentration (MBC), aliquots from wells showing no visible growth were plated on solid medium and further incubated for 24 h at the optimal growing temperature.

Bacterial membrane permeabilization was determined by flow cytometry, by measuring propidium iodide (PI) uptake [25]. Briefly, mid-log phase bacterial cultures, diluted to 1 x 10^6 CFU/mL in 5% TSB in 10 mM SPB, were incubated at 37°C (*E. coli* and *S. aureus*) or 22°C (*Y. ruckeri* and *L. garvieae*) for different times with increasing peptide concentrations. PI was then added to a final concentration of 10 µg/mL and incubation was prolonged for 4 min before analysis.

Measurements were performed using a Cytomics FC 5000 instrument (Beckman-Coulter, Inc., Fullerton, CA, USA) equipped with an argon laser (488 nm, 5 mW) and a fluorescence detector for orange filtered light (610 nm) set on logarithmic amplification. For each sample, at least 10000 events were acquired. Data analysis was performed with the FCS Express3 software (De Novo Software, Los Angeles, CA, USA).

In some experiments, the antimicrobial activity towards *E. coli* ATCC 25922 was evaluated under the same conditions used in phagocytosis assays (see below). In this case, a mid-log phase bacterial

198 culture was diluted to 1 x 10^8 CFU/mL in Live Cell Imaging Solution supplemented with 0.2%
199 glucose (LCIS-glu) and incubated at 18°C for 3 h with 8 µM of the indicated peptides. Samples
200 were analyzed for PI uptake as above or, alternatively, were diluted in PBS and plated on MH agar
201 to allow colony counts.
202
203 *Hemolytic assay*
204 For hemolytic assays on human erythrocytes, a 0.5% erythrocyte suspension from healthy donors
205 was prepared in PBS as described [26]. Different concentrations of peptides, or Triton X-100 at 1%,
206 were incubated with fresh suspension for 30 min at 37°C. Lysis was then blocked by adding a five-
207 fold excess of ice-cold PBS. Samples were immediately centrifuged at 5000 rpm for 5 min.
208 Hemolytic activities on trout erythrocytes were determined according to [15] with slight
209 modifications. Briefly, heparinized blood samples (approximately 3 mL) were washed three times
210 with PBS by centrifuging at 500 x g for 10 min at room temperature. Cells were resuspended in 30
211 mL PBS and incubated for 30 min at 18°C with the peptides or with 0.2% (final concentration)
212 Triton X-100. Samples were then cleared by centrifugation at 300 x g for 10 min.
213 Absorption of supernatants was measured at 405 nm. Percentages of hemolysis were calculated as
214 $[(A^{peptide}-A^{blank})/(A^{triton\ X\text{-}100} - A^{blank})]$ x 100, where $A^{peptide}$ was the absorption in the presence of
215 peptide, A^{blank} was that in the absence of peptide and $A^{triton\ X\text{-}100}$ the absorption of the erythrocyte
216 suspension treated with Triton X-100 to achieve complete lysis.
217
218 *RTG-2 cell line culture and stimulation*
219 The rainbow trout (*O. mykiss*) gonadal fibroblast cell line RTG-2 was obtained from the Istituto
220 Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (Brescia, Italy) and
221 maintained in Leibovitz-15 (L-15) medium supplemented with 10% (v/v) FBS, 2 mM L-glutamine,
222 100 units/mL penicillin and 100 µg/mL streptomycin (complete medium) at 22°C. For cytotoxicity
223 and cell viability assays, cells were seeded in 96-well plates at a density of 2.5 x 10^4 and 5 x 10^3 per
224 well, respectively. Cells were grown for 24 h before being incubated in the absence and presence of
225 increasing peptide concentrations in fresh complete medium for 60 min (lactate dehydrogenase
226 release) or 72 h (cell viability).
227
228 *Fish handling and leukocyte purification*

Female adult rainbow trout (*O. mykiss*) were obtained from a commercial farm and maintained in 3-m^3 fiberglass tanks in an open system supplied with filtered freshwater. The physico-chemical parameters of tank water were periodically measured throughout the experimental period and maintained at optimum levels for this fish species (temperature 12.5°C, pH 8.0, dissolved oxygen 9.5 mg/L, natural photoperiod). Trout were fed a commercial pellet diet (Skretting Italia, Mozzecane, Italy) according to the manufacturer's recommendations, and kept under veterinary control. The fish were always handled under sedation and the experimental procedures were performed in such way so as to minimize suffering and pain. The number of fish used was kept to a minimum where possible. All the procedures were performed according to the EU Directive 2010/63/EU for animal experiments.

Trout, weight range 400-800 g, were anaesthetized, bled from the caudal vein and euthanized by an overdose of MS-222 prior to sterile excision of the head kidney. Blood samples were allowed to clot at 4°C, centrifuged, and the serum obtained stored at 4°C until use in phagocytosis assays.

Head kidney leukocytes (HKL) were isolated according to [27]. Briefly, the head kidney tissue was gently pressed with a syringe plunger in cold Hanks' Balanced Salt Solution supplemented with 12.5 UI/mL heparin (HBSS-hep). After washing with HBSS-hep, the resulting cell suspension was layered onto Histopaque™-1077 and centrifuged at 300 x g for 25 min at 4°C. Leukocytes were then collected from the Histopaque-medium interface, washed twice with cold HBSS and counted by the Trypan Blue exclusion method. Cell density was adjusted to 10^7/mL either in L-15 medium supplemented with 2% (v/v) FBS for phagocytosis experiments or in HBSS supplemented with 0.125% (w/v) bovine serum albumin (HBSS-BSA) for respiratory burst assays. Leukocytes from single fish were maintained as individual cell cultures throughout the experiments, and comparisons between *in vitro* treatments were done among cells from a single individual.

Cytotoxicity and cell viability assays

Cell membrane damage in RTG-2 cells and HKL was evaluated as leakage of the cytosolic enzyme lactate dehydrogenase (LDH). LDH activity was quantified spectrophotometrically in cell-free supernatants and cell lysates from duplicate wells using the CytoTox-96™ non-radioactive cytotoxicity assay kit (Promega, Madison, WI, USA). Data were calculated as percent of total cellular LDH activity.

Cell viability was assessed on triplicate wells using the resazurin-based PrestoBlue™ metabolic dye (Invitrogen/Thermo Fisher Scientific) according to the manufacturer's instructions.

Phagocytosis assay

Phagocytic activity was analyzed by a microplate fluorometric assay using pHrodo™ Green *E. coli* BioParticles™ Conjugate (Molecular Probes/Thermo Fisher Scientific), consisting of inactivated *E. coli* labeled with a pH-sensitive fluorophore. The assay is based on the increased fluorescence of ingested particles in the acidic environment of the phagosomes, which enables a quantification of phagocytic uptake.

Particles were suspended in Live Cell Imaging Solution (Molecular Probes/Thermo Fisher Scientific) supplemented with 0.2% (w/v) glucose (LCIS-glu) at a density of 1 mg/mL, vortexed for 2 min and sonicated for 5 min at room temperature in a water-bath sonicator.

After separation, HKL were seeded at 10^6/well in 96-well plates in L-15 medium supplemented with 2% FBS and incubated overnight at 18°C. Non-adherent cells were then removed by gently rinsing with HBSS and the resulting phagocyte-enriched populations were preincubated for 30 min at 18°C with 50 µL LCIS-glu prior to the addition of 50 µL bioparticle suspension. When required, peptides and β-glucan, at the indicated concentrations, were added to cells in combination with particles. In some experiments, aliquots of particle suspensions were preincubated for 30 min at 18°C in the presence of 5% (v/v) fresh autologous serum prior to cell stimulation. In the latter case, serum at 5% was also added to the cell preincubation medium so as to keep the serum concentration constant throughout the assay. No-cell control wells containing bioparticles only were included in each plate to allow subtraction of the background fluorescence of pHrodo-particles at neutral pH. The microtubule poison colchicine [28, 29] (concentration range 125-1000 µg/mL, not causing detectable LDH release - data not shown) was used as a validation control to verify that the increase in particle fluorescence depended on an active internalization process. Upon bioparticles addition, plates were spun at 400 x g for 5 min to maximize particle contact with the cell layer, and subsequently incubated for 3 h at 18°C in the dark.

Fluorescence was recorded using an Enspire 2300 multiplate reader (Perkin Elmer, Waltham, MA, USA) in well-area scan mode, with excitation and emission wavelengths set at 509 and 533 nm respectively. Assays were performed in duplicate. In some cases data were expressed as blank (LCIS-glu) -subtracted mean fluorescence intensity (MFI) values. Alternatively, data were calculated as percent phagocytosis relative to control cell samples incubated with bioparticles in the absence of stimuli, after subtraction of background particle fluorescence (no-cell control).

Intracellular killing assay

The killing activity of HKL against engulfed bacteria was evaluated by the gentamicin protection method [30]. Cell stimulation with bacteria and peptide or β-glucan was performed as described for phagocytosis assays, by adding 50 µL of a viable *E. coli* ATCC 25922 suspension (3 x 10^8 CFU/mL in LCIS-glu) instead of pHrodo-*E. coli* bioparticles. Each condition was tested in triplicate wells. Phagocytosis was allowed to occur for 1 h at 18°C followed by the addition of 100 µg/mL gentamicin to kill extracellular bacteria. After additional 2-h incubation at 18°C, cells were washed twice with HBSS and lysed by a 30-min treatment at room temperature with 0.1% Triton X-100 in LCIS-glu. The latter treatment does not affect *E. coli* viability (data not shown). Lysates were serially diluted in PBS, plated on MH plates and incubated overnight at 37°C to allow colony counts. The number of recovered bacteria was expressed as percent survival relative to control cells incubated with *E. coli* in the absence of peptide or β-glucan.

Measurement of respiratory burst activity

ROS production was measured by a real-time luminol-enhanced chemiluminescence assay as described in [27] with minor modifications. Freshly-purified HKL, suspended in HBSS-BSA as specified above, were dispensed into black, flat-bottomed 96-well microtiter plates at 0.5 x 10^6/well and added with 0.5 mM luminol, to a final volume of 100 µL. ROS production was triggered by the addition of 100 µL HBSS containing 1 µg/mL PMA or 250 µg/mL β-glucan. Control samples were added with 100 µL HBSS. When required, STF(1-37) was added in a volume of 5 µL/well. Each sample was run in triplicate. Chemiluminescence emission was recorded at 2-4 -min intervals for 20-40 min at room temperature using a microplate luminometer (Tecan Italia S.r.l., Milano, Italy). Results are reported as relative luminescence units (RLU). For quantification and statistical analysis of data, fold-increase values were calculated as: $RLU_{combination}/(RLU_{peptide} + RLU_{stimulus})$.

Statistical analysis

Data are presented as the means ± SD or as representative results from at least three independent experiments. Statistical evaluation was performed using the GraphPad Prism version 5.01 software (GraphPad Software, Inc., San Diego, CA, USA) by one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test. The level of significance was set at 0.05.

Results and discussion

Analysis and synthesis of C-terminal peptides from CATH1 and CATH2 cathelicidins

The C-terminal peptides from fish cathelicidins CATH1 and CATH2 were selected for synthesis according to the predicted amino acid sequences encoded by exon IV of the respective cathelicidin genes (AMP domain, see **Figure 1**) [8]. Due to the length of the putative AMPs, fragments including different motifs or conserved sequences from both types of cathelicidins were selected for functional evaluation of their relative importance in antimicrobial activity. Three peptides, one from CATH1 of *Salmo trutta fario* [STF(1-37)], and two from CATH2 of *Thymallus thymallus* [TT(1-24) and TT(1-35)] started directly from the putative N-terminus of the AMP domain, at the very beginning of exon IV, corresponding to the lysine residue present in the highly conserved QKIRTRR motif of Salmonidae, Osmeridae and Gadidae AMPs [8] (see **Figure 1**). STF(1-37) was chosen because it contains a disulfide motif. The shorter TT(1-24) and longer TT(1-35) fragments were selected to probe the importance of length on activity. Furthermore, TT(1-35) contains the IAGA hydrophobic motif at its C-terminus [8]. Two *Oncorhynchus mykiss* peptides were also synthesized, one beginning at the putative N-terminal Lys residue [rtCATH2(1-40)] and the second starting from Arg 5 in the QKIRTRR motif [rtCATH2(5-40)] according to a previously published sequence, based on an alternative cleavage site [6]. The C-terminal AMP domain of *Salvelinus fontinalis* is the longest among salmonid cathelicidins, and contains several tandem repeats. For this reason, the SF(18-45) fragment comprises the central region of this domain with two RXGGGS repeats with an intercalated LIG hydrophobic motif (see **Figure 1**).

All peptide fragments were selected to have a positive charge of at least +5. As indicated in **Table 1**, they are all rich in glycine and serine residues, and show a relatively high isoelectric point due to many positively charged residues at physiological pH. Their secondary structure was evaluated by circular dichroism (CD) measurements, performed in sodium phosphate buffer (SPB), in the presence of helix-favouring solvent TFE (50% in SPB), of large unilamellar vesicles (LUV) or of lipopolysaccharide (LPS). These conditions mimic different aspects of the bacterial membrane environment, which is known to trigger transitions from a random coil to amphipathic active form in membrane-active AMPs [31]. As shown in **Figure 2**, CD spectra recorded in SPB displayed a negative peak at around 200 nm for all peptides, which is typical of random coiled structures [32]. The presence of 50% TFE did not have a marked effect on the shape of the spectra, indicating that they do not have a propensity to adopt a helical conformation. TT(1-35) and SF(18-45) spectra also show little variation in the presence of LUVs that mimic the bacterial membrane (**Figures 2B** and

2D), whereas the spectra of rtCATH2(1-40) and STF(1-37) (**Figures 2A** and **2C**) show variations in intensity and shifts that are consistent with some form of conformational transition on membrane interaction. For rtCATH2(1-40) it is not possible to surmise what the membrane-bound conformation might be. For STF(1-37), a shift of the trough to longer wavelengths could be consistent with a hairpin like structure with some β-sheet content, stabilized by the disulfide bond. Similar results were also observed in the presence of LPS micelles (data not shown).

These results were not unexpected, as the peptide sequences present few features indicating a propensity to adopt ordered conformations, but rather the high glycine content suggests they are low complexity, intrinsically disordered sequences. Recently, Zhang *et al.* [14] have indicated that even for longer salmonid cathelicidin-derived peptides the conformational change on membrane interaction resulted in less helical than beta-sheet content, but with a predominant contribution from random coil, confirming a low tendency for adopting regular structures.

Antimicrobial activity in complete and diluted medium

Antimicrobial activity has been determined as MIC against a panel of bacteria including standard laboratory strains as well as known trout pathogens such as the Gram-negatives *Yersinia ruckeri*, *Aeromonas salmonicida*, *Aeromonas hydrophila*, *Vibrio anguillarum*, and the Gram-positive *Lactococcus garvieae*. Surprisingly, none of the peptides showed activity when assayed in complete Mueller-Hinton broth (MH, MIC >64 µM) or in complete tryptic soy broth (TSB) (data not shown). On the other hand they generally exhibited significant activity when tested under low medium and salt conditions (5% TSB in SPB, see **Table 2**). MBC values were equal to the MICs, indicating that under these conditions all peptides had a bactericidal action (data not shown). Marked differences in potency and activity spectra were observed among the different peptides tested in this medium (**Table 2**)**,** with MIC values ranging from 1 to >64 µM. TT(1-35) showed a potent antimicrobial activity against *A. salmonicida*, *L. garvieae,* and *E. coli* and to a lesser extent also against *Y. ruckeri*. Conversely, the shorter fragment TT(1-24) exhibited a generally reduced activity indicating that a minimum length, or the presence of the C-terminal residues, are important for activity. rtCATH2(1-40) and STF(1-37) seemed, on the whole, to be as active as TT(1-35) towards most strains. The N-terminus truncated fragment rtCATH2(5-40) was remarkably less active underlining the importance of the highly conserved KIRTRR sequence for activity. The SF(18-45) fragment, containing RXGGGS repeats typical of CATH1, showed a weak activity against all the strains tested. None of

the tested peptides inhibited the growth of *V. anguillarum* or *A. hydrophila* (MIC >32 μM). Assays were also repeated using the somewhat more robust conditions (20% MH in SPB) used for assaying the activity of the medium-sensitive human cathelicidin LL-37 [31], but this resulted in a 4-8 -fold increase in MIC values, confirming the medium sensitivity of fish cathelicidin AMPs. To understand whether the inhibitory effect was due to salt concentrations or some medium components, we tested the activity in M9 minimal medium having an osmolarity equal to that of MH broth, but lacking peptone and lipids. The low activity of all peptides suggests that salt concentration is the main factor affecting antibacterial activity. The low susceptibility of *V. anguillarum*, which requires 2% NaCl for growth, also finds an explanation in this observation.

Data on antibacterial properties of fish cathelicidins towards various pathogens, using various types of synthetic fragments, have previously been reported for rainbow trout [6, 14, 15], atlantic salmon [16], the salmonid lenok [9], ayu [11], and cod [12, 17]. In these studies different cathelicidin peptides and assay conditions were used so that a direct comparison of activities is difficult, but in most cases antibacterial activity was measured in dilute media. Some authors used 20-25% MH broth but a non-canonical and low-stringency definition of MIC (i.e., the concentration causing 50% reduction of bacterial growth compared to control) [6, 14, 15, 33]. Others do not clearly specify the salt concentrations with which assays have been performed, but the antimicrobial activity of the human cathelicidin LL-37, reported for comparison [9], is consistent with assays performed in low-salt medium [31]. Overall, none of these studies showed data on antibacterial activity using complete media, an aspect that we recognize to be important to understand the biological functions of these molecules. Furthermore, the mature cathelicidin peptide from Atlantic cod, codCath, also shows a reduced capacity to kill *B. megaterium* cells on increasing salt concentrations [17]. Overall these results indicate that salt dependent-activity is a common and distinctive feature of fish cathelicidins not present in other type of fish AMPs [4, 34].

Kinetics of bacterial membrane permeabilization

The mode of action of CATH AMP fragments was investigated by flow cytometry, assessing membrane damage by determining the percentage of bacterial cells in which cytoplasmic penetration of propidium iodide occurs (PI positive cells, PI+). The most active peptides, STF(1-37), TT(1-35) and rtCATH2(1-40) (see **Table 2**) were selected for this analysis. Under these conditions, all peptides used at concentrations corresponding to the MIC or ½ the MIC were able to

consistently and rapidly damage the membranes of both reference strains *E. coli* (**Figure 3A**) and *S. aureus* (**Figure 3B**), and also of the fish pathogens *Yersinia ruckeri* (**Figure 3C**) and *Lactococcus garvieae* (**Figure 3D**). Permeabilization of the Gram-positive *S. aureus* and *Lactococcus garvieae* were particularly rapid, with all cells becoming PI+ within 15 min of incubation (**Figure 3B** and **3D**). However, one should consider that in the low medium and salt conditions employed the stability of both bacteria is compromised, as indicated by a measureable positivity also in the absence of peptides (**Figure 3B**). Repeating the assay in complete MH broth however abrogated permeabilization for all strains also in the presence of peptides.

This type of bacterial killing mechanism has also been shown for trout cathelicidins by Zhang *et al.* with respect to *Edwardsiella ictaluri*, using both the PI permeability assay and SEM observations [14]. Our data are in agreement with these results and, furthermore, they highlight the correspondence between killing and membrane damage under conditions in which the peptides are active, and the absence of both under conditions in which they are inactive. Overall these results confirm that bacterial membrane is a target of fish cathelicidins, but also that membrane interaction and damaging are quite medium-dependent activities.

Several studies have shown that salmonid cathelicidin genes are expressed in immune-related tissues such as spleen, head kidney and gills, and are upregulated upon infection [6, 8, 15, 16], which is consistent with a role of salmonid cathelicidins in defense against infection. Our results however raise doubts as to whether salmonid cathelicidins could retain significant antibacterial activity in body fluids at physiological osmolarity (300 mOsm) [35] although it cannot be excluded that an inhibitory effect on bacteria is exerted under particular physiological conditions that are difficult to reproduce with *in vitro* assays. This apparent contradiction has also been found for mammalian α- and β-defensins, that have poor antimicrobial activities in the presence of physiological salt concentration (cf. 150 mM NaCl) [36]. However, defensins have important immunomodulatory properties that are unaffected by physiological ion concentrations, and it may be that these are the predominant functions of these peptides *in vivo* [37]. Similar considerations may also pertain to fish cathelicidins.

Effects on cell membrane integrity and viability in eukaryotic cells

Accumulating evidence suggests that, like mammalian cathelicidins [1, 38], fish family members may modulate host cell functions relevant to innate and/or adaptive immunity [14, 16, 18, 39]. The

ability of the peptides under consideration to interact with eukaryotic cells was preliminarily investigated by assessing their membrane-perturbing potential towards either human and trout erythrocytes or the trout RTG-2 cell line [40], as well-established models for toxicity studies. None of the peptides showed any hemolytic effects towards human red blood cells, up to 100 µM concentration (**Figure S1**). Similarly, STF(1-37), TT(1-35) and rtCATH2(1-40) were not hemolytic to trout cells (**Figure 4A**) and, when tested on RTG-2 cells up to a concentration of 32 µM, did not cause significant lactate dehydrogenase (LDH) release (**Figure 4B**). In both cellular models, this behaviour is markedly different with respect to membrane active alpha-helical AMPs used for comparison (**Figure 4A** and **B**), in keeping with previous reports on salmonid cathelicidins [14-16].

To further investigate potential effects on cell viability, beyond direct membrane damage, resazurin-based metabolic assays were conducted after 72-h exposure of low-density RTG-2 cultures to low micromolar concentrations of STF(1-37), TT(1-35) or rtCATH2(1-40). As shown in **Figure 4C**, none of the peptides impaired cellular viability under these conditions. Interestingly, STF(1-37) and TT(1-35) exhibited a growth-promoting effect, since a dose-dependent increase in total metabolic activity was observed in peptide-treated *versus* control samples (**Figure 4C**). TT(1-35) showed the highest activity, resulting in a nearly 30% increase in resazurin reduction at 4 µM, while an approximate 20% increase was achieved with STF(1-37) at the highest concentration used (8 µM) (**Figure 4C**). Conversely, rtCATH2(1-40) was inactive at the tested concentrations (**Figure 4C**). These findings support the ability of STF(1-37) and TT(1-35) to functionally interact with the host cells, and are consistent with published evidence on similar proliferation-inducing effects on epithelial and fibroblast cells by mammalian cathelicidins [41-44].

Stimulation of phagocytosis in trout leukocytes

The immunomodulatory potential of the CATH peptides was investigated by examining their effects on phagocyte functions, given the central role of these cells in fish immunity [45, 46]. Indeed, phagocytes have been shown to be targets of multiple activities of cathelicidins in mammals [38, 47]. Phagocytosis assays were performed on head kidney leukocytes (HKL) from rainbow trout using pHrodo Green-conjugated *E. coli* (pHrodo-*E. coli*) bioparticles as phagocytic targets. As shown in **Figure 5A**, STF(1-37), TT(1-35) and rtCATH2(1-40), added simultaneously with these particles, enhanced pHrodo-*E. coli* uptake by phagocyte-enriched HKL cultures in a dose-dependent manner. The effect of STF(1-37) and TT(1-35) peaked at 2 µM, with a roughly 30% and 35% increase, respectively, compared to particle uptake in the absence of peptides. A comparable

enhancing effect was observed at 8 µM in the case of rtCATH2(1-40) (**Figure 5A**). LDH release and resazurin-reduction assays conducted in parallel on cells treated with the peptides in the absence of particles did not reveal any toxic effects of the peptides on HKL (**Figure 5B** and data not shown). Interestingly, peptide treatment resulted in 11-22% -increased cellular metabolic activity which may suggest an activating effect of the peptides on HKL (**Figure 5B**).

In intracellular killing assays on HKL infected with live *E. coli*, a reduced number of live bacteria (about 20% decrease) was recovered from cell samples exposed to *E. coli* in the presence of STF(1-37) compared to cells incubated in peptide-free medium (**Figure 5C**). STF(1-37) was selected for these assays and further investigation as the presence of a disulfide bond as constraining element could allow improvement of pharmacological features in view of future applications [48]. The well-known fish immunostimulant β-glucan [49, 50], used for comparison, caused a roughly 33% decrease in bacterial survival in the same experiments (**Figure 5C**). The potentiating effect of STF(1-37) on bacterial clearance was likely independent from direct killing, since the CATH peptides did not display any antibacterial activity under these experimental conditions (**Figure S2**).

The capacity to modulate the uptake of pHrodo-*E. coli* particles was further analyzed in the presence of autologous serum to more closely reproduce the physiological environment, since cathelicidin activities may be affected by serum components [21, 51]. As reported in **Figure 5D**, serum itself increased particle uptake by HKL, consistent with an opsonizing effect of serum components. Although the stimulatory effect of serum varied remarkably among individual fish (data not shown), STF(1-37) showed a comparable potentiating activity on particle uptake in the absence and presence of serum (**Figure 5D** and **E**), supporting the relevance of this effect under *in vivo* conditions.

To further explore the potential for application of STF(1-37) in aquaculture, its effect on phagocytosis was then evaluated in combination with β-glucan, given the growing interest in this immunostimulant in the fish farming industry [49, 50]. As reported in **Figure 5F**, β-glucan by itself produced an approximately 60% increase in pHrodo-*E. coli* internalization at 62.5 µg/mL. The addition of STF(1-37) further enhanced phagocytic uptake compared to cells incubated with β-glucan alone. In this respect, the effect of the combination of 2 and 8 µM peptide with β-glucan was significantly higher as compared to each stimulus alone (**Figure 5F**) and appeared to be greater than additive (see Supplementary **Figure S3** for a comparative analysis of the experimental and calculated combined effect of STF(1-37) and β-glucan).

Effects of AMPs, both from mammals and fish, on the phagocytic process have been described in

514 several reports [39, 51-55]. It is worth noting, however, that these activities are often observed after
515 prolonged preincubation of phagocytes with the peptides before the addition of the phagocytic
516 target [39, 52-54]. Such potentiating effects may thus be ascribed to modulation of the expression of
517 cellular receptors involved in phagocytic uptake [52, 54]. This mechanism however appears
518 unlikely for cells co-stimulated with peptides and bacterial particles, as in this study. Potentiation of
519 phagocytic uptake under similar conditions has been reported in a few studies with cathelicidin or
520 defensin peptides [51, 55, 56], but the underlying mechanisms have yet to be elucidated. The fact
521 that the metabolic activity of HKL increases on incubation with the CATH peptides, as shown in
522 this study (**Figure 5B**) would support a mechanism involving direct activation of phagocytes.

523

524 *Potentiation of respiratory burst activity in trout leukocytes*

525 To further explore the capacity of STF(1-37) to activate fish leukocytes, we investigated its effect
526 on the respiratory burst response as a crucial phagocyte microbicidal function [45, 46]. ROS
527 production was analyzed on freshly-purified HKL stimulated with the peptide alone and in
528 combination with β-glucan or phorbol 12-myristate 13-acetate (PMA) as reference stimuli [57, 58].
529 The kinetics of the respiratory burst responses obtained in representative experiments upon cell
530 stimulation with STF(1-37) at 2 µM and β-glucan or PMA are reported in **Figure 6** (respectively, in
531 panels **6A** and **6C**). Cell stimulation with STF(1-37) alone resulted in a slight ROS increase that
532 peaked at 4 min and dropped to resting levels at approximately 12 min (**Figure 6A** and **6C**).
533 Notably, when the peptide was added in combination with β-glucan or PMA, it markedly modified
534 the cellular response to each co-stimulus. Specifically, the peptide strongly affected the kinetics of
535 the β-glucan-induced response, with a clear potentiating effect at early time points (≤ 8 min)
536 (**Figure 6A**), while in the combination with PMA, besides a similar accelerating effect, it also
537 produced an increase in the maximum intensity of the burst response (**Figure 6C**). Figures **6B** and
538 **6D** show fold-increase values calculated from three independent experiments as the ratio of the
539 effect of the combination of STF(1-37) with β-glucan or PMA to the sum of individual effects of the
540 stimuli at each time point. According to statistical analysis of these data, the effect of the
541 combination with both stimuli at early time points appears to be synergistic rather than additive
542 (fold-increase values of combined *vs* summed effects are significantly higher than one) (**Figure 6B**
543 and **D**).

544 The ability of STF(1-37) to affect the burst response of HKL adds to previous reports on
545 mammalian cathelicidins. In this regard, the human LL-37 has been shown to stimulate ROS

generation in neutrophils by itself [59], and to amplify the burst response to microbial stimuli or PMA in neutrophils and macrophages [51, 60]. In fish, a priming effect on the burst response to a fish pathogen has been recently reported for the ayu cathelicidin following 8-h cell preincubation with the peptide before bacterial challenge [39].

In the present study, the rapid burst response elicited by STF(1-37) itself, that peaked even earlier with respect to the reference stimuli, may indicate rapid interaction of the peptide with cellular components. Although the underlying mechanism has still to be defined, these data further support a modulatory role of salmonid cathelicidins on key phagocyte functions in fish. Despite the modest entity of the effect of STF(1-37) alone, the intracellular events triggered by the peptide may facilitate the cellular response to concomitant stimuli, which may result in enhanced phagocyte responses in complex environments such as those of infection sites.

Conclusions

Overall the data presented in this study point to a role of the salmonid cathelicidins in activation of phagocyte-mediated microbicidal mechanisms rather than as canonical AMPs principally showing direct antimicrobial activity. Salmonid CATHs are characterized by unique, intrinsically disordered sequences, and kill or inhibit bacterial pathogens only in low-salt conditions unlikely to occur physiologically. Given the prominent function of phagocytic leukocytes in fish defense against infection, the capacity of these salmonid CATHs to potentiate bacterial uptake and to boost the burst response makes these molecules attractive candidates as novel immunostimulant anti-infectives for fish. The synergistic effect of STF(1-37) and β-glucan with respect to both phagocytic uptake and respiratory burst activity is particularly appealing, and deserves further investigation in view of potential applications in aquaculture.

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Figure legends

Figure 1. Peptide sequences. Amino acid sequences of the full-length cathelicidin antimicrobial domain from CATH1 and CATH2 of *S. fontinalis*. *S. trutta* and *O. mykiss* (rainbow trout, rt), and peptide fragments selected for synthesis and functional evaluation (showing charged residues). Conserved residues within each type of cathelicidin peptide are highlighted in grey. Gaps were introduced to improve alignments.

Figure 2. Circular dichroism spectra of cathelicidin peptides. The spectra of rtCATH2(1-40) (A), TT(1-35) (B), STF(1-37) (C) and SF(18-45) (D) were recorded in phosphate buffer [——], in buffer plus 50% TFE [- - - -] or buffer plus LUVs (4 mM phospholipid) [·····]. Spectra are the mean from the accumulation of three scans.

Figure 3. Kinetics of permeabilization of *E. coli* ATCC 25922 (A), *S. aureus* ATCC 25923 (B), *Yersinia ruckeri* NCIMB 1315 (C) and *Lactococcus garvieae* ATCC 49156 (D) cells. Analyses have been performed in 5% TSB in SPB. Bacterial cells (1 x 10^6/mL) were incubated with each peptide at 2 µM (for *E. coli*) and 8 µM (for *S. aureus*, *Y. ruckeri* and *L. garvieae*). Tests were performed at 37°C except with *Y. ruckeri* and *L. garvieae* which were tested at 22°C. Data are a mean ± SD of three independent measurements.

Figure 4. Effects on cell membrane integrity and viability on trout cells. A) Hemolysis of trout erythrocytes. Erythrocyte suspensions were incubated for 30 min at 18°C in the presence of 100 µM STF(1-37), TT(1-35) and rtCATH2(1-40), or with 0.2% Triton X-100 to achieve complete lysis. The membrane-active peptides BMAP-27 and SMAP-29, at the indicated concentrations, were used for comparison. Data are expressed as percent hemolysis with respect to samples incubated with Triton X-100, and are the means ± SD of three independent experiments. B) Lactate dehydrogenase (LDH) release. RTG-2 cells were seeded as described in the method section and incubated 60 min in the presence of the indicated peptide concentrations in complete L-15 medium. LDH release in cell culture supernatants was calculated as percent of total LDH cellular activity. Data are the means ± SD of three independent experiments performed in duplicate. C) Cell viability. Cells, seeded as described in the method section, were incubated 72 h in complete L-15 medium in the absence and

773 presence of STF(1-37) (open bars), TT(1-35) (grey bars) and rtCATH2(1-40) (closed bars) at the
774 indicated concentrations. Percent viability relative to untreated cells was determined by the
775 PrestoBlue metabolic assay. The means ± SD of four independent experiments performed in
776 triplicate are reported. Asterisks denote statistical significance *vs* control. * $P < 0.05$, ** $P < 0.01$,
777 *** $P < 0.001$.

778

779 **Figure 5. Effects on phagocytic activity of trout head kidney leukocytes (HKL).** A) Rainbow
780 trout HKL were incubated for 3 h with pHrodo Green -conjugated *E. coli* bioparticles, in the
781 absence or presence of STF(1-37) (open bars), TT(1-35) (grey bars) and rtCATH2(1-40) (closed
782 bars) at the indicated concentrations. Incubations were carried out at 18°C in Live Cell Imaging
783 Solution supplemented with 0.2% (w/v) glucose (LCIS-glu), with simultaneous addition of peptides
784 and particles to cell samples. Phagocytosis in the absence of peptides was set at 100%. B) Metabolic
785 activity of HKL incubated with the peptides as above, in the absence of bioparticles. Data were
786 obtained using the PrestoBlue reagent and are expressed as percent relative to untreated cells. C)
787 The intracellular killing activity of HKL was determined by a gentamicin protection assay. HKL
788 were exposed to live *E. coli* as described in the method section, in the absence and presence of 2
789 µM STF(1-37) or 62.5 µg/mL β-glucan. The numbers of viable bacteria recovered from peptide-
790 and β-glucan -treated cells after a total 3 h incubation are reported as percent survival compared to
791 control HKL samples. D, E) Phagocytosis in the presence of trout serum. HKL samples were co-
792 stimulated with pHrodo-*E. coli* and STF(1-37) in the absence (open bars) and presence (closed bars)
793 of 5% autologous serum as described in the method section. Mean Fluorescence Intensity (MFI)
794 values recorded in one representative experiment out of four are reported in D. Panel E shows the
795 means ± SD of data obtained in the same experiments, normalized to the effect of serum alone
796 (100%). F) Phagocytosis in the presence of β-glucan. HKL were incubated with pHrodo-*E. coli*
797 bioparticles in the absence (open bars) and presence (closed bars) of 62.5 µg/mL β-glucan and the
798 indicated concentrations of STF(1-37). Data are expressed as percent relative to cells incubated with
799 bioparticles only. Data in panels A, B, E, F are the means ± SD of four independent experiments
800 performed at least in duplicate. Panel C, means ± SD of three independent experiments run in
801 triplicate. Asterisks denote statistical significant differences compared to phagocytosis in the
802 absence of stimuli (A, C, E) or between the indicated samples (F). * $P < 0.05$, ** $P < 0.01$, *** $P <$
803 0.001.

804

805 **Figure 6. Effect of STF(1-37) on respiratory burst activity of HKL.** Freshly purified HKL
806 suspended in HBSS-BSA were stimulated with 2 µM STF(1-37), alone and in combination with 125
807 µg/mL β-glucan (A, B) or 0.5 µg/mL PMA (C, D), and with β-glucan or PMA only. ROS production
808 was measured at the indicated time points by a luminol-based chemiluminescence assay. A, C) RLU
809 values recorded in one representative experiment out of three showing similar results are reported.
810 B, D) The effects of the combination of STF(1-37) and stimuli are expressed as fold-increase values
811 calculated as $RLU_{combination}/(RLU_{STF} + RLU_{stimulus})$. Data are means ± SD of three independent
812 experiments conducted in triplicate. ** $P < 0.01$, *** $P < 0.001$ *vs* the sum of individual effects of
813 peptide and β-glucan/PMA.

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Table 1. Principal features of cathelicidin peptides used in this study.

Peptide	MW (Da)	Net charge	pI	% K+R	% G	% S
SF(18-45)	2611.8	+5	12.48	18.5	37	18.5
TT(1-35)	3451.8	+10	12.61	28.6	25.7	25.7
TT(1-24)	2510.8	+9	12.49	37.5	25	25
STF(1-37)	3809.4*	+10	12.22	27	29.7	8.1
rtCATH2(5-40)	3686.1	+9	12.31	27.8	33.3	13.9
rtCATH2(1-40)	4184.7	+11	12.40	30	30	12.5

* oxidised form

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819 **Table 2. Minimum inhibitory concentrations of cathelicidin peptides against different bacteria.**

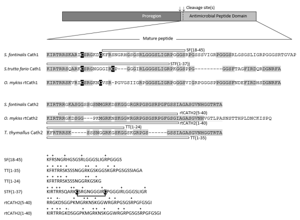
Strains	MIC (μM)*					
	TT (1-24)	TT (1-35)	STF (1-37)	rtCATH2 (5-40)	rtCATH2 (1-40)	SF (18-45)
E. coli ATCC 25922	32	4	2	16	4	16
S. aureus ATCC 25923	64	16-8	16-8	64	16-8	64
A. salmonicida ATCC 33658	32-16	2	8-4	16	8-4	64
Y. ruckeri NCIMB 1315	32	8	16	16	16	32
L. garvieae ATCC 49156	16-8	1	8	N/D	16-8	N/D
V. anguillarum ATCC 43305[§]	>32	>32	>32	N/D	>32	N/D
A. hydrophila ATCC 7966	>32	>32	>32	N/D	>32	N/D

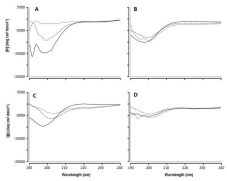
820 * This test was performed in 5% (v/v) TSB in 10 mM SPB pH 7.4. MIC values are representative of three independent experiments giving comparable results.

821 N/D: not determined.

822 [§] With addition of 2% NaCl

823


Cleavage site(s)
Proregion
Antimicrobial Peptide Domain
Mature peptide
SF(18-45)
S. fontinalis Cath1
KIRTRRSKARICSRGKDCKFRSNGRHGSGSRLGGGSLIGRPGGGSRPGSSSVIGRPGGGSRLGSGSLIGRPGGGSRTGVAP
STF(1-37))
S.trutta fario Cath1
KIRTRRSQARKCSRGNGGGIRCP---GGGIRLGGGSLIGRPKGGSPPG---------GGSFTAGFIRDQRDGNRFA
O. mykiss rtCath1
KIRTRRSKVRICSRGKNCVSR-PGVGSIIGRPGGGSLIGRPGGGSVIGRPGGGSPPGGGSFNDEFIRDHSDGNRFA
S. fontinalis Cath2
KIRTRRGKASGGSSGSNMGRKDSKGGGRGRPGSGSRPGFGSSIAGASGVNHGGTRTA
rtCATH2(5-40)
O. mykiss rtCath2
KIRTRRGKDSGG---PKMGRKNSKGGWRGRPGSGSRPGFGSGIAGASGVNHVGTLPASNSTTHPLDNCKISPQ
rtCATH2(1-40)
TT(1-24)
T. thymallus Cath2
KFRTRRSK-----SSSNGGRKGSKGGSKGRPGS------GSSIAGASGVNHGGTRTA
TT(1-35)
SF(18-45)
KFRSNGRHGSGSRLGGGSLIGRPGGGS
TT(1-35)
KFRTRRSKSSSNGGRKGSKGGSKGRPGSGSSIAGA
TT(1-24)
KFRTRRSKSSSNGGRKGSKG
STF(1-37)
KIRTRRSQARKCSRGNGGGIRCPGGGIRLGGGSLIGR
rtCATH2(5-40)
RRGKDSGGPKMGRKNSKGGWRGRPGSGSRPGFGSGI
rtCATH2(1-40)
KIRTRRGKDSGGPKMGRKNSKGGWRGRPGSGSRPGFGSGI



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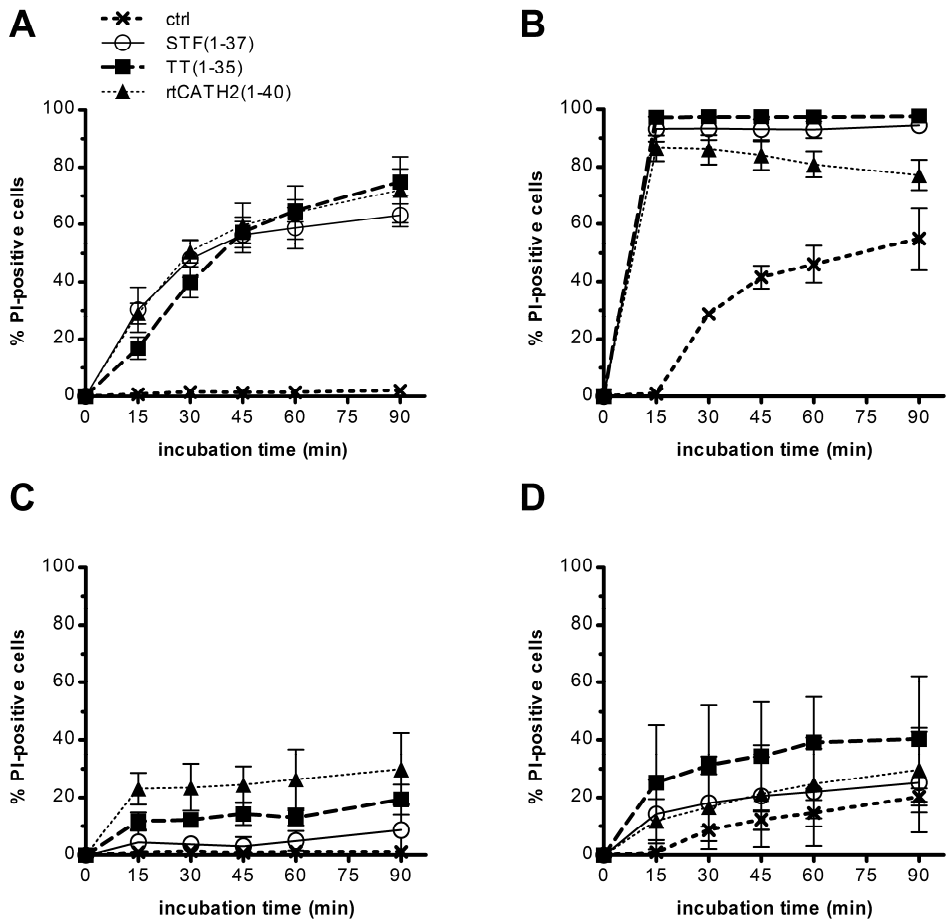
A
ctrl
STF(1-37)
TT(1-35)
rtCATH2(1-40)
% PI-positive cells
incubation time (min)
B
% PI-positive cells
incubation time (min)
C
% PI-positive cells
incubation time (min)
D
% PI-positive cells
incubation time (min)

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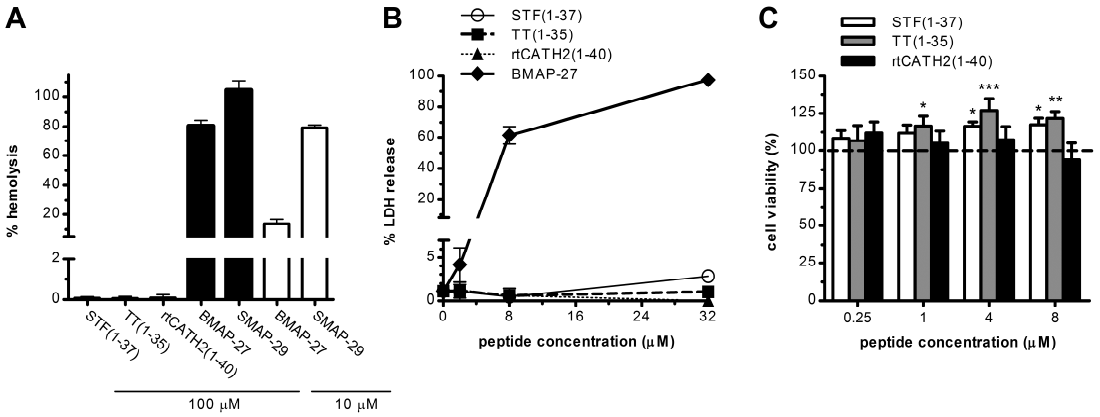

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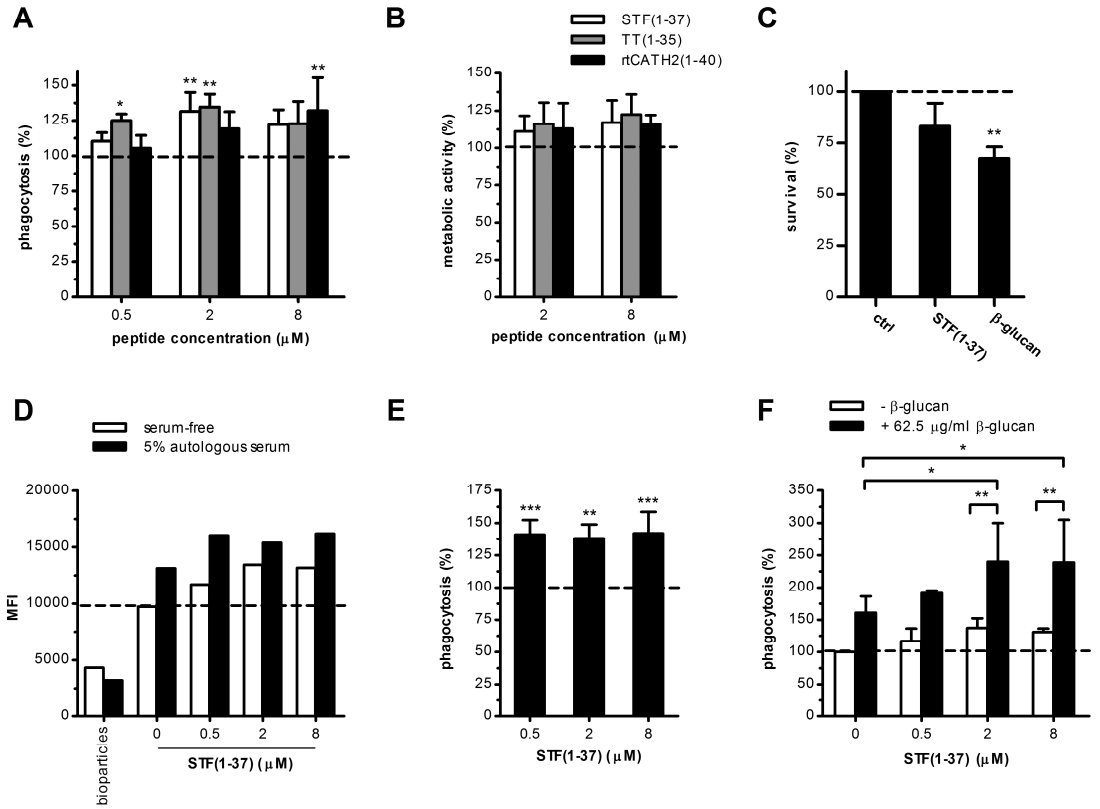

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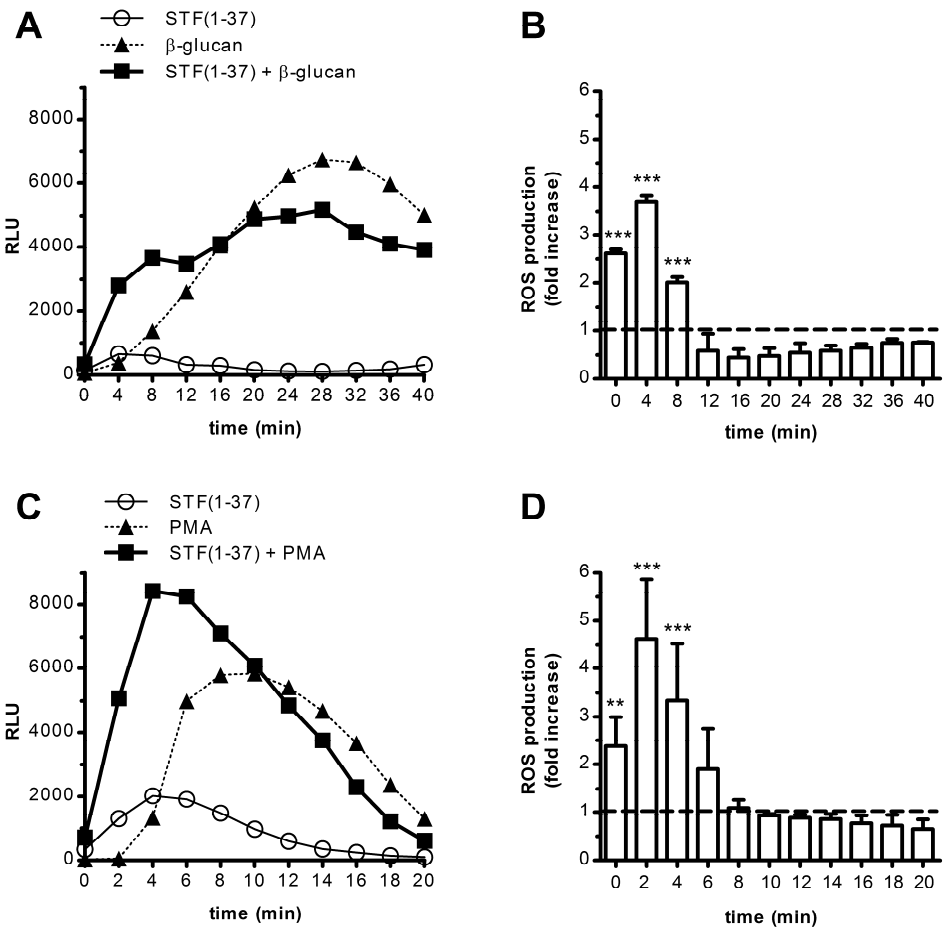

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