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A novel antimicrobial function for a ribosomal peptide from rainbow trout skin☆,☆☆

Jorge M.O. Fernandes and Valerie J. Smith*

Gatty Marine Laboratory, School of Biology, University of St. Andrews, Fife KY16 8LB, UK

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Abstract

An antimicrobial peptide was purified from skin secretions and epithelial cells of rainbow trout by cation exchange and reversed phase chromatography. Partial N-terminal amino acid sequence of the purified peptide revealed 100% identity with the first 11 residues of a 40S ribosomal peptide from medaka fish. Its molecular mass, determined by matrix-associated laser desorption/ionisation mass spectrometry, was found to be 6676.6 Da. These results indicate that this antimicrobial peptide is likely to be the 40S ribosomal protein S30. It is active at submicromolar concentrations, with an effective 50% reduction concentration of 0.02–0.04 μM against *Planococcus citreus*. Thus, in addition to its conventional function in the cell as part of the small ribosomal subunit, this peptide may play a role in protection against intracellular or extracellular pathogens.

Keywords: Antimicrobial; Rainbow trout; Ribosomal protein; Mucosal immunity

Gene-encoded antimicrobial peptides are crucial components of the innate immune system. Their broad-spectrum antibacterial activity, allied to their strategic location in phagocytes or epithelial surfaces, makes them central effector molecules of innate immunity [1]. Antimicrobial peptides are expressed by numerous organisms throughout the animal and plant kingdoms, although the majority of reports are from insects, amphibians, and mammals. Many have been isolated from mucosal surfaces [2–4] and are likely to be especially important epithelial defence factors for aquatic animals because these surfaces are in constant contact with an environment crowded with a wide variety of opportunists or potential pathogens.

Relatively few antimicrobial peptides have been purified from mucosa of aquatic organisms, particularly teleosts. Nevertheless, some of the teleost peptides are unusual in being peptides or protein fragments with known function, not previously directly associated with immunity [5]. For instance, parasins are potent antimicrobial peptides derived from catfish histone H2A [6].

With regard to salmonids, antibacterial activity has been detected in skin exudates of rainbow trout (*Oncorhynchus mykiss*) [7] and two antimicrobial peptides have been isolated: oncorhyncin a 3 kDa peptide from rainbow trout [7], and HSDF, a 26-residue fragment of histone H1 from coho salmon (*Oncorhynchus kisutch*) [8]. We have also recently purified and characterised a histone H2A-like protein with antimicrobial activity from skin of *O. mykiss* (Fernandes et al., unpublished). Evidence for the presence of at least one additional antimicrobial factor was obtained during that study. In the present investigation, we describe the purification and characterisation of this additional factor.

☆ Protein sequence data herein reported are available in the Swiss-Prot database under the Accession No. P83328.

☆☆ *Abbreviations used:* ATCC, American Type Culture Collection; BLAST, Basic Local Alignment Search Tool; Cfu, colony forming units; EC_{50}, effective concentration, 50% reduction; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HPLC, high performance liquid chromatography; MALDI-TOF MS, matrix-associated laser desorption/ionisation time-of-flight mass spectrometry; MBC, minimal bactericidal concentration; MHB, Mueller–Hinton broth; NCIMB, National Collections of Industrial, Food, and Marine Bacteria; OD, optical density; Rp, ribosomal protein; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid.

* Corresponding author. Fax: +44-1334-463443.
E-mail address: vjs1@st-and.ac.uk (V.J. Smith).

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Materials and methods

Animals. Adult female rainbow trout, 400–500 g in weight, were obtained from College Mill Trout Farm (Almondbank, Perthshire, UK). They were maintained in open circulation freshwater tanks (14 ± 1 °C) and fed daily with an amount of Dynamic Red M trout pellets (Ewos, West Lothian, UK) equivalent to 1% (w/w) of their body weight.

Preparation of skin mucus and epidermal cell extracts. Mucous skin secretions and associated epidermal cells were collected from the dorso-lateral surfaces of 10 freshly sacrificed fish 4 h after gentle surface stimulation with ultrafine sandpaper to enhance mucus secretion without causing visible bleeding. A total of ca. 150 ml mucus was homogenised 1:4 (v/v) in a solution of 50% (v/v) ethanol (Merck, Dorset, UK), 3.3% (v/v) trifluoroacetic acid (Sigma, Dorset, UK), and 2% (v/v) general-use protease inhibitor cocktail (Sigma), containing 4-(2-aminoethyl) benzenesulphonyl fluoride trans-epoxysuccinyl-L-leucylamido (4-guanidino) butane, bestatin, leupeptin, aprotinin, and sodium EDTA. The homogenate was then centrifuged at 29,000*g* for 60 min at 4 °C and the supernatant was lyophilised. The dried material was resuspended in 100 ml of 20 mM Hepes (Acros, Leicestershire, UK) and neutralised with 5 M NaOH (BDH, Dorset, UK) before final centrifugation at 29,000*g* for 30 min at 4 °C.

Microorganisms. The following bacterial strains were used: *Bacillus subtilis* (ATCC 6051), *Escherichia coli* (NCIMB 12210), *Listonella* (*Vibrio*) *anguillarum* (NCIMB 2129), and *Planococcus citreus* (NCIMB 1493). All were grown to logarithmic phase in Mueller–Hinton broth (MHB) (Oxoid, Basingstoke, UK) at the appropriate temperature (20 °C for *L. anguillarum* and *P. citreus*; 37 °C for *B. subtilis* and *E. coli*).

Antibacterial activity. Antibacterial activity was assessed using a modified version of the two-layer radial diffusion assay of Lehrer et al. [9] as described by Smith et al. [7], using the Gram-(+) bacterium, *P. citreus*, as the test organism during the protein purification procedure.

Determination of the effective 50% reduction concentration (EC_{50}) of the purified peptide against each of the bacteria listed above was performed using the microtitre broth dilution assay [10]. The peptides used were quantified by amino acid analysis as described below. One hundred microlitres of each bacterial suspension containing 10^5 colony forming units (cfu) per ml was mixed with serial twofold dilutions of test peptide in 0.2% (w/v) bovine serum albumin (Sigma), 0.01% (v/v) acetic acid (BDH) in sterile polypropylene 96-well microtitre plates (Corning Costar, Cambridge, UK). The positive control well contained bacteria and diluent only. The plates were incubated at the appropriate temperature for each bacterium (above) and the optical density (OD) was read at 570 nm using an MRX II microtitre plate reader (Dynex, West Sussex, UK) against a blank comprising diluent only. Values for experimental wells were recorded when the OD reached 0.2 in the positive control well. The EC_{50} was considered to be the lowest concentration of protein that reduced the growth by 50% relative to the control. The minimal bactericidal concentration (MBC) was obtained by plating out the contents of each well showing no visible growth. MBC was taken as the lowest concentration of protein that prevents any residual colony formation after incubation for 24 h at the appropriate temperature. Cecropin P1 (Sigma) was used as reference.

Muramidase activity. Muramidase activity was tested by radial diffusion assay as described in Smith et al. [7].

Peptide purification. The reconstituted lyophilised extract was fractionated by cation exchange chromatography on a CM Macro-Prep 1 cm × 10 cm Econo-column (Bio-Rad), previously equilibrated with 20 mM Hepes (Acros, Leicestershire, UK), 0.1 M NaCl, pH 7.0 (buffer A). Elution was performed with a linear AB gradient (where B is 20 mM Hepes, 1 M NaCl, pH 7.0) over 90 min, followed by 35 min of B, at a flow rate of 1 ml min^{-1}. Fractions eluting between 80% and 100% B were pooled and concentrated by solid-phase extraction using Sep-Pak Vac 5g $^tC_{18}$ cartridges (Waters, Herts, UK) equilibrated 0.15% (v/v) TFA in water. Two successive stepwise elutions were performed with 20 ml of 20% (v/v) and 70% (v/v) acetonitrile (BDH) in acidified water. The latter fraction was lyophilised, resuspended in acidified deionised water (Elga, Bucks, UK), and applied to an ODS2-Inertpak C_{18} reversed-phase HPLC column (particle size 5 μm, 4.6 mm × 250 mm, Capital HPLC, Broxburn, UK). Elution was executed at 25 °C with a biphasic gradient of 0.1% (v/v) TFA in water (solvent A) and 0.09% (v/v) TFA in acetonitrile (solvent B) at a flow rate of 1 ml min^{-1} as follows: wash with 100% A for 5 min, 0–20% B over 10 min, 20% B for 5 min, 20–60% B over 45 min, and 60% B for 5 min. Active fractions eluting between 25 and 40 min were lyophilised, reconstituted in 1 ml acidified water, and purified further by reversed-phase HPLC on the same column but under a shallower gradient (0–30% acetonitrile over 75 min at a flow rate of 1 ml min^{-1}). At each step, the purity and molecular weight of the proteins were estimated by high resolution polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS–PAGE) using a 16% separating gel, 14% spacer gel, and 5% stacking gel [11].

Protein quantification. Total protein was estimated by the method of Bradford [12] using bovine serum albumin (Pierce, Rockford, USA) as standard. Amino acid analysis of the purified peptide was obtained as a service from the Protein and Nucleic Acid Chemistry facility (University of Cambridge, UK) using the post-column ninhydrin method.

Matrix-associated laser desorptionlionisation time-of-flight mass spectrometry (MALDI-TOF MS). The molecular mass of the purified antimicrobial peptide was determined by MALDI-TOF MS at the Centre for Biomolecular Sciences (University of St. Andrews, UK) using a TofSpec 2E instrument (Micromass, Manchester, UK) operated in linear mode.

Partial primary structure determination. N-terminal amino acid sequencing was performed by standard automated Edman degradation on a Procise Sequencer (Applied BioSystems, Warrington, UK).

Sequence analysis. Homology searches against the SwissProt, NR, and Month databases were performed by the Basic Local Alignment Search Tool [13] at the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST). The Expert Protein Analysis System (ExPASy) proteomics server of the Swiss Institute of Bioinformatics (http://www.expasy.ch/) was used to predict amino acid compositions, protein masses, and isoelectric points. Sequence alignments were performed with the Omiga 2.0 sequence analysis software (Oxford Molecular/Accelrys, Cambridge, UK), employing the Clustal W 1.6 algorithm [14].

Proteolytic digestion. Susceptibility of the antimicrobial factor to proteolytic digestion was determined by incubation with 60 μg ml^{-1} (final concentration) Proteinase K (Sigma) for 60 min at 37 °C, followed by radial diffusion assay against *P. citreus*, as described above.

Results

The active fractions eluting between 0.8 and 1.0 M NaCl by cation exchange chromatography were concentrated by solid-phase extraction and subsequently subjected to C_{18} reversed-phase HPLC, resulting in two groups of active fractions. The fractions eluting between 24% and 38% acetonitrile were lyophilised, reconstituted in acidified deionised water, and re-chromatographed on the same column using a shallower water/acetonitrile gradient. Antibacterial activity against the test bacterium, *P. citreus*, was recovered in a single fraction coinciding with a peak eluting at 60.1 min (Fig. 1). Activity was abolished after proteolytic digestion with proteinase K, confirming that this antibacterial factor

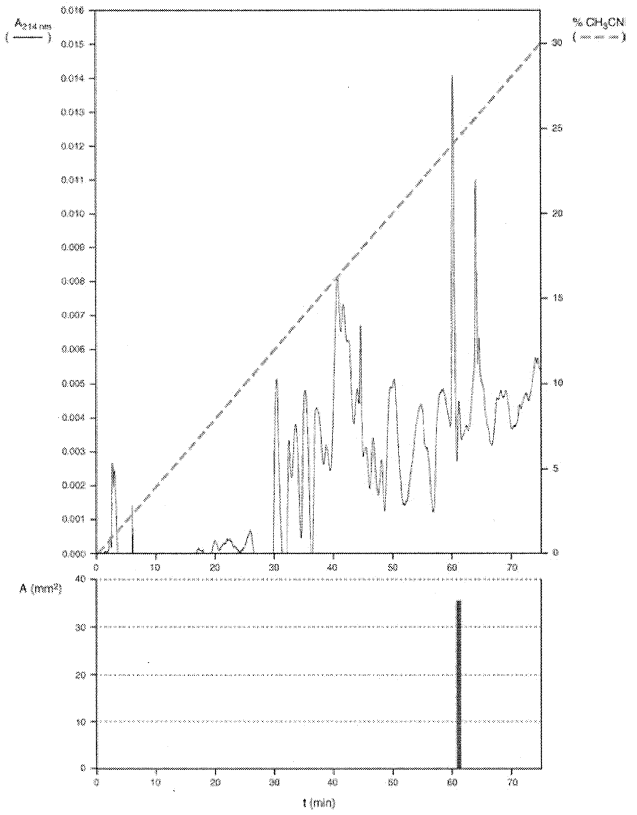

Fig. 1. Purification of an antimicrobial peptide from trout skin extracts by C_{18} reversed-phase chromatography. Acidic protein extracts of skin mucus and epidermal cells were fractionated by cation exchange chromatography on a CM Macro-Prep column and the active fractions were subjected to solid-phase extraction using $^{t}C_{18}$ Sep-Pak cartridges; the 70% acetonitrile eluate was then applied to a C_{18} reversed-phase column for HPLC. Fractions eluting between 24.4% and 37.8% acetonitrile were concentrated by lyophilisation, reconstituted in acidified water, and subjected to another C_{18} reversed-phase HPLC using a shallower water/acetonitrile gradient, as indicated by the dotted line. The solid line represents the absorbance, monitored at 214 nm. The peak eluting at 60.1 min was found to be antibacterial to *P. citreus* (histogram).

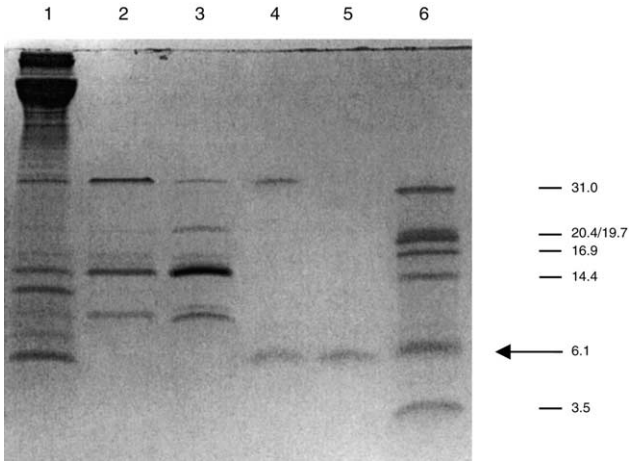

Fig. 2. Protein profile of the active fractions determined by Tris–Tricine SDS–PAGE analysis. Lane 1: crude extract; lane 2: pooled cation exchange fractions; lane 3: 70% acetonitrile eluate from solid-phase extraction; lane 4: active fractions after the first C_{18} reversed-phase HPLC; lane 5: purified antimicrobial peptide after second HPLC; lane 6: markers. Each lane contains 7.5 μl sample. The numbers on the right-hand side represent the molecular mass of the markers in kilodaltons. Peptide of interest in lane 5 is indicated by an arrow.

has a proteinaceous nature. Only one protein band with an electrophoretic mobility of ca. 6.5 kDa could be observed on a sodium dodecyl sulphate–polyacrylamide gel after silver staining (Fig. 2). The yield of purified peptide, estimated by amino acid analysis, was 2 ng g^{-1} mucus.

Partial N-terminal amino acid sequencing yielded the following sequence: KVHGSLARAGK. BLAST homology searches showed that the first 11 amino acids of the purified peptide are an exact match to those of 40S ribosomal protein S30 (40S Rp S30) from medaka fish *Oryzias latipes* (Fig. 3). The matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrum showed a single signal at 6676.6 Da (Fig. 4).

Quantification of activity using the microtitre broth dilution method revealed that it is active at submicromolar concentrations, with an EC_{50} of 0.02–0.04 μM and an MBC of 0.08 μM against *P. citreus* (Fig. 5, Table 1). It was not active against Gram-(−) bacteria at the concentrations tested (maximum 0.3 μM, Table 1) and it did not display muramidase activity.

Discussion

The present study describes the purification and partial characterisation of a cationic antimicrobial peptide from skin exudates and associated epidermal cells of rainbow trout, collected following gentle surface abrasion. It is unlikely that this peptide was derived from blood contamination, as great care was taken during sample collection to avoid surface bleeding. However, it may have been derived from lysed epithelial cells, a question currently under study.

The peptide displays potent antibacterial activity against the Gram-(+) bacteria tested, with an EC_{50} up to 60 times lesser than that of cecropin P1. The full identity between its partial primary structure and that of 40S Rp S30 from medaka fish indicates that the antimicrobial peptide purified from trout is identical or highly homologous to 40S Rp S30, a component of the small subunit of eukaryotic ribosomes. This finding is further supported by mass spectrometry analysis, which showed that this peptide's molecular mass (6676.6 Da) is very similar to that of 40S Rp S30 from medaka fish (6660 Da) [15]. Moreover, preliminary experiments performed in our laboratory have identified antibacterial activity in ribonucleoprotein particles obtained from liver cells of rainbow trout (data not shown) and we are

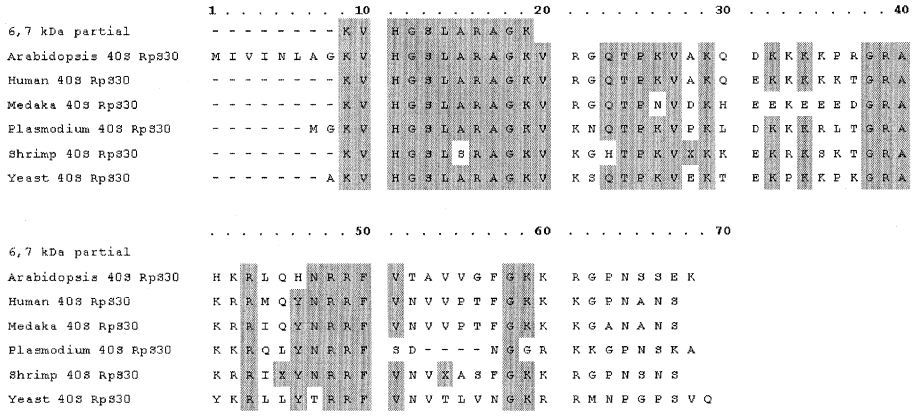

Fig. 3. Partial N-terminal sequence of the 6.7 kDa antimicrobial peptide purified from skin of rainbow trout and its homology with 40S ribosomal protein S30. Identical residues between the purified antimicrobial peptide and the deduced sequences for the 40S ribosomal protein S30 from *Arabidopsis thaliana* (Swiss-Prot P49689), human (Swiss-Prot Q05472), medaka fish (Swiss-Prot Q9W6Y0), *Plasmodium falciparum* (Swiss-Prot O96269), Atlantic white shrimp (GenBank BF024580), and *Saccharomyces cerevisiae* (Swiss-Prot Q12087) are shaded.

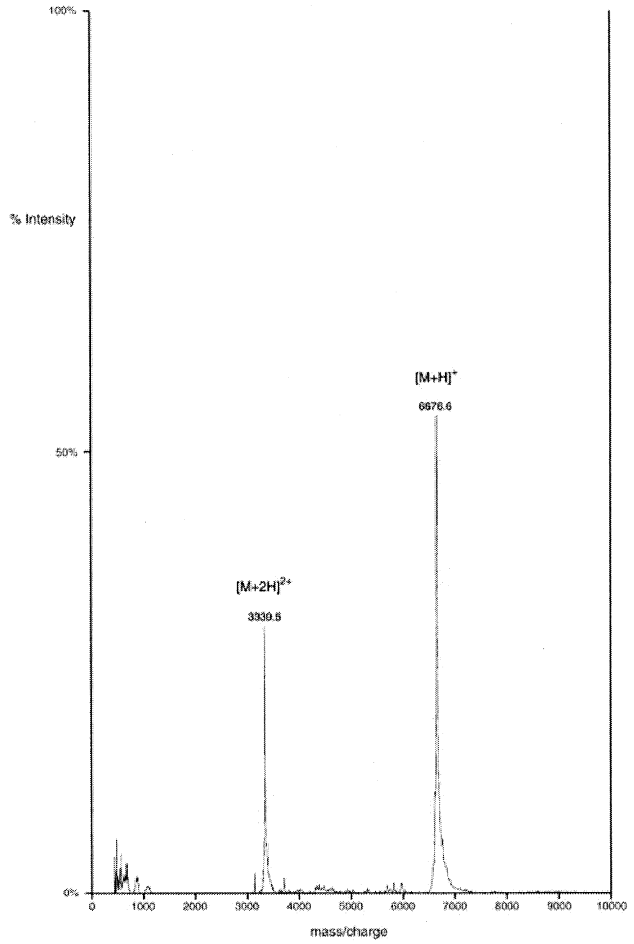

Fig. 4. Mass spectrum of the antibacterial peptide purified from trout skin exudates, determined by MALDI-TOF. The single ($[M+H]^+$) and double charged ($[M+2H]^{2+}$) molecular ions are labelled.

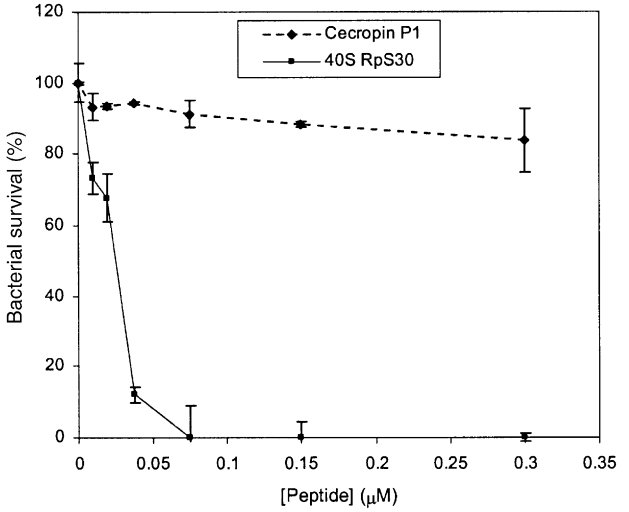

Fig. 5. Antibacterial activity of the purified antimicrobial peptide and cecropin P1 against *P. citreus*. Bacterial suspensions containing 10^5 *P. citreus* cfu ml^{-1} were incubated at 20 °C for 18 h with serially diluted antimicrobial peptide. Bacterial survival is expressed as the ratio of optical densities read at 570 nm (OD_{570}) between each test sample and the control (no peptide added). The average final OD_{570} of the control was 0.18. Data are represented as means ± SE, $n = 3$.

currently using these ribosomes to isolate the 6.7 kDa antibacterial peptide for further biochemical characterisation. To the best of our knowledge antimicrobial activity of ribosomal proteins from a non-mammalian animal has not been previously described.

Previous reports have shown that cecropin-like peptides found in *Helicobacter pylori* actually originate from the N-terminal region of the Rp L1 [16], indicating that cecropins may have evolved from Rp L1 of an ancestral intracellular pathogen [17]. The involvement of ribosomal proteins in innate immunity is corroborated by the recent finding of ubiquicidin in the cytosolic fraction of IFN-γ activated murine macrophages [18]. In this paper, Hiemstra et al. have shown that the antimicrobial peptide is likely to be Rp S30. The present finding of Rp S30 in the skin of an ecto-

Table 1
Bacteriostatic (EC_{50}, μM) and bactericidal (MBC, μM) activities of the purified antimicrobial peptide 40S Rp S30. Cecropin P1 was used as standard

Bacterium	Gram Staining	40S Rp S30		Cecropin P1	
		EC_{50}	MBC	EC_{50}	MBC
B. subtilis	+	0.15–0.3	>0.3	>2.4	>2.4
P. citreus	+	0.02–0.04	0.08	1.2–2.4	>2.4
E. coli	−	>0.3	>0.3	0.15–0.3	0.6
L. anguillarum	−	>0.3	>0.3	0.04–0.08	0.3

thermic lower vertebrate demonstrates that it is confined neither to mammals nor to peripheral leukocytes. As shown in Fig. 3, Rp S30 appears to be conserved throughout widely separated taxa, posing questions on its universal role in protection against intracellular or extracellular microbial infection. Possibly, it may act extracellularly following cell lysis or apoptosis. Importantly, this is one of the differentially expressed genes in immunostimulated Atlantic white shrimp *Litopenaeus setiferus* [19], a result that lends further credence to the potential role of Rp S30 as an antimicrobial defence factor.

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