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Innate Immune Responses of a Scleractinian Coral to Vibriosis

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Abstract :

Scleractinian corals are the most basal eumetazoan taxon and provide the biological and physical framework for coral reefs, which are among the most diverse of all ecosystems. Over the past three decades and coincident with climate change, these phototrophic symbiotic organisms have been subject to increasingly frequent and severe diseases, which are now geographically widespread and a major threat to these ecosystems. Although coral immunity has been the subject of increasing study, the available information remains fragmentary, especially with respect to coral antimicrobial responses. In this study, we characterized damicornin from *Pocillopora damicornis*, the first scleractinian antimicrobial peptide (AMP) to be reported. We found that its precursor has a segmented organization comprising a signal peptide, an acidic proregion, and the C-terminal AMP. The 40-residue AMP is cationic, C-terminally amidated, and characterized by the presence of six cysteine molecules joined by three intramolecular disulfide bridges. Its cysteine array is common to another AMP and toxins from cnidarians; this suggests a common ancestor, as has been proposed for AMPs and toxins from arthropods. Damicornin was active *in vitro* against Gram-positive bacteria and the fungus *Fusarium oxysporum*. Damicornin expression was studied using a combination of immunohistochemistry, reverse phase HPLC, and quantitative RT-PCR. Our data show that damicornin is constitutively transcribed in ectodermal granular cells, where it is stored, and further released in response to nonpathogenic immune challenge. Damicornin gene expression was repressed by the coral pathogen *Vibrio coralliilyticus*. This is the first evidence of AMP gene repression in a host-*Vibrio* interaction.

Keywords : Antimicrobial Peptides, Bacteria, Defensins, Immunology, Innate Immunity, Invertebrate Species, Cnidaria, Host-Vibrio Interaction, Microbiology

1 Introduction

Scleractinian corals are the biological, ecological and physical framework of tropical coral reefs, which are amongst the most diverse ecosystems on earth. Tropical coral reef ecosystems commonly occur adjacent to developing countries, and support major industries including food production, tourism and biotechnology development. However, with global change, natural disturbances and anthropogenic pressures that are increasing in frequency and severity (1-6), coral reefs are endangered. The reasons of this alarming status are multiple and include increasing water temperature, which disrupts the symbiosis and leads to coral bleaching, and anthropogenic pressures such as overfishing that leads to ecosystem disequilibrium. Amongst impacts on coral reefs, the incidence of coral disease appears to be increasing in frequency and severity (1,7). This phenomenon appears to be aggravated by global warming, and it has been suggested that high temperatures influence the outcome of bacterial infections by lowering the resistance of the coral to disease, and/or increasing pathogen growth, infectivity or virulence (8,9). Increased

virulence has been demonstrated in the bacterium *Vibrio coralliilyticus*, where it leads to bleaching and tissue lysis in *Pocillopora damicornis* (10), and in *V. shiloi*, which is the causative agent of bleaching in *Oculina patagonica* (11). It has been shown that an increase in temperature triggers bacteria adhesion, and toxin and enzyme production (12,13).

Although the central role of *Vibrio* species in several coral diseases has been widely documented, knowledge of the effects of *Vibrio* infection on coral physiology/immunity is rudimentary. One reason is the paucity of information on coral immunology, particularly with respect to defenses against infectious agents (1,14). As for all invertebrates, coral immunity is thought to rely on innate mechanisms involving pattern recognition receptors, and cellular and humoral responses directed against infectious agents (14-22).

There is virtually no information on the antimicrobial response of scleractinians. However, several recent studies have suggested the involvement of antibacterial agents. Thus, the mucus of several species of scleractinians has been shown to have antibacterial properties (23-26), and in a recent study on the transcriptomic response of *P. damicornis* to its specific pathogenic bacterium *V. coralliilyticus* (27), we identified an mRNA corresponding to a putative antimicrobial peptide (AMP).

We describe here the isolation and characterization of damicornin, the first AMP reported from a scleractinian coral. We report the structure of the damicornin precursor, its localization in coral tissues, its antimicrobial spectrum against a panel of microorganisms including its specific pathogenic bacterium *V. coralliilyticus*, and its expression in corals confronted with virulent and avirulent bacteria. Our results show that: (i) damicornin has a cysteine array common to other cnidarian AMPs and toxins; (ii) damicornin is expressed and released from coral ectodermal cells exposed to a non-pathogenic stimulus; and (iii) the gene for expression of damicornin is repressed concomitantly with the invasion of host ectodermal cells by the coral pathogen *V. coralliilyticus*.

EXPERIMENTAL PROCEDURES

Biological material

The *Pocillopora damicornis* (Linnaeus, 1758) isolate used in this study was collected from Lombock, Indonesia (CITES number: 06832/VI/SATS/LN/2001), propagated and maintained in aquaria, as described previously (22).

The filamentous fungus *Fusarium oxysporum* and strains of the Gram positive bacteria *Micrococcus luteus* (A270), *Bacillus megaterium* (IBMC), *Staphylococcus aureus* (SG511), *Brevibacterium stationis* (CIP 101282), *Microbacterium maritypicum* (CIP 105733T), and the Gram negative bacteria *Escherichia coli* (SBS 363), *V. aesturianus* (CIP 109791) and *V. splendidus* (CIP 107715) were the same as used in a previous study (28). *V. shiloi* (CIP 107136), and *V. coralliilyticus* strainYB1 (CIP 107925) were obtained from the Pasteur Institute (Collection de l'institut Pasteur). *V. coralliilyticus* was used in biotic stress and infection experiments with *P. damicornis* (29). For routine use *V. coralliilyticus* was cultured in 2216 Marine Broth medium (BD-DIFCO 279110) at 30°C under aerobic conditions with shaking (150 rpm). During experimental procedures (see below) it was used at the ambient coral maintenance temperature.

Experiments to determine which cells (host or symbiont) expressed the candidate genes involved the use of three zooxanthellae isolates; the origin of and culture conditions for the zooxanthellae have been reported elsewhere (22).

Stress protocol

The experiments were designed to investigate coral responses to bacterial challenge (bacterial stress and bacterial infection). Bacterial stress was induced by the addition of *V. coralliilyticus* at 25°C, whereas bacterial infection was induced by the addition of *V. coralliilyticus* under conditions of increasing water temperature (from 25°C to 32.5°C), which activated bacterial virulence. We recently reported that the bacterium becomes virulent at a temperature of 28°C (27).

Bacterial stress and infection treatments, and appropriate controls, were established in four separate 120 L tanks as follows: i) *V. coralliilyticus* added at a constant temperature of 25°C (Cb); ii) *V. coralliilyticus* added with a gradual temperature increase from 25°C to 32.5°C (Tb); iii) a constant temperature of 25°C without bacteria added (C); and iv) a gradual temperature increase from 25°C to 32.5°C in the absence of added bacteria (T). Nubbins of *P.*

damicornis (fixed piece of coral of approximately 10 g) were randomly placed in each experimental tank (n = 40 per tank) and acclimatized at 25°C for 2 weeks. Bacteria were added to the Cb and Tb treatment tanks every 3 days by balneation (12). Briefly, this involved washing the bacteria twice in filtered sea water (0.22 µm), and adding the washed cells to the tank to achieve a concentration of 10^3 cells/mL of tank water. Water circulation ensured the homogenous distribution of bacteria in the tank. The cultures of *V. coralliilyticus* were grown at 25°C for the Cb treatment, and at the temperature corresponding to that of the tank for Tb. For the Tb treatment and the T control the temperature was increased by 1.5°C every 3 days, beginning on day 3 (D3), until it reached 32.5°C. Three *P. damicornis* nubbins were randomly sampled from each tank every 3 days (D0, D3, D9, D12, D15, D18).

The tank temperature was controlled using an aquarium heater (600 W, Schego) connected to an electronic thermostat (Hobby Biotherm Professional). Illumination was supplied at an irradiance of 250 µmol photon/m^2/s (measured using a quantum meter; QMSW-SS, Apogee Instruments Inc.) using metal halide lamps (Iwasaki 6500 Kelvin, 400 W) set to a 12:12 h light:dark photoperiod. All other seawater parameters were held constant over time in the tanks. A water pump (IDRA, 1300 L/h) continuously recycled the tank seawater at a rate of 10.8 tank volumes/h, passing it through a biological filter and an Aquavie protein skimmer (EPS 600). A proportion of the tank water (2%) was replaced each day with natural filtered Mediterranean seawater heated to 25°C. To avoid the growth of bacterial blooms the water was continuously treated using a UVC filter (JBL: AquaCristal Series II, 5 W). At each time of addition of *V. coralliilyticus*, all equipment known to remove or kill bacteria (the protein skimmer and the UVC filter) was inactivated for 4 h to allow the bacteria to adhere to the coral tissues.

RNA extraction and complete open reading frame characterization of the putative AMP

RACE (rapid amplification of cDNA ends)-PCR experiments were performed to characterize the complete open reading frame (ORF) of the putative AMP. Tissue extraction, total RNA extraction, polyA+ purification and RACE-PCR were conducted using non-challenged *P. damicornis* nubbin, as described previously (22).

Quantitative RT-PCR analyses

Quantitative RT-PCR (q-RT-PCR) was used to analyze the expression profile of the putative AMP. Total RNA was extracted, and 2.5 µg was reverse transcribed using oligo dT primers and the Superscript II enzyme (Invitrogen). The resulting cDNA products were purified using a Nucleotrap Gel Extraction Trial kit (Clontech), and q-RT-PCR was performed using 2.5 µL of purified cDNA (diluted 1:50 in water) in a total volume of 10 µL containing 1× LightCycler® 480 SYBR Green I Master Mix (Roche Diagnostics) and 70 nM of each primer. The primers, which were designed using the Light Cycler Probe Design Software, version 1.0 (Roche Diagnostics), are shown in Table 1. Amplification was performed using a LightCycler 480 System (Roche Diagnostics) and the following reaction conditions: activation of the Thermo-Start® DNA polymerase at 95°C for 15 min, followed by 45 cycles of denaturation at 95°C for 30 s and annealing/extension at 60°C for 1 min. Melting curve profiles were assayed to ensure that a single product was amplified. Each run included a positive cDNA control that was sampled at the beginning of the experiment (D0) and also from each amplification plate; this positive control was also used as the calibrator sample, and blank controls (water) were included for each primer pair. The PCR products were resolved by electrophoresis, the bands were isolated directly from agarose gels, and DNA was extracted using the Gel Extraction PCR Purification System V (Promega). The resulting q-RT-PCR products were single-pass sequenced as described above.

For each reaction, the crossing point (C_p) was determined using the second derivative max method applied by the LightCycler Software, version 3.3 (Roche Diagnostics). The PCR efficiency (*E*) of each primer pair was calculated by determining the slope of standard curves obtained from serial dilution analysis of the cDNAs pooled from all experimental samples, as previously described (30). The individual q-RT-PCR efficiencies for the target or reference genes were calculated using the formula: $E = 10^{(-1/\text{slope})}$. The transcription level of the putative AMP was normalized using the mean geometric transcription rate of three reference genes encoding ribosomal proteins, obtained from *P. damicornis*: 60S ribosomal protein L22, 60S

ribosomal protein L40A and 60S acidic ribosomal phosphoprotein P0 (GenBank: HO112261, HO112283 and HO112666, respectively). The stable expression status of these 3 genes under non-stress, thermal stress, bacterial stress and bacterial infection conditions was recently demonstrated (27). The normalized relative quantities (NRQs) were calculated as previously described (31), using the equation:

$$NRQ = \frac{E_{\text{target}}^{\Delta C_p,\text{target}}}{\sqrt[3]{\prod_{i=1}^{3} E_{\text{ref}_i}^{\Delta C_p,\text{ref}_i}}}$$

where E_{target} is the amplification efficiency of the gene of interest; E_{ref} is the amplification efficiency of the reference gene; ΔCp, ref = Cp_{ref}(calibrator) – Cp_{ref}(sample); and ΔCp, target = Cp_{target}(calibrator) – Cp_{target}(sample).

Identification of the organism expressing the putative AMP

To determine which organism (host or symbiont) expressed the putative AMP gene, cross-PCR experiments were performed on DNA and RNA extracted from the holobiont (host plus symbiont) and from pure cultured zooxanthellae, as described previously (22). Briefly, PCR amplifications were performed with oligonucleotides amplifying the AMP, housekeeping genes, the gene encoding the small ribosomal subunit RNA of *Symbiodinium* spp. (32), and the cDNA encoding the major basic nuclear protein (GenBank accession number: HO112459) of *Symbiodinium* spp. (Table 1).

Production of synthetic peptide and antibodies

The putative peptide (ACADLRGKTFCRLFKSYCDKKGIRGRLMR DKCS-YSCGCR-NH2) was chemically synthesized (5 mg; GENEPEP, Saint-Clément de Rivière, France) in a C-terminally amidated form and the folding of the three disulphide bonds was performed. The HPLC purity of the peptide was 96%.

The synthetic peptide was used to assess the antimicrobial activity of the putative AMP, and to immunize New Zealand rabbits, as described previously (33). Serum from non-immunized and immunized rabbits was collected 70 days after initial injection, and tested for the presence of specific Igs (antibody or IgG) using ELISA (34) with uncoupled synthetic peptide adsorbed onto maxiSorp™ plates (Nunc). The IgG fraction was purified using affinity chromatography (35), and antibody specificity was tested by Western blot. Briefly, coral proteins and the synthetic peptide were subjected to Tris–tricine (16.5%) gel electrophoresis and electro-blotted onto a PVDF membrane. To verify the specificity, blots were probed with pre-immune and purified immune sera at a dilution of 1:1000. The remainder of the procedure was performed as described previously (36).

Immunolocalization experiments

Tissues of *P. damicornis* from unstressed coral colonies, or from coral colonies stressed with non-virulent bacteria, were processed following a procedure described elsewhere (37). Thin sections (7 µm) of tissues embedded in Paraplast were cut, mounted on silane-coated glass slides, and stored at 4°C in a dry atmosphere. The paraffin was removed from the sections, which were incubated for 1 h at room temperature (RT) in a saturating medium containing 2% BSA, 0.2% teleostean gelatin, 0.05% Tween 20 and 0.5% donkey serum in 0.05 mol/L phosphate-buffered saline (PBS) at pH 7.4. The sections were then incubated overnight at 4°C in a moist chamber with the purified antibodies raised against synthetic damicornin (85 µg/mL in saturating medium), or with the depleted purified antibodies raised against synthetic damicornin (depletion was performed by pre-incubating the purified antibodies for 1 h at RT with the synthetic damicornin). Excess antibodies were removed by repeated rinsing, and the sections were then incubated for 1 h at RT with biotinylated anti-rabbit antibodies (secondary antibodies; Amersham RPN1004) diluted 1:250 in the saturating medium. After incubation the sections were rinsed with PBS (pH 7.4), and stained for 15 min with streptavidin AlexaFluor 568 (Molecular Probes, S11226) diluted 1:50 in PBS and 4′,6-diamino-2-phenylindole (DAPI; Sigma D9542; 2 µg/mL). The sections were mounted in Pro-Long antifade medium (Molecular Probes, P7481) and observed using a confocal laser-scanning microscope (Leica, TCSSP5).

Analysis of P. damicornis tissues by RP-HPLC and MALDI-TOF MS

To detect the native antimicrobial peptide in coral tissues, 9 coral nubbins (sampled from the Cb and C tanks) were harvested and the tissue was extracted using a water pick (800 mL of 0.2 µm filtered seawater refrigerated at 4°C). The extracts were centrifuged at 3000 g for 10

min at 4°C. The extract supernatant was discarded and the pellet was resuspended in 10 volumes of 2 M acetic acid and homogenized (15 strokes) using a Dounce homogenizer (100 µm). The homogenate was placed in a 4°C water bath and sonicated (Vibra-cell™ 75185, medium power, 3 pulses of 30 s), then stirred over night at 4°C, and finally centrifuged at 10,000 g for 20 min at 4°C to remove cellular fragments. The supernatant was immediately collected and pre-fractionated using a Sep-Pak C18 Vac cartridge (Sep-Pac Vac 12cc, Waters Corporation). Briefly, the Sep-Pak column was washed using acidified water with Trifluoroacetic acid (TFA; 0.05%), and three successive elutions were performed with 10, 60 and 80% acetonitrile in acidified water. The fractions obtained were lyophilized and reconstituted with 1 mL of acidified water (0.05% TFA). The reconstituted extracts were centrifuged for 20 min at maximum speed and 4°C, and tested for antimicrobial activity as describe below.

All HPLC purification steps were performed using a Waters Breeze system (Waters 1525, binary HPLC pump) equipped with a UV detector (Waters 2487, dual λ absorbance detector). The column effluent was monitored by UV absorption at 224 and 280 nm. Fractions were hand-collected and tested for antimicrobial activity.

Aliquots (150 µL) of Sep-Pak fractions with antimicrobial activity were subjected to reverse phase HPLC using a Symmetry C18 column (250 mm × 4.6 mm; Waters). Elution was performed with a linear gradient of 15–85% acetonitrile in acidified water over 70 min at a flow rate of 1 mL/min. Fractions corresponding to absorbance peaks were collected in polypropylene tubes, freeze dried, reconstituted in 0.1 mL of acidified ultrapure water (UPW), and tested for antimicrobial activity as described below. The active fraction was again subjected to reverse phase HPLC using a Symmetry C8 column (150 mm × 2.1 mm; Waters). Elution was performed with a linear gradient of 45–55% acetonitrile in acidified water over 60 min at a flow rate of 0.3 mL/min. Fractions corresponding to absorbance peaks were collected in polypropylene tubes, freeze dried, reconstituted in 0.03 mL of acidified ultrapure water, and tested for antimicrobial activity or submitted to MS analysis.

The dried active fraction or 20 µg of synthetic peptide was resuspended in 10 µL of pure water (ULC/MS; Biosolve).

MALDI-TOF mass measurements were carried out using an Ultraflex™ TOF/TOF mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) at a maximum accelerating potential of 25 kV in positive mode, and in either linear or reflectron mode. Each sample (1 µL) was co-crystallized on stainless steel MALDI targets with 1 µL of 4-hydroxycinnamic acid (4HCCA; 10 mg/mL of acetonitrile in aqueous 0.1% TFA, 7:3 v/v) using the dried-droplet method of matrix crystallization.

External calibration of the MALDI mass spectra was carried out using singly charged monoisotopic peaks (Pepmix calibration standard; Bruker Daltonics, Wissembourg, France).

The same molecules were also treated by tryptic digestion prior to MALDI-TOF mass spectrometry. The tryptic digestion was conducted directly on stainless steel MALDI targets. A 1 µL sample was first reduced in 1 µL of a 2 × DTT solution (20 mM in NH_4HCOO_3, 50 mM) for 30 min at 55°C in a moist chamber. Secondly, alkylation was performed in the dark at RT in a moist chamber by adding 1 µL of a 2 × iodoacetamide solution (110 mM, in NH_4HCOO_3, 50 mM) and incubating for 30 min,. The protein samples were then digested by the addition of 2 µL of a trypsin (sequence grade; Promega, Charbonniere, France) solution (40 µg/mL, reconstituted just prior to use in 50 mM NH_4HCOO_3) and incubation overnight at 37°C in a moist chamber. For MALDI-MS analysis 1 µL of 4HCCA was spotted onto the digest and dried.

Disulfide bond assignment of damicornin

The experiments to establish the positions of the disulphide bonds in the putative AMP were performed using a MALDI LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) with automatic gain control turned on. The signal was optimized by adjusting the laser energy to 6–8 µJ. The default target values were used in all experiments. Both MS and MS^2 experiments were acquired in centroid mode. A 2 Da mass window was used for MS/MS precursor selection. Qualitative data were obtained using Xcalibur™ software. The Orbitrap analyzer was calibrated with the aid of a calibrated peptide mixture (MSCAL4, Sigma Aldrich, St. Louis, MO) for optimization in the mass range 200–4000.

Native peptide was digested with chymotrypsin without prior reduction and

alkylation. A sample (2 µL) was digested with 2 µl of a chymotrypsin (sequence grade; Promega, Charbonniere, France) solution (0.04 µg/µL in 50 mM NH_4HCOO_3). Digestion was performed overnight at 30°C, and spotted onto a stainless steel MALDI target, as described previously. Controls for chymotrypsin digestion were conducted in water or with the synthetic peptide.

Antimicrobial assays

Antibacterial activity of HPLC fractions. Following each HPLC purification step the antibacterial activity was assessed using a liquid growth inhibition assay (38). Briefly, 10 µL aliquots of the resuspended fractions were incubated in microtiter plates with 100 µL Luria-Bertani broth (LB) suspensions of each of *M. luteus* (starting OD_{600} 0.001) and *E. coli* (starting OD_{600} 0.001). Antibacterial activity was assayed by measurement of bacterial growth (OD_{600}) following incubation for 12 h at 30°C for *M. luteus* and 37°C for *E. coli*.

Determination of minimal bactericidal concentration (MBC). Antibacterial activity was assayed against several bacteria. MBCs were determined as described previously (39). A solution of 0.01% acetic acid and 0.2% bovine serum albumin (BSA) was used to dissolve and prepare a series of two-fold dilutions of the synthetic peptide. Aliquots (10 µL) from each dilution were incubated in sterile 96 well polypropylene microtiter plates with 100 µL of a suspension of test bacteria (starting OD_{600} 0.001) in Poor Broth (PB; 1% Bacto tryptone), or PB supplemented with NaCl (PB-NaCl; 15 g/L) for marine bacteria, or in Marine Broth 2216 for *Vibrio* species. Bacterial growth was assessed after incubation with agitation at 30°C for 18 h, or at 23°C and 30°C for *V. shiloi* and *V. coralliilyticus*. The MBC was determined by plating the contents of the first three wells having no visible bacterial growth onto LB agar plates and incubating at 30°C for 18 h. The lowest concentration of synthetic peptide that prevented colony formation was recorded as the MBC.

Determination of minimal inhibitory concentration (MIC). MICs were determined using a liquid growth inhibition assay based on a procedure described previously (40). Marine Broth 2216 was used for *Vibrio* sp., PB-NaCl was used for other marine bacteria, and PB was used for the remaining microorganisms. Briefly, 10 µL from each dilution of the synthetic peptide was incubated in a microtiter plate with a 100 µL suspension of each of the bacteria at a starting OD_{600} of 0.001. The MIC was recorded as the lowest dilution inhibiting bacterial growth (measured at OD_{600}) after incubation for 18 h at 30°C, or 30°C and 23°C for *V. shiloi* and *V. coralliilyticus*.

Bactericidal assay. Synthetic peptide (10 µL) at a concentration 10-fold that of the MIC (12.5 µM) was mixed with 90 µL of an exponential phase PB culture of *M. luteus* (starting OD_{600} 0.01). Following incubation at 30°C for 0, 1, 3, 10 and 30 min, and 2, 6 and 24 h, aliquots (10 µL) were plated onto LB agar and the number of colony forming units was counted after overnight incubation at 30°C. Controls consisted of bacterial culture incubated with 10 µL of sterile water.

Antifungal assay. Antifungal activity was monitored against *F. oxysporum* using a liquid growth inhibition assay as described previously (41).

Hemolysis assay. A solution of 0.01% acetic acid and 0.2% BSA was used to dissolve synthetic peptide (400 µM) and prepare a series of two-fold dilutions. An aliquot (20 µL) from each dilution was added to 180 µL of a PBS (pH 7.4) solution containing sheep erythrocytes (5%, v/v). As a positive control for hemolysis, 20 µL of 10% Triton X-100 in PBS replaced the peptide solution. The negative control consisted of 20 µL of the 0.01% acetic acid and 0.2% BSA solution. Following incubated for 2 h at 37°C the test solutions and controls were centrifuged for 3 min at 10,000g. The absorbance of the supernatants was measured at 570 nm (AD340, Beckman Coulter), and the percentage hemolysis was calculated as:

% hemolysis = (A_{570} sample – A_{570} negative control) / (A_{570} positive control – A_{570} negative control) × 100.

Statistical analysis

Variations in gene expression were analyzed separately all along the non-virulent (Cb set) and virulent (Tb set) treatments using Grubbs' test (42,43), which detects kinetic points that deviate significantly from the others (i.e. outliers). Statistical tests were performed using JMP software (SAS Institute, Inc.), and differences were considered statistically significant at the 5% level.

RESULTS

Characterization of the damicornin precursor

In a study of the transcriptomic response of *P. damicornis* to bacterial stress or infection (27) we identified an EST with amino acid sequence similarities to the prepro-aurelin gene (GenBank accession number, DQ837210), which encodes the precursor of an AMP in the jellyfish *Aurelia aurita* (BLASTX, E value = 1.4; amino acid alignment shown in Fig. 1). The complete cDNA, which was obtained by RACE-PCR (Fig. 2), consists of 751 nucleotides and contains an ORF encoding a 107 amino acid (AA) precursor sequence. This sequence has the canonical prepropeptide organization of many AMP precursors. It consists of a 22 AA N-terminal sequence (Met1–Ala22), which is highly hydrophobic and corresponds to a putative signal peptide (prepeptide), as predicted by the Signal3.0 software. This is followed by a highly acidic 45 AA sequence (Ala23–Arg67) with a calculated pI of 3.56. Anionic AAs (Asp and Glu) were found at 16 positions in this proregion, which ends with a dibasic motif (Arg66–Arg67) consistent with the putative cleavage site. The C-terminal sequence (Ala68–Gly107) corresponds to the putative AMP, and has an identical cysteine array and 37.3% AA sequence identity with aurelin from *A. aurita* (Fig. 1). The putative AMP of *P. damicornis* has several features of eukaryotic AMPs: (i) a high content of basic AAs (pI 9.64); (ii) 6 Cys residues apparently involved in disulphide bond formation; and (iii) a C-terminal Gly residue that could be a signal of amidation (44). This putative AMP was termed damicornin, and the complete cDNA sequence was submitted to GenBank (GenBank accession number, HQ825099).

Isolation, biochemical characterization and disulfide assignment of native damicornin

To demonstrate the presence of damicornin in coral tissues we prepared an acidic extract of corals that had been maintained at 25°C and exposed to *V. coralliilyticus* for 9 days. Unchallenged controls were also prepared. Following an initial prefractionation step, which was applied to each acidic extract using Sep-Pak cartridges (see Experimental Procedures), only the 60% acetonitrile fraction of the extract of *V. coralliilyticus*-exposed corals had antibacterial activity. This fraction was further separated using RP-HPLC. All fractions were tested for activity against *M. luteus* A270 (a sensitive Gram positive strain) and *E. coli* SBS 363 (a sensitive Gram negative strain). Only one fraction (Fig. 3A) was active, against *M. luteus*. This was eluted in 51% acetonitrile and subjected to a further RP-HPLC separation step. Only one fraction was active, against *M. luteus* (Fig. 3B). The MALDI mass spectrum of the active fraction (acquired in positive linear mode) showed a major ion at m/z = 4492.740 (Fig. 4A), which corresponds to the calculated average mass of damicornin (4492.35 Da) starting with an alanine residue at position 68 of the damicornin preprosequence, ending with C-terminal amidated arginine residue (resulting from Gly107 removal), and displaying oxidized cysteines. A peptide corresponding to this mature sequence was obtained by chemical synthesis, and had a mass identical to that of the active peptide, as determined by MALDI-TOF-MS (Fig. 4A). The active peptide from *P. damicornis* and the synthetic damicornin were subjected to tryptic digestion and mass spectrometry analysis. The molecular mass fingerprints of both digests were similar. The molecular mass fingerprints of both trypsic digests presented a similar pattern with 7 common peptides identified and corresponding to damicornin (Fig. 4B). Altogether, these data show that damicornin is expressed in coral tissues and is processed as it was hypothesized above. Damicornin contains 6 cysteine residues involved in three intramolecular disulphide bonds, and is C-terminally amidated by removal of the C-terminal glycine.

For the determination of disulfide pairing between the six cysteine residues of the damicornin, we first digest native peptide with chymotrypsin, omitting the reduction alkylation steps to preserve disulfide bridges. For three disulfide bridges, 15 possible disulfide bond pairing schemes can be predicted. The peptidic fragments resulting from the chymotrypsin digest were analysed by MALDI LTQ Orbitrap mass spectrometer. As illustrated in figure S1 (panel A), the presence of pseudomolecular ions $[M + H]^+$ at *m/z* 1437.64, 1667.83 and 1766.71 were consistent with a possible pairing scheme C_{11} - C_{32} , C_{18} – (C_{36} or C_{38}) and C_2 – (C_{36} or C_{38}). In order to confirm this possible pairing scheme, ion fragmentation reactions were conducted by collision induced dissociation (CID). Fragmentation of ion at *m/z* 1437.64 (figure S1 panel B) confirmed the C_{11} - C_{32} pairing. As the C-terminal fragment $SC_{36}GC_{38}R\text{-}NH_2$ obtained by chymotrypsin digestion includes two cysteines, the assignment of the two other disulfide bridge was partial and results did not

provide an unambiguous distinction between bonding to C_{36} or to C_{38}. Nevertheless, fragmentation of ions at *m/z* 1766.71 and *m/z* 1667.84 (figure S1 panel C and D) confirmed the pairing scheme C_2 – (C_{36} or C_{38}) and C_{18} – (C_{36} or C_{38}). The same cysteine connections were also obtained for the synthetic damicornin (data not shown).

In addition, figure 1 gives the alignment of Damicornin with the anemonia potassium channel toxins ShK, BgK and HmK (identified in the anemones *Stichodactyla helianthus*, *Bunodosoma granulifera* and *Heteractis magnifica*, respectively (45-47)). This alignment shows that all these molecules share the same cysteine array. To note that the data obtained on damicornin cysteine connectivity (Fig S1) were consistent with those obtained for these anemonia toxins (given in figure 1).

Antimicrobial activity of damicornin

Because only small amounts of purified native damicornin were obtained, the synthetic peptide was used in antimicrobial assays. In liquid growth inhibition assays damicornin showed potent antifungal activity against the filamentous fungus *F. oxysporum*, with an MIC of 1.25 µM (Table 2). It was also active against Gram positive bacteria.

The MBC was 2.5 µM against *M. luteus*, and varied from 5 to 20 µM against the other Gram positive bacteria. However, no activity was observed against most of the Gram-negative bacteria, even at the highest concentration tested (20 µM); the exception was *E. coli* SBS 363 (MBC = 20 µM).

The bactericidal effect of synthetic damicornin against Gram positive bacteria was tested in kinetic experiments. Damicornin was incubated with *M. luteus* at a concentration 10-fold higher than the MIC, and the inhibition of bacterial growth was monitored over time. After 6 h *M. luteus* had lost the ability to grow on LB agar (Table 3). We therefore concluded that damicornin was bactericidal against *M. luteus.*

We found that sheep red blood cells were not affected by exposure to damicornin at concentrations as high as 80 µM for 24 h (data not shown). This indicates that damicornin has no hemolytic activity.

Localization of damicornin in holobiont tissues

The sequence similarities between damicornin and the jellyfish aurelin suggest that damicornin is expressed by coral cells and not by the symbiont. To verify that the preprodamicornin encoding gene is expressed by the cnidarians host we developed cross-PCR experiments using DNA and cDNA extracted from the holobiont (host plus symbiont) and from pure cultures of *Symbiodinium* spp. clades B, C and D (Fig. 5). The PCRs were performed using primers amplifying (i) the damicornin gene, (ii) housekeeping genes, (iii) small ribosomal subunit RNA genes from *Symbiodinium* spp. (32), and (iv) the major basic nuclear protein gene (MBNP) of *Symbiodinium* spp. The damicornin-specific primers gave amplicons for DNA and cDNA from holobionts only (Fig. 5). In contrast, the small ribosomal subunit RNA and MBNP primers amplified DNA and cDNA from both holobiont and symbiont cultures. As with the damicornin gene, the three qRT-PCR reference genes (encoding the ribosomal proteins L22, L40A and P0) were only expressed in samples containing coral cells. This indicates that the damicornin and reference genes are expressed by coral cells.

Damicornin expression was monitored in coral tissue sections using antibodies raised against the synthetic peptide. The antibody specificity was tested using Western blotting (Fig. S2). This experiment, performed on unstressed coral, showed there was a molecular weight difference between the synthetic damicornin and the band found in coral extracts (around 10 kDa). This suggests that damicornin may be stored as a precursor in coral tissues. The expected molecular weight of the prodamicornin was 9.3 kDa. As antibacterial activity was detected by RP-HPLC fractionation of extracts of coral tissue stressed by bacterial exposure, the localization of damicornin was investigated in unstressed corals as well as those subjected to bacterial stress. This enabled visual assessment of the localization and concentration of damicornin in corals of different immune status. To impose bacterial stress the corals were exposed to non-virulent *V. coralliilyticus* for 9 days prior to tissues fixation. Similar results were obtained under either condition. Corals are diploblastic animals composed of two tissue types: oral and aboral (Fig. 6 A1). Tissue sections stained with DAPI are shown in Figure 6 A2. The reduced background obtained with this blue staining of the nucleus highlights the two cellular layers constituting each tissue type. The 4 different layers/epithelia can be easily distinguished. Briefly, the oral layer is composed of oral ectoderm (exposed to the seawater) and

oral endoderm (exposed to the coelenteron or gastric cavity). The aboral layer is composed of aboral endoderm (exposed to the coelenteron) and the aboral ectoderm (in contact with the skeleton), which is also referred to as the skeletogenic tissue. The ectoderm and endoderm (oral and aboral) are separated by an acellular layer, the mesoglea. Damicornin-specific antibodies specifically labeled cells in the oral ectoderm (Fig. 6 C1). At higher magnification the staining was associated with cells containing intracellular granules (Fig. 6, C2 and C3). Controls treated with the depleted antibody (i.e. antibodies pre-incubated with synthetic damicornin) showed faint tissue autofluorescence but no specific labeling (Fig. 6, B1 and B2), demonstrating that the staining of the ectodermal granular cells was specific.

Damicornin gene expression following bacterial challenge

The regulation of damicornin gene expression on exposure to non-virulent and virulent *V. coralliilyticus* was studied. Using qRT-PCR we analyzed the relative amount of preprodamicornin transcripts in coral tissues 0, 3, 6, 9, 12, 15 and 18 days after bacterial challenge. There was no significant variation in transcript abundance after challenge with the non-virulent bacteria (Fig. 7 A), but large variation was evident after challenge with the virulent *V. coralliilyticus* (Fig. 7 B). Damicornin transcripts markedly increased at day 6 (9.6-fold), and then declined significantly from day 12 to the end of the experiment (more than 44.5-fold at day 18 compared with day 0; Fig. 7 B). This strongly suggests that the coral pathogen *V. coralliilyticus* alters expression of the damicornin gene.

DISCUSSION

This report is the first concerning characterization, purification and expression of an antimicrobial peptide (AMP) from a scleractinian coral. It the most basal eumetazoan AMP characterized to date, and the only antimicrobial agent identified in a scleractinian coral. The AMP (damicornin, from the coral *P. damicornis*) had antimicrobial activity against Gram positive bacteria and the filamentous fungus *F. oxysporum*, but had little activity against Gram negative bacteria including *V. coralliilyticus*, a specific pathogen of *P. damicornis.*

RACE-PCR experiments and MS-MS characterization of native damicornin showed that it is a 39 residue cationic AMP (theoretical pI = 9.64) containing 11 basic residues. Its measured molecular mass (m/z = 4492.740) indicates that damicornin is folded by 3 intramolecular disulfide bridges involving the 6 cysteine residues in its sequence, and that it has C-terminal amidation resulting from the removal of an end glycine residue. AMPs folded by 3 intramolecular disulfide bridges have been reported in many invertebrate and vertebrate species, and often belong to the defensin super family (48). According to cysteine pairing, animal defensins are classified into four subfamilies, namely the vertebrate α-, β-, and θ-defensins, and the invertebrate defensins. The inclusion of damicornin in these families appears inappropriate because of its specific cysteine array. This also applies to AMPs from other marine invertebrates (e.g. penaeidins) that contain 3 disulfide bridges and, like damicornin, have a cysteine array that differs from that of invertebrate defensins (49). Despite this difference damicornin shares several features in common with invertebrate defensins: (i) it is particularly active against Gram positive bacteria and filamentous fungi, but has limited activity against Gram negative bacteria; (ii) it is characterized by a lack of hemolytic activity (48); and (iii) in terms of structural features, it can also have C-terminal amidation (50,51). The latter is common among cationic AMPs; it makes them more resistant to proteolysis and increases their net positive charge (49,52-56).

From the complete ORF obtained in the present study, damicornin is generated from a 107 residue precursor that we have termed preprodamicornin. This includes in sequence a putative signal peptide (22 amino acids), an anionic proregion (45 amino acids), and a cationic damicornin sequence in the C-terminal position (40 amino acids). Thus, from the structure of its precursor, damicornin is probably generated sequentially as follows: i) the signal peptide translocates preprodamicornin to the lumen of the rough endoplasmic reticulum, and is cleaved off by a signal peptidase; ii) the anionic proregion of predamicornin is removed by proteolytic cleavage of the Arg67–Ala68 bond by a processing enzyme that recognizes the dibasic motif (Arg–Arg) located ahead of the observed cleavage site; iii) the C-terminal extended glycine peptide substrate is hydroxylated by a peptidylglycine-α-

hydroxylating mono-oxygenase, and the intermediate is cleaved by a peptidyl-α-hydroxyglycine-α-amidating lyase, which leads to the formation of the mature α-amidated damicornin and the release of a glyoxylate. The first two steps of the process have been commonly reported in the maturation of AMP precursors (57-60). The mechanism occurring during the third step was described by Kolhekar *et al*. (61), and has been found in the processing of various AMPs (49,50).

Damicornin shares several key features with invertebrate defensins: i) it contains 6 cysteine residues involved in intramolecular disulphide bonds; ii) it is mainly active against Gram positive bacteria and filamentous fungi; iii) it has no hemolytic activity; iv) it has a classical precursor structure with a segmented organization containing a signal peptide followed by an anionic proregion and the cationic active peptide; v) its precursor is processed by mechanisms found for other defensin precursors; and vi) it has a C-terminal amidation typical of several invertebrate defensins and other AMPs of animal origin. However, damicornin is more similar (cysteine array and sequence similarities) to aurelin from the jellyfish *A. aurita* (62). As with other invertebrate defensins having the same cysteine array (the so-called CSαβ motif), including scorpion toxins (63,64), damicornin and aurelin have a common cysteine array with anemonia potassium channel toxins of type 1 (Fig. 1). Aurelin has additional structural similarities with anemonia toxins: it has a Lys residue at position 28 followed by an essential hydrophobic residue, both of which have been shown to be crucial for toxin activity by blocking voltage-gated K^+ channels (62,65-67). This essential dyad is not present in damicornin (Fig. 1). In addition, only damicornin has C-terminal amidation. These data suggest that disulfide-containing AMPs (damicornin, aurelin) and toxins from cnidarians originated from the same molecular ancestor, but have evolved independently to acquire specific molecular features and function.

The results show that damicornin is expressed by coral oral ectodermal cells, and is located within intracellular granules. AMP expression in granular epithelial cells has been reported in both vertebrates (68-70) and invertebrates (71-73); this facilitates the apical release of AMP in mucus, and thus its participation in mucosal defense and prevention of pathogen invasion. Our data suggest that the release of damicornin could be part of the coral epithelial defense. Whereas mature and active damicornin was isolated from corals challenged with non-virulent bacteria, no antibacterial activity could be detected in unchallenged controls. However, damicornin was expressed in both sets of animals, as evidenced by (i) similar transcription levels, and (ii) similar immunostaining of ectodermal cell granules. This suggests that the inactive damicornin precursor is stored in ectodermal cells, and is activated by post-translational processing upon release when triggered by an immune challenge. Our Western blotting results support this hypothesis; a band of approximately 10 kDa was detected by anti-damicornin antibody in unstressed coral extracts (Fig. S2). This band may correspond to prodamicornin, which has a theoretical molecular weight of 9.3 kDa. The hypothesis that active damicornin is matured and released in response to external signals is supported by previous studies showing the release of antibacterial molecules immediately after injury in *P. damicornis* and *Stylophora pistillata* (23,24).

A major finding of this study was that the expression of damicornin was repressed in *P. damicornis* exposed to the virulent pathogen *V. coralliilyticus*. After a transient (10-fold) increase in damicornin transcript abundance during the first 6 days following infection, a dramatic decrease (50-fold) was observed from day 9 to day 18. In contrast, no transcriptional change was observed when *P. damicornis* was exposed to the non-virulent bacterial state. In a recent study of infection by *V. coralliilyticus* (27) we showed that the bacteria enter coral tissues 6 days after challenge. This suggests that the first phase of infection involves bacterial recognition by host cells, which triggers a non-specific inflammatory response that activates damicornin gene transcription. In a second phase, following bacterial invasion, the pathogen suppresses damicornin transcription. Similar mechanisms of immune suppression have been reported in several intracellular bacteria including *Shigella flexneri*, which suppresses the transcription of several genes encoding AMPs following entry into intestinal cells (74). While not reported to directly affect AMP expression, several marine *Vibrio* species have been shown to suppress or modulate host immune defenses (75-79).

In conclusion, this report is the first to characterize a scleractinian AMP (damicornin). Damicornin has several features in common with

invertebrate defensins, and shares a specific cysteine array found in other cnidarian AMPs (aurelin from the jellyfish *A. aurita*) and toxins produced (anemonia). Structural similarities between AMPs and toxins have also been described for defensins and toxins of arthropods. This strongly suggests that AMPs and toxins have evolved from common molecular ancestors in diverse phyla. Damicornin was shown to be expressed and released from coral ectodermal cells in animals exposed to a non-pathogenic stimulus. Conversely, damicornin gene expression was repressed concomitantly with the entry of the coral pathogen *V. coralliilyticus* into host ectodermal cells. This is the first evidence of AMP gene repression in a host-*Vibrio* interaction. Future studies will be necessary to assess whether this immune suppression accounts for the success of the coral pathogen.

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79. Mateo, D. R., Siah, A., Araya, M. T., Berthe, F. C. J., Johnson, G. R., and Greenwood, S. J. (2009) *J. Invertebr. Pathol.* **102**(1), 50-56

FIGURE LEGENDS

Fig. 1. Damicornin, aurelin and anemonia toxins share the same cysteine array. The putative cleavage site is indicated by the double-ended arrow. Conserved amino acids are highlighted in grey. The conserved cysteine array is highlighted in bold grey and outlined in black. The anemonia toxins ShK, BgK and HmK are produced by the anemones *Stichodactyla helianthus*, *Bunodosoma granulifera* and *Heteractis magnifica*, respectively. The essential dyad for toxins blocking voltage-gated K^+ channels is underlined. Anemonia toxin disulfide pairing (C1-C6, C2-C4, C3-C5) is indicated under toxin sequences.

Fig. 2. cDNA and deduced amino acid sequences of preprodamicornin. The ORF sequence is shown in capital letters. The EST obtained from the SSH (Subtractive subtraction hybridization) library is highlighted in grey. The deduced amino acid sequence of the ORF is indicated above the nucleotide sequence. The asterisk indicates the stop codon. The arrow identifies the cleavage site of the signal peptide. The dibasic cleavage site between the acidic N-terminal proregion and the cationic C-terminal region is outlined in black. The damicornin active peptide is underlined in black. The cysteine residues and glycine amidation signal are shown in bold.

Fig. 3. Purification of damicornin from acidic extracts obtained from challenged coral tissue. A: Following prepurification by solid phase extraction, the material eluted from the fraction with 60% acetonitrile was loaded onto a C_{18} column. In this HPLC step elution was performed with a linear gradient of 15 to 85% acetonitrile over 60 min at a flow rate of 1 mL/min. Absorbance peaks were monitored at 224 nm. The fraction containing the antimicrobial activity is indicated by an arrow. B: Chromatogram from the last reverse phase purification of damicornin on a C_8 column; the arrow indicates the fraction containing the purified antimicrobial peptide of interest.

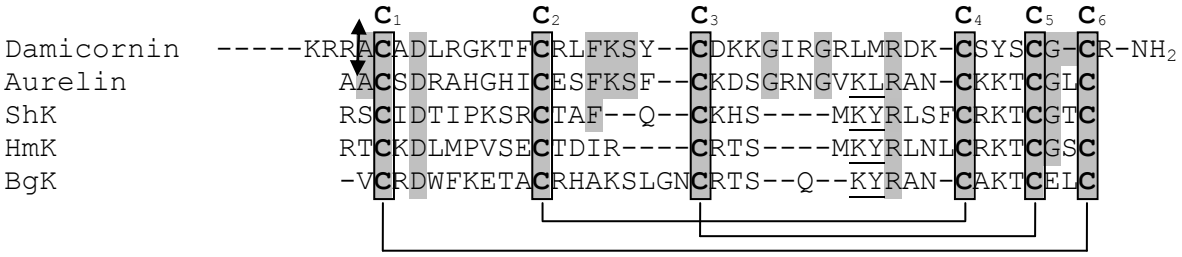
Fig. 4. Identification of damicornin by mass spectrometry analysis. A: Superimposed linear mode MALDI mass spectra of purified damicornin and synthetic peptide, showing a major peak at 4492.740 Da. B: Analysis of tryptic digests of purified peptide and synthetic peptide by MALDI-TOF MS. The peptides identified by MS analysis are underlined and reported to the damicornin sequence. The theoretical masses are shown, and the corresponding m/z ratios are boxed on each MS spectrum.

Fig. 5. The preprodamicornin gene is expressed by the coral host. The presence of preprodamicornin, the reference gene for q-RT-PCR, the small ribosomal subunit RNA of the zooxanthellae (Zx ssRNA), and the gene corresponding to the major basic zooxanthellae nuclear protein were investigated by PCR **(**using specific primers) on DNA and cDNA extracted from holobionts (corals plus zooxanthellae) or from pure cultures of clade B, C and D zooxanthellae. HgDNA: holobiont genomic DNA; ZgDNA: zooxanthellae genomic DNA; HcDNA: holobiont cDNA; ZcDNA: zooxanthellae cDNA.

Fig. 6. Immunolabeling of damicornin in ectodermal granular cells in oral tissue.
(A1) Bright field transmitted light image and (A2) an image of the DAPI-stained nucleus showing the four tissue layers and the coelenteron (i.e. the gastric cavity), and their position in relation to the seawater (SW) environment and the coral skeleton (Sk). The labeled damicornin appears bright orange (C1), (C2), (C3). (B1) and (B2) represent control experiments performed using anti-damicornin antibodies that were pre-adsorbed with the synthetic peptide used for immunization. (B2) and (C2) are magnifications of (B1) and (C1), respectively. Both show granular ectodermal cells (GC), with labeling (C2) and without labeling in the control experiment (B2). (C3) shows another area of the oral ectoderm (OEc) with 3 labeled granular cells. Other abbreviations: Zx, zooxanthellae; OEn, oral endoderm; Me, mesoglea.

Fig. 7. Disturbance of preprodamicornin expression following infection by virulent bacteria. The transcription rate of preprodamicornin was measured by q-RT-PCR for samples at D0, D3, D6, D9, D12, D15 and D18 from the non-virulent (A) and the virulent (B) treatments. The relative expression was normalized using the geometric mean of three reference genes (normalized relative quantity). The grey and black histograms represent the relative expression of the coral preprodamicornin gene in the

non-virulent and virulent treatments, respectively. The bars represent the mean of replicates, and the error bars represent the SEM. * indicates observations that deviated significantly ($P < 0.05$) from others in the same treatment (the non-virulent and the virulent treatments).


C1
C2
C3
C4
C5
C6
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Aurelin AACSDRAHGHICESFKSF--CKDSGRNGVKLRAN-CKKTCGLC
ShK RSCIDTIPKSRCTAF--Q--CKHS----MKYRLSFCRKTCGTC
HmK RTCKDLMPVSECTDIR----CRTS----MKYRLNLCRKTCGSC
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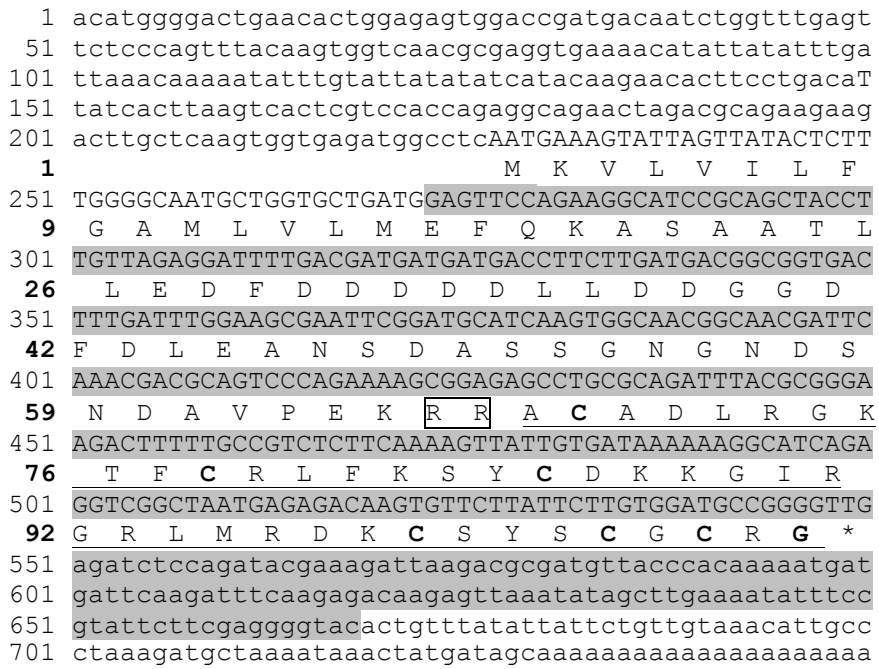
Figure 1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151 tatcacttaagtcactcgtccaccagaggcagaactagacgcagaagaag
201 acttgctcaagtggtgagatggcctcAATGAAAGTATTAGTTATACTCTT
1 M K V L V I L F
251 TGGGGCAATGCTGGTGCTGATGGAGTTCCAGAAGGCATCCGCAGCTACCT
9 G A M L V L M E F Q K A S A A T L
301 TGTTAGAGGATTTTGACGATGATGATGACCTTCTTGATGACGGCGGTGAC
26 L E D F D D D D D L L D D G G D
351 TTTGATTTGGAAGCGAATTCGGATGCATCAAGTGGCAACGGCAACGATTC
42 F D L E A N S D A S S G N G N D S
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59 N D A V P E K R R A C A D L R G K
451 AGACTTTTTGCCGTCTCTTCAAAAGTTATTGTGATAAAAAAGGCATCAGA
76 T F C R L F K S Y C D K K G I R
501 GGTCGGCTAATGAGAGACAAGTGTTCTTATTCTTGTGGATGCCGGGGTTG
92 G R L M R D K C S Y S C G C R G *
551 agatctccagatacgaaagattaagacgcgatgttacccacaaaaatgat
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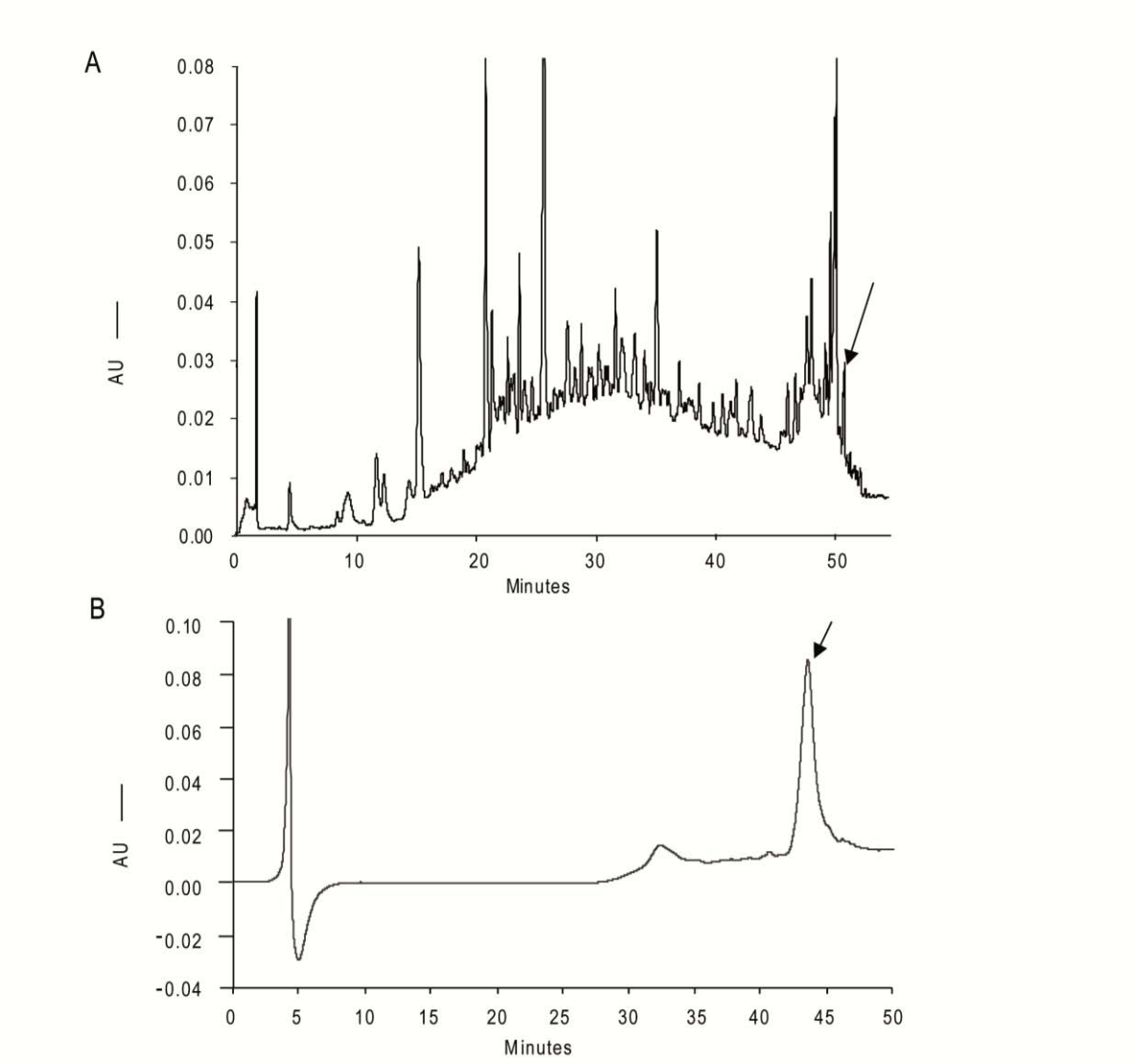
Figure 2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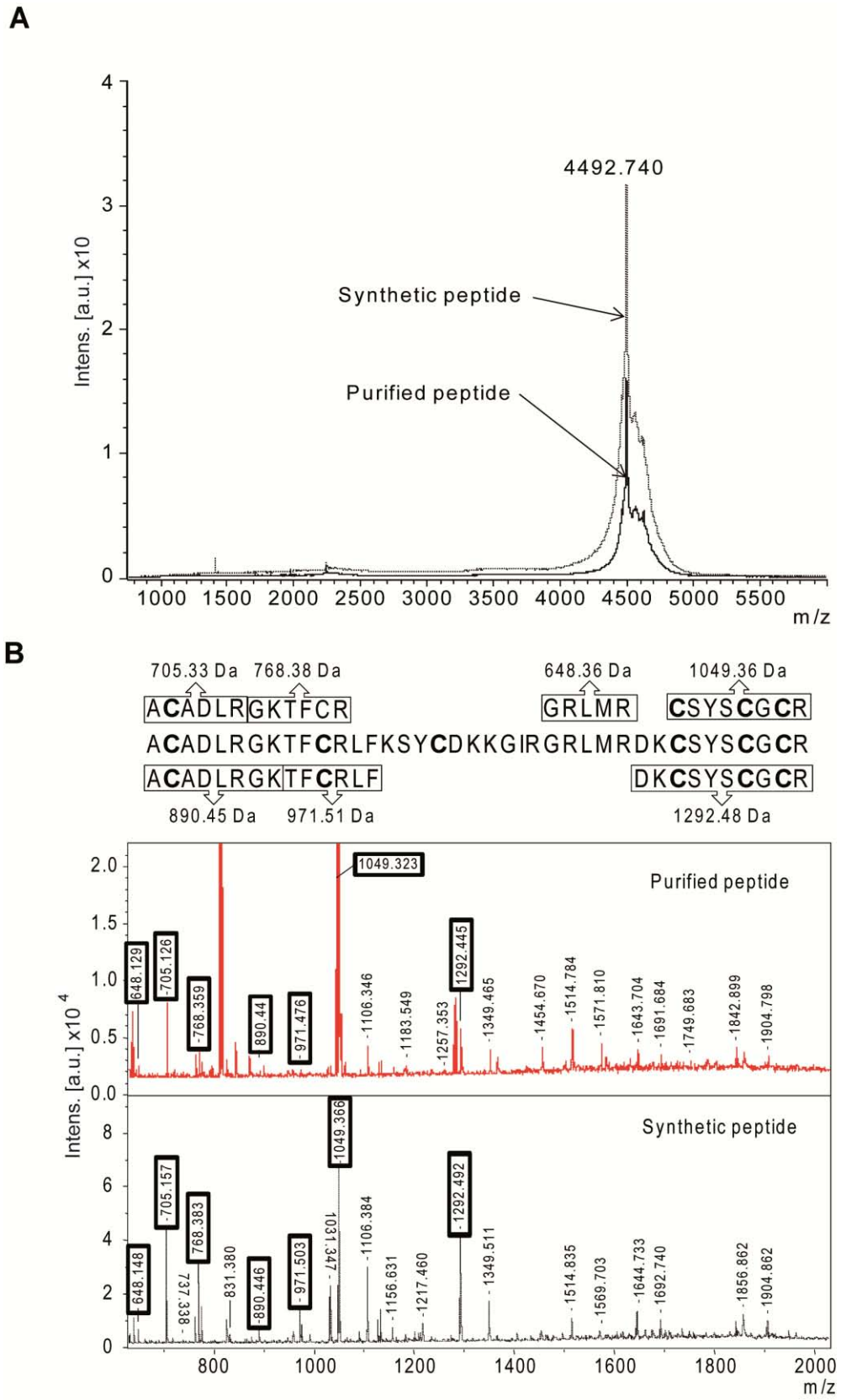
AU
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
0
10
20
30
40
50
Minutes
B
AU
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
-0.04
0
5
10
15
20
25
30
35
40
45
50
Minutes

Figure 3


A
4492.740
Synthetic peptide
Purified peptide
Intens. [a.u.] x10
m/z
B
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768.38 Da
648.36 Da
1049.36 Da
ACADLRGKTFCR
GRLMR
CSYSCGCR
ACADLRGKTFCRLFKSYCDKKGIRGRLMRDKCSYSCGCR
ACADLRGKTFCRLF
DKCSYSCGCR
890.45 Da
971.51 Da
1292.48 Da
Purified peptide
Synthetic peptide
Intens. [a.u.] x10 4
m/z

Figure 4

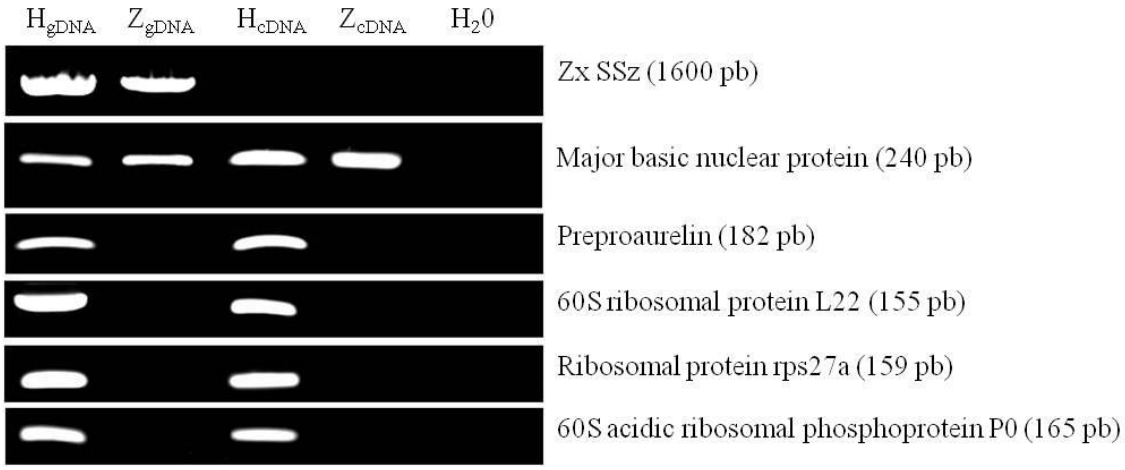
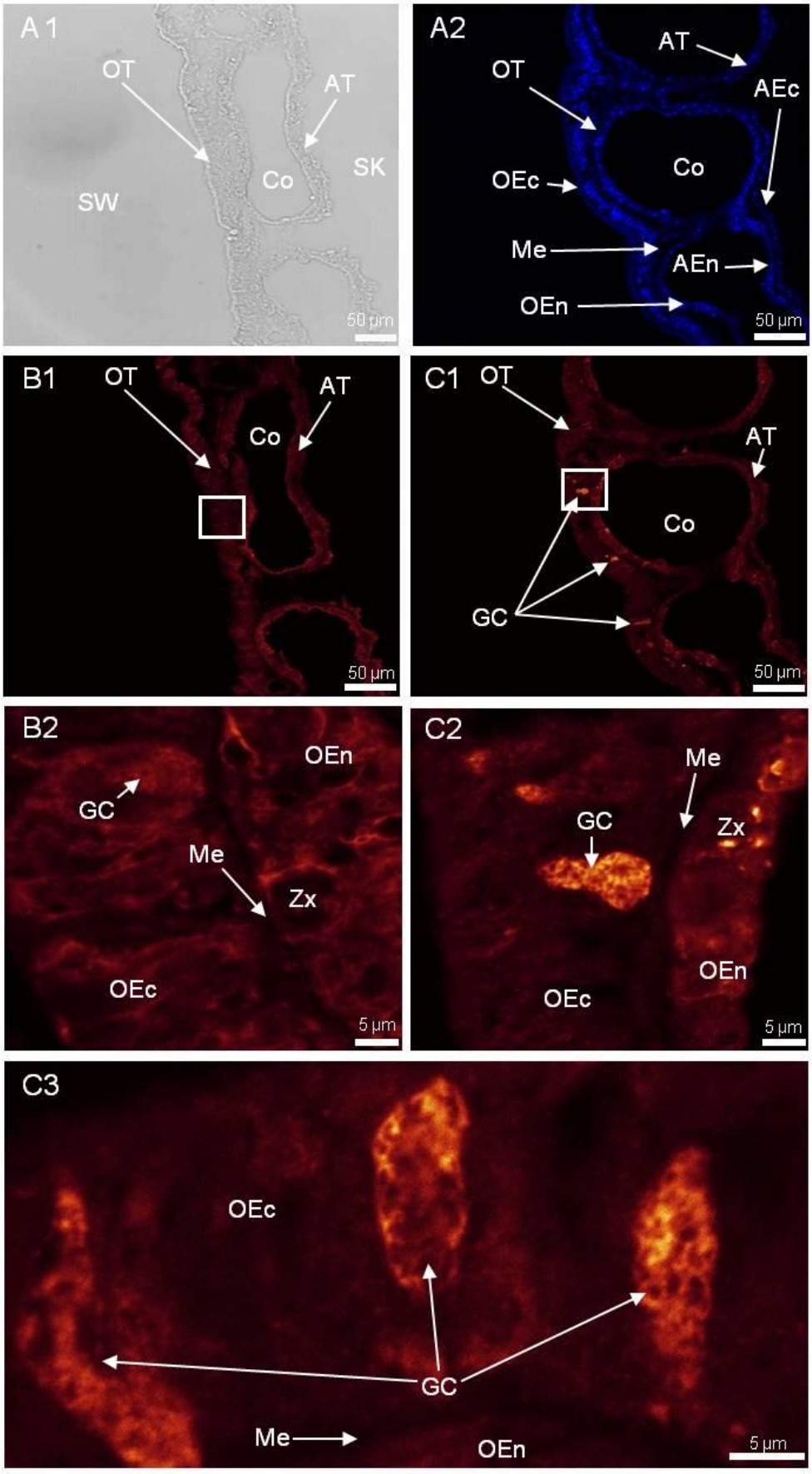

H_{gDNA}
Z_{gDNA}
H_{cDNA}
Z_{cDNA}
H_20
Zx SSz (1600 pb)
Major basic nuclear protein (240 pb)
Preproaurelin (182 pb)
60S ribosomal protein L22 (155 pb)
Ribosomal protein rps27a (159 pb)
60S acidic ribosomal phosphoprotein P0 (165 pb)

Figure 5


A 1
OT
AT
SK
Co
SW
50 µm
A2
AT
OT
AEc
Co
OEc
Me
AEn
OEn
50 µm
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OT
AT
Co
50 µm
C1
OT
AT
Co
GC
50 µm
B2
OEn
GC
Me
Zx
OEc
5 µm
C2
Me
GC
Zx
OEn
OEc
5 µm
C3
OEc
GC
Me
OEn
5 µm

Figure 6

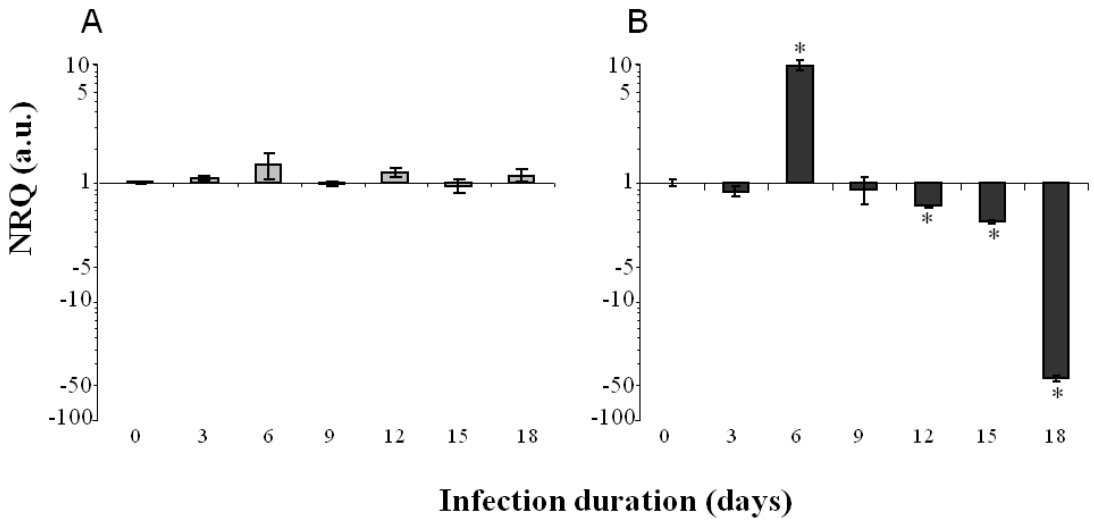
A
B
NRQ (a.u.)
10
5
1
-5
-10
-50
-100
0
3
6
9
12
15
18
*
Infection duration (days)

Figure 7

Table 1: Primers used for q-RT-PCR

Gene name	*Forward primer (5'→ 3')*	*Reverse primer (5'→ 3')*
Preprodamicornin	AGTCCCAGAAAAGCGG	GTGGGTAACATCGCGT
Major basic nuclear protein	GGTACAGCAAACTGCG	TTGGAAACGTCCGACC
60S ribosomal protein L22	TGATGTGTCCATTGATCGTC	CATAAGTAGTTTGTGCAGAGG
60S ribosomal protein L40A	CGACTGAGAGGAGGAGC	CTCATTTGGACATTCCCGT
60S acidic ribosomal phosphoprotein P0	GCTACTGTTGGGTAGCC	CTCTCCATTCTCGTATGGT

Table 2: Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration of damicornin.

Microorganisms	MIC (μM)	MBC (μM)
Gram-positive bacteria		
B. megaterium (souchier IBMC)	20	>20
S. aureus (SG511)	5	>20
M. luteus (A270)	1.25	2.5
B. stationis (CIP 101282)	10	10
M. maritypicum (CIP 105733T)	20	>20
Gram-negative bacteria		
E. coli (SBS 363)	10	20
V. aesturianus (CIP 109791)	>20	>20
V. shilonii (CIP 107136)*	>20	>20
V. coralliilyticus strainYB1 (CIP 107925)*	>20	>20
V. splendidus (CIP 107715)	>20	>20
Fungi		
F. oxysporum	1.25	ND

MBC were determined by testing different concentrations of synthetic peptide in liquid growth inhibition assays against different bacterial strains according to Hancock method. MBC values are expressed in μM. ND is used for Not Done. Marine bacteria are indicated in bold.* Indicate that for this strains the MIC and MBC were test either at 23 and 30°C, results were the same at both temperature.

Table 3: Bacteriolytic effect of damicornin on *M. luteus*

Time of incubation	Control	AMP
	10^4 CFU.mL^{-1}	
0 min	2.40	2.46
1 min	2.94	2.86
3 min	2.96	2.89
10 min	3.68	2.58
30 min	3.81	1.95
2 h	3.86	1.30
6 h	9.60	0.00
24 h	22.40	0.00