

Innate Immune Responses of a Scleractinian Coral to Vibriosis^{*[S]}

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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.

Scleractinian corals are the biological, ecological, and physical framework of tropical coral reefs, which are among 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 for this alarming status are multiple and include increasing water temperature, which disrupts symbiosis and leads to coral bleaching, and anthropogenic pressures such as overfishing that lead to ecosystem disequilibrium. Among 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 *Vibrio 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

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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).³

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 nonpathogenic 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 *P. damicornis* (Linnaeus, 1758) isolate used in this study was collected from Lombok, 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* (IBM), *Staphylococcus aureus* (SG511), *Brevibacterium stationis* (CIP 101282), and *Microbacterium maritipicum* (CIP 105733T) and the Gram-negative bacteria *Escherichia coli* (SBS 363), *Vibrio aesturianus* (CIP 109791), and *Vibrio splendidus* (CIP 107715) were the same as used in a previous study (28). *V. shiloi* (CIP 107136) and *V. coralliilyticus* strain YB1 (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 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-liter 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 to 32.5 °C (Tb); (iii) a constant temperature of 25 °C without bacteria added (C); and (iv) a gradual temperature increase from 25 to 32.5 °C in the absence of added bacteria (T). Nubbins of *P. damicornis* (fixed piece of coral of ~10 g) were randomly placed in each experimental tank ($n = 40/\text{tank}$) 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, and 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 $\mu\text{mol photon}/\text{m}^2/\text{s}$ (measured using a quantum meter; QMSW-SS, Apogee Instruments Inc.) using metal halide lamps (Iwasaki 6500 Kelvin, 400 W) set to a 12-h light:12-h dark photoperiod. All other seawater parameters were held constant over time in the tanks. A water pump (IDRA, 1300 liters/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 of the 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-PCR experiments were performed to characterize the complete ORF of the putative AMP. Tissue extraction, total RNA extraction, poly(A)⁺ purification, and RACE-PCR were conducted using nonchallenged *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

³ The abbreviations used are: AMP, antimicrobial peptide; MIC, minimal inhibitory concentration; D_n, day *n*; RACE, rapid amplification of cDNA ends; q-RT-PCR, quantitative RT-PCR; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; MBC, minimal bactericidal concentration; PB, poor broth.

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	TTGGAACGTCGACC
60 S ribosomal protein L22	TGATGTGTCCATTGATCGTC	CATAAGTAGTTTGTGCAGAGG
60 S ribosomal protein L40A	CGACTGAGAGGAGGAGC	CTCATTTGGACATTCGCCGT
60 S acidic ribosomal phosphoprotein P0	GCTACTGTTGGGTAGCC	CTCTCCATTCTCGTATGGT

using 2.5 μ l of purified cDNA (diluted 1:50 in water) in a total volume of 10 μ l containing 1 \times LightCycler[®] 480 SYBR Green I Master Mix (Roche Applied Science) and 70 nM of each primer. The primers, which were designed using the Light Cycler Probe Design Software, version 1.0 (Roche Applied Science), are shown in Table 1. Amplification was performed using a Light-Cycler 480 system (Roche Applied Science) 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 Light-Cycler Software, version 3.3 (Roche Applied Science). 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 described previously (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*: 60 S ribosomal protein L22, 60 S ribosomal protein L40A, and 60 S acidic ribosomal phosphoprotein P0 (GenBank[™] accession numbers HO112261, HO112283, and HO112666, respectively). The stable expression status of these three genes under nonstress, thermal stress, bacterial stress, and bacterial infection conditions was recently demonstrated (27). The normalized relative quantities (NRQ) were calculated as described previously (31), using the equation,

$$NRQ = \frac{E_{\text{target}}^{\Delta C_{p,\text{target}}}}{3 \sqrt[3]{\prod_{i=1}^3 E_{\text{ref}_i}^{\Delta C_{p,\text{ref}_i}}}} \quad (\text{Eq. 1})$$

where E_{target} is the amplification efficiency of the gene of interest; E_{ref} is the amplification efficiency of the reference gene; $\Delta C_{p,\text{ref}} = C_{p,\text{ref}}(\text{calibrator}) - C_{p,\text{ref}}(\text{sample})$; and $\Delta C_{p,\text{target}} = C_{p,\text{target}}(\text{calibrator}) - C_{p,\text{target}}(\text{sample})$.

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 (ACADLRGKTFCLFKSYCDKKGIR-GRLMRDKCSYSCGCR-NH₂) was chemically synthesized (5 mg; Genepep, Saint-Clément de Rivière, France) in a C-terminally amidated form, and the folding of the three disulfide 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 nonimmunized 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 electroblotted onto a PVDF membrane. To verify the specificity, the blots were probed with preimmune 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 nonvirulent bacteria were processed following a procedure described elsewhere (37). Thin sections (7 μ m) 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 in a saturating medium containing 2% BSA, 0.2% teleostean gelatin, 0.05% Tween 20, and 0.5% donkey serum in 0.05 mol/liter 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 μ g/ml in saturating medium) or with the depleted purified antibodies raised against synthetic damicornin (depletion was performed by preincubating the purified

antibodies for 1 h at room temperature with the synthetic damicornin). Excess antibodies were removed by repeated rinsing, and the sections were then incubated for 1 h at room temperature with biotinylated anti-rabbit antibodies (secondary antibodies; Amersham Biosciences 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 (S11226; Molecular Probes) diluted 1:50 in PBS and DAPI (D9542; Sigma; 2 $\mu\text{g}/\text{ml}$). The sections were mounted in Pro-Long antifade medium (P7481; Molecular Probes) and observed using a confocal laser-scanning microscope (TCSSP5; Leica).

Analysis of *P. damicornis* Tissues by Reverse Phase HPLC and MALDI-TOF MS

To detect the native antimicrobial peptide in coral tissues, nine 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 $3000 \times g$ 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, three pulses of 30 s), then stirred over night at 4 °C, and finally centrifuged at $10,000 \times g$ for 20 min at 4 °C to remove cellular fragments. The supernatant was immediately collected and prefractionated using a Sep-Pak C18 Vac cartridge (Sep-Pac Vac 12cc; Waters Corporation). Briefly, the Sep-Pak column was washed using acidified water with 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 described below.

All of the 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 \times 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, 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 \times 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 (ultra liquid chromatography/mass spectrometry solvent; 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 (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 $2 \times$ DTT solution (20 mM in NH_4HCO_3 , 50 mM) for 30 min at 55 °C in a moist chamber. Secondly, alkylation was performed in the dark at room temperature in a moist chamber by adding 1 μl of a $2 \times$ iodoacetamide solution (110 mM, in NH_4HCO_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 $\mu\text{g}/\text{ml}$, reconstituted just prior to use in 50 mM NH_4HCO_3) and incubation overnight at 37 °C in a moist chamber. For MALDI-MS analysis, 1 μl of 4-hydroxycinnamic acid was spotted onto the digest and dried.

Disulfide Bond Assignment of Damicornin

The experiments to establish the positions of the disulfide 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/MS 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) 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 $\mu\text{g}/\mu\text{l}$ in 50 mM NH_4HCO_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 of Luria-Bertani broth (LB) suspensions of each of *M. luteus* (starting A_{600} , 0.001) and *E. coli* (starting A_{600} , 0.001). Antibacterial activity was assayed by measurement of

Scleractinian AMP Immune Responses during Vibriosis

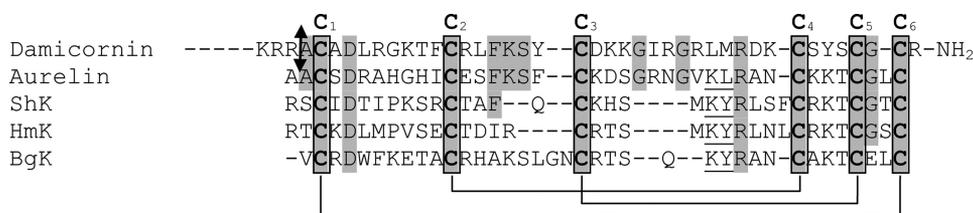


FIGURE 1. **Damicornin, aurelin, and anemone toxins share the same cysteine array.** The putative cleavage site is indicated by the *double-ended arrow*. Conserved amino acids are highlighted in *gray*. The conserved cysteine array is highlighted in *bold gray* and outlined in *black*. The anemone toxins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

bacterial growth (A_{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% BSA was used to dissolve and prepare a series of 2-fold dilutions of the synthetic peptide. Aliquots (10 μ l) from each dilution were incubated in sterile 96-well polypropylene microtiter plates with 100 μ l of a suspension of test bacteria (starting A_{600} , 0.001) in poor broth (PB; 1% Bacto tryptone) or PB supplemented with NaCl (PB-NaCl; 15 g/liter) 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 μ l from each dilution of the synthetic peptide was incubated in a microtiter plate with a 100- μ l suspension of each of the bacteria at a starting A_{600} of 0.001. The MIC was recorded as the lowest dilution inhibiting bacterial growth (measured at A_{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 μ l) at a concentration 10-fold higher than the MIC (12.5 μ M) was mixed with 90 μ l of an exponential phase PB culture of *M. luteus* (starting A_{600} , 0.01). Following incubation at 30 °C for 0, 1, 3, 10, and 30 min and 2, 6, and 24 h, aliquots (10 μ l) 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 μ l 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 μ M) and prepare a series of 2-fold dilutions. An aliquot (20 μ l) from each dilution was added to 180 μ l of a PBS (pH 7.4) solution containing sheep erythrocytes (5%, v/v). As a positive control for hemolysis, 20 μ l of 10% Triton X-100 in PBS replaced the pep-

ptide solution. The negative control consisted of 20 μ l of the 0.01% acetic acid and 0.2% BSA solution. Following incubation for 2 h at 37 °C, the test solutions and controls were centrifuged for 3 min at 10,000 \times g. 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) \times 100.

Statistical Analysis

Variations in gene expression were analyzed separately all along the nonvirulent (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 expressed sequence tag with amino acid sequence similarities to the prepro-aurelin gene (GenBankTM 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 precursor sequence. This sequence has the canonical prepropeptide organization of many AMP precursors. It consists of a 22-amino acid N-terminal sequence (Met-1 to Ala-22), 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-amino acid sequence (Ala-23 to Arg-67) with a calculated pI of 3.56. Anionic amino acids (Asp and Glu) were found at 16 positions in this proregion, which ends with a dibasic motif (Arg-66 to Arg-67) consistent with the putative cleavage site. The C-terminal sequence (Ala-68 to Gly-107) corresponds to the putative AMP and has an identical cysteine array and 37.3% amino acid 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 amino acids (pI 9.64); (ii) six Cys residues apparently involved in disulfide 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 GenBankTM (accession number HQ825099).

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1  acatggggactgaacactggagagtgaccgatgacaatctggtttgagt
51  tctcccagtttacaagtgggtcaacgcgaggtgaaaacatattatattga
101 ttaaacaaaaatattgtattatatacatacaagaacacttccctgacat
151 tatcacttaagtcaactcgccaccagaggcagaactagacgcagaagaag
201 acttgctcaagtggtgagatggcctcAATGAAAGTATTAGTTATACTCTT
1  M K V L V I L F
251 TGGGGCAATGCTGGTGCTGATGAGTTCCAGAAGGCATCCGCAGCTACCTI
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 S Y 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
401 AAACGACGCAGTCCAGAAAAGCGGAGAGCCTGCGCAGATTTACGCGGGA
59  N D A V P E K R R A C A D L R G K
451 AGACTTTTGGCGTCTCTTCAAAGTTATTGTGATAAAAAAGGCATCAGA
76  T F C R L F K S Y C D K K G I R
501 GGTTCGGCTAATGAGAGACAAGTCTTATTCTTGTGGATGCGCGGGTTC
92  G R L M R D K S Y S C G C R G *
551 agatctccagatacgaagattaagacgcgatgttaccacaaaaatgat
601 gattcaagatttcaagagacaagatgtaaatatagcttgaaaatatttcc
651 gtattcttcgaggggtacactgtttatatttctgttgtaaacattgcc
701 ctaaatgatgctaaaaataactatgatagcaaaaaaaaaaaaaaaaaaaaa

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FIGURE 2. cDNA and deduced amino acid sequences of preprodamicornin. The ORF sequence is shown in *capital letters*. The expressed sequence tag obtained from the subtractive subtraction hybridization library is highlighted in *gray*. 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*.

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 reverse phase HPLC. All of the 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 reverse phase 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 Gly-107 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 tryptic digests presented a similar pattern with seven 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 six cysteine residues

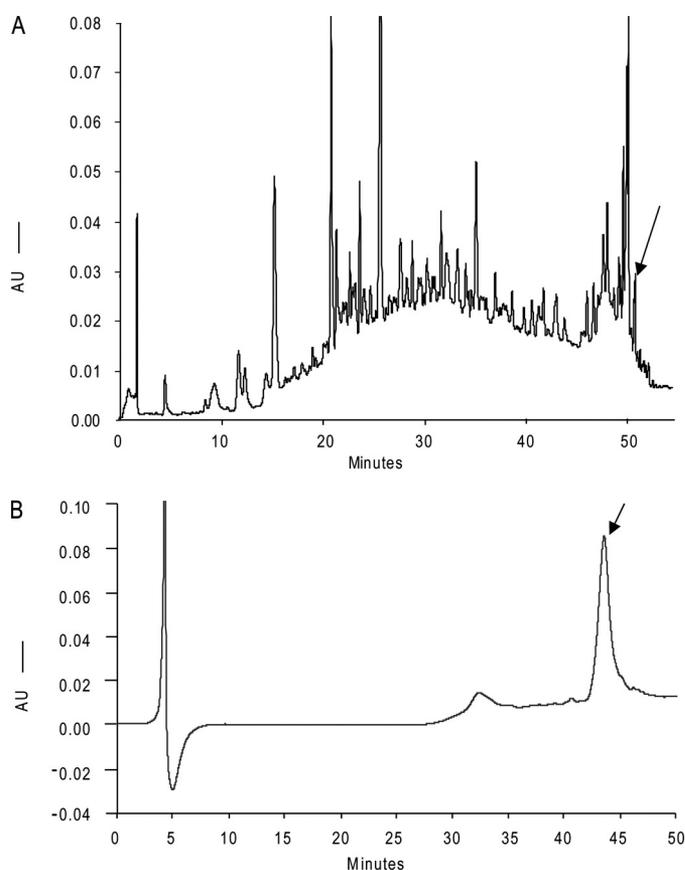


FIGURE 3. Purification of damicornin from acidic extracts obtained from challenged coral tissue. A, following prepurification by solid phase extraction, the material eluted from the fraction with 60% acetonitrile was loaded onto a C_{18} column. In this HPLC step, elution was performed with a linear 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.

involved in three intramolecular disulfide 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 analyzed by MALDI LTQ Orbitrap mass spectrometer. As illustrated in [supplemental Fig. S1A](#), the presence of pseudo-molecular ions $[M + H]^+$ at m/z 1437.64, 1667.83, and 1766.71 were consistent with a possible pairing scheme C_{11} with C_{32} , C_{18} with C_{36} or C_{38} , and C_2 with C_{36} or C_{38} . To confirm this possible pairing scheme, ion fragmentation reactions were conducted by collision-induced dissociation. Fragmentation of ion at m/z 1437.64 ([supplemental Fig. S1B](#)) confirmed the C_{11} - C_{32} pairing. Because the C-terminal fragment $SC_{36}GC_{38}R-NH_2$ obtained by chymotrypsin digestion includes two cysteines, the assignment of the two other disulfide bridges 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 ([supplemental Fig. S1, C and D](#)) confirmed the pairing scheme C_2 -(C_{36} or C_{38}) and C_{18} -(C_{36} or

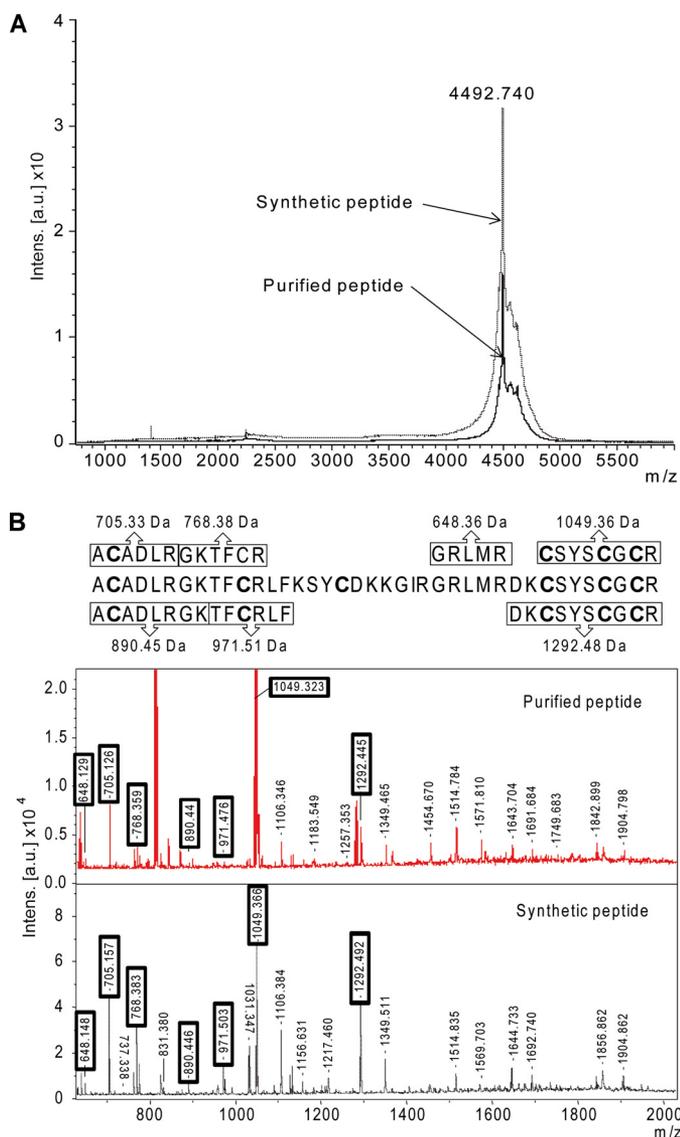


FIGURE 4. Identification of damicornin by mass spectrometry analysis. A, superimposed linear mode MALDI mass spectra of purified damicornin and synthetic peptide, showing a major peak at 4492.740 Da. B, analysis of tryptic digests of purified peptide and synthetic peptide by MALDI-TOF MS. The peptides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

C₃₈). The same cysteine connections were also obtained for the synthetic damicornin (data not shown).

In addition, Fig. 1 gives the alignment of damicornin with the anemone 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 of these molecules share the same cysteine array. Note that the data obtained on damicornin cysteine connectivity (supplemental Fig. S1) were consistent with those obtained for these anemone toxins (given in Fig. 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.

TABLE 2
Minimal inhibitory concentration and minimal bactericidal concentration of damicornin

MBC were determined by testing different concentrations of synthetic peptide in liquid growth inhibition assays against different bacterial strains according to the Hancock method. ND, not done.

Microorganisms	MIC	MBC
	μM	μM
Gram-positive bacteria		
<i>B. megaterium</i> (souchier IBMC)	20	>20
<i>S. aureus</i> (SG511)	5	>20
<i>M. luteus</i> (A270)	1.25	2.5
<i>B. stationis</i> (CIP 101282) ^a	10	10
<i>M. maritopicum</i> (CIP 105733T) ^a	20	>20
Gram-negative bacteria		
<i>E. coli</i> (SBS 363)	10	20
<i>V. aesturianus</i> (CIP 109791) ^a	>20	>20
<i>V. shiloi</i> (CIP 107136) ^{a,b}	>20	>20
<i>V. corallilyticus</i> strain YB1 (CIP 107925) ^{a,b}	>20	>20
<i>V. splendidus</i> (CIP 107715) ^a	>20	>20
Fungi		
<i>F. oxysporum</i>	1.25	ND

^a Marine bacteria.

^b For these strains, the MIC and MBC were tested at either 23 or 30 °C. The results were the same at both temperatures.

TABLE 3
Bacteriolytic effect of damicornin on *M. luteus*

Time of incubation	Control	AMP
	10 ⁴ CFU·ml ⁻¹	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

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

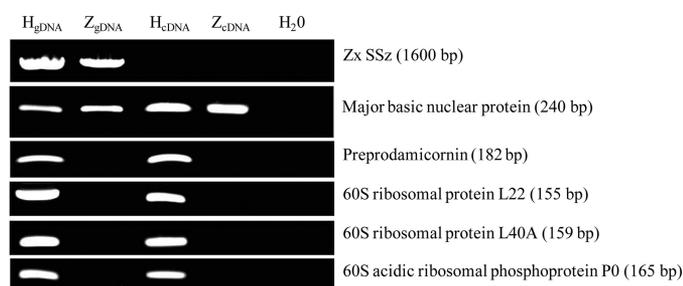


FIGURE 5. The preprodamicornin gene is expressed by the coral host. The presence of preprodamicornin, the reference gene for q-RT-PCR, the small ribosomal subunit RNA of the zooxanthellae (Zx ssRNA), and the gene corresponding to the major basic zooxanthellae nuclear protein were investigated by PCR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

basic nuclear protein gene 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 major basic nuclear protein primers amplified DNA and cDNA from both holobiont and symbiont cultures. As with the damicornin gene, the three q-RT-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 (supplemental Fig. S2). This experiment, performed on unstressed coral, showed there was a molecular mass difference between the synthetic damicornin and the band found in coral extracts (~10 kDa). This suggests that damicornin may be stored as a precursor in coral tissues. The expected molecular mass of the prodamicornin was 9.3 kDa. Because antibacterial activity was detected by reverse phase 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 nonvirulent *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, panel A1). Tissue sections stained with DAPI are shown in Fig. 6 (panel A2). The reduced background obtained with this blue staining of the nucleus highlights the two cellular layers constituting each tissue type. The four 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, panel C1). At higher magnification, the

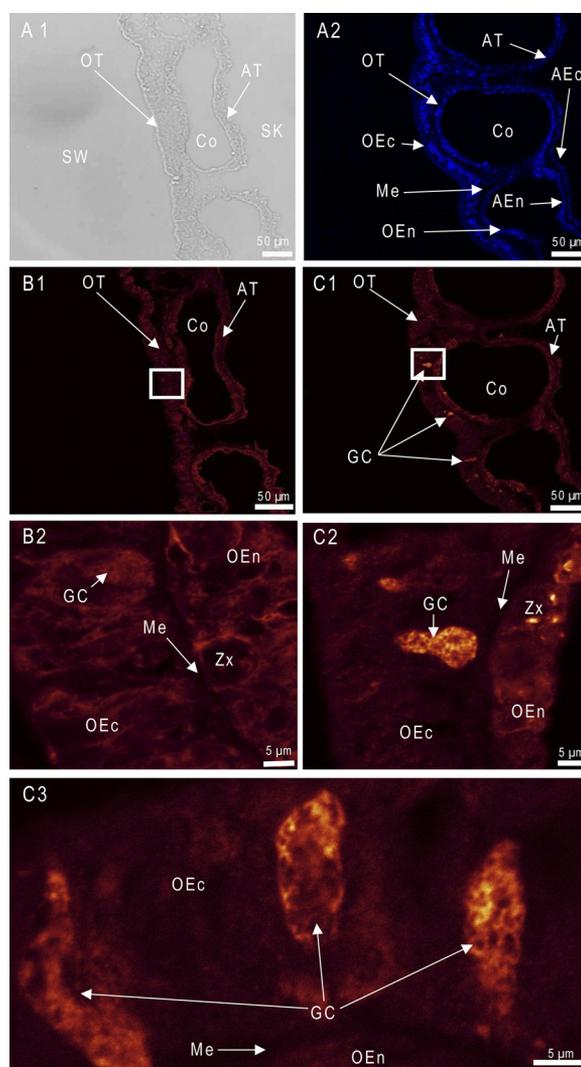


FIGURE 6. Immunolabeling of damicornin in ectodermal granular cells in oral tissue. A1 and A2, bright field transmitted light image (A1) and an image of the DAPI-stained nucleus (A2) showing the four tissue layers and the coelenteron (i.e. the gastric cavity) and their position in relation to the seawater (SW) environment and the coral skeleton (SK). B1 and B2, control experiments performed using anti-damicornin antibodies that were preadsorbed with the synthetic peptide used for immunization. C1, C2, and C3, the labeled damicornin appears bright orange. 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 three labeled granular cells. Zx, zooxanthellae; OEn, oral endoderm; Me, mesoglea.

staining was associated with cells containing intracellular granules (Fig. 6, panels C2 and C3). Controls treated with the depleted antibody (i.e. antibodies preincubated with synthetic damicornin) showed faint tissue autofluorescence but no specific labeling (Fig. 6, panels B1 and B2s), 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 nonvirulent and virulent *V. coralliilyticus* was studied. Using q-RT-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 nonvirulent bacteria (Fig. 7A), but large variation was evident after challenge

Scleractinian AMP Immune Responses during Vibriosis

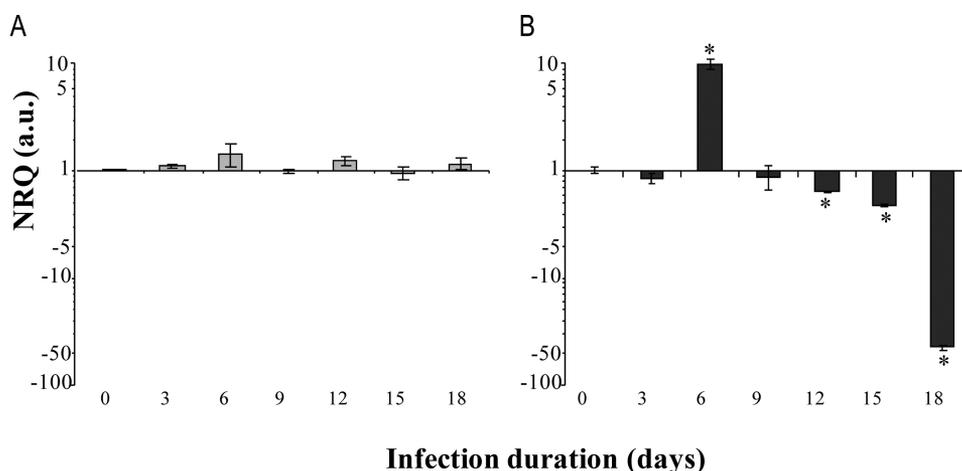


FIGURE 7. **Disturbance of preprodamicornin expression following infection by virulent bacteria.** The transcription rate of preprodamicornin was measured by q-RT-PCR for samples at D0, D3, D6, D9, D12, D15, and D18 from the nonvirulent (A) and the virulent (B) treatments. The relative expression was normalized using the geometric mean of three reference genes (normalized relative quantity, NRQ). The gray and black histograms represent the relative expression of the coral preprodamicornin gene in the nonvirulent and virulent treatments, respectively. The bars represent the mean of replicates, and the error bars represent the S.E. *, observations that deviated significantly ($p < 0.05$) from others in the same treatment (the nonvirulent and the virulent treatments).

with the virulent *V. coralliilyticus* (Fig. 7B). 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. 7B). 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 AMP from a scleractinian coral. It is 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 three intramolecular disulfide bridges involving the six cysteine residues in its sequence and that it has C-terminal amidation resulting from the removal of an end glycine residue. AMPs folded by three intramolecular disulfide bridges have been reported in many invertebrate and vertebrate species and often belong to the defensin superfamily (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 three 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 preprodamicornin is removed by proteolytic cleavage of the Arg-67–Ala-68 bond by a processing enzyme that recognizes the dibasic motif (Arg-Arg) located ahead of the observed cleavage site; and (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 six cysteine residues involved in intramolecular disulfide 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 CS $\alpha\beta$ 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 and 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 nonvirulent 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 ~10 kDa was detected by anti-damicornin antibody in unstressed coral extracts (supplemental Fig. S2). This band may correspond to prodamicornin, which has a theoretical molecular mass 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 days 9 to 18. In contrast, no transcriptional change was observed when *P. damicornis* was exposed to the nonvirulent 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 nonspecific 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). Although 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 nonpathogenic 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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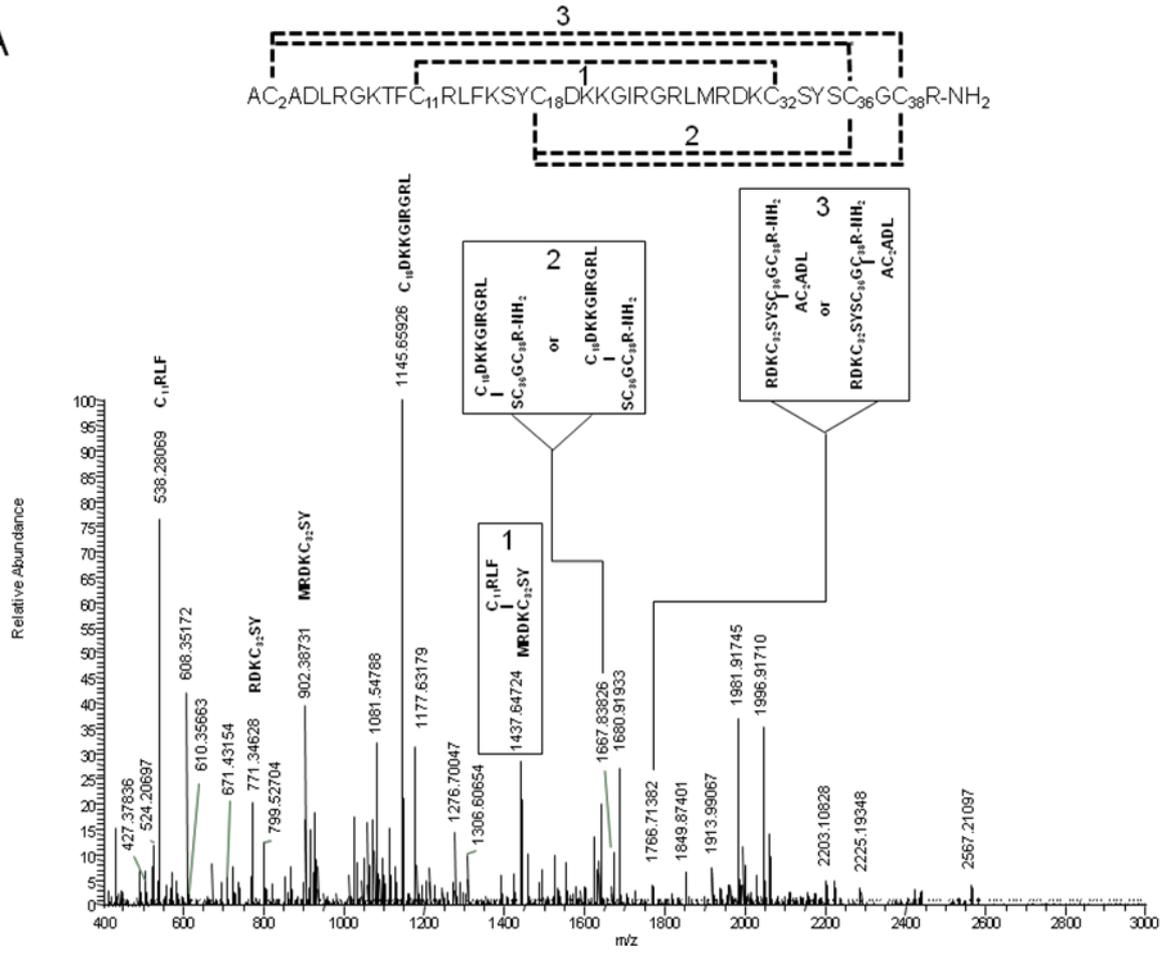
REFERENCES

- Bourne, D. G., Garren, M., Work, T. M., Rosenberg, E., Smith, G. W., and Harvell, C. D. (2009) *Trends Microbiol.* **17**, 554–562
- Donner, S. D., Skirving, W. J., Little, C. M., Oppenheimer, M., and Hoegh-Guldberg, O. V. (2005) *Glob. Chang. Biol.* **11**, 2251–2265
- Hoegh-Guldberg, O., Mumby, P. J., Hooten, A. J., Steneck, R. S., Greenfield, P., Gomez, E., Harvell, C. D., Sale, P. F., Edwards, A. J., Caldeira, K., Knowlton, N., Eakin, C. M., Iglesias-Prieto, R., Muthiga, N., Bradbury, R. H., Dubi, A., and Hatziolos, M. E. (2007) *Science* **318**, 1737–1742
- Hughes, T. P., Baird, A. H., Bellwood, D. R., Card, M., Connolly, S. R., Folke, C., Grosberg, R., Hoegh-Guldberg, O., Jackson, J. B., Kleypas, J., Lough, J. M., Marshall, P., Nyström, M., Palumbi, S. R., Pandolfi, J. M., Rosen, B., and Roughgarden, J. (2003) *Science* **301**, 929–933
- Lesser, M. P. (2007) *Proc. Natl. Acad. Sci. U.S.A.* **104**, 5259–5260
- Ward, J. R., and Lafferty, K. D. (2004) *PLoS Biol.* **2**, E120
- Weil, E., Smith, G., and Gil-Agudelo, D. L. (2006) *Dis. Aquat. Org.* **69**, 1–7
- Rodriguez-Lanetty, M., Harii, S., and Hoegh-Guldberg, O. (2009) *Mol. Ecol.* **18**, 5101–5114
- Ward, J. R., Kim, K., and Harvell, C. D. (2007) *Mar. Ecol.-Prog. Ser.* **329**, 115–121
- Ben-Haim, Y., Thompson, F. L., Thompson, C. C., Cnockaert, M. C., Hoste, B., Swings, J., and Rosenberg, E. (2003) *Int. J. Syst. Evol. Microbiol.* **53**, 309–315
- Kushmaro, A., Rosenberg, E., Fine, M., Ben Haim, H., and Loya, Y. (1998) *Mar. Ecol.-Prog. Ser.* **171**, 131–137
- Ben-Haim Rozenblat, Y., and Rosenberg, E. (2004) in *Coral Health and Disease* (Rosenberg, E., and Loya, Y., eds) pp. 301–324, Springer-Verlag, New York

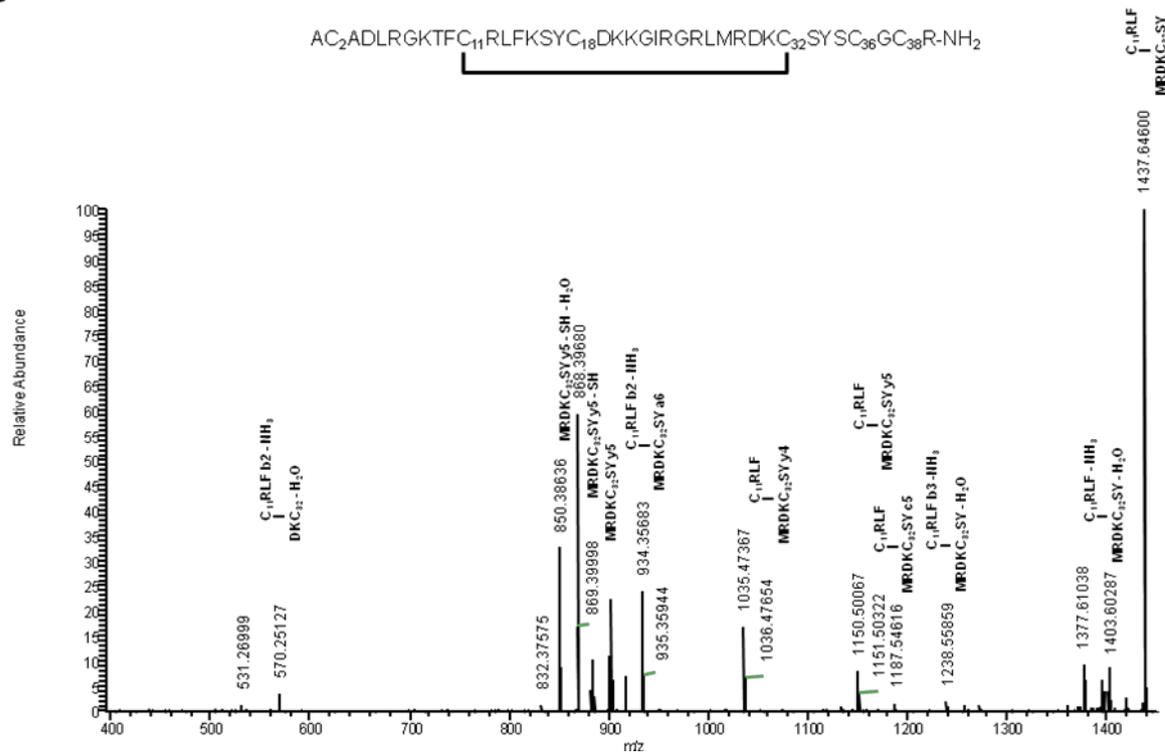
13. Rosenberg, E. (2004) in *Coral Health and Disease* (Rosenberg, E., and Loya, Y., eds) pp. 445–462, Springer-Verlag, New York
14. Mydlarz, L. D., Jones, L. E., and Harvell, C. D. (2006) *Annu. Rev. Ecol. Evol. Syst.* **37**, 251–288
15. Kvennefors, E. C., Leggat, W., Hoegh-Guldberg, O., Degnan, B. M., and Barnes, A. C. (2008) *Dev. Comp. Immunol.* **32**, 1582–1592
16. Kvennefors, E. C., Leggat, W., Kerr, C. C., Ainsworth, T. D., Hoegh-Guldberg, O., and Barnes, A. C. (2010) *Dev. Comp. Immunol.* **34**, 1219–1229
17. Mullen, K. M., Peters, E. C., and Harvell, C. D. (2004) in *Coral Health and Disease* (Rosenberg, E., and Loya, Y., eds) pp. 377–400, Springer-Verlag, New York
18. Mydlarz, L. D., Couch, C. S., Weil, E., Smith, G., and Harvell, C. D. (2009) *Mar. Ecol.-Prog. Ser.* **371**, 67–78
19. Palmer, C. V., Bythell, J. C., and Willis, B. L. (2010) *FASEB J.* **24**, 1935–1946
20. Palmer, C. V., Modi, C. K., and Mydlarz, L. D. (2009) *Plos One* **4**, e7298
21. Palmer, C. V., Mydlarz, L. D., and Willis, B. L. (2008) *Proc. R. Soc. Lond. B Biol. Sci.* **275**, 2687–2693
22. Vidal-Dupiol, J., Adjeroud, M., Roger, E., Foure, L., Duval, D., Mone, Y., Ferrier-Pages, C., Tambutte, E., Tambutte, S., Zoccola, D., Allemand, D., and Mitta, G. (2009) *BMC Physiol.* **9**, 14
23. Geffen, Y., Ron, E. Z., and Rosenberg, E. (2009) *FEMS Microbiol. Lett.* **295**, 103–109
24. Geffen, Y., and Rosenberg, E. (2005) *Mar. Biol.* **146**, 931–935
25. Gochfeld, D. J., and Aeby, G. S. (2008) *Mar. Ecol.-Prog. Ser.* **362**, 119–128
26. Kelman, D., Kashman, Y., Rosenberg, E., Kushmaro, A., and Loya, Y. (2006) *Mar. Biol.* **149**, 357–363
27. Vidal-Dupiol, J., Ladrrière, O., Meistertzheim, A. L., Fouré, L., Adjeroud, M., and Mitta, G. (2011) *J. Exp. Biol.* **214**, 1533–1545
28. Schmitt, P., Wilmes, M., Pugnère, M., Aumelas, A., Bachère, E., Sahl, H. G., Schneider, T., and Destoumieux-Garzón, D. (2010) *J. Biol. Chem.* **285**, 29208–29216
29. Ben-Haim, Y., and Rosenberg, E. (2002) *Mar. Biol.* **141**, 47–55
30. Yuan, J. S., Reed, A., Chen, F., and Stewart, C. N., Jr. (2006) *BMC Bioinformatics* **7**, 85–97
31. Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., and Vandesomepele, J. (2007) *Genome Biol.* **8**, R19
32. Rowan, R., and Powers, D. A. (1991) *Mar. Ecol.-Prog. Ser.* **71**, 65–73
33. Vaitukaitis, J., Robbins, J. B., Nieschlag, E., and Ross, G. T. (1971) *J. Clin. Endocrinol. Metab.* **33**, 988–991
34. Hancock, C. D., and Evans, G. I. (1992) in *Methods in Molecular Biology: Immunochemical Protocols* (Manson, M., eds) pp. 33–41, Humana Press, Totowa, NJ
35. Porath, J., and Olin, B. (1983) *Biochemistry* **22**, 1621–1630
36. Zoccola, D., Tambutté, E., Kulhanek, E., Puverel, S., Scimeca, J. C., Allemand, D., and Tambutté, S. (2004) *Biochim. Biophys. Acta* **1663**, 117–126
37. Puverel, S., Tambutte, E., Zoccola, D., Domart-Coulon, I., Bouchot, A., Lotto, S., Allemand, D., and Tambutte, S. (2005) *Coral Reefs* **24**, 149–156
38. Bulet, P., Cocianich, S., Dimarcq, J. L., Lambert, J., Reichhart, J. M., Hoffmann, D., Hetru, C., and Hoffmann, J. A. (1991) *J. Biol. Chem.* **266**, 24520–24525
39. Destoumieux-Garzón, D., Thomas, X., Santamaria, M., Goulard, C., Barthélémy, M., Boscher, B., Bessin, Y., Molle, G., Pons, A. M., Letellier, L., Peduzzi, J., and Rebuffat, S. (2003) *Mol. Microbiol.* **49**, 1031–1041
40. Hetru, C., and Bulet, P. (1997) in *Antibacterial Peptide Protocols* (Shafer, W. M., ed) pp. 35–50, Humana Press, Totowa, NY
41. Fehlbaum, P., Bulet, P., Michaut, L., Lagueux, M., Broekaert, W. F., Hetru, C., and Hoffmann, J. A. (1994) *J. Biol. Chem.* **269**, 33159–33163
42. Grubbs, F. E. (1969) *Technometrics* **11**, 1–21
43. Stefansky, W. (1972) *Technometrics* **14**, 469–479
44. Murthy, A. S., Mains, R. E., and Eipper, B. A. (1986) *J. Biol. Chem.* **261**, 1815–1822
45. Cotton, J., Crest, M., Bouet, F., Alessandri, N., Gola, M., Forest, E., Karlsson, E., Castañeda, O., Harvey, A. L., Vita, C., and Ménez, A. (1997) *Eur. J. Biochem.* **244**, 192–202
46. Gendeh, G. S., Young, L. C., de Medeiros, C. L., Jeyaseelan, K., Harvey, A. L., and Chung, M. C. (1997) *Biochemistry* **36**, 11461–11471
47. Pohl, J., Hubalek, F., Byrnes, M. E., Nielsen, K. R., Woods, A., and Pennington, M. W. (1995) *Let. Peptide Sci.* **1**, 291–297
48. Bulet, P., Stöcklin, R., and Menin, L. (2004) *Immunol. Rev.* **198**, 169–184
49. Destoumieux, D., Bulet, P., Loew, D., Van Dorsseleer, A., Rodriguez, J., and Bachère, E. (1997) *J. Biol. Chem.* **272**, 28398–28406
50. Klaidiny, J., Albert, S., Bachanová, K., Kopernický, J., and Simúth, J. (2005) *Insect Biochem. Mol. Biol.* **35**, 11–22
51. Rees, J. A., Moniatte, M., and Bulet, P. (1997) *Insect Biochem. Mol. Biol.* **27**, 413–422
52. Baumann, T., Kämpfer, U., Schürch, S., Schaller, J., Largiadèr, C., Nentwig, W., and Kuhn-Nentwig, L. (2010) *Cell. Mol. Life Sci.* **67**, 2787–2798
53. Herbinère, J., Braquart-Varnier, C., Grève, P., Strub, J. M., Frère, J., Van Dorsseleer, A., and Martin, G. (2005) *Dev. Comp. Immunol.* **29**, 489–499
54. Lorenzini, D. M., da Silva, P. I., Jr., Fogaça, A. C., Bulet, P., and Daffre, S. (2003) *Dev. Comp. Immunol.* **27**, 781–791
55. Nakamura, T., Furunaka, H., Miyata, T., Tokunaga, F., Muta, T., Iwanaga, S., Niwa, M., Takao, T., and Shimonishi, Y. (1988) *J. Biol. Chem.* **263**, 16709–16713
56. Silva, P. I., Jr., Daffre, S., and Bulet, P. (2000) *J. Biol. Chem.* **275**, 33464–33470
57. Boulanger, N., Lowenberger, C., Volf, P., Ursic, R., Sigutova, L., Sabatier, L., Svobodova, M., Beverley, S. M., Späth, G., Brun, R., Pesson, B., and Bulet, P. (2004) *Infect. Immun.* **72**, 7140–7146
58. De Zoysa, M., Whang, I., Lee, Y., Lee, S., Lee, J. S., and Lee, J. (2010) *Fish Shellfish Immunol.* **28**, 261–266
59. Li, C., Haug, T., Styrvold, O. B., Jørgensen, T. Ø., and Stensvåg, K. (2008) *Dev. Comp. Immunol.* **32**, 1430–1440
60. Mitta, G., Hubert, F., Noël, T., and Roch, P. (1999) *Eur. J. Biochem.* **265**, 71–78
61. Kolhekar, A. S., Roberts, M. S., Jiang, N., Johnson, R. C., Mains, R. E., Eipper, B. A., and Taghert, P. H. (1997) *J. Neurosci.* **17**, 1363–1376
62. Ovchinnikova, T. V., Balandin, S. V., Aleshina, G. M., Tagaev, A. A., Leonova, Y. F., Krasnodembsky, E. D., Men'shenin, A. V., and Kokryakov, V. N. (2006) *Biochem. Biophys. Res. Commun.* **348**, 514–523
63. Bontems, F., Roumestand, C., Boyot, P., Gilquin, B., Doljansky, Y., Menez, A., and Toma, F. (1991) *Eur. J. Biochem.* **196**, 19–28
64. Bontems, F., Roumestand, C., Gilquin, B., Ménez, A., and Toma, F. (1991) *Science* **254**, 1521–1523
65. Alessandri-Haber, N., Lecoq, A., Gasparini, S., Grangier-Macmath, G., Jacquet, G., Harvey, A. L., de Medeiros, C., Rowan, E. G., Gola, M., Ménez, A., and Crest, M. (1999) *J. Biol. Chem.* **274**, 35653–35661
66. Dauplais, M., Lecoq, A., Song, J., Cotton, J., Jamin, N., Gilquin, B., Roumestand, C., Vita, C., de Medeiros, C. L., Rowan, E. G., Harvey, A. L., and Ménez, A. (1997) *J. Biol. Chem.* **272**, 4302–4309
67. Rauer, H., Pennington, M., Cahalan, M., and Chandy, K. G. (1999) *J. Biol. Chem.* **274**, 21885–21892
68. Cole, A. M., Weis, P., and Diamond, G. (1997) *J. Biol. Chem.* **272**, 12008–12013
69. Karlsson, J., Pütsep, K., Chu, H., Kays, R. J., Bevins, C. L., and Andersson, M. (2008) *BMC Immunology* **9**, 37
70. Wehkamp, J., Chu, H., Shen, B., Feathers, R. W., Kays, R. J., Lee, S. K., and Bevins, C. L. (2006) *FEBS Lett.* **580**, 5344–5350
71. Bosch, T. C., Augustin, R., Anton-Erxleben, F., Fraune, S., Hemmrich, G., Zill, H., Rosenstiel, P., Jacobs, G., Schreiber, S., Leippe, M., Stanisak, M., Grötzinger, J., Jung, S., Podschun, R., Bartels, J., Harder, J., and Schröder, J. M. (2009) *Dev. Comp. Immunol.* **33**, 559–569
72. Ferrandon, D., Jung, A. C., Criqui, M., Lemaitre, B., Uttenweiler-Joseph, S., Michaut, L., Reichhart, J., and Hoffmann, J. A. (1998) *EMBO J.* **17**, 1217–1227
73. Mitta, G., Vandenbulcke, F., Hubert, F., and Roch, P. (1999) *J. Cell Sci.* **112**, 4233–4242
74. Sperandio, B., Regnault, B., Guo, J., Zhang, Z., Stanley, S. L., Jr., Sansonetti, P. J., and Pédrón, T. (2008) *J. Exp. Med.* **205**, 1121–1132
75. Allam, B., and Ford, S. E. (2006) *Fish Shellfish Immunol.* **20**, 374–383
76. Choquet, G., Soudant, P., Lambert, C., Nicolas, J. L., and Paillard, C. (2003) *Dis. Aquat. Org.* **57**, 109–116
77. Labreuche, Y., Lambert, C., Soudant, P., Boulo, V., Huvet, A., and Nicolas, J. L. (2006) *Microbes Infect.* **8**, 2715–2724
78. Labreuche, Y., Soudant, P., Gonçalves, M., Lambert, C., and Nicolas, J. L. (2006) *Dev. Comp. Immunol.* **30**, 367–379
79. Mateo, D. R., Siah, A., Araya, M. T., Berthe, F. C., Johnson, G. R., and Greenwood, S. J. (2009) *J. Invertebr. Pathol.* **102**, 50–56

Fig. S1. Damicornin disulphide bond assignment. Disulfide bond assignment was undertaken using a combination of endoprotease digestion and MS experiments. Panel (A) corresponds to MS spectrum obtained using a MALDI LTQ Orbitrap XL mass spectrometer. Interesting masses were associated with the putative peptides (boxes 1, 2 and 3). The various possible combinations of cysteine connectivity are given and reported on the damicornin sequence. The possible connectivities corresponding to each box number are reported (dotted lines) with the same number on the damicornin sequence. To confirm the identity of the putative peptide combination, MS/MS fragmentation of the 3 molecules of interest was undertaken. Panel (B) shows the MS-MS analysis (1437.64 Da) of the peptide of C₁₁RLF and MRDKC₃₂SY (Box 1, panel A). Panel (C) shows the MS-MS analysis (1766.88 Da) of the peptide of RDKC₃₂SYSC₃₆GC₃₈R and AC₂ADL (Box 3, panel A). Panel (D) shows the MS-MS analysis (1667.83 Da) of the peptide of C₁₈DKKGIRGRL and SC₃₆GC₃₈R (Box 2, panel A). "?" indicates that the cysteines implicated in the connectivity of 2 peptide could not be identified.

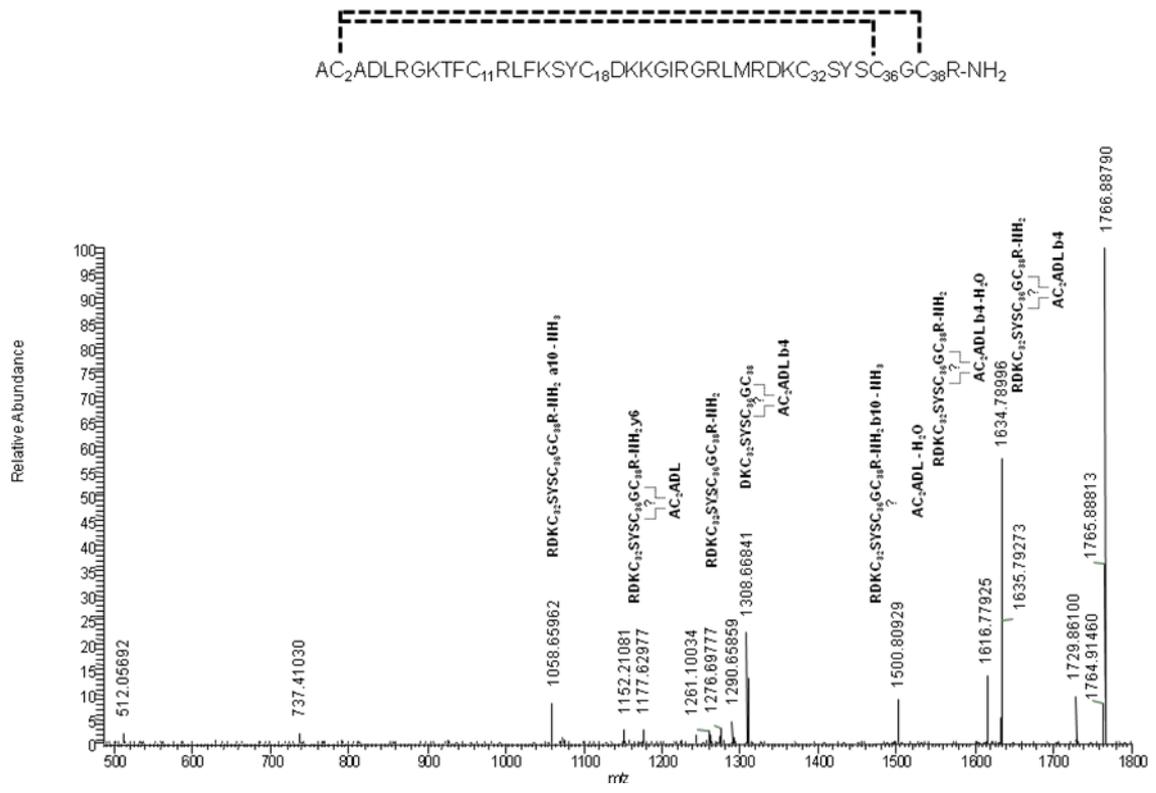
A



B



C



D

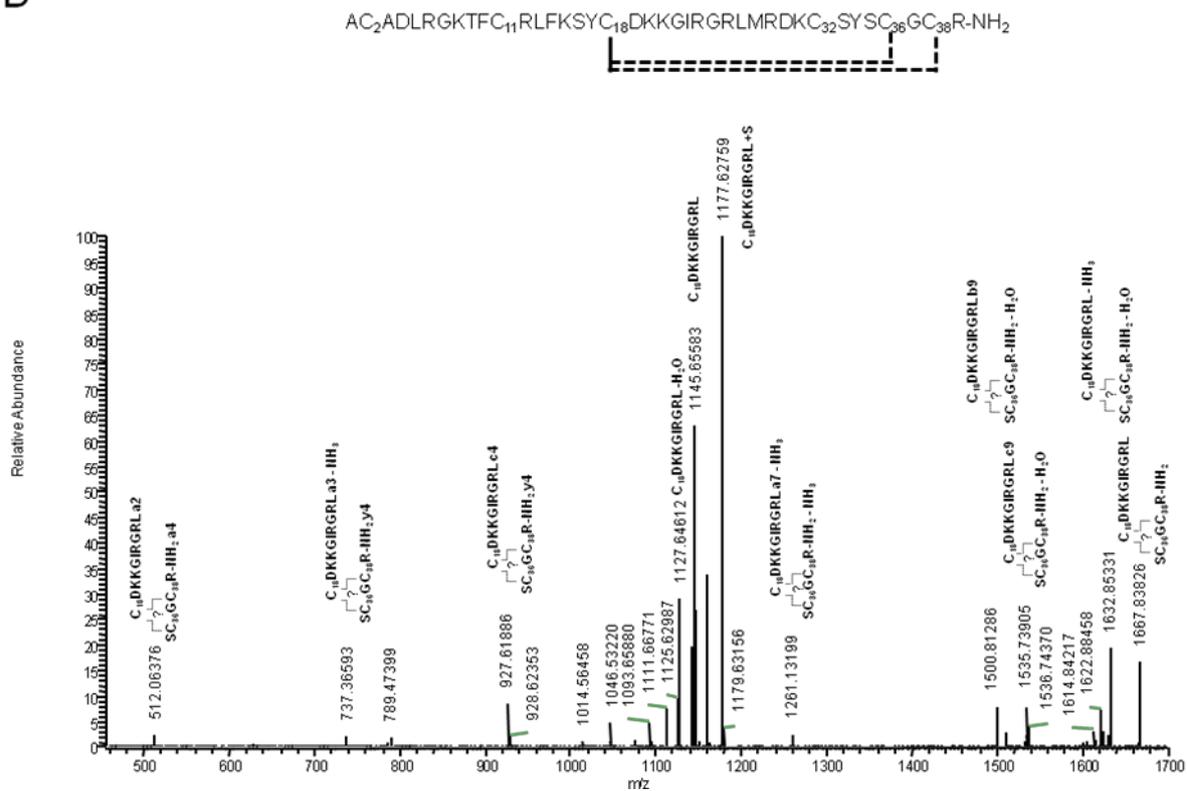


Fig. S2. Anti-damicornin antibody specificity tested by Western blotting. The specificity of the anti-damicornin antibody was tested on coral protein extract and on the synthetic damicornin used for immunization, following Tris-tricine (16.5%) electrophoresis. Coral protein extract and synthetic damicornin were probed with the purified antibody and the pre-immune sera.

