Coral-associated bacteria, quorum sensing disrupters, and the regulation of biofouling

Karina Golberg, Valentina Pavlov, Robert S. Marks, and Ariel Kushmaro

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Marine biofouling, the settlement of microorganisms and macroorganisms on structures submerged in seawater, although economically detrimental, is a successful strategy for survival in hostile environments, where coordinated bacterial communities establish biofilms via the regulation of quorum sensing (QS) communication systems. The inhibition of QS activity among bacteria isolated from different coral species was investigated to gain further insight into its potency in the attenuation, or even the prevention, of undesirable biofouling on marine organisms. It is hypothesized that coral mucus/microorganism interactions are competitive, suggesting that the dominant communities secrete QS disruptive compounds. One hundred and twenty bacterial isolates were collected from healthy coral species and screened for their ability to inhibit QS using three bioreporter strains. Approximately 12, 11, and 24% of the isolates exhibited anti-QS activity against Escherichia coli pSB1075, Chromobacterium violaceum CV026, and Agrobacterium tumefaciens KYC55 indicator strains, respectively. Isolates with positive activity against the bioluminescent monitor strains were scanned via a cytotoxic/genotoxic, E. coli TV1061 and DPD2794 antimicrobial panel. Isolates detected by C. violaceum CV026 and A. tumefaciens KYC55 reporter strains were tested for their ability to inhibit the growth of these reporter strains, which were found to be unaffected. Tests of the Favia sp. coral isolate Fav 2-50-7 (>98% similarity to Vibrio harveyi) for its ability to attenuate the formation of biofilm showed extensive inhibitory activity against biofilms of Pseudomonas aeruginosa and Acinetobacter baumannii. To ascertain the stability and general structure of the active compound, cell-free culture supernatants exposed to an increasing temperature gradient or to digestion by proteinase K, were shown to maintain potent QS attenuation and the ability to inhibit the growth of biofilms. Mass spectrometry confirmed the presence of a low molecular mass compound. The anti-QS strategy exemplified in the coral mucus is a model with potentially wide applications, including countering the ecological threat posed by biofilms. Manipulating synchronized bacterial behavior by detecting new QS inhibitors will facilitate the discovery of new antifouling compounds.

Keywords: quorum sensing inhibitors; biofilm; bioreporters; Acinetobacter baumannii; Pseudomonas aeruginosa

Introduction

Marine biofouling, defined as the rapid and extensive growth of marine organisms on submerged inanimate and living surfaces, is a serious problem worldwide. The establishment of biofilms in the aquatic environment typically begins with the settlement of microorganisms (bacteria and unicellular eukaryotes), followed by the recruitment of macrofouling species (invertebrate larvae and algal spores; Dobretsov et al. 2006, 2009), whose activities engender considerable economic consequences for aquaculture equipment, naval vessels, and a diversity of industrial structures (Braithwaite & McEvoy 2005; Dobretsov et al. 2006; Schultz et al. 2011; Fitridge et al. 2012). Although biocide treatments, including antifouling paints (Voulvoulis et al. 2002) are effective against fouling, many of the toxins are highly destructive pollutants of marine ecosystems (Yebra et al. 2004; Thomas & Brooks 2010). Thus, there is a need to pursue novel, non-toxic compounds that are capable of hindering the development of marine biofilms.

The marine environment is an abundant source of natural products that possess anti-biofilm properties. There is growing evidence that a variety of marine residents, particularly bacteria isolated from coral reefs, are capable of producing such compounds (Thenmozhi et al. 2009; Bakkiraraj & Pandian 2010; Nithya & Pandian 2010; Qian et al. 2010). Coral reefs are ecosystems with high biodiversity, reflected mostly by symbioses with microorganisms. While providing shelter, nutritional advantages, and a physical scaffold for biofilm formation, corals and other sedentary reef organisms are protected from deleterious pathogens by the activities of their resident microbes, which secrete antimicrobial compounds and engage in nitrogen fixation, nitrogen cycling, sulfur cycling, and physiology maintenance (Lesser et al. 2004; Shnit-Orland & Kushmaro 2009; Medina 2011).

*Corresponding authors. Email: rsmarks@bgu.ac.il; arielkus@bgu.ac.il

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Few, if any, such activities that are especially pertinent to the coral mucus microbial community would be possible without a biofilm, the preferential microbial lifestyle in general and probably of the coral mucus microbial community in particular (del Pozo & Patel 2007). Microbiological assessments indicate that 99% of the bacterial world exists in a biofilm mode of growth (Potera 1998), implying the paradox of both expressing and inhibiting the same structural phenotype. However, it is precisely this paradox that illustrates the complexity within the bacterial communities embedded in self-produced biofilm matrices and their relationships with their coral hosts and/or new, potentially harmful residents. Specific bacteria–coral associations may play pivotal roles in the state of coral health or disease levels. A beneficial bacterial community may possess potent deterrence mechanisms against pathogenic fouling organisms, and as such, it may protect the coral holobiont (Rohwer et al. 2001; Ritchie 2006; Bourne et al. 2007; Shnit-Orland & Kushmaro 2009).

Elucidating the mechanism of biofilm regulation may offer a novel inhibition strategy. Biofilm formation is cooperatively regulated by synchronized community behavior. The bacterial paradigm, which describes the world as non-cooperative with every bacterium behaving as an independent unit, was undermined when quorum sensing (QS) ascribed the behavior to higher order, multicellular organisms of the prokaryote kingdom (Williams 2007). Bacterial intracellular or intercellular QS communication is based on the production, detection, and subsequent response to signal molecules (Chen et al. 2011). Chemical language regulates the expression of particular genes in the entire community, such as those active during biofilm development, to help it survive in and adapt to changing environments (Hoiby et al. 2001; Atkinson & Williams 2009).

QS is achieved through the production of two classes of molecules: cell density-population dependent signals, known as auto-inducers, which provide the population with a numerical cell assessment, and different growth stage signal metabolites such as indoles (Lee et al. 2007). Probably the best characterized auto-inducers are low molecular weight acyl-homoserine lactone (AHL) molecules that are regularly produced at basal level and subsequently received by ‘speaking-listening’ bacteria (Adonizio et al. 2006). When high population densities are achieved, signals accumulate in the extracellular milieu until they reach threshold concentrations that initiate synchronized bacterial behavior through a multistage mechanism of regulation (Waters & Bassler 2005). Understanding the molecular regulatory mechanism behind biofilm initiation and maturation may lead to a novel anti-pathogen strategy where virulent bacterial phenotypes are inhibited without inhibition of growth. Indeed, growth limitation was found to stimulate the appearance of multi-resistance traits in bacteria (Lipsitch 2001). The disruption (or even prevention) of biofouling is feasible at two levels of QS attenuation: the first is signal reception and the second focuses on signal synthesis pathways (Whitehead et al. 2001). An alternative strategy is biological signal inactivation by AHL degrading enzymes, which fall into two categories, AHL lactonases and AHL acylases, and which cleave the lactone ring and the amide bond, respectively (Park et al. 2005; Czajkowski & Jafra 2009). Blocking one of the key steps of the QS mechanisms of pathogenic bacteria may tune down virulence factor production and make biofilms more susceptible to treatment with antibiotics.

The evidence gathered thus far emphasizes the need for better insight into the forces driving coral ecology in the light of the ability of different species of coral-associated bacteria to interfere with QS signaling mechanisms and biofilm formation. However, despite the abundance of active compounds from marine environments, to date, the discovery and isolation of anti-QS compounds from these sources has been slow compared with the synthetic chemistry approach (Dobretsov et al. 2011). The present study stresses the importance of the marine environment, especially corals and their corresponding microbial communities, as a potential model for naturally occurring products with anti-QS properties. In light of the significance of QS in the formation of biofilms, coral-associated bacteria and their ability to inhibit QS communication were examined. More specifically, given the limited information available on the production of these cues by marine bacteria, the purpose of this study was to gain a clearer understanding of the ecological role of the anti-QS substances secreted by coral-associated bacteria.

Materials and methods

Field sampling and isolation of bacteria

Bacterial isolates were collected from a reef adjacent to the Inter-University Institute for Marine Science in the Gulf of Eilat, Israel (29° 30.211’ N, 34° 55.068’ E). The samples were obtained from the coral mucus layer of different species growing at depths of 3–10 m (Harel et al. 2008). The coral samples included seven stony coral species (Platygryra sp., Porites sp., Fungia granulosa, Favia sp., Stylphora sp., Acanthastrea sp., and Pocillopora sp.) and one soft coral species (Xenia sp.). Additional isolates were obtained from corals sampled in a previous study (Shnit-Orland & Kushmaro 2009). With the exception of bacteria from Acanthastrea sp., all bacteria were designated using the first three initials of the specific host coral name. The former was designated by the first letter of the genus.
Bacterial cultures were isolated as reported previously (Golberg et al. 2011). Briefly, mucus samples were collected from the upper portion of the healthy coral colony or polyp using plastic bacteriological loops to rub off the mucus layer. Bacteria were isolated via a serial dilution and plating technique as follows: the mucus sample tubes were centrifuged and then diluted 10-fold, after which the samples were spread at concentrations of 10% over marine agar plates (Himedia Laboratories). After incubation at 22 °C for 2–3 weeks, unique colonies were isolated by repeated streaking steps.

**Bacterial strains, culture media and growth conditions**

The bioreporters used in this study included the *Escherichia coli* strains pSB1075, obtained from J. Davies (University of Calgary, Calgary, Canada), DPDP2794 and TV1061, obtained from S. Belkin (Hebrew University, Jerusalem, Israel). Strain *E. coli* pSB1075 carries the pSB1075 plasmid that contains a fusion of lasRI in which the lasI promoter is transcriptionally activated in response to the presence of long chain AHLs (containing 10–14 carbons in the acyl chain) (Winson et al. 1998). The *E. coli* TV1061 monitor strain harbors a plasmid encoding the gfpE stress heat-shock promoter, which is present in the absence of cytotoxic metabolites and elevated temperatures (Arsene et al. 2000). The bioreporter *E. coli* DPDP2794 carries the DNA damage promotor recA, which is involved in the cell repair system when exposed to genotoxic substances (Vollmer et al. 1997). Each of the bioreporters mentioned above expresses the luxCDABE reporter gene fused to an appropriate promoter.

The *E. coli* strains were cultivated in LB medium (Difco Luria-Bertani medium, BD, France) supplemented with ampicillin (100 μg ml⁻¹). The *E. coli* strain pSB1075 was grown overnight at 30 °C; the temperature for the other bioreporters was kept at 37 °C. All three strains were cultivated in a rotary thermo-shaker (Gerhardt, Germany) at 120 rpm. Overnight inocula were diluted into fresh LB medium to a density of approximately 10⁷ cells ml⁻¹ for re-growth at 30 °C, without shaking or the addition of antibiotics, to the early exponential phase (OD₆₀₀ ~0.2) as determined in an Ultrospec 2100 Pro spectrophotometer (Amersham, Berks, UK).

In addition to the *E. coli* strains, the reporter strain *Agrobacterium tumefaciens* KYC55 (carrying the pJZ372 (tral-lacZ), pJZ384 (T7 promoter and traR) and pJZ410 (T7 RNA polymerase and cI857 genes)) was provided by Prof. J. Zhu (University of Pennsylvania, Philadelphia, PA). This strain responds most strongly to AHL with side chains of between 4-12 carbons in length and with reduced acyl chain or 3-oxo/hydroxyl substitution as reflected by β-galactosidase activity (Zhu et al. 2003). *A. tumefaciens* KYC55 was grown under constant agitation in AT medium for 18 h without the addition of antibiotics (Zhu et al. 2003).

Another indicator strain, *Chromobacterium violaceum* CV026, was kindly provided by Prof. P. Williams (University of Nottingham, Nottingham, UK). Unsubstituted AHL chains varying in size from 4 to 8 carbons can up-regulate violet pigment production (violacein) via the cognate receptor CviR, while long-chain AHLs (10-14 carbons), detected by their ability to inhibit violacein, can function as QS inhibitor biosensors (McClean et al. 1997). *C. violaceum* CV026 was grown in LB medium at 30 °C, whilst *Acinetobacter baumannii* and the GFP-tagged *Pseudomonas aeruginosa* PA01 strains were grown in the same medium at 37 °C. All three strains were subjected to agitation at 120 rpm for 24 h.

Bacteria isolated from different corals were cultivated at 26 °C using 100% marine broth (MB) or LBN medium (Luria-Bertani medium supplemented with NaCl to a final concentration of 2%), the latter of which was used for *A. tumefaciens* KYC55 reporter strain screening because of the potential that the MB medium could inhibit the generation of the blue coloration.

**Bioluminescence assay for anti-QS activity**

Bacteria isolated from different corals were screened for their ability to inhibit QS activities using the *E. coli* pSB1075 sensor strain. After incubation at 26 °C in MB for 24 h, cultures were centrifuged at 14,870 × g for 10 min and filtered through 0.2 μm membranes. Bacterial bioluminescence was measured in relative light units using a luminometer (Thermo Fisher Scientific) set at 490 nm (ie a wavelength compatible with the emission spectrum of bacterial luciferase; Hakkila et al. 2002) in a transparent 96-well microtiter plate. QS inhibitory activity was assessed by comparing the bioluminescence signal observed in wells containing 90 μl of the bioreporter strain and 10 μl of the bacterial cell-free culture supernatant with that obtained from the same reaction mixture with the addition of 10 μl of 1.7 nM N-(3-Oxododecanoyl)-l-homoserine lactone (3-oxo-C12) (O9139, Sigma-Aldrich). Inhibition was indicated when the addition of 3-oxo-C12 resulted in a reduction in bioluminescence. During measurement, the sample temperature was maintained at 26 °C. Bacterial bioluminescence was expressed as the induction factor (IF), a normalized value calculated using Equation (1):

$$ IF = \frac{Bi}{Bc} $$

where Bi represents the maximum bioluminescent signal obtained from a specific coral cell-free culture isolate and Bc represents the maximum signal of the control reporter bacteria strain alone. An equivalent symbol designated Bia was used in Equation (2) for the AHL supplementation reactions in the sample well:
Therefore it cannot produce its own autoinducer signals, A. tumefaciens inhibition of QS activity. The bioreporter strain of the assay used in the present study to estimate the lacZ repression by cell-free cultures C. violaceum effect of bacteria cell-free culture supernatant on the purple pigment was determined. The antibacterial activity of extracts was tested against a panel of cytotoxic/genotoxic bioreporter bacteria comprising E. coli TV0161 and DPD2794. The luminescent emission was measured as above. Mytomicin C and 2% ethanol were used as positive controls for E. coli DPD2794 and TV0161, respectively. Values of IF > 1.5 were considered to represent induction, and therefore indicate metabolic or mutagenic damage by substances present in the cell-free culture supernatant.

Bioluminescence assay for cytotoxic and genotoxic activity
The activity of coral-associated bacterial cell-free culture supernatants was tested against a panel of cytotoxic/genotoxic bioreporter bacteria comprising E. coli TV0161 and DPD2794. The luminescent emission was measured as above. Mytomicin C and 2% ethanol were used as positive controls for E. coli DPD2794 and TV0161, respectively. Values of IF > 1.5 were considered to represent induction, and therefore indicate metabolic or mutagenic damage by substances present in the cell-free culture supernatant.

Flask incubation assay and quantification of violacein
QS inhibition activity by cell-free coral-associated bacterial cultures was quantified by a flask incubation assay using activated C. violaceum CV026, a Cvi mutant as a bioreporter as described previously (Choo et al. 2006) with a few modifications. This LuxI homolog mutant exhibits a non-pigmented QS controlled phenotype unless provided with exogenous AHL. An inoculum of C. violaceum CV026 (OD600 ~0.2) was added to flasks containing 32 μM N-hexanoyl-DL-homoserine lactone (HHL) (O9926, Sigma-Aldrich) and 1% bacterial cell-free culture supernatant; LB medium was added to obtain a total volume of 10 ml. The flasks were incubated for 24 h at 30 °C with constant agitation at 100 rpm. In the quantitative violacein assay, 1 ml of each reaction culture was centrifuged at 13,840 × g for 10 min, the cell-free culture supernatants were removed and 1 ml DMSO was added to the pellet. The tubes were vortexed vigorously for 5 min followed by centrifugation at 13,840 × g for 5 min before the absorbance (585 nm) of the purple pigment was determined. The antibacterial effect of bacteria cell-free culture supernatant on C. violaceum CV026 growth was monitored by turbidity measurements at 595 nm.

lacZ repression by cell-free cultures
The lawn plate assay (Golberg et al. 2011) was the basis of the assay used in the present study to estimate the inhibition of QS activity. The bioreporter strain A. tumefaciens KYC55 lacks the Ti plasmid, and therefore it cannot produce its own autoinducer signals, a critical criterion for a biosensor (Zhu et al. 2003). Cell-free culture supernatants from the isolates of coral bacteria were analyzed to determine the extent of inhibition compared to the activated bioreporter. The presence of inhibitor compounds was assessed by their reduction of β-galactosidase transcription when they competitively bound the receptor of the cognate AHL. β-galactosidase expression was evaluated by a complex culture mixture assay containing 10 ml AT medium, 10 ml of an overnight culture of A. tumefaciens KYC55, and an equivalent volume of AT buffer supplemented with 60 μg ml⁻¹ 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and 2.3 μM N-(3-Oxoctanoyl)-L-homoserine lactone (3-oxo-C8) (O1764, Sigma-Aldrich). The mixture was added to a 96-well plate along with the tested cell-free culture supernatant at a ratio of 19:1. The plates were incubated at 30 °C for 7 h without agitation and screened for the reduction of blue emission indicative of attenuation in β-galactosidase transcription by absorbance measurements at 635 nm. Controls consisted of LBN medium, distilled water (DW) and MB. Absorbance at 595 nm was used to monitor the effect that coral isolated cell-free culture supernatant activity had on the growth of A. tumefaciens KYC55.

Preparation of extracts of the cell-free culture supernatant
A 15 ml volume of LBN or MB culture was grown at 26 °C for 24 h with agitation at 120 rpm, after which extraction was performed with equal volumes of ethyl acetate acidified with formic acid (0.5%) or dichloromethane (DCM). For high-performance thin layer chromatography (HPTLC) and scanning electron microscopy (SEM) analyses, only DCM was used. The mixture was shaken vigorously for 15 min, the organic phase was then removed and extraction was repeated. The combined extracts were evaporated to dryness by exposure to a flow of nitrogen and the residue was resuspended in 1 ml acidified ethyl acetate, transferred to HPLC glass vials, and re-evaporated. The dry extracts were resuspended in 150 μl of acidified ethyl acetate and stored at −20 °C.

Inhibition of QS by extracts of the cell-free culture supernatant
Crude extracts of the cell-free culture were applied to 6 mm sterile disks which, after evaporation, were subsequently placed onto plates with the activated indicator strain, C. violaceum CV026 or A. tumefaciens KYC55. For the overlay technique, 200 μl of C. violaceum CV026 (OD600 ~0.9) were added with 2.5 μM N-(3-oxo-hexanoyl)-L-homoserine lactone (3-oxo-C6) (K3007, Sigma-Aldrich) to 5 ml 0.6% soft agar, and the mixture was poured into pre-heated LB agar plates. The inhibitory activities of extracts were also estimated by disk diffusion assays coupled to the A. tumefaciens KYC55.
plate assay. A detection solution containing equivalent aliquots of 0.8% water agar (Bacto agar, BD), an over-night culture of strain A. tumefaciens KYC55, AT buffer, 60 μg ml^–1 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), and 3-oxo-C8 (0.5 μM) was poured into a square Petri dish. The plates were incubated at 30 °C for 24 h and screened for zones of inhibition of violet or blue pigmentation around the disks.

**Thermal and protease K stability**

The activity of the potent coral bacterial isolate Fav 2-50-7 was evaluated by a temperature gradient and enzymatic effectors. Cell-free bacterial culture supernatants were exposed to a rising temperature gradient for 10 min (26, 40, 50, 65, 80, and 95 °C) or protease K (100 μg ml^–1) treatment for 1 h at 37 °C. The cell-free culture supernatants obtained were extracted twice with DCM as described above, and their stabilities were determined using activated C. violaceum CV026 coupled to HPTLC and an anti-biofilm assay using the PA01 strain of P. aeruginosa. In both cases, MB extract was used as a negative control. The extract of each sample was spotted onto C18 reverse phase HPTLC (HPTLC glass plates 20 × 10 cm, RP-18, 05914, Merck 64271 Darmstadt, Germany), and the chromatogram was developed with a mixture of methanol/propanol/water (40:20:40 (v/v)). The separated extract was visualized by exposing the HPTLC plate to a UV lamp (Sigma-Aldrich) and overlaying it with a culture of activated A. tumefaciens KYC55 or C. violaceum CV026 as described above for the detection of non-pigmented zones.

**Biofilm formation assay and microscopic visualization**

Cell-free culture supernatants and selected extracts were tested in vitro for their anti-biofilm properties against biofilms of A. baumannii and P. aeruginosa strain PA01. Biofilms produced by both bacteria were allowed to form on standard microscope slides or on 1 cm² glass slides. Sterile polystyrene tubes containing the microscope slides or 24-well microplates with the glass slides were inoculated with 2% bacterial cell-free culture supernatant and a 1% culture (OD600 ~1) of either PA01 or A. baumannii. Following static incubation at 37 °C for 24 h, the turbidity of the planktonic culture was measured. Subsequently, the microscope slides and the glass slices were rinsed gently with DW, dried and stained for 15 min with a 0.2% crystal violet solution, after which the biofilms were washed again in DW. Crystal violet was extracted by incubation with ethanol (99%) for 1 h, and the absorbance at 595 nm was determined with a plate reader and used as a proxy for relative biomass. Extracts were tested by directly applying 1 μl samples to glass slides sealed with silicone isolators (GBL665201, Sigma-Aldrich) and the organic solvent was evaporated before addition of PA01 and incubation for 24 h. The formation of biofilms on the extract-treated surfaces was visualized by SEM.

**The formation of biofilm determined in a dose dependent manner**

The minimal active threshold concentration of Fav 2-50-7 cell-free culture supernatant that affected the formation of biofilm of PA01 and A. baumannii was determined in a dose dependent manner using dilutions of 1/10, 1/50, 1/10^2, 1/10^3, 1/10^4, and 1/10^5. The lowest concentrations that were able to attenuate the formation of biofilms (ie biofilm inhibition concentrations) were established by relative spectrophotometric quantification. The extent of biofilm inhibition was quantified relative to the MB control and expressed in terms of percentage inhibition. The unstained biofilm of PA01 at a 1/50 dilution of Fav 2-50-7 cell-free culture supernatant and the MB control were visualized by confocal scanning laser microscopy (CSLM; Zeiss LSM510). The excitation: emission wavelengths of the GFP-tagged PA01 strains were 488: 517 nm, respectively. The images were acquired with a 40 ×.30 Plan-Neofluar oil immersion objective. Simulated three-dimensional (3D) images and sections were constructed using IMARIS software (Bitplane AG).

**SEM**

The biofilms of P. aeruginosa PA01 generated on glass surfaces with Fav 2-50-7 extracts derived from thermal and protease K supernatant of cell-free culture treatments were imaged using SEM. After incubation for 24 h, samples of biofilm were prepared for SEM. After fixation in Karnovsky’s fixative (2% glutaraldehyde, 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2–7.4)) at 4 °C overnight, the samples were subsequently dehydrated in an ascending ethanol series (30, 50, 70, 90, and 100%; 10 min at each concentration) before immersion in a hexamethyldisilazane/ethanol solution (25, 33, 50, 66, 75, and 100%) at room temperature for 5 min per concentration. The treated specimens were air dried for 4 h and prior to SEM (JSM-7400F, JEOL), a 20 nm layer of gold was deposited using a EMITECH K575x sputtering device (Emitech Ltd, UK).

**Purification and mass spectrometry (MS) analysis of the active compound**

The coral bacterial isolate extract Fav 2-50-7 was separated with several repetitions of HPTLC as described above. Following the development of the chromatogram, only part of the HPTLC plate was visualized by the activated C. violaceum CV026 overlay, and the preparative region was scraped from the silica matrix corresponding to the QS attenuator compound retention factor (Rf). The isolated fraction was extracted with 1 ml acetonitrile and the supernatant was collected after centrifugation at
4000 \times g \text{ for 5 min.} \text{ The resultant active fraction was further purified on reversed-phase preparative HPLC (Waters Delta Prep 4000, Mckinley Scientific, US) using a C18 column and a linear water/acetonitrile gradient containing 0.1\% trifluoroacetic acid. The purity of the fraction was tested by analytical HPLC (Agilent 1100, US) with the same gradient. The fraction collected was lyophilized and the residue was dissolved in 1 ml of acetonitrile/water (1:1 (v/v)) to determine the molecular weight by MS on a LTQ XL Orbitrap using a static nanospray (Thermo-Fisher, San Jose, CA) in positive ion mode.}

16S rRNA gene sequencing and bacterial identification

Bacterial DNA was extracted from pure cultures using an UltraClean Microbial DNA Isolation kit (MoBio Laboratories, Solana Beach, CA). Nearly complete genomic 16S rRNA was amplified by PCR with a Mastercycler gradient thermocycler (Eppendorf, Westbury, NY) using the universal forward and reverse primers 8F and 1512R, respectively. An initial 4 min denaturation step at 95 °C was followed by 30 cycles of the following program: 30 s at 94 °C, 40 s at 54 °C, 70 s at 72 °C, and a final extension step at 72 °C for 20 min concluded the reaction. The PCR products, with sizes of approximately 1500 bp, were separated by electrophoresis on a 0.8\% agarose gel. Sequencing was performed using the ABI PRISM dye terminator cycle sequencing ready reaction kit with AmpliTaq DNA polymerase FS and an ABI model 373A DNA sequencer (Perkin-Elmer). 16S rRNA gene sequences were aligned with the best matched relative sequences in the GenBank database using the basic alignment nucleotide search tool (BLAST) at the National Center for Biotechnology Information (NCBI) website.

Statistics

Values were expressed as mean±standard deviation (SD). Differences between the test samples and the control were analyzed using one-way ANOVA followed by Dunnett’s test. In the case of the calculation of QS inhibition, tests were represented by either a bioluminescence signal or a colorimetric reaction obtained from cell-free culture supernatants, while the activation of each bioreporter strain with the appropriate AHL molecule served as a control. In assays monitoring the growth of bioreporter strains, bioreporter bacteria supplemented with cell-free culture supernatant were compared to the growth of the bioreporter control.

Results

One hundred and twenty strains were isolated from the mucus layers of different corals and were then examined for their QS inhibition activities using three different bioreporter strains.

Bioluminescence assay

The rationale for the activity inhibition assay is that some bacteria secrete inhibitory compounds, which in turn, substitute as competitive inhibitors of AHL by binding to its cognate receptor protein, the result of which is a declining bioluminescent signal. Out of the 120 coral-associated bacteria, 12\% showed significant inhibition of bioluminescence activity in a 96-well microtiter plate assay (Table 1, Figure S1 in the Supplementary information) supported by three independent experiments, (ANOVA, Dunnett’s test, p < 0.05). [Supplementary material is available via a multimedia link on the online article webpage.] Interestingly, strains Fav 2-50-7-5 and Fav 2-50-9, affiliated with Vibrio sp. (originating from Favia sp. coral), exhibited relatively strong inhibition of bioluminescence; 82 and 81\%, respectively.

To rule out the possibility that the culture tested contained an antimicrobial agent that inhibited the growth of the bioreporter strain regardless of the QS inhibition activity, coral-associated bacterial cell-free culture supernatants were tested using a bioluminescent bacterial panel against cytotoxic/genotoxic substances (Figure S2). The results, supported by IF values in the range of 1, indicated that there was no extensive effect on the panel strains. However, the positive controls, mytomycin C and 2\% ethanol, substantially activated the cytotoxic/genotoxic strains, lending further support to the presence of QS inhibition activity while rejecting the possibility of an antibiotic effect.

Flask incubation assay and the quantification of violacein

QS inhibitory activity by diverse coral isolates is presented as the inhibition (%) of violacein pigment production cf the control strain C. violaceum CV026 supplemented with HHL (Table 1, Figure S3A). Overall, screening demonstrated an 11\% inhibition of QS activity with the efficacy of cell-free culture supernatants ranging from 37 to 91\%. Among the active coral-associated bacteria, the Por 3-10-10 isolate from Porites sp. coral showed marked QS attenuation activity, as expressed by a 91\% decline in pigmentation. The observed inhibitory activity by the coral isolates cannot be ascribed to antimicrobial agents, as the growth of strain C. violaceum CV026 was not significantly affected (ANOVA, Dunnett’s test, p > 0.05), (Figure S3B).

Repression of lacZ by cell-free cultures

Inhibition of expression of β-galactosidase by the A. tumefaciens KYC55 reporter strain was verified in 24\% of the coral bacterial isolates (ANOVA, Dunnett’s
Table 1. QS attenuation activity by coral-associated bacteria using three bioreporter systems.

| Coral host       | Bacterial isolate | QS inhibition activity (%) |               |               |
|------------------|-------------------|-----------------------------|---------------|---------------|
|                  |                   | pSB1075                     | CV026         | KYC55         |
| Acanthastrea sp. | 1A 1-10-2         | 42 ± 1*                     | 26 ± 13*      | 26 ± 13*      |
|                  | 1A 1-10-3         | 34 ± 13*                    |               |               |
|                  | 3A 1-10-1         | 40 ± 7*                     |               |               |
|                  | 3AT 1-10-2        | 35 ± 1*                     |               |               |
|                  | 3AT 1-10-3        | 37 ± 5*                     |               |               |
| Fungia granulosa | 4Fug 1-10-2       | 66 ± 2*                     | 51 ± 7*       | 39 ± 5*       |
|                  | 4Fug 1-10-3       |                           | 45 ± 6*       |               |
|                  | 4Fug 1-10-4       |                           |               |               |
|                  | 4Fug 1-10-5       | 64 ± 0.4*                   |               |               |
|                  | 4Fug 1-10-6       |                           | 42 ± 7*       |               |
|                  | 4Fug 1-10-7       | 68 ± 0.2*                   |               |               |
|                  | Fug 3-10-01       | 64 ± 4*                     | 39 ± 7*       |               |
|                  | Fug 3-10-3        |                           | 39 ± 6*       |               |
|                  | Fug 3-10-7        |                           | 46 ± 8*       |               |
|                  | Fug 3-10-12       | 55 ± 2*                     |               |               |
|                  | Fug 3-10-13       |                           | 41 ± 6*       |               |
|                  | Fug 3-10-14       | 59 ± 2*                     |               |               |
| Favia sp.        | Fav 2-50-07       | 63 ± 11*                    | 39 ± 9*       |               |
|                  | Fav 2-50-75       | 82 ± 3*                     | 41 ± 8*       |               |
|                  | Fav 2-50-06       | 48 ± 2*                     | 48 ± 10*      |               |
|                  | Fav 2-50-65       | 58 ± 5*                     |               |               |
|                  | Fav 2-50-6-3      | 52 ± 8*                     |               |               |
|                  | Fav 2-50-7-3      | 46 ± 2*                     |               |               |
|                  | Fav 2-50-09       | 81 ± 3*                     | 18 ± 10*      |               |
|                  | Fav 3-10-02       | 59 ± 1*                     |               |               |
|                  | Fav 1-10-13       | 37 ± 10*                    |               |               |
|                  | 2FavT 1-10-1      |                           | 45 ± 5*       |               |
|                  | 2Fav 1-10-3       |                           | 27 ± 8*       |               |
|                  | 2Fav 1-10-7       |                           | 32 ± 8*       |               |
|                  | Fav 3-10-5        | 52 ± 15*                    |               |               |
| Stylophora sp.   | Sty 2-10-05       | 52 ± 4*                     |               |               |
|                  | Sty 1-10-1        |                           | 61 ± 5*       |               |
|                  | Sty 1-10-2        | 73 ± 1*                     |               |               |
| Platygyra sp.    | Pla 1-10-05 (1)   | 20 ± 3*                     | 3 ± 2         |               |
|                  | Pla 1-10-05 (2)   |                           | 25 ± 4*       |               |
|                  | Pla 3-50-1 (2)    | 59 ± 4*                     |               |               |
|                  | Pla 3-50-1 (3)    | 80 ± 18*                    |               |               |
| Porites sp.      | Por 3-10-10       | 91 ± 3*                     |               |               |
|                  | Por 3-10-9        | 59 ± 1*                     |               |               |
|                  | Por 3-10-8        | 75 ± 13*                    |               |               |
|                  | Por 3-50-5        | 64 ± 9*                     |               |               |
|                  | Por 3-50-4        | 64 ± 5*                     |               |               |
|                  | Por 3-10-02       | 75 ± 10*                    |               |               |
|                  | Por 2-10-05       | 37 ± 6*                     |               |               |
| Pocillopora sp.  | Poc 1-10-22       | 47 ± 12*                    |               |               |
| Xenia sp.        | Xen 2-10-48       | 51 ± 8*                     |               |               |
| Total isolates   |                   | 14                          | 13            | 29            |

Note: *Values with a significant difference (p < 0.05) from those of the control.
test, $p < 0.05$) (Table 1, Figure S4A). The *Stylopora* isolate Sty 1-10-1 caused the maximum inhibition of QS; 61% compared to the controls, which included the growth medium LBN, DW, and MB, with inhibition levels of 12, 2, and 46%, respectively (Figure S4A). Inhibition of QS was also indicated by data showing that cell-free culture supernatants of the isolates tested either did not influence, or even accelerated the growth of *A. tumefaciens* KYC55 (Figure S4B). Also notable is that the LBN and DW controls had no effect on the sensor bacteria (Figure S4A and B). Taken together, these data strongly indicate the existence of a non-antimicrobial mode of inhibition.

**Inhibition of QS by extracts of the cell-free cultures**

Supernatants of bacterial cell-free cultures, previously screened by all the indicator strains and found to have QS attenuation abilities, underwent additional organic extraction. Conjugation results based on the absorbance readings for crystal violet extracted from each dilution. Because cell-free culture supernatant supplementation did not affect the viability of either PA01 or *A. baumannii*, the observed reduction in biofilm attachment was not due to growth limitation. The formation of biofilm was further analyzed via 3D simulation (images acquired by CSLM) of the treated and untreated biofilms. In comparison to PA01 alone (Figure 2A), which established nucleation aggregates all over the glass, Fav 2-50-7, when added to the 1/50 dilution, significantly disrupted the formation of a biofilm, which appeared as scattered, individually attached cells (Figure 2B).

To determine the minimal active dilution, Fav 2-50-7 cell-free culture supernatant was examined in a dose dependent manner (Figure 1) on biofilms of both PA01 and *A. baumannii*. Attachment of biofilm of PA01 was attenuated in line with ascending dilutions, the strongest anti-biofilm activity being observed at dilutions of 1/10, 1/50, and 1/100 (Figure 1A). Decreasing concentrations of cell-free culture were progressively less effective in reducing biofilm formation, but each maintained a certain level of inhibition whereas the highest dilution (1/100) displayed only negligible activity. Although a similar trend was observed for *A. baumannii* (Figure 1B), the biomass of the biofilm increased incrementally in all dilutions >1/50 in contrast to the findings for PA01. However, the formation of biofilm was greatest for both strains of bacteria under the highest dilutions and *A. baumannii* showed similar, strong activity at the 1/10 and 1/50 dilutions of the cell-free culture supernatants (Figure 1B).

Photographic images supported the relative quantification results based on the absorbance readings for crystal violet extracted from each dilution. Because cell-free culture supernatant supplementation did not affect the viability of either PA01 or *A. baumannii*, the observed reduction in biofilm attachment was not due to growth limitation. The formation of biofilm was further analyzed via 3D simulation (images acquired by CSLM) of the treated and untreated biofilms. In comparison to PA01 alone (Figure 2A), which established nucleation aggregates all over the glass, Fav 2-50-7, when added to the 1/50 dilution, significantly disrupted the formation of a biofilm, which appeared as scattered, individually attached cells (Figure 2B).

Subsequently, extracts from the cell-free culture supernatant of Fav 2-50-7 were tested for their ability to inhibit the formation of biofilm after exposure to an increasing temperature gradient or proteinase K digestion, as described above. The data obtained from SEM (Figure 3) indicated apparent differences in both the bio-

| Sample no. | Coral isolate (accession no.) | Best match isolate (accession no.), % similarity | CV026 | KYC55 | Biofilm inhibition activity |
|------------|-------------------------------|-----------------------------------------------|-------|-------|-----------------------------|
| 1          | 4Fug1-10-2 (HM346577)         | *Vibrio braziliensis* (HM584046), 98%          | +++  | +++  | +++                         |
| 2          | Fav 3-10-05 (JX266849)       | *Pseudoalteromonas nigrifaciens* (HM055757), 99% | NA   | +++  | NA                          |
| 3          | Fav 2-50-65 (JX266847)       | *Vibrio communis* (JF36185), 99%              | ++   | +++  | ++                          |
| 4          | Fav 2-50-75 (HM346588)       | *Vibrio owensii* (HQ908717), 99%              | ++   | +++  | +++                         |
| 5          | Fav 2-50-7 (HM346580)        | *Vibrio harveyi* (DQ146936), 99%              | ++   | +++  | +++                         |
| 6          | 4Fug 1-10-7 (HM346578)       | *Vibrio rotiferianus* (GQ454985), 98%         | +++  | +    | +++                         |
| 7          | Fug 3-10-01 (JX266848)       | *Vibrio campbellii* (JN547725), 100%          | +++  | NA   | NA                          |
| 8          | Fug 3-10-03 (JX266846)       | *Vibrio agarivorans* (NR_028946), 97%         | +++  | NA   | ++                          |
| 9          | Por 3-50-05 (FJ041089)       | *Pseudoalteromonas ganghensis* (DQ011614), 99% | ++   | +    | NA                          |
| 10         | Fav 2-50-09 (HM346581)       | *Vibrio alginolyticus* (EU333997), 99%        | ++   | +    | +                           |

Notes: (+++) strong, (++) medium, and (+) low activity, (NA) no activity.
mass and the structure of the biofilms formed on the thermal gradient and proteinase K vs the MB-treated surfaces. SEM images were acquired for all thermal treatments, but only that at 95 °C is shown in Figure 3 because all images revealed a similar pattern of biofilm. Neither heating nor enzymatic digestion affected the activity of the Fav 2-50-7 extract (Figure 3A and B) compared to the MB control (Figure 3C).

Characterization of the chemical properties of the active compound

Investigation of the migration pattern of the Fav 2-50-7 cell-free culture supernatant extract using HPTLC coupled to each of the activated indicator strains (*A. tumefaciens* KYC55 or *C. violaceum* CV026) enabled visualization of the active compound (Figure 4B and C).
Compounds which inhibited QS migrated with characteristic Rf values and showed a repeating pattern of untailed spots 2 cm above the base line, which simultaneously deactivated both biosensors. The migration distance of the active compound was in the range of standard AHL molecules, corresponding to N-octanoyl-homoserine lactone (C8) and N-(3-oxodecanoyl)-L-homoserine lactone (3-oxo-C10) (data not shown). In addition, visualization of the pre-overlaid HPTLC plates under UV light revealed three distinct compounds capable of emitting quanta of light as a result of the short excitation. The fluorescent compounds either migrated to the solvent migration line or to the same location as the QS inhibition compound, or they did not migrate at all and remained at the origin (Figure 4A). To provide additional support for competitive association between the detected active compound secreted by the Fav 2-50-7 isolate and that of the AHL molecule on binding to its cognate receptor, the mass of the compound was determined. The mass spectra of the purified HPLC fraction was dominated by one abundant ion (100%; relative abundance), m/z of the [M+H] is 235.1228 (Figure 5).

Figure 3. SEM images of biofilm formed by PAO1 on glass coated with Fav 2-50-7 cell-free culture supernatant extracts after (A) heating, (B) proteinase K digestion, and (C) shows the control MB extract. Scale bars = 1 μm.

Figure 4. Activity analysis of Fav 2-50-7 extract that was pretreated with heat (26–95 °C) and proteinase K (PK) on a reverse phase HPTLC plate developed with the A. tumefaciens KYC55 and C. violaceum CV026 bioreporters. (A) Migration patterns of samples seen under UV. (B) Visualization with A. tumefaciens KYC55 strain; this image is representative of all the samples and is shown for an extract at 26 °C. (C) C. violaceum CV026 overlay of the thermal gradient and proteinase K (PK) extracts with a MB control. Arrow indicates the direction of development of the chromatogram; the active compounds are framed by dotted lines. Scale bar = 1 cm.

Figure 5. MS of the active fraction produced by the Fav 2-50-7 isolate. The compound analyzed by MS was previously purified by HPTLC, followed by HPLC from spent culture supernatant extract.
Discussion

Marine habitats encompass an enormous variety of surfaces including plants and animals as potential host niches for diverse microbial communities to reach a ‘quorum’ at high cell population densities. Numerous bacteria exploit a wide array of living surfaces in the marine environment primarily because such surfaces provide a physical scaffold for biofilm establishment under QS control (Dunne 2002). Therefore, the increasing evidence that various marine bacteria, particularly coral-associated bacteria, exploit an AHL-based communication system is not surprising (Mohamed et al. 2008; Thanmoozhi et al. 2009). Coral mucus provides a rich source of nutrients, both to beneficial as well as to potentially pathogenic bacteria attracted from the relatively nutrient-poor seawater surrounding the coral environment (Brown & Bythell 2005; Mao-Jones et al. 2010). Thus, it is reasonable to hypothesize that the maintenance of coral homeostasis probably reflects the domination of particular species, via the synthesis of QS inhibition compounds, as a survival strategy that may maintain coral health or drive the disease process. Indeed, the complexity of the host–bacterial relationship is illustrated by the existence of pathogenic strains that can secrete QS antagonistic compounds in order to penetrate the beneficial community. Likewise, corals may produce AHL analogs to repel destructive bacteria. However, further investigation is needed to elucidate the mechanism of action.

The hypothesis that coral-associated bacteria possess QS inhibition abilities is supported by several recent observations suggesting that coral-associated bacteria, especially Vibrio sp., can interfere with QS and subsequently inhibit the formation of biofilm (Thanmoozhi et al. 2009; Tait et al. 2010; Alagely et al. 2011). Additional evidence was obtained in the present study when screening revealed that approximately 12, 11, and 24% of the isolates exhibited QS inhibition activity against E. coli pSB1075, C. violaceum CV026, and A. tumefaciens KYC55 indicator strains, respectively (Table 1). These findings correlate with evidence that the marked detection of inhibition (24%) by the sensitive A. tumefaciens KYC55 indicator strain was due to over expression of the TraR receptor protein (Zhu et al. 2003). In addition, the growth of A. tumefaciens KYC55 (Figure S4B) was significantly enhanced in the presence of a few coral-associated bacteria cell-free culture supernatants (ANOVA, Dunnett’s test, p < 0.05). The results presented here suggest that bacteria produced different exudates, including AHL molecules (which could be degraded by the bioreporter and used as a source of nutrient) or growth factors, and that these exudates were possibly present in the supernatant of the cell-free cultures. Previous screening revealed that about 30% of the coral isolates produced different AHL molecules (Golberg et al. 2011). Other studies have shown that soil strains of Variovorax paradoxus, the soil Pseudomonas strain PAI-A and PA01 are all capable of metabolizing AHL as their sole source of energy and nitrogen for growth via an acylase enzyme (Leadbetter & Greenberg 2000; Huang et al. 2003). Increasing molar growth yields of V. paradoxus were linearly correlated to acyl side chain length, and the PA01 and PAI-A strains preferentially degraded long-chain AHLS (8-carbon acyl chains or longer) for growth.

The minor overlap observed in the inhibition activities of the bioreporter strains indicated the importance of using several different bacterial sensors to cover a broad range of detection. Isolates that inhibited the activity of C. violaceum CV026 did not overlap with the E. coli pSB1075 biosensor. However, only one isolate was detected by both the C. violaceum CV026 and A. tumefaciens KYC55 reporters, implying the existence of a different range of detection. In addition, A. tumefaciens KYC55 and E. coli pSB1075 detected eight isolates in common. Nonetheless, the A. tumefaciens KYC55 and E. coli pSB1075 bioreporters detected mainly anti-QS activity from V. alginolytica and Favia sp. coral isolates while the C. violaceum CV026 strain was sensitive particularly to strains from the Porites sp. coral. Different coral hosts are perhaps colonized by characteristic ‘lingual’ members of the microbial community (ie they secrete similar chemical languages) and therefore, the competitive language inhibitor of coral isolates may structurally resemble the chemical signals of the microbial community and be detected by specific bioreporters.

Of equal importance is the exposure of bacteria to damaging agents (eg antibacterial substances), which by inhibition of protein synthesis decelerate bacterial growth, a result that can be falsely interpreted as QS inhibition rather than the effect of the antibiotic (Bjarnsholt & Givskov 2007). However, based on the present observations, the relatively low IF assessed by the cytotoxic/genotoxic panel (Figure S2), together with data-monitoring growth (Figures S3B and S4B) imply that inhibition probably occurs as a result of QS attenuation and not because of an antimicrobial compound. It is also noteworthy that in terms of the bioluminescent monitor strain, expression of the rsaL gene encoded between the lasR and lasI constructs significantly repressed transcription of the P. aeruginosa 3-oxo-C12 signal (de Kievit et al. 1999).

More recent work has confirmed the existence of a competitive relationship between the RsaL and LasR proteins over lasI promoter binding except under high cell density, which allows LasR to overcome competitor repression activity. Stimulation of LasR transcription by elevated levels of 3-oxo-C12 signals and the reduction in RsaL concentration due to degradation or transcriptional down-regulation are two theoretical explanations for this response (Rampioni et al. 2006). It was further...
demonstrated that non-AHL compounds, namely, nitrofuran derivatives, activate the bioluminescence reaction of the *E. coli* pSB1075 reporter strain without manipulation of QS (Zaitseva et al. 2010). These reports highlight the lack of reliability and reproducibility associated with biosensors harboring luminescence reporter genes, particularly in the case of screening for inhibitors of QS by exogenous AHL supplementation. Results in the latter case may falsely demonstrate reduced inhibition activity due to enhanced reporter gene expression by different compounds.

The adaptive biofilm structures observed in stressed, unstable aquatic environments are characterized by a high degree of resistance to antibiotic compounds (Nickel et al. 1985; Stewart & Costerton 2001; Costerton et al. 2003; Drenkard 2003; Rasmussen et al. 2005). Consistent with these observations, it was found that using antimicrobial agents to control pathogenic bacteria in aquaculture farms prompts the appearance of resistant strains in response to the selection pressure (Alderman & Hastings 1998; Akinbowale et al. 2006). To bypass this undesirable scenario, several compounds which attenuate QS were found to directly control the formation of microfouling. Modification of the composition of the bacterial community and the abundance of the existing biofilms indirectly influenced the attachment of larvae of macrofouling organisms, suggesting a promising strategy to prevent biofouling of aquaculture equipment (Dobretsov et al. 2007, 2011).

Numerous aquatic organisms have co-evolved while establishing symbiotic associations with bacteria, advancing the notion that the need to possess an antifouling mechanism is not host-restricted. This is further supported by reports showing that both coral- and sponge-associated bacteria were found to produce a wide range of anti-quorum, and subsequently antifouling, compounds that mediated the defense systems of their hosts (Thennmozhi et al. 2009; Kiran et al. 2010; Alagely et al. 2011; Dusane et al. 2011). Despite the fact that many active compounds have been isolated, the mode of action has only been elucidated for a few. The red algae *Delisea pulchra* defends itself from fouling by bacteria by producing halogenated furanones, which are LuxR competitive AHL mimics (Manefield et al. 1999). The same concept was also demonstrated in the present study by an anti-biofilm assay against PA01 and *A. baumannii* using coral bacteria cell-free culture supernatant and extracts (Figures 1–3; Table 2). Care must be exercised when correlating the anti-QS and anti-biofilm properties of certain coral isolates, since detection of an anti-QS compound does not necessarily indicate the presence of activity that prevents the establishment of a biofilm. Among the coral isolates, the most potent extracts exhibited positive inhibition of QS activity for at least one of the bioreporters, although several extracts possessed no anti-biofilm properties. These unexpected results are compatible with previous findings showing a conditional role for QS in the establishment of a biofilm (Heydorn et al. 2002; Purevdorj et al. 2002). A hydrodynamic study that also examined the effect of cell signaling regulation demonstrated unambiguous conclusions. It was found that QS was not required to ensure that mature biofilm structures, in terms of thickness and roughness parameters, were established (Heydorn et al. 2002). High shear hydrodynamic conditions did not generate marked structural differences between the biofilms formed by wild-type *P. aeruginosa* and the *lasI* mutant (Purevdorj et al. 2002). However, these findings contrasted with the results of a previous study (Davies et al. 1998), which found that QS played a crucial role in the development of a biofilm; the isogenic *P. aeruginosa lasI* mutant formed flat, undifferentiated structures unless provided with the cognate signal molecule 3-oxo-C12.

Regarding the most active coral bacterial isolate, Fav 2-50-7; in spite of the elevated dilution indexes of 1/104 and 1/105 (Figure 1) tested on PA01 and *A. baumannii*, respectively, the Fav 2-50-7 cell-free culture supernatant was relatively active. Moreover, that activity remained stable when the cell-free culture supernatant was exposed to either an increasing thermal gradient or proteinase K digestion.

The data obtained infer that some similarities may exist between the chemical structures of AHL molecules and the active compounds tested in this study, based on similar size and polarity to AHLs. The MS analyses of the active fraction confirmed the presence of a low molecular weight compound, which was found to coincide with the mass range of AHL molecules (Figure 5). Moreover, since physiological temperature influences the rate of AHL turnover, which can cause elevated AHL ring hydrolysis, it stands to reason that the active compounds tested possess a different structure from that of the lactone moiety (Yates et al. 2002). On the other hand, *Vibrio* spp. demonstrated consistent AHL production with almost no temperature dependence and enhanced inhibition of QS with increasing temperature (Tait et al. 2010). The results of the present study imply that *Vibrio* spp. can maintain their activity irrespective of either temperature increases or enzymatic digestion (Figure 4), and this ability probably reflects their domination in the marine environment as temperature increases (Alves et al. 2010). The inclusion of treated Fav 2-50-7 extracts (Figure 3) considerably decreased biofilm formation of PA01 by reducing microcolony number and size, probably during the QS controlled attachment phase (Costerton et al. 1999).

The present study led to the conclusion that coral-associated bacteria are capable of producing compounding which inhibit QS and prevent the formation of biofilm. Research will be continued on the purification and identification of the active compounds, with an emphasis on elucidating the mechanism of action.
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