A peer-reviewed version of this preprint was published in PeerJ on 18 August 2016.

View the peer-reviewed version [peerj.com/articles/2275](peerj.com/articles/2275), which is the preferred citable publication unless you specifically need to cite this preprint.

Raina J, Tapiolas D, Motti CA, Foret S, Seemann T, Tebben J, Willis BL, Bourne DG. (2016) Isolation of an antimicrobial compound produced by bacteria associated with reef-building corals. PeerJ 4:e2275
https://doi.org/10.7717/peerj.2275
Bacterial communities associated with healthy corals produce antimicrobial compounds that inhibit the colonization and growth of invasive microbes and potential pathogens. To date, however, bacteria-derived antimicrobial molecules have not been identified in reef-building corals. Here we report the isolation of an antimicrobial compound produced by *Pseudovibrio* sp. P12, a common and abundant coral-associated bacterium. This strain was capable of metabolizing dimethylsulfoniopropionate (DMSP), a sulfur molecule produced in high concentrations by reef-building corals and playing a role in structuring their bacterial communities. Bioassay-guided fractionation coupled with nuclear magnetic resonance (NMR) and mass spectrometry (MS), identified the antimicrobial as tropodithietic acid (TDA), a sulfur-containing compound likely derived from DMSP catabolism. TDA was produced in large quantities by *Pseudovibrio* sp., and prevented the growth of two previously identified coral pathogens, *Vibrio coralliilyticus* and *V. owensii*, at very low concentrations (0.5 µg/mL) in agar diffusion assays. Genome sequencing of *Pseudovibrio* sp. P12 identified gene homologs likely involved in the metabolism of DMSP and production of TDA. These results provide additional evidence for the integral role of DMSP in structuring coral-associated bacterial communities and underline the potential of these DMSP-metabolizing microbes to contribute to coral disease prevention.
Isolation of an antimicrobial compound produced by bacteria associated with reef-building corals

Jean-Baptiste Raina\textsuperscript{a,b,c,d,e,*}, Dianne M. Tapiolas\textsuperscript{b}, Cherie A. Motti\textsuperscript{b}, Sylvain Forêt\textsuperscript{d,f}, Torsten Seemann\textsuperscript{g}, Jan Tebben\textsuperscript{c,h,i}, Bette L. Willis\textsuperscript{a,d} & David G. Bourne\textsuperscript{a,b}

\textsuperscript{a}James Cook University, Marine Biology and Aquaculture, College of Science and Engineering, Townsville 4810, QLD, Australia.
\textsuperscript{b}AIMS@JCU, James Cook University, Townsville 4810, QLD, Australia.
\textsuperscript{c}Australian Institute of Marine Science, Townsville 4810, QLD, Australia.
\textsuperscript{d}Australian Research Council Centre of Excellence for Coral Reef Studies, James Cook University, Townsville 4810, QLD, Australia.
\textsuperscript{e}Climate Change Cluster (C3), University of Technology Sydney, Sydney 2007, NSW, Australia.
\textsuperscript{f}Australian National University, Research School of Biology, Canberra 2601, ACT, Australia.
\textsuperscript{g}Victorian Life Sciences Computation Initiative, University of Melbourne, Melbourne 3168, VIC, Australia.
\textsuperscript{h}University of New South Wales, Sydney 2052, NSW, Australia.
\textsuperscript{i}Alfred Wegener Institute, Section Chemical Ecology, Bremerhaven 27570, Germany.

*Corresponding author
Tel: +61 295 144 092
Email: Jean-Baptiste.Raina@uts.edu.au
Abstract

Bacterial communities associated with healthy corals produce antimicrobial compounds that inhibit the colonization and growth of invasive microbes and potential pathogens. To date, however, bacteria-derived antimicrobial molecules have not been identified in reef-building corals. Here we report the isolation of an antimicrobial compound produced by *Pseudovibrio* sp. P12, a common and abundant coral-associated bacterium. This strain was capable of metabolizing dimethylsulfoniopropionate (DMSP), a sulfur molecule produced in high concentrations by reef-building corals and playing a role in structuring their bacterial communities. Bioassay-guided fractionation coupled with nuclear magnetic resonance (NMR) and mass spectrometry (MS), identified the antimicrobial as tropodithietic acid (TDA), a sulfur-containing compound likely derived from DMSP catabolism. TDA was produced in large quantities by *Pseudovibrio* sp., and prevented the growth of two previously identified coral pathogens, *Vibrio coralliilyticus* and *V. owensii*, at very low concentrations (0.5 µg/mL) in agar diffusion assays. Genome sequencing of *Pseudovibrio* sp. P12 identified gene homologs likely involved in the metabolism of DMSP and production of TDA. These results provide additional evidence for the integral role of DMSP in structuring coral-associated bacterial communities and underline the potential of these DMSP-metabolizing microbes to contribute to coral disease prevention.
Introduction

Coral reefs are one of the most biologically diverse ecosystems on the planet (Bellwood & Hughes 2001; Knowlton 2001; Pauley 1997). Each square centimeter of a coral’s surface harbors several thousand species of microbes (Sunagawa et al. 2010) and up to $10^7$ bacterial cells (Garren & Azam 2010; Koren & Rosenberg 2006), an abundance ten times greater, on average, than the surrounding sea water. These microbial assemblages are often highly specific to their coral host and include large numbers of rare and sometimes even unique taxa (Sunagawa et al. 2010). Although the phylogenetic diversity and dynamics of coral-associated bacterial communities have been studied for more than a decade (Bourne & Munn 2005; Bourne et al. 2013; Littman et al. 2009; Rohwer et al. 2001; Sunagawa et al. 2010), their ecological and functional roles in the biology and health of corals are still poorly understood.

Recent studies have started to unravel the roles that coral-associated bacteria and their interactions with their coral hosts are likely to play within the coral holobiont. For example, some members of the Cyanobacteria, Rhizobiales and Vibrionaceae taxa are likely to fix dissolved nitrogen, a particularly important process in oligotrophic environments such as coral reefs (Lema et al. 2012; Lesser et al. 2004; Olson et al. 2009). Others, like Roseobacter, Pseudomonas and Oceanospirillales, can metabolize dimethylsuloniopropionate (DMSP), an organic sulfur compound produced in large quantities by corals and suspected to play a role in structuring coral-associated bacterial communities (Raina et al. 2010; Raina et al. 2013). It has also been hypothesized that bacteria act as a line of defense against invasive pathogens, either by competing for space and occupying coral niches (Ritchie & Smith 2004), or by directly
producing antimicrobial compounds that inhibit the growth of invasive microbes in coral mucus 
(Ritchie 2006).

In artificial culture conditions, approximately 25% of the cultivable coral bacteria 
produce antimicrobial compounds that prevent the growth of pathogenic micro-organisms 
(Ritchie 2006; Shnit-Orland & Kushmaro 2008). Several of these antimicrobial-producing taxa, 
such as *Pseudoalteromonas*, *Pseudomonas*, and the *Roseobacter* clade are commonly found in 
association with numerous coral species (Nissimov et al. 2009; Radjasa et al. 2008; Rypien et al. 
2010; Shnit-Orland & Kushmaro 2009). Although the presence of antimicrobial defences in reef-
building corals has been reported (Geffen et al. 2009; Geffen & Rosenberg 2005; Gochfeld & 
Aeby 2008; Koh 1997), only few active compounds – all produced by the coral animal itself – 
have been isolated to date (Fusetani et al. 1996; Kodani et al. 2013; Vidal-Dupiol et al. 2011).

The aim of this study was to identify specific antimicrobial compounds and thereby enhance our 
understanding of the functional roles played by coral-associated bacteria. Our specific objectives 
were to: (i) isolate a common coral-associated bacterium with antimicrobial activity and identify 
the compound(s) responsible for the activity; (ii) evaluate the susceptibility of the coral 
pathogens *Vibrio coralliilyticus* and *Vibrio owensii* to the isolated compound; (iii) determine the 
effect that thermal stress might have on its production; and (iv) investigate the natural abundance 
of the antimicrobial compound in coral extracts.

**Materials and Methods**

**Bacterial isolation**
Healthy colonies of the corals *Pocillopora damicornis*, *Acropora millepora* and *Montipora aequituberculata* (one colony per species) were collected in November 2011 from Davies Reef, Great Barrier Reef, Australia (latitude, 18°51'S; longitude, 147°41'E, Great Barrier Reef Marine Park Authority permit G12/35236.1) and maintained in aquaria for 6 days at the Australian Institute of Marine Science (Townsville, Queensland, Australia). Five replicate coral fragments (approximately 25 mm in length, containing 60 to 70 polyps) were collected from each colony and washed in sterile artificial seawater (ASW) to remove loosely attached microbes. Tissue slurries were produced by airbrushing (80 lb/in$^2$) each coral fragment into 5 mL of ASW to remove coral tissues and associated microbes. These tissue slurries were homogenized to break down tissue clumps, and a dilution series was plated immediately on bacteriological agar (1%) in 1 L ASW supplemented with 0.3% casamino acids and 0.4% glucose (Hjelm et al. 2004). After 2 days of incubation at 28°C, single colonies were transferred into Marine Broth (MB; Difco, BD, Franklin Lakes, NJ) and grown overnight. Liquid cultures were re-plated on minimal marine agar and the procedure was repeated until pure cultures were obtained.

**Well diffusion assay with bacterial isolates**

Fifty bacteria isolated from the coral tissue slurries of the 3 species (*A. millepora* = 16, *P. damicornis* = 17, *M. aequituberculata* = 17) were tested for growth-inhibitory activity against the known coral pathogens *Vibrio coralliilyticus* P1 (LMG23696) and *V. owensii* DY05 (LMG25443) in a well diffusion agar assay. In brief, the *Vibrio* strains were seeded into two different batches of minimal marine agar (after the agar temperature cooled to 40°C). Following solidification, wells (diameter 5 mm) were cut into the agar and loaded with 20 μL of overnight
cultures (10^8 cells/ml) of the test isolates grown in MB (28°C, 170 rpm). Plates were incubated at 28°C and monitored every 24 h for a period of 72 h for inhibition zones. *Phaeobacter* strain 27-4 was used as a positive antagonistic control on each plate because of its broad spectrum inhibitory activity against *Vibrio* (Bruhn et al. 2007; Hjelm et al. 2004).

**DNA extraction, gene sequencing genomic analyses**

One strain, P12 isolated from *Pocillopora damicornis*, produced the strongest growth-inhibitory activity against the two target *Vibrio* strains. High molecular weight genomic DNA from P12 was extracted using a miniprep phenol-chloroform based extraction. Briefly, 5 mL of overnight liquid culture of P12 (10^8 cells/ml) were spun in a micro-centrifuge (10,000 rcf) for 2 min. The pellet was then resuspended in 567 µL of TE buffer, 30 µL of 10% SDS and 3 µL of 20 mg/mL proteinase K. The tube was shaken thoroughly and incubated for 1 hr at 37°C. One hundred microliters of 5M NaCl was subsequently added and the sample thoroughly mixed before adding 80 µL of CTAB/NaCl (10% CTAB in 0.7 M NaCl). The solution was incubated for 10 min at 65°C, extracted with an equal volume of phenol/chloroform/isoamyl alcohol and centrifuged for 10 mins (10,000 rcf). The supernatant was then extracted with an equal volume of chloroform/isoamyl alcohol and centrifuged again for 10 min. The aqueous phase was transferred to a new tube, DNA precipitated with equal volume of ice-cold isopropanol, washed with 70% ethanol and dried.

The near complete 16S rRNA gene of the strain was PCR amplified with bacterial specific primers 63F and 1387R, as outlined in Marchesi et al. (Marchesi et al. 1998). Amplified PCR products were visualized by electrophoresis on 1% agarose gel stained with ethidium bromide. The amplified DNA was dried in a vacuum centrifuge (Savant DNA 120) and
sequenced (Macrogen, Inc., Seoul, Korea). The 16S rRNA gene sequence of isolate P12 was used for phylogenetic comparisons and Maximum Likelihood trees were constructed using the ARB software.

We produced a draft genome assembly of P12. A paired-end library was prepared using the Illumina Truseq protocol (Illumina, San Diego, CA, USA), with an insert size of 169 bp and a read size of 150 bp. The library was sequenced on an Illumina MiSeq instrument at Monash University (Melbourne, Australia). The genome was assembled with the SPAdes assembler (v2.4.0) (Bankevich et al. 2012) and annotated with the Prokka software (v1.5.2) (Seeman 2014). The presence of the genes involved in DMSP metabolism (dmdA, dddD, dddL, dddP, dddY, dddQ, dddW) and TDA production (tdaA-tdaH) was investigated by searching for homologs of the corresponding genes using reciprocal best blast hits.

**DMSP metabolic capabilities of the isolate P12**

Two different minimal media were used to examine the DMSP metabolic capabilities of P12: a modified marine ammonium salt medium (MAMS) (Raina et al. 2009) lacking a carbon source, and a modified basal salt medium lacking a sulfur source (Fuse et al. 2000) (25 g of NaCl, 0.7 g of KCl, 0.05 g of KH$_2$PO$_4$, 1 g of NH$_4$NO$_3$, 0.2 g of MgCl$_2$·H$_2$O, 0.02 g of CaCl$_2$·2H$_2$O, 0.005 g of FeEDTA, 1 g of Tris, 5 g of sodium succinate, 1.35 g of glucose in 1 L of distilled water). DMSP was added to both media (1 mM), acting either as the sole carbon or sulfur source. Five milliliters of each culture media were inoculated in triplicate with single P12 colonies and incubated at 28°C for 6 days. Negative controls containing only the basal media and DMSP were used to account for possible chemical breakdown of DMSP. Bacterial growth was assessed via optical density measurement (NanoDrop, Thermo Fisher, Waltham, MA). DMSP
metabolism was assessed by $^1$H Nuclear Magnetic Resonance spectroscopy (NMR). Methanol (CH$_3$OH; 40 mL) was added to each culture tube, the mixture shaken vigorously and sonicated for 10 mins before being dried in vacuo using a rotary evaporator (Buchi, Flawil, Switzerland). The dried extracts were resuspended in a mixture of deuterium oxide (D$_2$O; 250 µL) and deuterated methanol (CD$_3$OD; 750 µL) (Cambridge Isotope Laboratories, Andover, MA, USA). A 750-mL aliquot of the particulate-free extract was transferred into a 5-mm Norell tube (Norell Inc., Landisville, NJ, USA) and analyzed immediately using quantitative NMR (Tapiolas et al. 2013).

Preparation of crude extracts for antagonist assays

An overnight culture of P12 (8 mL) was used to inoculate 4×250 mL of MB (total culture volume = 1L). Bacterial cells were incubated for two days at 28°C (120 rpm); the culture broth was then acidified to pH 2 with sulphuric acid before being exhaustively extracted with ethyl acetate ($3 \times 1.5$ L). The extract was washed three times with MilliQ H$_2$O and dried in vacuo using a rotary evaporator (Buchi). The dried extract was then weighed and resuspended in CH$_3$OH (which was chosen for its ability to solubilize a wide range of compounds, its volatility and its innocuity in small volume towards both V. coralliilyticus and V. owensii) and tested in well-diffusion assays to confirm the extraction of the antimicrobial compound(s).

Purification and characterization of active compound

Purification of the crude extract was carried out using solid phase extraction on a reversed phase C$_{18}$ flash vacuum column (Septra C$_{18}$-E, Phenomenex, Torrance, CA, USA). Eleven fractions were eluted sequentially with 20%, 40%, 60%, 80%, 90% CH$_3$OH in H$_2$O and
100% CH$_3$OH, followed by 20%, 50% and 100% dichloromethane (CH$_2$Cl$_2$) in CH$_3$OH, 40%
hexane in CH$_2$Cl$_2$ and finally 100% hexane. Each fraction was dried and resuspended in CH$_3$OH
(1 mg mL$^{-1}$). Well diffusion assays were prepared as described above. On each plate, test wells
were inoculated with 20 µL of each chromatographic fraction, or 20 µL of CH$_3$OH as a control,
and Vibrio growth monitored. The most active faction (80% CH$_3$OH) presented an intense
yellow color. Fine orange-red needles were crystallized from this active fraction to yield
compound 1 (2.1 mg, 1.7 % dry weight of organic extract).

NMR and FTMS analysis

Identification and structural elucidation of compound 1 was achieved using liquid
chromatography - mass spectrometry (LC-MS), NMR, and Fourier Transform mass spectrometry
(FTMS). Likewise these techniques were used to monitor for the presence of compound 1 in
extracts and fractions. LC-MS analyses were performed on a Thermo Fisher Scientific Ultra
High Performance Liquid Chromatography system connected to an LTQ Orbitrap XL mass
spectrometer (Thermo Fisher Scientific, San Jose, CA). Samples were separated on a ACQUITY
UPLC BEH RP-C$_{18}$ column (130Å, 1.7 µm, 2.1 mm x 100 mm, solvents A = aqueous 0.1%
formic acid and B = acetonitrile, gradient elution 80% A:20% B for 0.5 min ramped up to 100%
B over 10 min, then held for 4 min, 400 µL) and detected by positive mode electrospray
ionisation using two different m/z ranges: 150-1500 and 170-400. $^1$H and $^{13}$C NMR spectra of
compound 1 were acquired in a 5 mm 509-UP Norell NMR tube on a Bruker Avance 600 MHz
NMR spectrometer (Bruker, Germany) with a TXI cryoprobe using standard Bruker pulse
sequences. NMR spectra were referenced to residual $^1$H and $^{13}$C resonances in deuterated
chloroform (CDCl$_3$). High resolution mass spectra of compound 1 were measured with a Bruker
BioApex 47e Fourier Transform Mass Spectrometer (FTMS) fitted with an Analytica of Branford ESI source; ions were detected in negative mode within a mass range \( m/z \) 200-1,000 via direct infusion at 120 \( \mu l \) h\(^{-1}\).

Temperature-dependent activity

The antimicrobial activity of P12 grown at 32°C (upper limit of coral thermal tolerance) was compared to that of the control incubated at 28°C. The two cultures were grown overnight in MB at the two different temperatures, and their densities were determined by flow-cytometry (BD Accuri C6, Beckman Coulter, Brea, CA). Cell numbers were normalized prior to inoculation into agar wells, and their activities against the two pathogens were compared using well-diffusion assays as described above. The same procedure was repeated with compound 1: two vials containing equal concentrations (2 \( \mu M \) of 1 in CH\(_3\)OH) were incubated overnight at 28°C or 32°C and their antimicrobial activities compared using the well diffusion assay.

Preparation of coral extracts

The coral species *Montipora aequituberculata, M. turtlensis, Pocillopora damicornis, Acropora millepora*, and *Porites cylindrica* (one colony each; 500 g of dry skeleton per species) were collected in July 2012 from Orpheus Island, Great Barrier Reef, Australia (latitude, 18°35’S; longitude, 146°20’E, Great Barrier Reef Marine Park Authority permit G12/35236.1). Coral tissues were airbrushed (80 lb/in\(^2\)) into 1µm filtered seawater (FSW) (total volume = 500 mL), acidified to pH 2 with sulphuric acid and the solution exhaustively extracted with equal volumes of ethyl acetate (3 × 750 mL). The combined organic layers were partitioned with MilliQ H\(_2\)O, dried and tested in well-diffusion assays, as previously described for the bacterial
isolate extracts. The extracts of those coral species that exhibited antimicrobial activity were subsequently fractionated as described above for the crude extract from P12 and tested in well-diffusion assays. The active fractions were analyzed using $^1$H NMR, FTMS and LC-MS.

Results

Isolate P12: antimicrobial production, taxonomy and metabolic capabilities

A total of 50 coral-associated bacterial isolates were obtained from tissue slurry homogenates of the three coral species. Twelve of the 50 strains tested against the two pathogenic Vibrios (V. coralliilyticus and V. owensii) inhibited their growth in well diffusion assays. The bioactive isolate that exhibited the strongest in vitro activity against both pathogens, isolate P12, originated from Pocillopora damicornis and produced growth inhibition zones of 5 mm (±0.07 mm, n=20) against V. owensii and 2 mm (±0.09 mm, n=20) against V. coralliilyticus.

The activity of P12 was temperature-dependent (Figure 1 A-B) and was significantly reduced when grown at 32°C compared to 28°C (Unpaired T-Test, $n=20$, df=38, $t=30.61$, *$p<0.001$ for V. owensii and $n=20$, df=38, $t=10.49$, *$p<0.001$ for V. coralliilyticus; Figure 1C). Based on its bioactivity, the isolate P12 was selected for bioassay-guided fractionation.

According to its 16S rRNA gene sequence (NCBI accession number: KX198136), isolate P12 is an alphaproteobacterium belonging to the Rhodobacteraceae family and the Pseudovibrio genus. Its most closely related species is Pseudovibrio denitrificans (100% identity to the type strain; Figure 2). Like other P. denitrificans strains (Enticknap et al. 2006), P12 colonies formed brown mucoid colonies when grown on Marine Agar. The brown color was absent when the strain was grown on minimal marine agar, with colonies appearing white. This strain was able
use DMSP as either a sole carbon or sole sulfur source (Figure 3). The complete utilization of DMSP from the liquid media after 2 to 3 days of incubation, as well as the presence of its metabolic byproduct dimethylsulfide (DMS), were confirmed by $^1$H NMR. However acrylate, another possible byproduct of DMSP metabolism, was not observed.

Among the seven different DMSP degradation pathways currently identified (Moran et al. 2012), the full DMSP cleavage pathway ($dddD$, $dddB$, $dddC$, $dddT$, $dddR$; Table 1), involved in the conversion of DMSP into DMS without formation of acrylate (Todd et al. 2007) (Table 1), was identified in P12. We also identified possible orthologs for the demethylation pathway ($dmdA$, $dmdB$, $dmdC$ and $dmdD$) used by marine bacteria to assimilate sulfur from DMSP, though these gene have low sequence identity to the genes originally identified in Ruegeria pomeroyi DSS-3 (Howard et al. 2006; Reisch et al. 2011) (Table 1). The presence of these two gene pathways corroborates the $^1$H NMR measurements: the observed production of DMS without acrylate formation following DMSP metabolism (DddD pathway); and the ability to use DMSP as sole sulfur source (DmdA pathway) (Table 1).

Identification of antimicrobial compounds produced by P12

Well diffusion assays revealed that the crude extract from P12 retained the antimicrobial properties of the strain against both Vibrio species. Purification of the active fractions using reverse phase liquid chromatography yielded compound 1: optically inactive orange-red crystals; 2.1 mg (1.7% dry weight); IR (film) $v_{\text{max}}$ 3420, 1660, 1280 cm$^{-1}$; UV (PDA, CH$_3$OH) $\lambda_{\text{max}}$ 512 nm; $^1$H NMR spectrum (600 MHz, CD$_3$Cl): $\delta$ 7.12, 7.44, 7.45 and 16.7; $^{13}$C NMR (150 MHz, CD$_3$Cl): $\delta$ 120.3, 132.0, 136.0, 138. 7, 149.5, 168.7, 171.7, and 183.5; HRESIMS $m/z$ found
278 210.9534 (calculated for C₈H₃O₃S₂⁻ 210.9529, Δ 2 ppm). Combined spectroscopic techniques revealed that compound 1 was tropodithietic acid (TDA) (Brinkhoff et al. 2004; Penesyan et al. 2011) (Figure 4A).

282 Orthologs for 11 genes involved in TDA biosynthesis (Geng et al. 2008) were present in the *Pseudovibrio* sp. P12 genome (Table 1). The biosynthesis of TDA correlated with production of the yellow-brown pigmentation in the culture medium and antimicrobial activity, similar to that previously reported (Brinkhoff et al. 2004; Bruhn et al. 2005; Porsby 2010). Both coral pathogens were highly sensitive to TDA, with the pure compound still visually inhibiting their growth at 0.5 µg/mL (2.35 µM; Figure 4B). In contrast to the decrease in antimicrobial activity exhibited by *Pseudovibrio* sp. P12 after incubation at 32°C, TDA activity was not affected by exposure to this temperature (Unpaired T-Test, \( n=20, df=38, t=-0.94, p=0.355 \) for *V. owensii* and \( n=20, df=38, t=0.632, p=0.531 \) for *V. coralliilyticus*; Figure 1B-C).

292 *Investigating the presence of TDA in coral samples*

All of the extracts derived from the coral species investigated exhibited antimicrobial activity against the two pathogens, with the inhibition zones for *P. cylindrica*, *M. aequituberculata*, *M. turtlensis* and *P. damicornis* ranging from 3 to 5 mm in radius whilst the inhibition zones for *A. millepora* were much smaller (1 mm on average). \(^1\)H NMR, LC-MS and FTMS analyses of the extracts and the active fractions of all coral species did not confirm the presence of TDA. The purified TDA could be detected by LC-MS in femtomolar concentrations when the coral fractions were artificially spiked, indicating that this lack of detection was not due...
to preferential ionization. Thus, TDA was either not present in the coral fractions tested or in concentrations below the LC-MS detection threshold.

Discussion

While previous studies have identified corals as a source of bacteria with antimicrobial activity (Radjasa et al. 2008; Sulistiyani et al. 2010), this study represents the first isolation and identification of an antimicrobial compound produced by a reef building coral-associated bacterium with activity against coral-associated pathogens. Tropodithietic acid (TDA) was characterized from a pure culture of *Pseudovibrio* sp. P12 previously isolated from corals and strongly inhibited the growth of two coral pathogens. Although we could not confirm the presence of this molecule in the corals tested, TDA has the capacity to provide protective antimicrobial properties to the coral host and prevent colonization by invasive bacterial species.

The strain P12 strongly inhibited the growth of *Vibrio coralliilyticus* and *V. owensii*, two coral pathogens causing white syndrome (a collective term describing rapidly progressing tissue loss, exposing band-like areas of white skeleton) (Ben-Haim et al. 2003; Sussman et al. 2008; Ushijima et al. 2012; Willis et al. 2004). *Vibrio coralliilyticus* exhibits antimicrobial resistance to a wide range of commercial antibiotics and is also resistant to the activities of a large number of coral-associated bacteria (Rypien et al. 2010; Shnit-Orland & Kushmaro 2009; Vizcaino et al. 2010). Its resistance to commercial antibiotics is considerably greater than that of other marine pathogens such as *V. parahaemolyticus* or *V. vulnificus*, and may contribute to its competitive advantage within the coral holobiont, as well as its ability to infect corals (Vizcaino et al. 2010).
However, whilst *V. coralliilyticus* is resistant to many coral-associated bacteria, its growth was strongly inhibited by the strain P12, emphasizing its antimicrobial capabilities.

The isolate P12 belongs to the bacterial genus *Pseudovibrio* (Shieh et al. 2004), and is ubiquitously found in association with healthy sponges (Enticknap et al. 2006; Thiel & Imhoff 2003; Webster & Hill 2001) and corals (see Table 2). *Pseudovibrio* are thought to be involved in symbiotic relationships with various organisms; they are vertically transmitted in large densities by adult sponges to their larvae (Enticknap et al. 2006) and their presence is required for the growth of the sulfur-oxidizing bacteria *Beggiatoa* in culture (Bondarev et al. 2013). Furthermore, their genome is organized similarly to that of *Rhizobia*, a well-characterized symbiotic bacterium (Bondarev et al. 2013; Enticknap et al. 2006; Kennedy et al. 2009). The full genome sequences of *Pseudovibrio* FO-BEG1 (KEGG genome T01669; isolated from a Caribbean coral, and sharing 100% sequence similarity with P12 based on its 16S rRNA gene sequence) reveal the presence of genes involved in host-cell adhesion, interactions with eukaryotic cell machinery, and production of secondary metabolites (Bondarev et al. 2013), further suggesting that this bacterium is involved in symbiotic relationships with its hosts.

The *Pseudovibrio* genus is also known for its antimicrobial properties, especially against human pathogens such as *Mycobacterium tuberculosis*, *Bacillus cereus*, *Yersinia enterocolitica*, *Listeria monocytogenes* or methicillin-resistant *Staphylococcus aureus* (O'Halloran et al. 2011; Sulistiyani et al. 2010). To date, three active compounds have been isolated from different *Pseudovibrio* strains: heptylprodigiocin in tunicate-associated *P. denitrificans* Z143-1 (Sertan-de Guzman et al. 2007), pseudovibrocin in *P. denitrificans* strain PaH3.28 (Vizcaino 2011) and
tropodithietic acid (TDA) from red alga-associated *P. ascidiaceicola* D323 (Penesyan et al. 2011). In the present study, we isolated TDA from P12, a strain closely related to *P. denitrificans*. The ability of P12 to produce TDA was further supported by the presence of the genes essential for TDA biosynthesis in the genome of strain P12 (Geng et al. 2008). TDA inhibits the growth of a wide range of marine pathogens (Bruhn et al. 2007; Bruhn et al. 2005) and is produced almost exclusively by bacteria from the *Roseobacter* clade, especially the genera *Phaeobacter*, *Silicibacter*, and *Ruegeria* (Brinkhoff et al. 2004; Bruhn et al. 2005; Geng & Belas 2010; Geng et al. 2008; Wilson et al. 2016) that are commonly associated with DMSP-producing dinoflagellates (Miller et al. 2004; Wagner-Dobler & Biebl 2006) and reef-building corals (Bourne et al. 2013; Littman et al. 2009; Raina et al. 2009).

Many members of the *Roseobacter* clade, including coral-associated isolates, have been implicated in sulfur cycling (Miller et al. 2004; Moran et al. 2003; Raina et al. 2010). Interestingly, TDA contains two sulfur atoms (C_8H_4O_3S_2) and *Pseudovibrio* sp. P12 was able to use DMSP either as sole carbon or sole sulfur source, a common trait among *Alphaproteobacteria* and especially the *Roseobacter* clade (Bruhn et al. 2005; Wagner-Dobler & Biebl 2006). Bacteria from this clade preferentially metabolize DMSP rather than sulphate (SO_4^{2-}), despite the latter being between 10^6 to 10^7-fold more abundant in seawater (Geng & Belas 2010; Kiene et al. 1999). Based on genomic and chemical analyses, DMSP metabolism in P12 could occur via two likely routes: the cleavage pathway (encoded by *dddD*, (Todd et al. 2007)) that releases the climate-regulating molecule DMS, and the demethylation pathway (encoded by *dmdA*, (Howard et al. 2006)) by which the bacterium can retain the sulfur contained in DMSP molecules.
The biosynthetic pathway of TDA has not been fully elucidated (Brock et al. 2014). Both labeling (Cane et al. 1992; Thiel et al. 2010) and genetic dissection (Geng & Belas 2010) studies have shown that its aromatic skeleton is derived from phenylacetyl-CoA produced by the shikimate pathway. However, the sulfur donor allowing the incorporation of the two sulfur atoms into the TDA molecule has not been clearly identified. It has been proposed that sulfur originating from DMSP metabolism might be used to synthesize TDA (Bruhn et al. 2007; Bruhn et al. 2005; Geng & Belas 2010; Porsby 2010; Wagner-Dobler & Biebl 2006). For example, DMSP increases TDA synthesis two-fold in comparison to other sulfur sources (Geng & Belas 2010), suggesting that DMSP is a preferred source of sulfur for TDA biosynthesis. Even though other sources of sulfur, such as the amino-acids cysteine and methionine present in artificial media like Marine Broth, might be used to synthesize TDA (Geng & Belas 2010), DMSP is by far the most readily available reduced sulfur source in the marine environment (Simo 2001). It is therefore likely that in DMSP-rich environments, such as reef-building corals, DMSP metabolism provides the sulfur needed to produce TDA via the demethylation pathway (Howard et al. 2006).

The presence of TDA in extracts derived from five coral species (Montipora aequituberculata, M. turtlensis, Pocillopora damicornis, and Porites cylindrica) could not be confirmed. Three possibilities can explain this lack of detection: (i) TDA is not synthesized in corals; (ii) TDA is present in corals in concentrations below the detection limit of our instruments (which would imply that this compound has a very limited role in coral defense); (iii) our sampling effort was not sufficient. Indeed, we only sampled one colony per species,
from a location more than 100 km away from the site where the TDA-producing bacteria was isolated and without prior characterization of the bacterial communities present in the colony sampled. Given that TDA-producing Roseobacters are among the first bacteria to colonize the surface of marine microalgae and corals (Apprill et al. 2009; Dang & Lovell 2000; Miller et al. 2004) and can be highly abundant in some coral species (Raina et al. 2009), it would be premature to rule out possible TDA biosynthesis in corals.

The activity of P12 against V. coralliilyticus sharply decreased at elevated temperatures (32°C), however, the activity of the purified TDA did not. This reveals that the loss of antimicrobial activity observed for P12 at 32°C is not due to thermal sensitivity of TDA but likely to a decrease in its production. Our results are in line with previously reported decline in the antibacterial activity of other TDA-producing Roseobacter with temperature increase (Bruhn et al. 2005). Clear links have previously been identified between warm thermal anomalies and outbreaks of white syndromes (Bruno et al. 2007; Heron et al. 2010; Maynard et al. 2011). If indeed TDA is synthesized in vivo, a decrease in its production during anomalously high seawater temperatures could facilitate pathogen outbreaks in corals following thermal stress, especially since the virulence of some disease-causing bacteria (i.e. V. coralliilyticus) increase at 32°C (Sussman et al. 2008).

This study demonstrates that a common coral-associated bacterium, Pseudovibrio sp. P12, produces TDA, a potent antimicrobial compound that inhibits the growth of marine and coral pathogens, including V. coralliilyticus. The bacterium can use DMSP as a sole sulfur or carbon source and potentially as a precursor in the biosynthesis of TDA. The production of TDA
by *Pseudovibrio* sp. P12 is greatly reduced at temperatures causing thermal stress in corals, potentially providing a window of opportunity for the growth of pathogens. These results provide additional evidence for the integral role of DMSP in structuring healthy, coral-associated bacterial communities and suggest that these DMSP-metabolizing communities may contribute to the prevention of coral diseases.

**Acknowledgments**

The authors would like to thank E. Botté, C. Gao and M. Garren for their laboratory assistance.

**Figures**
Figure 1: Representative well diffusion assays of (A) *Pseudovibrio* sp. P12 and (B) pure TDA, incubated at two different temperatures (28°C and 32°C) and then inoculated onto agar plates with embedded *Vibrio owensii* [(-) Negative control]. (C) Comparison of the radius of inhibition zones between the two temperature treatments for both *Pseudovibrio* sp. P12 (Unpaired T-Test, \(n=20\), \(df=38\), \(t=30.61\), \(*p<0.001\) for *V. owensii* and \(n=20\), \(df=38\), \(t=10.49\), \(*p<0.001\) for *V. coralliilyticus*) and pure TDA (2 µM, Unpaired T-Test, \(n=20\), \(df=38\), \(t=-0.94\), \(p=0.355\) for *V. owensii* and \(n=20\), \(df=38\), \(t=0.632\), \(p=0.531\) for *V. coralliilyticus*).
**Figure 2**: Maximum likelihood phylogenetic tree based on 16S rRNA gene sequences showing the isolate used in this study (P12 in red) and closely associated *Pseudovibrio* spp. Note: the strain FO-BEG1 has been fully sequenced. *Phaeobacter* sp. 27-4 [AJ536669] was used as outgroup. Maximum parsimony bootstrap values (10,000 replicates) are given when different from 100. The scale bar indicates the number of substitution per nucleotide position.
Figure 3: $^1$H NMR spectra showing DMSP utilization as (A) sole carbon source and (B) sole sulfur source in minimal media at the end of a 6-day incubation. The “control” lines in all cases are the growth medium (with no bacterial inoculation). The black and green spectra show the results from inoculation with *Pseudovibrio* sp. P12 and *V. coralliilyticus* (negative control), respectively. In both cases, the DMSP signals (within the three boxes, see Tapiolas et al. 2013)) disappeared in the *Pseudovibrio* treatment and remain unchanged between the no-bacteria
control and the *V. coralliilyticus* treatment. In the case of DMSP as a sole sulfur source, *Pseudovibrio* consumed the DMSP and other carbon sources present and produced secondary metabolites (appearance of new signals). a: solvent peak (methanol).

**Figure 4**: (A) Tropodithietic acid (TDA). (B) Dilution series of pure TDA, showing zones of growth inhibition against *Vibrio coralliilyticus*: [(1): 500 μg/mL, (2): 50 μg/mL, (3): 5 μg/mL, (4): 500 ng/mL, (5): 50 ng/mL, (6): 5 ng/mL, (7): 500 pg/mL, (8): 50 pg/mL, (-) negative control with solvent only].
Table 1: Orthologous genes involved in DMSP degradation and TDA biosynthesis in *Pseudovibrio* sp. P12 genome. Accession numbers available in NCBI (http://www.ncbi.nlm.nih.gov/genbank/). Percent of similarity compared to *a* *Marinomonas* sp. MWY11 (Todd et al. 2007); *b* *Ruegeria pomeroyi* DSS-3 (Reisch et al. 2011); *c* *Pseudovibrio* sp. FO-BEG1 (Alcolombri et al. 2015); *d* *Phaeobacter inhibens* (Brock et al. 2014); *e* *Nesiotobacter exalbescens*; *f* *Rhodopseudomonas palustris*; *g* *Stappia stellulata*.

| Gene      | Function                                      | Percent of identity | Accession number |
|-----------|-----------------------------------------------|---------------------|------------------|
| **DddD**  | L-carnitine dehydratase                      | 70% *a*             | KM819464         |
| **DddT**  | BCCT transporter                              | 70% *a*             | KM819465         |
| **DddB**  | Alcohol dehydrogenase                         | 70% *a*             | KM819466         |
| **DddC**  | Methylmalonate-semialdehyde dehydrogenase    | 56% *a*             | KM819467         |
| **DddR**  | Transcriptional regulator, LysR family protein| 53% *a*             | KM819468         |
| **DmdA**  | Aminomethyl transferase family protein        | 24% *b*             | KU521525         |
| **DmdB**  | Acyl-CoA synthetase                           | 30% *b*             | KU521526         |
| **DmdC**  | MMPA-CoA dehydrogenase                        | 43% *b*             | KU521527         |
| **DmdD**  | MTA-CoA hydratase                             | 32% *b*             | KU521528         |
| **Alma1** | Hypothetical Alma1 ortholog                   | 99% *c*             | KU521524         |
| **tdaA**  | Transcriptional regulator, LysR family protein| 66% *d*             | KU760700         |
| **tdaB**  | Glutathione S-transferase domain protein      | 67% *d*             | KU760701         |
| **tdaC**  | Prephenate dehydratase                        | 67% *d*             | KU760702         |
| **tdaD**  | Acyl-CoA thioester hydrolase                  | 81% *d*             | KU760703         |
| **tdaE**  | Acyl-CoA dehydrogenase                        | 69% *d*             | KU760704         |
| **tdaF**  | Phosphopantothenoylcysteine decarboxylase     | 72% *d*             | KU760705         |
| **cysL**  | Sulfite reductase hemoprotein beta-component  | 76% *e*             | KU760706         |
| **malY**  | Cystathione beta-lyase                        | 68% *e*             | KU760707         |
| **paal**  | Phenylacetate-CoA oxygenase                   | 59% *f*             | KU760708         |
| **paaJ**  | Phenylacetate-CoA oxygenase, PaaJ subunit     | 73% *g*             | KU760709         |
| **paaK**  | Phenylacetic acid degradation oxidoreductase  | 57% *g*             | KU760710         |
### Table 2: Summary of *Pseudovibrio* isolated or sequenced from corals, accession numbers are displayed when available (NA: not available).

| Host             | Location | Method   | Reference                  | Accession numbers          |
|------------------|----------|----------|----------------------------|----------------------------|
| Acropora palmata | Panama   | Amplicon | (Sunagawa et al. 2010)     | GU118050, GU118108, GU119014 |
| Porites astreoides | Panama   | Amplicon | (Sunagawa et al. 2010)     | GU118050, GU118108, GU119014 |
| Acropora cervicornis | Panama   | Amplicon | (Sunagawa et al. 2010)     | GU118050, GU118108, GU119014 |
| Montastrea franksi | Panama   | Amplicon | (Sunagawa et al. 2010)     | GU118050, GU118108, GU119014 |
| Tubastrea coccinea | China     | Amplicon | (Yang et al. 2013)         | JF925014                   |
| Pseudopterogorgia americana | Puerto Rico | Isolated | (Vizcaino et al. 2010)     | GQ406787, GQ406798, GQ391966, GQ406786 |
| Platygyra carnosus | Hong Kong | Isolated | (Chiu et al. 2012)         | JF411474, JF411466, JF411439, JF411464 |
| Oculina patagonica | Israel    | Isolated | (Koren & Rosenberg 2006)   | DQ416557, AY654776         |
| Montastrea anularis | Florida   | Isolated | (Rypien et al. 2010)       | FJ952798, FJ952774, FJ952804 |
| Sinularia sp.     | Indonesia | Isolated | (Sulistiyani et al. 2010)   | NA                        |
| Acropora almata   | Florida   | Isolated | (Ritchie 2006)             | DQ530540                   |
| Sarcophytton sp.  | Java      | Isolated | (Sabdono & Radjasa 2006)   | NA                        |
| Oculina patagonica | Israel    | Isolated | (Nissimov et al. 2009)     | NA                        |
| Lobophytum sp.    | Taiwan    | Isolated | (Chen et al. 2012)         | JQ342682, JQ342695, JQ342696, JQ342697 |
| Hard coral        | Florida   | Isolated | (Bondarev et al. 2013)     | CP003147                   |

### References

- Alcolombri U, Ben-Dor S, Feldmesser E, Levin Y, Tawfik DS, and Vardi A. 2015. Identification of the algal dimethyl sulfide-releasing enzyme: a missing link in the marine sulfur cycle. *Science* 348:1466-1469.
- Apprill A, Marlow HQ, Martindale MQ, and Rappe MS. 2009. The onset of microbial associations in the coral *Pocillopora meandrina*. *ISME J* 3:685-699.
- Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, and Pevzner PA. 2012. SPAdes: a new genome assembly algorithm and its application to single-cell sequencing. *Journal of Computational Biology* 19:455-477. doi: 10.1089/cmb.2012.0021
- Bellwood DR, and Hughes TP. 2001. Regional-scale assembly rules and biodiversity of coral reefs. *Science* 292:1532-1535.
Ben-Haim Y, Thompson FL, Thompson CC, Cnockaert MC, Hoste B, Swings J, and Rosenberg E. 2003. *Vibrio coralliilyticus* sp. nov., a temperature dependent pathogen of the coral *Pocillopora damicornis*. *IJSEM* 53:309-315.

Bondarev V, Richter M, Romano S, Piel J, Schwedt A, and Schulz-Vogt HN. 2013. The genus *Pseudovibrio* contains metabolically versatile bacteria adapted for symbiosis. *Environ Microbiol* 15:2095-2113.

Bourne D, and Munn C. 2005. Diversity of bacteria associated with the coral *Pocillopora damicornis* from the Great Barrier Reef. *Environ Microbiol* 7:1162-1174.

Bourne DG, Dennis PG, Uthicke S, Soo RM, Tyson GW, and Webster NS. 2013. Coral reef invertebrate microbiomes correlate with the presence of photosymbionts. *ISME J* In press. 10.1038/ismej.2012.172

Brukhoff T, Bach G, Heidorn T, Liang L, Schlingloff A, and Simon M. 2004. Antibiotic production by a *Roseobacter* clade-affiliated species from the German Wadden Sea and its antagonistic effects on the indigenous isolates. *Appl Environ Microbiol* 70:2560-2565.

Brock NL, Nikolay A, and Dickschat JS. 2014. Biosynthesis of the antibiotic tropodithietic acid by the marine bacterium Phaeobacter inhibens. *Chemical Communications* 50:5487-5489. 10.1039/C4CC01924E

Bruhn JB, Gram L, and Belas R. 2007. Production of antibacterial compounds and biofilm formation by *Roseobacter* species are influenced by culture conditions. *Appl Environ Microbiol* 73:442-450.

Bruhn JB, Nielsen KF, Hjelm M, Hansen M, Bresciai J, Schulz S, and Gram L. 2005. Ecology, inhibitory activity, and morphogenesis of a marine antagonistic bacterium belonging to the *Roseobacter* clade. *Appl Environ Microbiol* 71:7263-7270.

Bruno JF, Selig ER, Casey KS, Page CA, Willis BL, Harvell DC, Sweatman H, and Melendy AM. 2007. Thermal stress and coral cover as driver of coral disease outbreaks. *PLoS Biol* 5:e124. 10.1371/journal.pbio.0050124

Cane DE, Wu Z, and van Epp JE. 1992. Thiotropocin biosynthesis. Shikimate origin of a sulfur-containing tropolone derivative. *J Am Chem Soc* 114:8479-8483.

Chen Y-H, Kuo J, Sung P-J, Chang Y-C, Lu M-C, Wong T-Y, Liu J-K, Weng C-F, Twan W-H, and Kuo F-W. 2012. Isolation of marine bacteria with antimicrobial activities from cultured and field-collected soft corals. *World Journal of Microbiology and Biotechnology* 28:3269-3279. 10.1007/s11274-012-1138-7

Chiu JMY, Li S, Li A, Po B, Zhang R, Shin PKS, and Qiu J-W. 2012. Bacteria associated with skeletal tissue growth anomalies in the coral *Platygyra carnosus*. *FEMS Microbiology Ecology* 79:380-391. 10.1111/j.1574-6941.2011.01225.x

Dang HY, and Lovell CR. 2000. Bacterial primary colonization and early succession on surfaces in marine waters as determined by amplified rRNA gene restriction analysis and sequences analysis of 16S rRNA genes. *Appl Environ Microbiol* 66:467-475.

Enticknap JJ, Kelly M, Peraud O, and Hill RT. 2006. Characterization of a culturable Alphaproteobacterial symbiont common to many marine sponges and evidence for vertical transmission via sponge larvae. *Appl Environ Microbiol*.

Fuse H, Takimura O, Murakami K, Yamaoka Y, and Omori T. 2000. Utilization of dimethyl sulfide as a sulfur source with the aid of light by *Marinobacterium* sp. strain DMS-S1. *Appl Environ Microbiol* 66:5527-5532.
Fusetani N, Toyoda T, Asai N, Matsunaga S, and Maruyama T. 1996. Montiporic acids A and B, cytotoxic and antimicrobial polyacetylene carboxylic acids from eggs of the scleractinian coral Montipora digitata. J Nat Prod 59:796-797.

Garren M, and Azam F. 2010. New method for counting bacteria associated with coral mucus. Appl Environ Microbiol 76:6128-6133.

Geffen Y, Ron EL, and Rosenberg E. 2009. Regulation of release of antibacterials from stressed scleractinians corals. FEMS Microbiology Letters 295:103-109.

Geffen Y, and Rosenberg E. 2005. Stress-induced rapid release of antibacterials by scleractinian corals. Mar Biol 146:931-935.

Geng H, and Belas R. 2010. Expression of tropodithietic acid biosynthesis is controlled by a novel autoinducer. J Bacteriol 192:4377-4387.

Geng H, Bruhn JB, Nielsen KF, Gram L, and Belas R. 2008. Genetic dissection of tropodithietic acid biosynthesis by marine roseobacters. Appl Environ Microbiol 74:1535-1545.

Gochfeld D, and Aeby GS. 2008. Antibacterial chemical defences in Hawaiian corals provide possible protection from disease. MEPS 362:119-128.

Heron SF, Willis BL, Skirving WJ, Eakin M, Page CA, and Miller IR. 2010. Summer hot snaps and winter conditions: modelling white syndrome outbreaks on Great Barrier Reef corals. PLoS One 5:e12210. 10.1371/journal.pone.0012210

Hjelm M, Bergh O, Riaza A, Nielsen J, Melchiorsen J, Jensen S, Duncan H, Ahrens P, Birkbeck H, and Gram L. 2004. Selection and identification of autochthonous potential probiotic bacteria from turbot larvae (Scophthalmus maximus) rearing units. Syst Appl Microbiol 27:360-371.

Howard EC, Henriksen JR, Buchan A, Reisch CR, Burgmann H, Welsh R, Ye W, González JM, Mace K, Joye SB, Kiene RP, Whitman WB, and Moran MA. 2006. Bacterial taxa that limit sulfur flux from the ocean. Science 314:649-652.

Kennedy J, Baker P, Piper C, Cotter PD, Walsh M, Mooij MJ, Bourke MB, Rea MC, O’Connor PM, Ross RP, Hill C, O’Gara F, Marchesi JR, and Dobson ADW. 2009. Isolation and analysis of bacteria with antimicrobial activities from the marine sponge Haliclona simulans collected from Irish waters. Mar Biotechnol 11:384-396.

Kiene RP, Linn LJ, Gonzalez J, Moran MA, and Bruton JA. 1999. Dimethylsulfiniopropionate and methanethiol are important precursors of methionine and protein-sulfur in marine bacterioplankton. Appl Environ Microbiol 65:4549-4558.

Knowlton N. 2001. Coral reef biodiversity - habitat size matters. Science 292:1493-1494.

Kodani S, Sato K, Higuchi T, Casareto BE, and Suzuki Y. 2013. Montiporic acid D, a new polyacetylene carboxylic acid from scleractinian coral Montipora digitata. Natural Product Research 27:1859-1862.

Koh EGL. 1997. Do scleractinian corals engage in chemical warfare against microbes. Journal of Chemical Ecology 23:379-398.

Koren O, and Rosenberg E. 2006. Bacteria associated with mucus and tissues of the coral Oculina patagonica in summer and winter. Appl Environ Microbiol 75:254-259.

Lema KA, Willis BL, and Bourne DG. 2012. Corals form characteristic associations with symbiotic nitrogen-fixing bacteria. Appl Environ Microbiol 78:3136-3144.

Lesser MP, Mazel CH, Gorbunov MY, and Falkowski PG. 2004. Discovery of symbiotic nitrogen-fixing cyanobacteria in corals. Science 305:997-1000.
Littman RA, Willis BL, Pfeffer C, and Bourne DG. 2009. Diversity of coral-associated bacteria differ with location but not species for three Acroporids on the Great Barrier Reef. *FEMS Microbiology Letters* 68:152-163.

Marchesi JR, Sato T, Weightman AJ, Martin AT, Fry JC, and Wade WG. 1998. Design and evaluation of useful bacterium-specific primers that amplify genes coding for 16S rRNA. *Appl Environ Microbiol* 64:795-799.

Maynard JA, Anthony KRN, Harvell DC, Burgman MA, Beeden R, Sweatman H, Heron SF, Lamb JB, and Willis BL. 2011. Predicting outbreaks of a climate-driven coral disease in the Great Barrier Reef. *Coral Reefs* 30:485-495.

Miller TR, Hnilicka K, Dziedzic A, Desplats P, and Belas R. 2004. Chemotaxis of *Silicibacter* sp. strain TM1040 toward dinoflagellate products. *Appl Environ Microbiol* 70:4692-4701.

Moran MA, González JM, and Kiene RP. 2003. Linking a bacterial taxon to sulfur cycling in the sea: studies of the marine *Roseobacter* group. *Geomicrobiol J* 20:375-388.

Moran MA, Reisch CR, Kiene RP, and Whitman WB. 2012. Genomic insights into bacterial DMSP transformations. *Annual Review of Marine Science* 4:523-542.

Nissimov J, Rosenberg E, and Munn CB. 2009. Antimicrobial properties of resident coral mucus bacteria of *Oculina patagonica*. *FEMS Microbiol Lett* 292:210-215.

O'Halloran JA, Barbosa TM, Morrissey JP, Kennedy J, O'Gara F, and Dobson ADW. 2011. Diversity and antimicrobial activity of *Pseudovibrio* spp. from Irish marine sponges. *J Appl Microb* 110:1495-1508.

Olson ND, Ainsworth TD, Gates RD, and Takabayashi M. 2009. Diazotrophic bacteria associated with Hawaiian *Montipora* corals: diversity and abundance in correlation with symbiotic dinoflagellates. *J Exp Mar Biol and Ecol* 371:140-146.

Pauley G. 1997. Diversity and distribution of reef organisms. In: Birkeland C, ed. *Life and death of coral reefs*. New York: Chapman and Hall, 298-345.

Penesyan A, Tebben J, Lee M, Thomas T, Kjelleberg S, Harder T, and Egan S. 2011. Identification of the antibacterial compound produced by the marine epiphytic bacterium *Pseudovibrio* sp. D323 and related sponge-associated bacteria. *Mar Drugs* 9:1391-1402.

Porsby CH. 2010. Antagonism of *Roseobacter* clade bacteria against pathogenic bacteria PhD Thesis. Technical University of Denmark.

Radjasa OK, Wiese J, Sabdono A, and J.F. I. 2008. Coral as source of bacteria with antimicrobial activity. *J Coastal Development* 11:121-130.

Raina JB, Dinsdale EA, Willis BL, and Bourne DG. 2010. Do the organic sulfur compounds DMSP and DMS drive coral microbial associations? *Trends in Microbiology* 18:101-108.

Raina JB, Tapiolas D, Willis BL, and Bourne DG. 2009. Coral-associated bacteria and their role in the biogeochemical cycling of sulfur. *Appl Environ Microbiol* 75:3492-3501.

Reisch CR, Stoudemayer MJ, Varaljaj VA, Amster JJ, Moran MA, and Whitman WB. 2011. Novel pathway for assimilation of dimethylsuloniopropionate widespread in marine bacteria. *Nature* 473:208-211.

Ritchie KB. 2006. Regulation of microbial populations by coral surface mucus and mucus-associated bacteria. *Marine Ecology Progress Series* 322:1-14.
Ritchie KB, and Smith GW. 2004. Microbial communities of coral surface mucopolysaccharide layer. In: Rosenberg E, and Loya Y, eds. *Coral health and disease*. Berlin: Springer-Verlag, 259-263.

Rohwer F, Breitbart M, Jara J, Azam F, and Knowlton N. 2001. Diversity of bacteria associated with the Caribbean coral *Monastera franksi*. *Coral Reefs* 20:85-91.

Rypien KL, Ward JR, and Azam F. 2010. Antagonistic interactions among coral-associated bacteria. *Environmental Microbiology* 12:28-39.

Sabdono A, and Radjasa OK. 2006. Antifouling activity of bacteria associated with soft coral *Sarcophyton* sp. against marine biofilm-forming bacteria. *J Coastal Development* 10:55-62.

Seeman T. 2014. Prokka: rapid prokariotic genome annotation. *Bioinformatics* 30:2068-2069.

Sertan-de Guzman AA, Predicala RZ, Bernardo EB, Neilan BA, Elardo SP, Mangalindan GC, Tasdemir D, Ireland CM, Barraquio WL, and Concepcion GP. 2007. *Pseudovibrio denitrificans* strain Z143-1, a heptylprodigiosin-producing bacterium isolated from a Philippine tunicate. *FEMS Microbiol Lett* 277:188-196.

Shieh WY, Lin Y, and Jean WD. 2004. *Pseudovibrio denitrificans* gen. nov., sp. nov., a marine, facultative anaerobic, fermentative bacterium capable of denitrification. *IJSEM* 54:2307-2312.

Shnit-Orland M, and Kushmaro A. 2008. Coral mucus bacteria as a source of antibacterial activity. *Proceedings of the 11th International Coral Reef Symposium*:257-259.

Shnit-Orland M, and Kushmaro A. 2009. Coral mucus-associated bacteria: a possible first line of defence. *FEMS Microbiol Lett* 67:371-380.

Simo R. 2001. Production of atmospheric sulfur by oceanic plankton: biogeochemical, ecological and evolutionary links. *Trends in Ecology and Evolution* 16:287-294.

Sulistiyani S, Nugraheni A, Radjasa OK, Sabdono A, and Khoeri MM. 2010. Antibacterial activities of bacterial symbionts of soft coral *Sinularia* sp. against tuberculosis bacteria. *J Coastal Development* 14:45-50.

Sunagawa S, Woodley CM, and Medina M. 2010. Threatened corals provide underexplored microbial habitats. *PLoS One* 5:e9554. 10.1371/journal.pone.0009554

Sussman M, Willis BL, Victor S, and Bourne DG. 2008. Coral pathogens identified for white syndrome epizootic in the indo-pacific. *PLoS One* 3:e2393.

Tapiolas DM, Raina JB, Lutz A, Willis BL, and Motti CA. 2013. Direct measurement of dimethylsulfoniopropionate (DMSP) in reef-building corals using quantitative nuclear magnetic resonance (qNMR) spectroscopy. *JEMBE* 443:85-89.

Thiel V, Brinkhoff T, Dickschat JS, Wickel S, Grunenberg J, Wagner-Dobler I, Simon M, and Schulz S. 2010. Identification and biosynthesis of tropone derivatives and sulfur volatiles produced by bacteria of the marine *Roseobacter* clade. *Org Biomol Chem* 2010:234-246.

Thiel V, and Imhoff JF. 2003. Phylogenetic identification of bacteria with antimicrobial activities isolated from Mediterranean sponges. *Biomolecular Engineering* 20:421-423.

Todd JD, Rogers R, Li YG, Wexler M, Bond PL, Sun L, Curson ARJ, Malin G, Steinke M, and Johnston AWB. 2007. Structural and regulatory genes required to make the gas dimethylsulfide in bacteria. *Science* 315:666-669.

Ushijima B, Smith A, Aeby GS, and Callahan SM. 2012. *Vibrio owensii* induces the tissue loss disease *Montipora* white syndrome in the Hawaiin reef coral *Montipora capitada*. *PLoS One* 7:10.
Vidal-Dupiol J, Ladriere O, Destoumieux-Garzon D, Sautiere PE, Meistertzheim AL, Tambutte E, Tambutte S, Duval D, Foure L, Adjeroud M, and Mitta G. 2011. Innate immune responses of a scleractinian coral to vibriosis. *J Biol Chem* 286:22688-22698.

Vizcaino MI. 2011. The chemical defense of *Pseudopternogorgia americana*: a focus on the antimicrobial potential of a *Pseudovibrio* sp. PhD Thesis. University of South Carolina.

Vizcaino MI, Johnson WR, Kimes NE, Williams K, Torralba M, Nelson KE, Smith GW, Weil E, Moeller PDR, and Morris PJ. 2010. Antimicrobial resistance of the coral pathogen *Vibrio coralliilyticus* and sister phylotypes isolated from a diseased Octocoral. *Microb Ecol* 59:646-657.

Wagner-Dobler I, and Biebl H. 2006. Environmental biology of the marine Roseobacter lineage. *Annual Reviews Of Microbiology* 60:255-280.

Webster NS, and Hill RT. 2001. The culturable microbial community of the Great Barrier Reef sponge *Rhopaloeides odorabile* is dominated by an Alphaproteobacterium. *Marine Biology* 138:843-851.

Willis BL, Page CA, and Dinsdale EA. 2004. Coral disease on the Great Barrier Reef. In: Rosenberg E, and Loya Y, eds. *Coral health and disease*: Springer, 69-104.

Wilson MZ, Wang R, Gitai Z, and Seyedsayamdost MR. 2016. Mode of action and resistance studies unveil new roles for tropodithietic acid as an anticancer agent and the γ-glutamyl cycle as a proton sink. *Proceedings of the National Academy of Sciences* 113:1630-1635. 10.1073/pnas.1518034113

Yang S, Sun W, Zhang F, and Li Z. 2013. Phylogenetically Diverse Denitrifying and Ammonia-Oxidizing Bacteria in Corals *Alcyonium* gracillimum and *Tubastraea coccinea*. *Marine Biotechnology* 15:540-551. 10.1007/s10126-013-9503-6