Isolation of an antimicrobial compound produced by bacteria associated with reef-building corals

Jean-Baptiste Raina [Corresp., 1, 2, 3, 4, 5], **Dianne Tapiolas** [2], **Cherie A Motti** [2], **Sylvain Foret** [3, 6], **Torsten Seemann** [7], **Jan Tebben** [8, 9], **Bette L Willis** [3, 4], **David G Bourne** [2, 4]

[1] Climate Change Cluster (C3), University of Technology Sydney, Sydney, NSW, Australia
[2] Australian Institute of Marine Science, Townsville, QLD, Australia
[3] James Cook University, Australian Research Council Centre of Excellence for Coral Reef Studies, Townsville, QLD, Australia
[4] Marine Biology and Aquaculture, College of Science and Engineering, James Cook University of North Queensland, Townsville, QLD, Australia
[5] James Cook University, AIMS@JCU, Townsville, QLD, Australia
[6] Research School of Biology, Australian National University, Canberra, ACT, Australia
[7] Victorian Life Sciences Computation Initiative, University of Melbourne, Melbourne, Victoria, Australia
[8] Section Chemical Ecology, Alfred Wegener Institute, Bremerhaven, Germany
[9] University of New South Wales, Sydney, NSW, Australia

Corresponding Author: Jean-Baptiste Raina
Email address: Jean-Baptiste.Raina@uts.edu.au

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.

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

[a]James Cook University, Marine Biology and Aquaculture, College of Science and Engineering, Townsville 4810, QLD, Australia.

[b]AIMS@JCU, James Cook University, Townsville 4810, QLD, Australia

[c]Australian Institute of Marine Science, Townsville 4810, QLD, Australia.

[d]Australian Research Council Centre of Excellence for Coral Reef Studies, James Cook University, Townsville 4810, QLD, Australia

[e]Climate Change Cluster (C3), University of Technology Sydney, Sydney 2007, NSW, Australia

[f]Australian National University, Research School of Biology, Canberra 2601, ACT, Australia.

[g]Victorian Life Sciences Computation Initiative, University of Melbourne, Melbourne 3168, VIC, Australia.

[h]University of New South Wales, Sydney 2052, NSW, Australia.

[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.

49

50 **Introduction**

51 Coral reefs are one of the most biologically diverse ecosystems on the planet (Bellwood
52 & Hughes 2001; Knowlton 2001; Pauley 1997). Each square centimeter of a coral's surface
53 harbors several thousand species of microbes (Sunagawa et al. 2010) and up to 10^7 bacterial cells
54 (Garren & Azam 2010; Koren & Rosenberg 2006), an abundance ten times greater, on average,
55 than the surrounding sea water. These microbial assemblages are often highly specific to their
56 coral host and include large numbers of rare and sometimes even unique taxa (Sunagawa et al.
57 2010). Although the phylogenetic diversity and dynamics of coral-associated bacterial
58 communities have been studied for more than a decade (Bourne & Munn 2005; Bourne et al.
59 2013; Littman et al. 2009; Rohwer et al. 2001; Sunagawa et al. 2010), their ecological and
60 functional roles in the biology and health of corals are still poorly understood.

61

62 Recent studies have started to unravel the roles that coral-associated bacteria and their
63 interactions with their coral hosts are likely to play within the coral holobiont. For example,
64 some members of the *Cyanobacteria*, *Rhizobiales* and *Vibrionaceae* taxa are likely to fix
65 dissolved nitrogen, a particularly important process in oligotrophic environments such as coral
66 reefs (Lema et al. 2012; Lesser et al. 2004; Olson et al. 2009). Others, like *Roseobacter*,
67 *Pseudomonas* and *Oceanospirillales*, can metabolize dimethylsulfoniopropionate (DMSP), an
68 organic sulfur compound produced in large quantities by corals and suspected to play a role in
69 structuring coral-associated bacterial communities (Raina et al. 2010; Raina et al. 2013). It has
70 also been hypothesized that bacteria act as a line of defense against invasive pathogens, either by
71 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

94 Healthy colonies of the corals *Pocillopora damicornis*, *Acropora millepora* and
95 *Montipora aequituberculata* (one colony per species) were collected in November 2011 from
96 Davies Reef, Great Barrier Reef, Australia (latitude, 18°51'S; longitude, 147°41'E, Great Barrier
97 Reef Marine Park Authority permit G12/35236.1) and maintained in aquaria for 6 days at the
98 Australian Institute of Marine Science (Townsville, Queensland, Australia). Five replicate coral
99 fragments (approximately 25 mm in length, containing 60 to 70 polyps) were collected from
100 each colony and washed in sterile artificial seawater (ASW) to remove loosely attached
101 microbes. Tissue slurries were produced by airbrushing (80 lb/in^2) each coral fragment into 5 mL
102 of ASW to remove coral tissues and associated microbes. These tissue slurries were
103 homogenized to break down tissue clumps, and a dilution series was plated immediately on
104 bacteriological agar (1%) in 1 L ASW supplemented with 0.3% casamino acids and 0.4%
105 glucose (Hjelm et al. 2004). After 2 days of incubation at 28°C, single colonies were transferred
106 into Marine Broth (MB; Difco, BD, Franklin Lakes, NJ) and grown overnight. Liquid cultures
107 were re-plated on minimal marine agar and the procedure was repeated until pure cultures were
108 obtained.
109
110 *Well diffusion assay with bacterial isolates*
111 Fifty bacteria isolated from the coral tissue slurries of the 3 species (*A. millepor*a = 16, *P.*
112 *damicornis* = 17, *M. aequituberculata* = 17) were tested for growth-inhibitory activity against the
113 known coral pathogens *Vibrio coralliilyticus* P1 (LMG23696) and *V. owensii* DY05
114 (LMG25443) in a well diffusion agar assay. In brief, the *Vibrio* strains were seeded into two
115 different batches of minimal marine agar (after the agar temperature cooled to 40°C). Following
116 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_2PO_4, 1 g of NH_4NO_3, 0.2 g of $MgCl_2 \cdot H_2O$, 0.02 g of $CaCl_2 \cdot 2H_2O$, 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

163 metabolism was assessed by ^{1}H Nuclear Magnetic Resonance spectroscopy (NMR). Methanol
164 (CH_3OH; 40 mL) was added to each culture tube, the mixture shaken vigorously and sonicated
165 for 10 mins before being dried *in vacuo* using a rotary evaporator (Buchi, Flawil, Switzerland).
166 The dried extracts were resuspended in a mixture of deuterium oxide (D_2O; 250 µL) and
167 deuterated methanol (CD_3OD; 750 µL) (Cambridge Isotope Laboratories, Andover, MA, USA).
168 A 750-mL aliquot of the particulate-free extract was transferred into a 5-mm Norell tube (Norell
169 Inc., Landisville, NJ, USA) and analyzed immediately using quantitative NMR (Tapiolas et al.
170 2013).
171
172 *Preparation of crude extracts for antagonist assays*
173 An overnight culture of P12 (8 mL) was used to inoculate 4×250 mL of MB (total culture
174 volume = 1L). Bacterial cells were incubated for two days at 28°C (120 rpm); the culture broth
175 was then acidified to pH 2 with sulphuric acid before being exhaustively extracted with ethyl
176 acetate (3 × 1.5 L). The extract was washed three times with MilliQ H_2O and dried *in vacuo*
177 using a rotary evaporator (Buchi). The dried extract was then weighed and resuspended in
178 CH_3OH (which was chosen for its ability to solubilize a wide range of compounds, its volatility
179 and its innocuity in small volume towards both *V. coralliilyticus* and *V. owensii*) and tested in
180 well-diffusion assays to confirm the extraction of the antimicrobial compound(s).
181
182 *Purification and characterization of active compound*
183 Purification of the crude extract was carried out using solid phase extraction on a
184 reversed phase C_{18} flash vacuum column (Septra C_{18}-E, Phenomenex, Torrance, CA, USA).
185 Eleven fractions were eluted sequentially with 20%, 40%, 60%, 80%, 90% CH_3OH in H_2O and

100% CH_3OH, followed by 20%, 50% and 100% dichloromethane (CH_2Cl_2) in CH_3OH, 40% hexane in CH_2Cl_2 and finally 100% hexane. Each fraction was dried and resuspended in CH_3OH (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_3OH as a control, and *Vibrio* growth monitored. The most active faction (80% CH_3OH) 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

209 BioApex 47e Fourier Transform Mass Spectrometer (FTMS) fitted with an Analytica of
210 Branford ESI source; ions were detected in negative mode within a mass range *m*/*z* 200-1,000 via
211 direct infusion at 120 μl h^{-1}.
212
213 *Temperature-dependent activity*
214 The antimicrobial activity of P12 grown at 32°C (upper limit of coral thermal tolerance)
215 was compared to that of the control incubated at 28°C. The two cultures were grown overnight in
216 MB at the two different temperatures, and their densities were determined by flow-cytometry
217 (BD Accuri C6, Beckman Coulter, Brea, CA). Cell numbers were normalized prior to
218 inoculation into agar wells, and their activities against the two pathogens were compared using
219 well-diffusion assays as described above. The same procedure was repeated with compound **1**:
220 two vials containing equal concentrations (2 μM of **1** in CH_3OH) were incubated overnight at
221 28°C or 32°C and their antimicrobial activities compared using the well diffusion assay.
222
223 *Preparation of coral extracts*
224 The coral species *Montipora aequituberculata, M. turtlensis, Pocillopora damicornis,*
225 *Acropora millepora,* and *Porites cylindrica* (one colony each; 500 g of dry skeleton per species)
226 were collected in July 2012 from Orpheus Island, Great Barrier Reef, Australia (latitude,
227 18°35'S; longitude, 146°20'E, Great Barrier Reef Marine Park Authority permit G12/35236.1).
228 Coral tissues were airbrushed (80 lb/in^2) into 1μm filtered seawater (FSW) (total volume = 500
229 mL), acidified to pH 2 with sulphuric acid and the solution exhaustively extracted with equal
230 volumes of ethyl acetate (3 × 750 mL). The combined organic layers were partitioned with
231 MilliQ H_2O, dried and tested in well-diffusion assays, as previously described for the bacterial

232 isolate extracts. The extracts of those coral species that exhibited antimicrobial activity were
233 subsequently fractionated as described above for the crude extract from P12 and tested in well-
234 diffusion assays. The active fractions were analyzed using ^{1}H NMR, FTMS and LC-MS.
235
236 **Results**
237 *Isolate P12: antimicrobial production, taxonomy and metabolic capabilities*
238 A total of 50 coral-associated bacterial isolates were obtained from tissue slurry
239 homogenates of the three coral species. Twelve of the 50 strains tested against the two
240 pathogenic *Vibrios* (*V. coralliilyticus* and *V. owensii*) inhibited their growth in well diffusion
241 assays. The bioactive isolate that exhibited the strongest *in vitro* activity against both pathogens,
242 isolate P12, originated from *Pocillopora damicornis* and produced growth inhibition zones of 5
243 mm (±0.07 mm, n=20) against *V. owensii* and 2 mm (±0.09 mm, n=20) against *V. coralliilyticus*.
244 The activity of P12 was temperature-dependent (Figure 1 A-B) and was significantly reduced
245 when grown at 32°C compared to 28°C (Unpaired T-Test, n=20, df=38, t=30.61, *p<0.001 for *V.*
246 *owensii* and n=20, df=38, t=10.49, *p<0.001 for *V. coralliilyticus*; Figure 1C). Based on its
247 bioactivity, the isolate P12 was selected for bioassay-guided fractionation.
248
249 According to its 16S rRNA gene sequence (NCBI accession number: KX198136), isolate
250 P12 is an alphaproteobacterium belonging to the *Rhodobacteraceae* family and the *Pseudovibrio*
251 genus. Its most closely related species is *Pseudovibrio denitrificans* (100% identity to the type
252 strain; Figure 2). Like other *P. denitrificans* strains (Enticknap et al. 2006), P12 colonies formed
253 brown mucoid colonies when grown on Marine Agar. The brown color was absent when the
254 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) ν_{max} 3420, 1660, 1280 cm^{-1}; UV (PDA, CH_3OH) λ_{max} 512 nm; ^{1}H NMR spectrum (600 MHz, CD_3Cl): δ 7.12, 7.44, 7.45 and 16.7; ^{13}C NMR (150 MHz, CD_3Cl): δ 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 $C_8H_3O_3S_2^-$ 210.9529, Δ 2 ppm). Combined spectroscopic techniques
279 revealed that compound **1** was tropodithietic acid (TDA) (Brinkhoff et al. 2004; Penesyan et al.
280 2011) (Figure 4A).
281
282 Orthologs for 11 genes involved in TDA biosynthesis (Geng et al. 2008) were present in
283 the *Pseudovibrio* sp. P12 genome (Table 1). The biosynthesis of TDA correlated with production
284 of the yellow-brown pigmentation in the culture medium and antimicrobial activity, similar to
285 that previously reported (Brinkhoff et al. 2004; Bruhn et al. 2005; Porsby 2010). Both coral
286 pathogens were highly sensitive to TDA, with the pure compound still visually inhibiting their
287 growth at 0.5 µg/mL (2.35 µM; Figure 4B). In contrast to the decrease in antimicrobial activity
288 exhibited by *Pseudovibrio* sp. P12 after incubation at 32°C, TDA activity was not affected by
289 exposure to this temperature (Unpaired T-Test, n=20, df=38, t=-0.94, p=0.355 for *V. owensii*
290 and n=20, df=38, t=0.632, p=0.531 for *V. coralliilyticus*; Figure 1B-C).
291
292 *Investigating the presence of TDA in coral samples*
293 All of the extracts derived from the coral species investigated exhibited antimicrobial
294 activity against the two pathogens, with the inhibition zones for *P. cylindrica*, *M.*
295 *aequituberculata*, *M. turtlensis* and *P. damicornis* ranging from 3 to 5 mm in radius whilst the
296 inhibition zones for *A. millepora* were much smaller (1 mm on average). ^{1}H NMR, LC-MS and
297 FTMS analyses of the extracts and the active fractions of all coral species did not confirm the
298 presence of TDA. The purified TDA could be detected by LC-MS in femtomolar concentrations
299 when the coral fractions were artificially spiked, indicating that this lack of detection was not due

300 to preferential ionization. Thus, TDA was either not present in the coral fractions tested or in
301 concentrations below the LC-MS detection threshold.
302
303 **Discussion**
304 While previous studies have identified corals as a source of bacteria with antimicrobial
305 activity (Radjasa et al. 2008; Sulistiyani et al. 2010), this study represents the first isolation and
306 identification of an antimicrobial compound produced by a reef building coral-associated
307 bacterium with activity against coral-associated pathogens. Tropodithietic acid (TDA) was
308 characterized from a pure culture of *Pseudovibrio* sp. P12 previously isolated from corals and
309 strongly inhibited the growth of two coral pathogens. Although we could not confirm the
310 presence of this molecule in the corals tested, TDA has the capacity to provide protective
311 antimicrobial properties to the coral host and prevent colonization by invasive bacterial species.
312
313 The strain P12 strongly inhibited the growth of *Vibrio coralliilyticus* and *V. owensii*, two
314 coral pathogens causing white syndrome (a collective term describing rapidly progressing tissue
315 loss, exposing band-like areas of white skeleton) (Ben-Haim et al. 2003; Sussman et al. 2008;
316 Ushijima et al. 2012; Willis et al. 2004). *Vibrio coralliilyticus* exhibits antimicrobial resistance to
317 a wide range of commercial antibiotics and is also resistant to the activities of a large number of
318 coral-associated bacteria (Rypien et al. 2010; Shnit-Orland & Kushmaro 2009; Vizcaino et al.
319 2010). Its resistance to commercial antibiotics is considerably greater than that of other marine
320 pathogens such as *V. parahaemolyticus* or *V. vulnificus*, and may contribute to its competitive
321 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

345 tropodithietic acid (TDA) from red alga-associated *P. ascidiaceicola* D323 (Penesyan et al.
346 2011). In the present study, we isolated TDA from P12, a strain closely related to *P.*
347 *denitrificans*. The ability of P12 to produce TDA was further supported by the the presence of
348 the genes essential for TDA biosynthesis in the genome of strain P12 (Geng et al. 2008). TDA
349 inhibits the growth of a wide range of marine pathogens (Bruhn et al. 2007; Bruhn et al. 2005)
350 and is produced almost exclusively by bacteria from the *Roseobacter* clade, especially the genera
351 *Phaeobacter*, *Silicibacter*, and *Ruegeria* (Brinkhoff et al. 2004; Bruhn et al. 2005; Geng & Belas
352 2010; Geng et al. 2008; Wilson et al. 2016) that are commonly associated with DMSP-producing
353 dinoflagellates (Miller et al. 2004; Wagner-Dobler & Biebl 2006) and reef-building corals
354 (Bourne et al. 2013; Littman et al. 2009; Raina et al. 2009).
355
356 Many members of the *Roseobacter* clade, including coral-associated isolates, have been
357 implicated in sulfur cycling (Miller et al. 2004; Moran et al. 2003; Raina et al. 2010).
358 Interestingly, TDA contains two sulfur atoms ($C_8H_4O_3\underline{S_2}$) and *Pseudovibrio* sp. P12 was able to
359 use DMSP either as sole carbon or sole sulfur source, a common trait among
360 *Alphaproteobacteria* and especially the *Roseobacter* clade (Bruhn et al. 2005; Wagner-Dobler &
361 Biebl 2006). Bacteria from this clade preferentially metabolize DMSP rather than sulphate (SO_4^{2-}
362), despite the latter being between 10^6 to 10^7-fold more abundant in seawater (Geng & Belas
363 2010; Kiene et al. 1999). Based on genomic and chemical analyses, DMSP metabolism in P12
364 could occur *via* two likely routes: the cleavage pathway (encoded by *dddD*, (Todd et al. 2007))
365 that releases the climate-regulating molecule DMS, and the demethylation pathway (encoded by
366 *dmdA*, (Howard et al. 2006)) by which the bacterium can retain the sulfur contained in DMSP
367 molecules.

368

369 The biosynthetic pathway of TDA has not been fully elucidated (Brock et al. 2014). Both
370 labelling (Cane et al. 1992; Thiel et al. 2010) and genetic dissection (Geng & Belas 2010) studies
371 have shown that its aromatic skeleton is derived from phenylacetyl-CoA produced by the
372 shikimate pathway. However, the sulfur donor allowing the incorporation of the two sulfur atoms
373 into the TDA molecule has not been clearly identified. It has been proposed that sulfur
374 originating from DMSP metabolism might be used to synthesize TDA (Bruhn et al. 2007; Bruhn
375 et al. 2005; Geng & Belas 2010; Porsby 2010; Wagner-Dobler & Biebl 2006). For example,
376 DMSP increases TDA synthesis two-fold in comparison to other sulfur sources (Geng & Belas
377 2010), suggesting that DMSP is a preferred source of sulfur for TDA biosynthesis. Even though
378 other sources of sulfur, such as the amino-acids cysteine and methionine present in artificial
379 media like Marine Broth, might be used to synthesize TDA (Geng & Belas 2010), DMSP is by
380 far the most readily available reduced sulfur source in the marine environment (Simo 2001). It is
381 therefore likely that in DMSP-rich environments, such as reef-building corals, DMSP
382 metabolism provides the sulfur needed to produce TDA *via* the demethylation pathway (Howard
383 et al. 2006).

384

385 The presence of TDA in extracts derived from five coral species (*Montipora*
386 *aequituberculata*, *M. turtlensis*, *Pocillopora damicornis*, and *Porites cylindrica*) could not be
387 confirmed. Three possibilities can explain this lack of detection: (*i*) TDA is not synthesized in
388 corals; (*ii*) TDA is present in corals in concentrations below the detection limit of our
389 instruments (which would imply that this compound has a very limited role in coral defense);
390 (*iii*) our sampling effort was not sufficient. Indeed, we only sampled one colony per species,

391 from a location more than 100 km away from the site where the TDA-producing bacteria was
392 isolated and without prior characterization of the bacterial communities present in the colony
393 sampled. Given that TDA-producing *Roseobacters* are among the first bacteria to colonize the
394 surface of marine microalgae and corals (Apprill et al. 2009; Dang & Lovell 2000; Miller et al.
395 2004) and can be highly abundant in some coral species (Raina et al. 2009), it would be
396 premature to rule out possible TDA biosynthesis in corals.
397
398 The activity of P12 against *V. coralliilyticus* sharply decreased at elevated temperatures
399 (32°C), however, the activity of the purified TDA did not. This reveals that the loss of
400 antimicrobial activity observed for P12 at 32°C is not due to thermal sensitivity of TDA but
401 likely to a decrease in its production. Our results are in line with previously reported decline in
402 the antibacterial activity of other TDA-producing *Roseobacter* with temperature increase (Bruhn
403 et al. 2005). Clear links have previously been identified between warm thermal anomalies and
404 outbreaks of white syndromes (Bruno et al. 2007; Heron et al. 2010; Maynard et al. 2011). If
405 indeed TDA is synthesized *in vivo*, a decrease in its production during anomalously high
406 seawater temperatures could facilitate pathogen outbreaks in corals following thermal stress,
407 especially since the virulence of some disease-causing bacteria (i.e. *V. coralliilyticus*) increase at
408 32°C (Sussman et al. 2008).
409
410 This study demonstrates that a common coral-associated bacterium, *Pseudovibrio* sp.
411 P12, produces TDA, a potent antimicrobial compound that inhibits the growth of marine and
412 coral pathogens, including *V. coralliilyticus*. The bacterium can use DMSP as a sole sulfur or
413 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.

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Figures

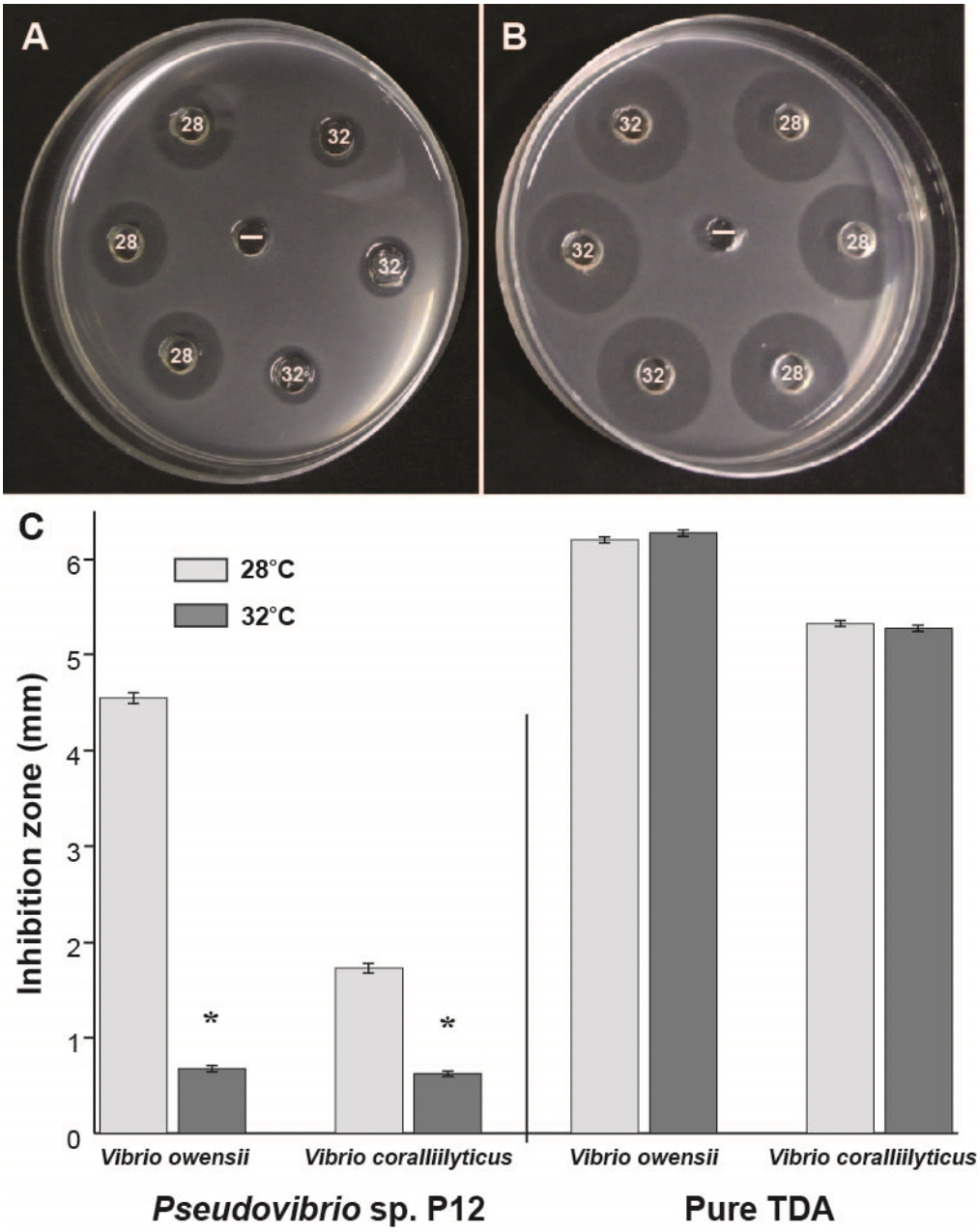

Figure 1: Representative well diffusion assays of (*A*) *Pseudovibrio* sp. P12 and (*B*) pure TDA, incubated at two different temperatures (28°C and 32°C) and then inoculated onto agar plates with embedded *Vibrio owensii* [(-) Negative control]. (*C*) Comparison of the radius of inhibition zones between 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*).

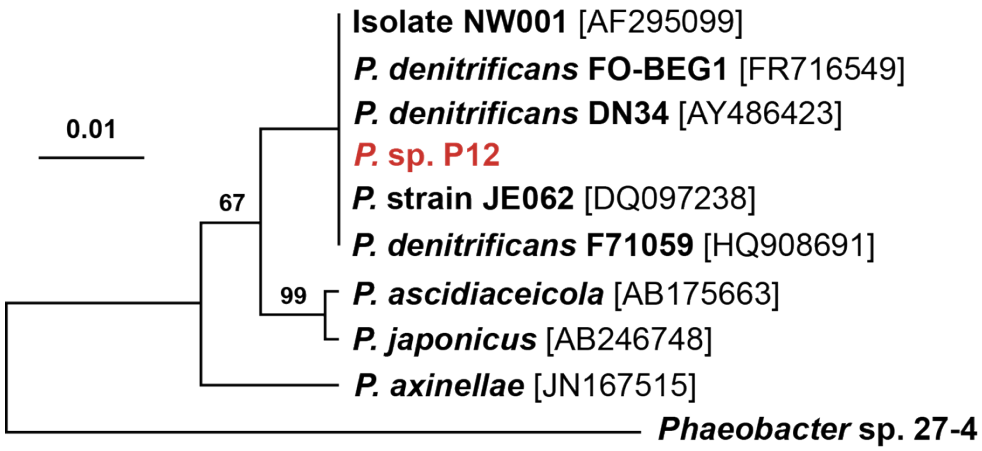

Figure 2: Maximum likelihood phylogenetic tree based on 16S rRNA gene sequences showing the isolate used in this study (P12 in red) and closely associated *Pseudovibrio* spp. Note: the strain FO-BEG1 has been fully sequenced. *Phaeobacter* sp. 27-4 [AJ536669] was used as outgroup. Maximum parsimony bootstrap values (10,000 replicates) are given when different from 100. The scale bar indicates the number of substitution per nucleotide position.

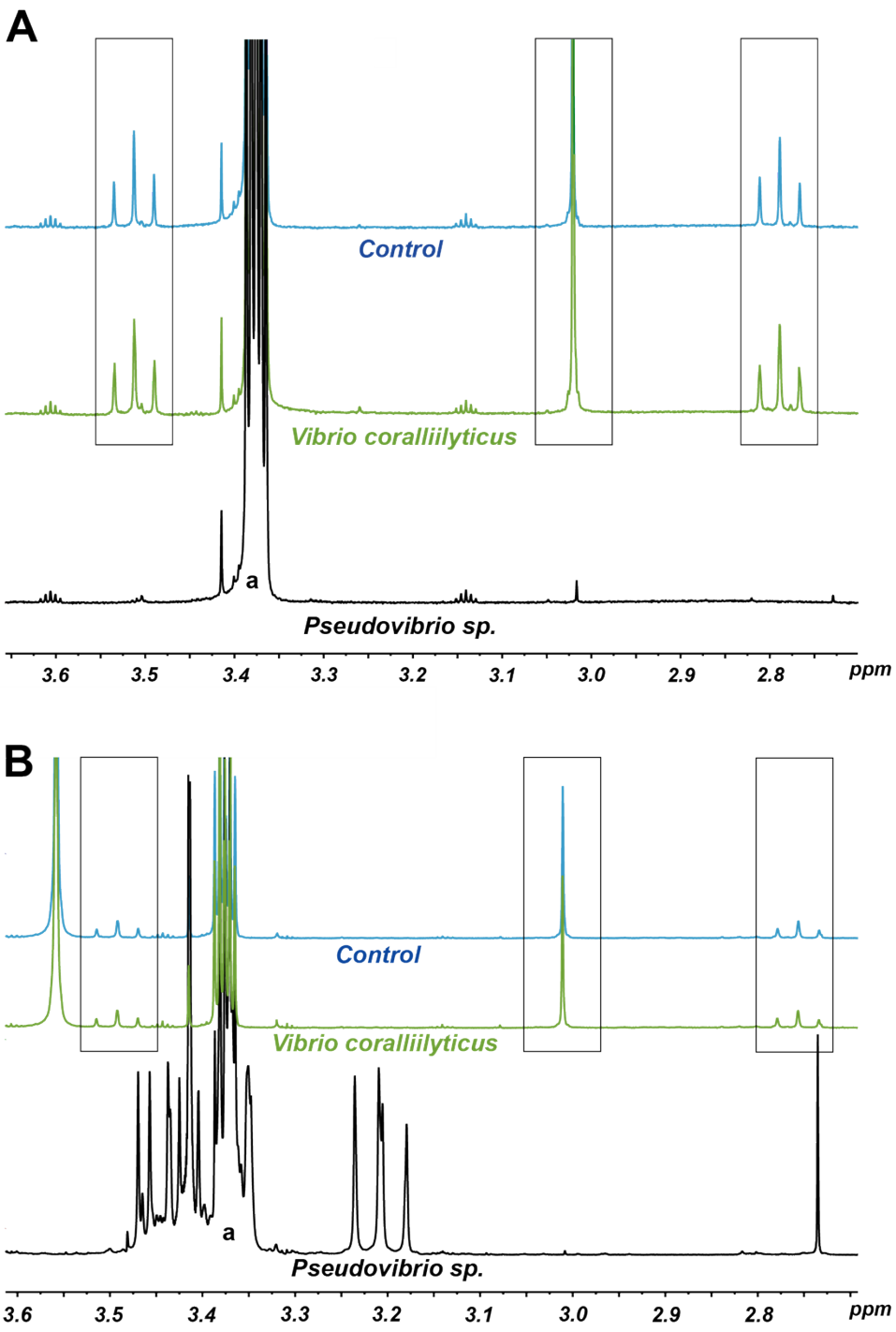

Figure 3: ^{1}H NMR spectra showing DMSP utilization as (*A*) sole carbon source and (*B*) sole sulfur source in minimal media at the end of a 6-day incubation. The "control" lines in all cases are the growth medium (with no bacterial inoculation). The black and green spectra show the results from inoculation 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).

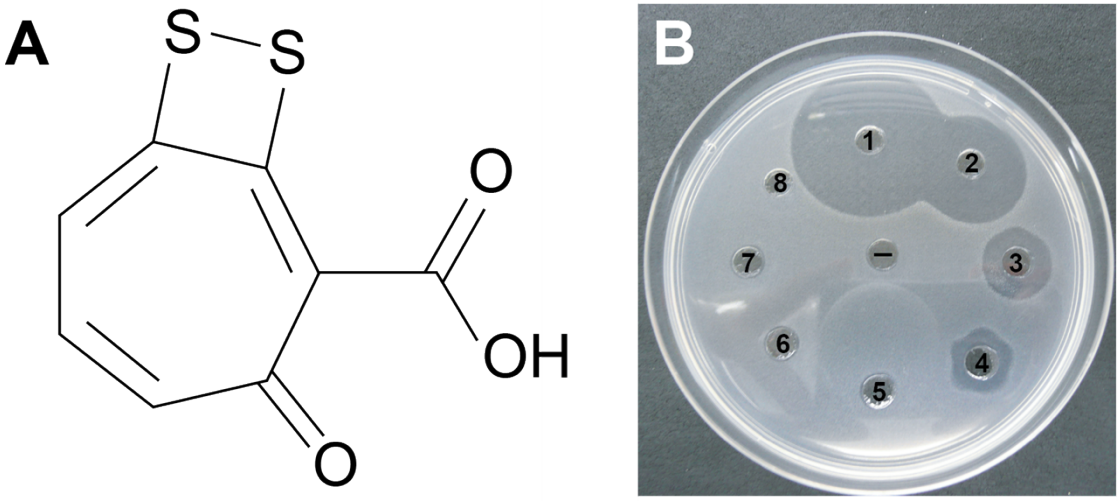

Figure 4: (*A*) Tropodithietic acid (TDA). (*B*) Dilution series of pure TDA, showing zones of growth inhibition against *Vibrio coralliilyticus*: [(1): 500 µg/mL, (2): 50 µg/mL, (3): 5 µg/mL, (4): 500 ng/mL, (5): 50 ng/mL, (6): 5 ng/mL, (7): 500 pg/mL, (8): 50 pg/mL, (-) negative control with solvent 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. MWYl1 (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
cysI	Sulfite reductase hemoprotein beta-component	76% [e]	KU760706
malY	Cystathione beta-lyase	68% [e]	KU760707
paaI	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
Tubastraea 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
Sarcophyton 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

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