Coral-Associated Bacteria and Their Role in the Biogeochemical Cycling of Sulfur[∇]

Jean-Baptiste Raina,[1,2] Dianne Tapiolas,[1] Bette L. Willis,[2] and David G. Bourne[1]*

Australian Institute of Marine Science, PMB3, Townsville MC, Queensland 4810, Australia,[1] and ARC Centre of Excellence for Coral Reef Studies and School of Marine and Tropical Biology, James Cook University, Townsville, Queensland 4811, Australia[2]

Marine bacteria play a central role in the degradation of dimethylsulfoniopropionate (DMSP) to dimethyl sulfide (DMS) and acrylic acid, DMS being critical to cloud formation and thereby cooling effects on the climate. High concentrations of DMSP and DMS have been reported in scleractinian coral tissues although, to date, there have been no investigations into the influence of these organic sulfur compounds on coral-associated bacteria. Two coral species, *Montipora aequituberculata* and *Acropora millepora*, were sampled and their bacterial communities were characterized by both culture-dependent and molecular techniques. Four genera, *Roseobacter*, *Spongiobacter*, *Vibrio*, and *Alteromonas*, which were isolated on media with either DMSP or DMS as the sole carbon source, comprised the majority of clones retrieved from coral mucus and tissue 16S rRNA gene clone libraries. Clones affiliated with *Roseobacter* sp. constituted 28% of the *M. aequituberculata* tissue libraries, while 59% of the clones from the *A. millepora* libraries were affiliated with sequences related to the *Spongiobacter* genus. *Vibrio* spp. were commonly isolated from DMS and acrylic acid enrichments and were also present in 16S rRNA gene libraries from coral mucus, suggesting that under "normal" environmental conditions, they are a natural component of coral-associated communities. Genes homologous to *dddD*, and *dddL*, previously implicated in DMSP degradation, were also characterized from isolated strains, confirming that bacteria associated with corals have the potential to metabolize this sulfur compound when present in coral tissues. Our results demonstrate that DMSP, DMS, and acrylic acid potentially act as nutrient sources for coral-associated bacteria and that these sulfur compounds are likely to play a role in structuring bacterial communities in corals, with important consequences for the health of both corals and coral reef ecosystems.

Dimethylsulfoniopropionate (DMSP) is an organic sulfur compound implicated in the formation of clouds via its cleavage product dimethyl sulfide (DMS) and therefore has the potential to exert major cooling effects on climate (9, 38). The production of DMSP is mainly restricted to a few classes of marine macro- and microalgae (27, 68), with the main producers being phytoplankton species belonging to prymnesiophyte and dinoflagellate taxa (28, 62, 67). Recently, significant concentrations of DMSP and DMS have been recorded in association with animals that harbor symbiotic algae such as scleractinian corals and giant clams (7, 8, 68), raising questions about the role of coral reefs in sulfur cycling. The densities of symbiotic dinoflagellates (genus *Symbiodinium*, commonly known as zooxanthellae) in coral tissues are similar to those recorded for dinoflagellates in phytoplankton blooms (11, 68). Since dinoflagellates are among the most significant producers of DMSP and high intracellular concentrations of DMSP have been found in both cultured zooxanthellae (26) and scleractinian corals (6–8, 25), these observations suggest that endosymbiotic zooxanthellae have an integral role in sulfur cycling in oligotrophic reef waters.

Most of the DMSP produced by planktonic dinoflagellates is exuded into the surrounding water, where it is degraded by bacteria via two possible pathways: the first one converts a large fraction (ca. 75%) of dissolved DMSP to methylmercaptopropionate, which is subsequently incorporated into the biomass of microbial cells (22, 27, 66). The second pathway transforms the remaining part of the dissolved DMSP to equimolar concentrations of DMS and acrylic acid (43, 66, 72). This metabolic pathway for DMSP degradation has been identified in the alphaproteobacterial species *Sulfitobacter* sp. and the enzyme involved (DMSP-dependent DMS lyase [DddL]) characterized (10). Another pathway for DMS formation (without production of acrylate) has been described for *Marinomonas* sp. and the gene responsible, *dddD*, identified. In addition, the protein DddR has been directly implicated in the regulation of the gene encoding DddD (66). The DMS produced by these enzymes are then released into the surrounding water (27). Prior to the 1980s, diffusion of supersaturated DMS from the oceans to the atmosphere was thought to be the major removal pathway of this compound from the oceans (35, 72). More recently, however, it has been estimated that between 50 and 80% of the DMS produced by DMSP-degrading bacteria is degraded directly by other types of bacteria (58, 59), although the populations and metabolic pathways involved in the degradation of DMS are still poorly understood.

Coral-associated bacterial communities are known to be diverse and highly abundant (12, 30, 48, 49, 52). These dynamic communities exploit a number of habitats associated with corals, including mucus on coral surfaces (48), intracellular niches within coral tissues (3, 16, 45, 47, 52), spaces within coral skeletons (15, 51), and seawater surrounding corals (16, 61).

TABLE 1. Basal medium composition used for the isolation of DMSP-, DMS-, and acrylic acid-degrading bacteria

Solution	Component(s) (g/liter)[a]	
	MAMS medium	MASW medium
1	NaCl (25.0), $(NH_4)_2SO_4$ (1.0), $CaCl_2 \cdot 2H_2O$ (0.2)	NaCl (25.0), $MgSO_4 \cdot 7H_2O$ (2.44), KCl (0.6), $NaNO_3$ (1.0), $CaCl_2 \cdot 2H_2O$ (0.3)
2	$MgSO_4 \cdot 7H_2O$ (1.0), $FeSO_4 \cdot 7H_2O$ (0.002), $Na_2MoO_4 \cdot 2H_2O$ (0.002)	KH_2PO_4 (0.5)
3	KH_2PO_4 (3.6)	K_2HPO_4 (0.9)
4	K_2HPO_4 (4.6)	NH_4Cl (0.267)
5	SL10 tr metal solution*	SL10 tr metal solution*
6	Pfennig's vitamin solution†	Pfennig's vitamin solution†

[a] The MAMS medium was modified from that of Goodwin et al. (22). For both media, the solutions were autoclaved separately before they were combined. *, SL10 trace metal solution (74) (1 ml/liter). †, Pfennig's vitamin solution (47) (1 ml/liter).

Each of these habitats is believed to harbor different bacterial populations (4, 52). Despite high bacterial diversity, corals have been reported to harbor species-specific microbial communities for beneficial effects; however, their role in coral health is poorly understood (47–50). In coral reef environments, bacteria are dependent upon organic compounds produced by photoautotrophic organisms such as endosymbiotic zooxanthellae (48); therefore, photosynthates translocated to coral tissues and mucus may determine microbial communities closely associated with corals (48, 52). The high levels of DMSP and DMS produced by corals, coupled with the dependence of DMSP and DMS conversion on processes typically involving bacteria, suggest that corals are likely to harbor bacterial species involved in the cycling of these compounds. To investigate the potential of the organosulfur compound DMSP and its breakdown products, DMS and acrylic acid, to drive coral-associated microbial communities, we used these compounds as sole carbon sources to isolate bacteria from two coral species (*Montipora aequituberculata* and *Acropora millepora*) and then directly compared these microbial communities with coral-associated microbiota identified using culture-independent analyses. Genes implicated in the metabolism of DMSP were also characterized from isolated strains, confirming that bacteria associated with corals have the potential to metabolize organic sulfur compounds present in coral tissues.

MATERIALS AND METHODS

Sample collections. Three colonies of the corals *A. millepora* and *M. aequituberculata* were collected from Davies Reef, Great Barrier Reef, Australia (latitude, 18°50.9′S; longitude, 147°41′E) and maintained in aquaria at the Australian Institute of Marine Science (Townsville, Queensland, Australia). Five replicate samples of coral mucus were taken from each colony using sterile 50-ml syringes. Samples (1 ml) were used immediately for selective enrichment cultures, and the remaining volume was filtered through 0.22-μm-pore-size Sterivex filter columns (Durapore; Millipore), which were filled with 1.6 ml of lysis buffer (0.75 M of sucrose, 40 mM EDTA, 50 mM Tris-base; pH 8.3), and stored at −80°C for subsequent molecular analyses. Seawater samples (1 liter each) were also taken from the aquaria and processed in the same manner. Coral tissue slurry samples were obtained from five replicate coral fragments (25 mm in length, 60 to 70 polyps) from each colony and washed in autoclaved, 0.22-μm-pore-size-filtered artificial seawater (ASW) to remove loosely attached microbes. The samples were airbrushed (80 lb/in²) to a slurry with 5 ml of ASW to remove coral tissues and associated microbes. This slurry was homogenized to break down aggregates and divided into 1-ml aliquots and either stored at −80°C for DNA extraction or used immediately for selective enrichment cultures. All corals appeared healthy when the samples were collected.

Bacterial enrichment cultures and isolation of bacterial strains using DMSP, DMS, and acrylic acid as carbon sources. After air brushing, the remaining coral skeleton was crushed by using a sterilized pestle and mortar. Dilution series in

basal medium were performed for each of the five replicates of mucus, tissue slurry, and crushed skeleton samples for each species. Sterile 125-ml crimp-top vials were used for the dilution series, and the vials were inoculated with coral bacteria to a final volume of 10 ml. All vials were sealed by using sterile Teflon-coated butyl rubber septa. Two different basal media (lacking a carbon source) were tested for their ability to support the growth of coral bacteria: a modified marine ammonium mineral salt (MAMS) medium and a modified ASW media (MASW) (Table 1). The carbon sources used for enrichments were DMSP (50 μM), DMS (50 μM), and acrylic acid (50 μM). These carbon sources were added aseptically through the septa of crimp-top vials with a syringe and needle. Control bottles containing only the basal medium and the carbon source were set up, along with enrichment cultures, to account for the chemical breakdown of the carbon sources. Cultures were incubated at 28°C for 7 days, and the presence of DMS in the headspace gas was monitored by gas chromatography (GC) analysis. Enrichments were respiked daily with an additional dose (20 μM) of the carbon source to avoid the potential toxic effect of high dosage concentrations. The degradation of acrylic acid was assessed by nuclear magnetic resonance (NMR) analysis.

A 50-μl aliquot of each enrichment culture was spread onto the appropriate basal medium plate (MAMS or MASW) containing the appropriate carbon source (DMSP, DMS, or acrylic acid). DMS enrichment plates were kept in gas-tight jars, and 200 μM DMS was added to each jar. The jars were regularly vented and replenished with DMS (every 3 days). All enrichment plates were incubated at 28°C in the dark for 14 days. To isolate single strains capable of metabolizing the carbon source for growth, well-separated individual colonies were picked from isolation plates and resuspended in 10 ml of MAMS or MASW medium containing the appropriate carbon source (50 μM DMSP, 50 μM DMS, or 1 mM acrylic acid). Degradation of DMSP and DMS by the isolates was monitored by GC analysis, while the degradation of acrylic acid was assessed by NMR analysis. A 50-μl aliquot of each liquid culture was then inoculated back onto appropriate plates as described above to confirm the cultures formed colonies with a consistent morphological appearance. Again, a single colony was picked and grown in liquid culture as described above, and degradation of the carbon source was confirmed.

DNA extraction and purification. DNA was extracted from isolated single-strain liquid cultures by using a DNA extraction kit (Promega, Madison, WI), according to the manufacturer's instructions and resuspended in 30 μl of sterile Milli-Q water. DNA from the seawater and coral mucus samples was extracted according to the procedure of Schauer et al. (53), resuspended in 30 μl of Milli-Q water, and used directly for PCR amplification. DNA from the coral tissue samples was extracted according to a modified urea extraction protocol (4). Extracted DNA (30 μl) from coral tissues was purified to remove coextracted PCR-inhibitory humic and phenolic materials by passage through a 0.7% agarose gel, and DNA fragments larger than 2 kb were cut from the gel and purified by using a QIAquick gel extraction kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. All DNA was resuspended in 50 μl of sterile Milli-Q water, quantified with a Nanodrop spectrophotometer (Amersham Pharmacia Biotech, Uppsala, Sweden), and stored at −20°C until required.

PCR amplification of bacterial 16S rRNA genes. Primers 63F and 1387R, specifically targeting a 1,300-bp section of the genomic bacterial 16S rRNA gene (39), were used for PCR amplification of DNA. The PCRs included 2.5 μmol of each deoxyribonucleotide triphosphate and 5× PCR buffer containing $MgCl_2$, 10 pmol of each primer, and 0.25 μl of *Taq* DNA polymerase, adjusted to a final volume of 50 μl with sterile Milli-Q water. The reaction conditions were as

follows: 94°C for 3 min; followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min; and then a final extension of 72°C for 7 min. Amplified PCR products were visualized by electrophoresis on 1% agarose gel stained with ethidium bromide.

PCR amplification of DMSP degradation genes. Homologous amino acid sequences of DddD, DddR, and DddL, implicated previously in the degradation of DMSP (10, 66), were aligned by using CLUSTALW (http://www.ebi.ac.uk /clustalw/; MEGA package), and potential active sites within the protein sequences were used to localize the most conserved regions within the DNA sequence of the respective genes. Primers were manually designed within these conserved regions.

The primer pairs dddDf (5′-ACCAACGTCATTGCAGGACC-3′) and dddDr (5′-TGTGCGTGTTCTTCCGGTG-3′), dddRf (5′-GGCGCGCAGCCAGTTC AG-3′) and dddRr (5′-GGCTATGAGGAGGGCTGG-3′), and dddLf (5′-CTG GGAATACGGCTACGAGA-3′) and dddLr (5′-GTTCAAGATCAGCGATCC GG-3′) were used on DMSP-degrading isolates only. The PCR reagent concentrations and reaction conditions were identical to those described above except that an annealing temperature of 60°C was used. PCR products were visualized by electrophoresis on a 1% agarose gel stained with ethidium bromide and purified with a gel purification kit (Qiagen) according to the manufacturer's instructions. The DNA was dried in a vacuum centrifuge (Savant DNA 120) and sequenced (Macrogen, Inc., Seoul, Korea).

Clone library construction and sequencing. PCR products from seawater, coral mucus, and coral tissue samples were purified by using a QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions. Purified DNA was ligated into a TOPO-TA cloning vector (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, and ligated vectors and inserts were sent to the Australian Genome Research Facility for clone library construction and sequencing. A total of 96 clones were sequenced from each library with inserts of the wrong size or chimeric sequences removed from subsequent analysis.

GC analysis. Determination of DMS in the vials was performed by injecting 50 μl of headspace gas into a GC-ECD gas chromatograph (HP GC-500; Hewlett-Packard, Palo Alto, CA) fitted with a 1-m-by-4 mm glass column containing Poropack-Q and using nitrogen as the carrier gas (flow rate, 30 ml/min) at 200°C. A flame ionization detector was used to detect the compound. A DMS standard (Fluka, Evry, France) was used to confirm retention times.

NMR analysis. High-pressure liquid chromatography-grade methanol (20 ml) was added to each vial containing the acrylic acid enrichments and subsequently transferred to a glass round-bottom flask. The mixtures were dried in vacuo using a Buchi rotary evaporator. Deuterated methanol (CD_3OD, 800 μl) and formic acid (1 μl) were added to each flask, and the dissolved extracts were transferred to NMR tubes. ^1H NMR spectra were recorded on a Bruker Avance 600 MHz NMR spectrometer with a cryoprobe. NMR spectra were referenced to the residual ^1H resonance in the deuterated solvent and were recorded using standard Bruker pulse sequences.

Data analysis. All sequence data were edited by using the Sequencher program (Gene Codes Corp.) for removal of the vector and primer sequences and assembled into a single file for BLAST search comparisons (http://www.ncbi.nlm .nih.gov/) to determine the closest sequence affiliation to reference organisms or previously sequenced 16S rRNA genes. Chimeric sequences (checked with the CHECK_CHIMERA software of the Ribosomal Database Project [37]) were removed from the analysis. Correspondence analysis was performed by using Statistica (StatSoft) software. This analysis was carried out at the genus level, based on a contingency table describing the abundance of the different bacterial genera retrieved in the clone libraries. Coverage values were calculated by the equation: $C = 1 − (n/N) \times 100$, where n is the number of unique clones, and N the total number of clones examined in the libraries. Simpson's (36) and Shannon-Weaver (55) diversity indices were generated by using DOTUR software (54).

Nucleotide sequence accession numbers. The nucleotide sequences obtained in the present study have been deposited in GenBank database (http://www.ncbi .nlm.nih.gov/) under accession numbers FJ463226 to FJ463252 (isolates), FJ462134 to FJ462141 (genes), and FJ809043 to FJ809713 (clone libraries).

RESULTS

Isolation of coral-associated bacteria involved in DMSP metabolism. From 14 single colonies picked from enrichment plates, 10 bacterial strains belonging to six different genera were isolated from coral mucus, tissue, and skeleton samples of *A. millepora* and *M. aequituberculata* and demonstrated to degrade DMSP. Some bacteria were isolated more than once and were retrieved in enrichments inoculated from different coral derived samples (e.g., mucus, tissue, and/or skeleton). Of two basal media formulations (MAMS and MASW), only enrichments grown on MAMS demonstrated the ability to degrade DMSP, as confirmed by DMS formation detected in the culture headspace by GC analysis, typically 24 to 48 h after culture inoculation. After 2 weeks, no DMS formation was observed in the headspace gas of the MASW enrichment cultures or in control uninoculated enrichments, and hence these samples were not analyzed further.

The majority of isolates with the ability to degrade DMSP belonged to the *Gammaproteobacteria* class as determined by 16S rRNA gene sequence affiliation, and included *Alteromonas*-, *Arhodomonas*-, *Idiomarina*-, *Pseudomonas*-, and *Spongiobacter*-related organisms (Table 2). Organisms related to *Roseobacter* (*Alphaproteobacteria*) were also isolated. *Arhodomonas* and *Spongiobacter* isolates were obtained from *A. millepora* mucus and tissue samples, respectively, while the *Idiomarina*-related isolates were obtained from both the tissue and the skeleton enrichments of *A. millepora* (Table 2). *Pseudomonas* and *Roseobacter* isolates, in contrast, were only obtained from *M. aequituberculata* coral tissue enrichments. The final isolates, related to *Alteromonas* spp., were recovered from *A. millepora* mucus and skeleton samples and *M. aequituberculata* tissue samples (Table 2). No difference was observed in the DMS production rate between the different isolates. *Arhodomonas*-, *Pseudomonas*-, and *Roseobacter*-related species harbored the *dddD* gene (or *dddR*, its regulator) previously implicated in the degradation of DMSP in *Marinomonas* sp. (66) (Table 2). Both *Pseudomonas*- and *Roseobacter*-related isolates possessed both genes, whereas only the *dddR* gene was detected in *Arhodomonas*-related species. *Pseudomonas*-related species also possessed the *dddL* gene, encoding for a DMSP-lyase enzyme (10). Target genes could not be amplified from *Spongiobacter*-, *Alteromonas*-, and *Idiomarina*-related organisms.

Isolation of coral-associated bacteria involved in DMS metabolism. In contrast to enrichments grown on DMSP, only samples inoculated into MASW medium were able to completely deplete the headspace gas of DMS, generally within 3 to 4 days after inoculation. Samples inoculated into the other medium did not degrade DMS (even after 2 weeks) and were not analyzed further. From 14 colonies picked from enrichment plates, 11 bacterial strains from six different genera demonstrated the ability to degrade DMS. All belonged to the *Gammaproteobacteria* class, and they included *Alteromonas*-, *Idiomarina*-, *Photobacterium*-, *Pseudoalteromonas*-, and *Vibrio*-related organisms (Table 2). These isolates have not previously been implicated in the degradation of DMS, and therefore the present study broadens the identity of taxonomic microbial species able to metabolize this organic sulfur compound. *Vibrio* and *Alteromonas* isolates were retrieved from tissue and mucus samples derived from both *A. millepora* and *M. aequituberculata* corals. Other isolated strains, such as *Pseudoalteromonas* and *Idiomarina* sp., were derived only from *A. millepora* samples, whereas the *Photobacterium* sp. was enriched only from *M. aequituberculata* coral tissues (Table 2). No difference was

TABLE 2. Bacteria isolated from DMSP, DMS, and acrylic acid enrichment cultures of mucus, tissue, and skeletal samples of the corals, *A. millepora* and *M. aequituberculata*, and PCR detection of genes homologous to *dddR* and *dddD* previously implicated in DMSP degradation

Enrichment[a]	Closest taxonomically related strain(s)[b]	Strain source[c]	Taxonomic description	Alignment (bp)	Sequence identity (%)	Gene(s) involved in DMSP degradation
DMSP	*Arhodomonas* sp. strain EL-201	*Acropora* mucus	*Gammaproteobacteria*	682/699	97	*dddR*
	Spongiobacter nickelotolerans	*Acropora* tissues	*Gammaproteobacteria*	667/699	95	Other pathway
	Pseudomonas sp. strain CJ11075	*Montipora* tissues	*Gammaproteobacteria*	666/670	99	*dddR, dddD, dddL*
	Idiomarina sp. strain PR53-12	*Acropora* tissues and skeleton	*Gammaproteobacteria*	666/670	99	Other pathway
	Alteromonas sp. strain S1613	*Acropora* mucus and skeleton, *Montipora* tissues	*Gammaproteobacteria*	700/700	99–100	Other pathway
	Roseobacter sp. strain SOEmb11	*Acropora* and *Montipora* tissues	*Alphaproteobacteria*	685/700	98	*dddR, dddD*
DMS	*Alteromonas* sp. strain S1613 and CF6-3	*Acropora* mucus and skeleton, *Montipora* mucus	*Gammaproteobacteria*	698/699	99–100	
	Pseudoalteromonas ruthenica	*Acropora* tissues and skeleton	*Gammaproteobacteria*	695/700	99	
	Vibrio tubiashi	*Acropora* tissues	*Gammaproteobacteria*	699/700	99	
	Vibrio sp. strains 6G8 and 1G4	*Montipora* tissues, *Acropora* tissues and skeleton	*Gammaproteobacteria*	700/700	100	
	Photobacterium sp. strain 3F8	*Montipora* tissues	*Gammaproteobacteria*	698/699	98	
	Idiomarina sp. strain JL110-118	*Acropora* skeleton	*Gammaproteobacteria*	700/700	100	
Acrylic acid	*Vibrio harveyi*	*Acropora* mucus, *Acropora* tissues	*Gammaproteobacteria*	700/700	100	
	Vibrio fischeri	*Acropora* tissues	*Gammaproteobacteria*	700/700	100	
	Vibrio fortis	*Acropora* mucus, *Acropora* tissues	*Gammaproteobacteria*	641/642	89–98	
	Photobacterium sp. strain 3F8	*Acropora* tissues	*Gammaproteobacteria*	694/695	99	
	Halomonas sp. strain s2151	*Acropora* tissues	*Gammaproteobacteria*	644/667	96	
	Shewanella piezotolerans	*Montipora* tissues	*Gammaproteobacteria*	676/698	96	

[a] All enrichments were performed on MAMS medium (see Table 1).
[b] Sequences were aligned to the closest relative by using BLAST (1). The similarity was calculated without gaps taken into account.
[c] That is, the material(s) from which the bacteria were isolated.

observed in the DMS degradation rate between the different isolates.

Bacterial 16S rRNA gene clone libraries. Comparison among bacterial 16S rRNA clone libraries constructed from mucus and tissue samples of *A. millepora* and *M. aequituberculata* revealed species-specific differences. The bacterial diversities of libraries from two independent *A. millepora* tissue samples were similar and were dominated by the class *Gammaproteobacteria*, representing 86 and 88% of the total number of affiliated sequences (Fig. 1b). Similarly, libraries constructed from *M. aequituberculata* tissues were highly consistent although, in contrast to the *A. millepora* tissue libraries, the most abundant class of bacteria was *Alphaproteobacteria* (Fig. 1a), which represented 62 and 57% of sample 1 and 2 tissues, respectively. Clones derived from mucus samples of both *A. millepora* and *M. aequituberculata* displayed a higher proportion of sequences that were affiliated with unidentified or unclassified bacterial groups (Fig. 1c and d); unidentified groups represented 22 and 43%, respectively, of sequences within these libraries. A control library derived from seawater in the coral holding tanks similarly contained a high proportion (53%) of clones that were affiliated with unidentified or unclassified 16S rRNA gene sequences (Fig. 1e).

Interestingly, diversity indices based on retrieved DNA sequences differed between the *M. aequituberculata* and *A. millepora* coral tissue libraries. *M. aequituberculata* tissue (sample 1) and *A. millepora* mucus libraries possessed the highest bacterial diversity, with 58 operational taxonomic units (OTU)

grouped over the 91 clones sequenced (Table 3). The libraries for *M. aequituberculata* mucus, tissue (sample 2), and seawater also had a high bacterial diversity. In contrast, both *A. millepora* tissue libraries (samples 1 and 2) demonstrated a smaller number of OTU groups. The Shannon-Weaver (55) and Simpson's (36) indices confirmed the difference in diversity between samples (Table 3). The estimated percentage of diversity covered by each library was relatively low, ranging from 33% for *A. millepora* mucus to 38% for the *M. aequituberculata* mucus library (Table 3). Both *A. millepora* tissues libraries had higher coverage estimates of 68 and 66% for samples 1 and 2, respectively.

Comparison of retrieved isolates and retrieved clone library sequences. All bacterial isolates that demonstrated the ability to metabolize DMSP also demonstrated 16S rRNA gene sequence affiliation with corresponding sequences within the clone libraries. For example, Spongiobacter- and *Roseobacter*-related organisms represented a large fraction of the clone sequences retrieved from the libraries. *Spongiobacter*-affiliated sequences accounted for 59% of the *A. millepora* tissues libraries and *Roseobacter* accounted for 28% of the *M. aequituberculata* tissues, 15% of the *M. aequituberculata* mucus, and 10% of the *A. millepora* mucus libraries. 16S rRNA gene sequences linked to other bacterial isolates represented between 1 and 4% of the retrieved clone library sequences (Fig. 1).

Similarly, all isolates that possessed the ability to metabolize DMS were also represented within the clone libraries (Fig. 1). Of particular interest were *Vibrio* isolates, which represented
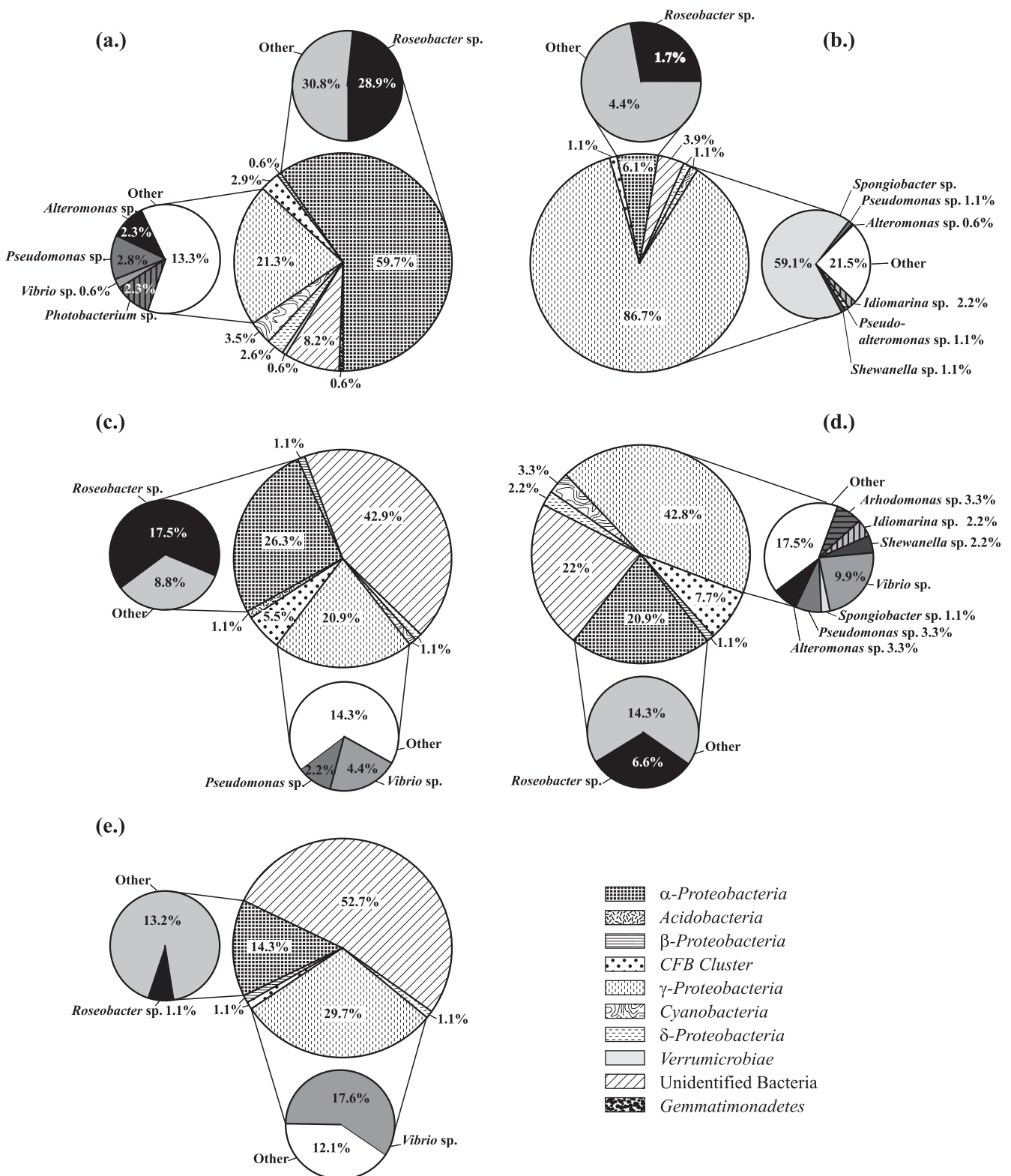
FIG. 1. Composition of the coral clone libraries. (a) *M. aequituberculata* tissues (181 clones); (b) *A. millepora* tissues (172 clones); (c) *M. aequituberculata* mucus (91 clones); (d) *A. millepora* mucus (91 clones); (e) seawater libraries (91 clones). The large pie charts represent the contents of the libraries at the class level. The smaller pies represent the percentage proportions of the different isolates (at the genus level). Replicate tissue libraries were highly similar, and therefore clone sequence data were pooled for these samples.

TABLE 3. Number of OTU and coverage estimates for clone libraries constructed for mucus and tissue samples of *A. millepora* and *M. aequituberculata* and for seawater

Clone library	No. of OTU	% of coverage estimated	Shannon-Weaver index	Simpson's index
A. millepora mucus	58	33	3.8	0.02
A. millepora tissues (sample 1)	21	68	2.19	0.165
A. millepora tissues (sample 2)	23	66	2.33	0.142
M. aequituberculata mucus	51	38	3.4	0.054
M. aequituberculata tissues (sample 1)	58	34	3.79	0.022
M. aequituberculata tissues (sample 2)	52	36	3.64	0.027
Seawater	53	35	3.62	0.028

10 and 4% of the *A. millepora* mucus and *M. aequituberculata* tissue libraries, respectively. Moreover, it is interesting that *Vibrio*-related organisms were dominant in the seawater samples, comprising 12% of DNA sequences retrieved from the library and represented the majority of sulfur-degrading bacteria reported in this library (Fig. 1e).

A correspondence analysis was performed at the genus level on the bacteria present in the clone libraries that were able to metabolize DMSP and DMS. The results confirmed the differences in bacterial communities observed between libraries at the class and genus level (Fig. 2). The bacteria able to degrade DMSP and DMS in *A. millepora* tissue libraries belonged almost exclusively to the genus *Spongiobacter*, whereas the sulfur-degrading communities were more diverse in the other coral libraries (Fig. 2). Indeed, the tissues and mucus of *M. aequituberculata* harbored predominantly *Roseobacter*, *Pseudomonas*, and *Alteromonas* spp., while the mucus of *A. millepora* was dominated by *Alteromonas* and *Shewanella* spp. and, to a lesser extent, by *Idiomarina* and *Pseudomonas* spp. In contrast, the seawater library was dominated by *Vibrio*-related sequences.

Acrylic acid. NMR analyses revealed that only enrichment cultures growing with MASW as the growth medium demonstrated degradation of acrylic acid. Interestingly, enrichment cultures derived from *A. millepora* mucus and tissues metabolized acrylic acid twice as fast as cultures derived from *M. aequituberculata*. A total of six isolates were recovered from coral enrichment cultures, and all of them belonged to the *Gammaproteobacteria* class. The isolates included organisms related to *Photobacterium*, *Halomonas*, and *Shewanella* species, as well as different vibrios, including *V. fortis*, *V. harveyi*, and *V. fischeri* (Table 2). All isolates showed rapid metabolism of acrylic acid, such that it was undetectable in NMR spectra 7 days after inoculation (Fig. 3). All isolates showed phylogenetic affiliation to sequences recovered from the coral clone libraries, with members of the *Vibrio* genus in particular being a major component of the *A. millepora* (10%) and *M. aequituberculata* mucus (4%) libraries (Fig. 1).

DISCUSSION

Bacterial strains metabolizing DMSP. Bacteria capable of metabolizing DMSP have been widely studied (1, 10, 17, 23, 73); however, the present study is the first report of isolation of

bacteria derived from corals that have this metabolic potential. Although the *Alteromonas* (1), *Pseudomonas* (33), and *Roseobacter* (18, 33) genera have previously been demonstrated to degrade DMSP, the *Spongiobacter*, *Arhodomonas*, and *Idiomarina* strains isolated from coral have never previously been directly implicated in the degradation of this compound. Since corals harbor both diverse and unique microbial diversity (29, 49, 50, 52), the discovery of new DMSP-degrading species is not surprising. Previously, the genus *Spongiobacter* has only been reported in association with sponges (42), acidians (40), and acroporid corals (5). This genus represented more than half of the clones in the *A. millepora* tissue libraries and has previously been found to be dominant in healthy *A. millepora* located at Magnetic Island, where it represented 41% of the retrieved DNA sequences (5). Being such a dominant member of the coral-associated microbial community indicates that it might play an important role in coral health, although the nature of this role is unknown at present. The discovery that *Spongiobacter* isolates possess the ability to metabolize DMSP indicates that it may be a dominant component of the microbial-coral sulfur cycling loop in corals such as *A. millepora*. Previous studies have shown that microbial communities in bleached corals shift away from a dominant *Spongiobacteria* community at the onset of bleaching but subsequently return to a *Spongiobacteria*-dominated profile as corals recover and regain their symbiotic dinoflagellates (5). Since DMSP production in corals is dependent on the dinoflagellate partner, loss of *Spongiobacter* from microbial profiles could be linked to the loss of dinoflagellates in bleached corals and consequent reductions in DMSP concentrations.

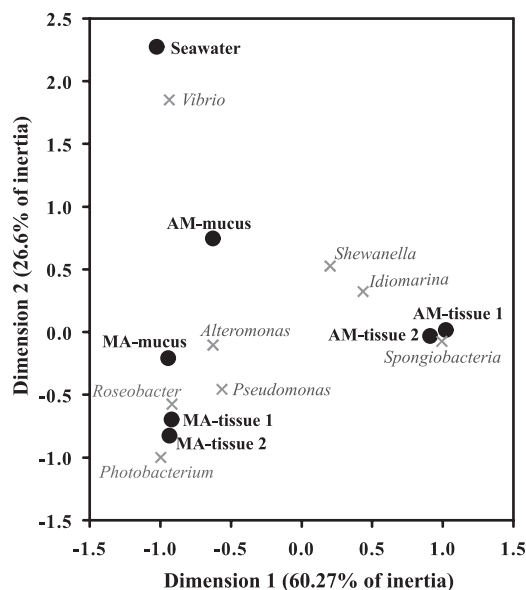*Roseobacter*-related organisms were isolated from both coral

FIG. 2. Correspondence analysis plot showing the relationships between clone libraries from mucus, tissue and skeletal samples of the corals, *A. millepora* and *M. aequituberculata*, and the main sulfur-degrading bacterial genera isolated in the present study. AM, *A. millepora* (tissue or mucus libraries); MA, *M. aequituberculata* (tissue or mucus libraries). Total inertia = 1.5586, chi² = 427.37, df = 42, P = 0.000.
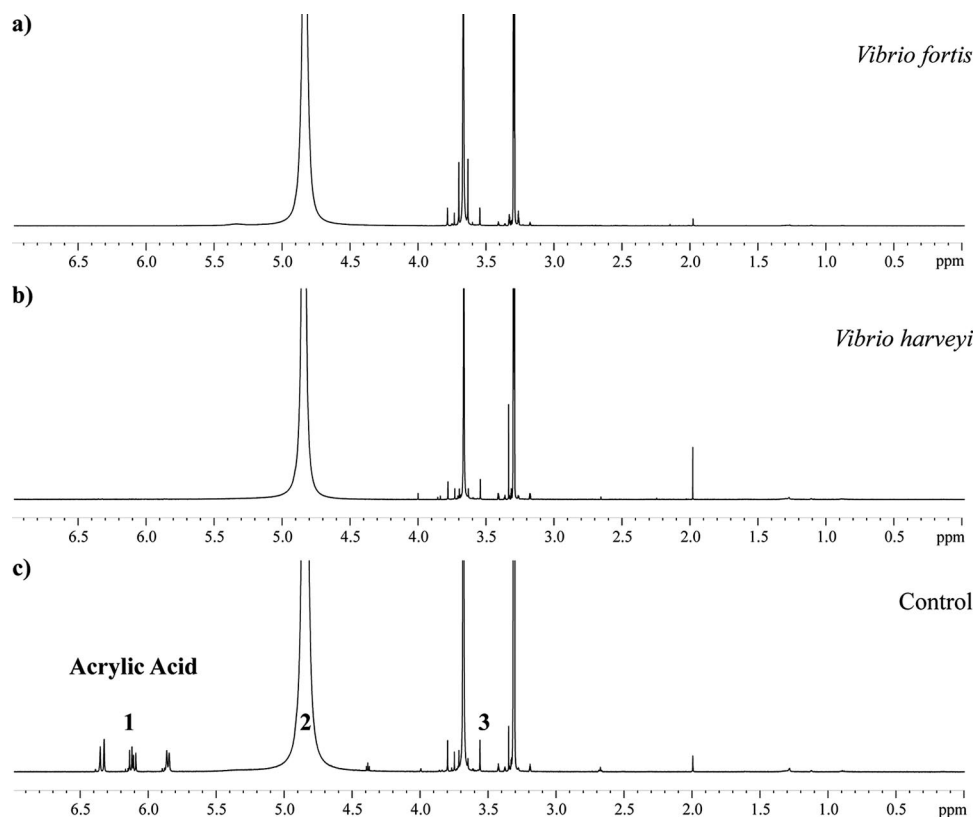
FIG. 3. ^{1}H NMR spectra of acrylic acid enrichment (50 μM) in MASW medium with *Vibrio fortis* (a) or *Vibrio harveyi* (b), both isolated from *A. millepora* tissues, and control (c) at 7 days after the acrylic acid inoculation. The three peaks of the acrylic acid signal (position 1) are only visible in the control; the other peaks represent the solvent (water) (position 2) and methanol (position 3).

species sampled in the present study and represented a large component (28%) of sequences retrieved from the clone library derived from *M. aequituberculata* tissues. The ability of this bacterial genus to degrade DMSP has been widely studied (18, 19, 32, 69), and it is known to dominate bacterioplankton communities in environments with high DMSP concentrations, such as phytoplankton blooms (20, 46) or polar waters (69). This genus has antibacterial activities against a wide range of fish and invertebrate pathogens (21) and, interestingly, one of the antibiotics produced by *Roseobacter*, thiotropocin, is a sulfur compound that might be derived from DMSP metabolism (70). Members of the *Roseobacter* genus are widely associated with corals (4, 5, 16, 30, 50) and suspected to be involved in a symbiotic relationship with coral-cultured zooxanthellae (K. Ritchie, unpublished data). Given its strong association with corals and the potential to produce antimicrobial compounds through the assimilation of DMSP, the *Roseobacter* genus is potentially central to the health of corals. Isolated DMSP-metabolizing *Pseudomonas* and *Alteromonas* strains are similarly often reported in coral diversity studies and associate with a variety of different coral species (4, 16, 30, 47).

Gene orthologues to *dddD*, *dddR*, and *dddL* genes, previously implicated in DMSP degradation in *Marinomonas* sp. strain MWYL1 (66) and *Sulfitobacter* sp. (*Alphaproteobacteria*) (10), were detected in half of the DMSP-degrading isolates recovered from corals in the present study and corroborate the degradation of DMSP observed by GC analysis. None of these

genes were detected in *Alteromonas*, *Idiomarina*, and *Spongiobacter* isolates. The absence of gene orthologues to *dddD*, *dddR*, and/or *dddL* in these bacteria may be due to a limitation of the designed primers to amplify their target sequences, or it might reflect that other DMSP degradation pathways may be present in these organisms. Indeed, orthologues to *dddD* and *dddL* have not been found in several other DMS-producing bacteria (10), suggesting that other key DMS-producing genes (such as the new gene, *dddP*, found in *Roseovarius* [65]) are yet to be discovered.

The present study specifically targeted the isolation of bacteria involved in this DMSP cleavage pathway, although not all of the DMSP-degrading bacteria possess this pathway (10, 22, 27, 66). The alternative demethylation/dethiolation pathway incorporates the derived sulfur into bacterial biomass without the associated formation of DMS (27). DMSP consumption cannot be assessed directly through GC analysis but must be measured indirectly through the detection of DMS production. Therefore, any isolates or enrichments that used the alternative demethylation/dethiolation pathway were not observed to form DMS and therefore were not examined further. Other techniques able to measure direct decreases in DMSP concentrations through time in enrichment bottles (rather than indirect measures of DMS production) are currently being investigated.

Bacterial strains metabolizing DMS. Despite the importance of DMS degradation mediated by bacteria (58), bacterial

populations and metabolic pathways involved in the degradation of this compound are still poorly understood. The dominant population of DMS-degrading organisms in the enrichments affiliated with members of the *Alteromonas*, *Idiomarina*, *Photobacterium*, *Pseudoalteromonas*, and *Vibrio* genera. The ability of these bacteria to degrade DMS has not been reported previously, emphasizing the need for further studies and a greater understanding of bacteria able to metabolize this compound. No gene involved in the cleavage of DMS has been identified to date; therefore, it is not possible to use genetic markers (such as *dddD*, *dddR*, or *dddL* genes for DMSP) to identify potential DMS-degrading bacteria. Since only a small percentage of marine bacteria have been grown on artificial culture media (14, 44), it is likely that only a small percentage of bacteria with the ability to metabolize DMS have been isolated.

Coral-associated microbial communities. Overlap between the bacterial diversity detected in coral clone libraries and bacteria implicated in organic sulfur metabolism highlights the potential importance of DMSP and DMS in structuring coral-associated bacterial communities. Differences between host metabolism and/or associated photosynthetic by-products exuded by corals may explain variations in the chemical compositions of both coral mucus and coral tissues (41, 48) and therefore contribute to the observed species-specific differences in dominant coral-bacterial associations. Correspondence analysis clearly differentiated the bacterial diversity associated with different coral species, indicating that sulfur-degrading communities might be species specific. For example, the *Spongiobacter* genus dominated sulfur-degrading genera in *A. millepora* tissue samples, whereas *Roseobacter* was the most abundant genus in *M. aequituberculata* tissue samples. In contrast, *Vibrio*-related organisms were present in the seawater sample. Coral-associated microbes also inhabit different niches within the coral (4, 52), and correspondence analysis also partitioned the libraries based on these different coral microenvironments (mucus versus tissue).

Previous studies have shown that consistent genera of bacteria are commonly associated with corals from geographically separated locations (34, 49), and some of these genera have the potential to metabolize DMSP and DMS. For example, the *Pseudomonas* and *Roseobacter* genera have been found in all coral species investigated to date (4, 5, 30, 31, 49, 50), providing further support that some bacterial populations implicated in DMSP/DMS degradation are conserved between coral species, and the availability of these organic sulfur compounds may drive the specificity of these coral-bacterium associations.

Acrylic acid, the forgotten story. DMSP and DMS have received much attention in geochemistry studies of global sulfur cycling, but acrylic acid, a breakdown product in some DMSP metabolism pathways, has not been investigated.

To date, only three studies have isolated bacteria involved in the metabolism of acrylic acid (2, 24, 71), highlighting a lack of knowledge in this area. The isolates derived from our coral samples were related to *Photobacterium*, *Halomonas*, and *Shewanella* spp., as well as to several *Vibrio* spp. Acrylic acid degradation has been recorded previously for *Halomonas* (24), and positive chemotactic responses to acrylic acid were observed for *Vibrio* (60). *Vibrio*-related organisms, known to be implicated in various coral diseases (64), were a major constit-

uent of our coral libraries, representing up to 10% of the clones in the *A. millepora* mucus. Similar results have been observed for the coral *Pocillopora damicornis*, with *Vibrio* species representing a proportion up to 38% of clone libraries, indicating that this group may constitute a natural part of healthy coral-associated microbial communities (4). The role of acrylic acid in the health corals is unknown and requires further study. The cleavage products of DMSP metabolism, DMS and acrylic acid, have both been reported to have antimicrobial properties (13, 56, 57, 63).

Conclusion. Microbial communities associated with corals have been shown to be highly diverse, and yet there is evidence of species specificity in some associations (47, 49, 50, 52). The factors that drive these species-specific associations are poorly understood, although the passage of nutrients between the holobiont's symbiotic partners, including both zooxanthellae and associated microbial communities, will be important. High concentrations of the organic sulfur compounds, DMSP and DMS, have previously been reported to be produced by dinoflagellates that are symbiotic with hard coral species, although the potential role of these compounds in structuring coral-associated microbial communities has not previously been investigated. The present study is the first to report the isolation of coral-associated bacteria that are capable of metabolizing DMSP and DMS organic sulfur compounds and, along with the study by Johnston et al. (24), one of the first to isolate acrylic acid-degrading bacteria in general. The findings presented here support the hypothesis that by-products of coral algal photosynthesis, which exude high concentrations of DMSP and DMS, can support bacteria capable of metabolizing DMSP, DMS, and acrylic acid. Four bacterial strains, including *Spongiobacter*, *Pseudomonas*, *Roseobacter*, and *Vibrio* spp., represented between 30 and 60% of the identified clones retrieved from the coral tissue and mucus libraries, and all were able to metabolize these sulfur compounds. Further studies are required to identify the full spectrum of bacteria able to degrade these compounds and to elucidate the genes and pathways involved in sulfur metabolism. The results of the present study confirm that a better understanding of coral-bacteria associations is a prerequisite for increasing our current knowledge of coral health.

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