

Diversity of the Bacterial Communities Associated with the Azooxanthellate Deep Water Octocorals *Leptogorgia minimata*, *Iciligorgia schrammi*, and *Swiftia exertia*

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Abstract

This study examined the microbiota associated with the marine azooxanthellate octocorals *Leptogorgia minimata*, *Swiftia exertia*, and *Iciligorgia schrammi* collected from moderate depths (45 m). Traditional aerobic plate culture, fluorescence in situ hybridization (FISH), and molecular identification of the 16S rDNA region were used for this purpose. In general, cultures were found to be selective for Gammaproteobacteria, Alphaproteobacteria, and Firmicutes. Interestingly, FISH counts for Firmicutes in the whole coral (holobiont) were near the detection limit of this assay, representing less than 6% of the total detectable microbiota in all counts. Proteobacteria, especially Alpha- and Gammaproteobacteria, made up the majority of the total microbiota in the holobionts. In addition, the absence of zooxanthellae in these three corals was confirmed by the use of polymerase chain reaction (PCR) and dinoflagellate-specific primers, and spectrophotometric chlorophyll pigment measurements. No evidence of zooxanthellae could be found in any of the corals by either of these techniques. This is the first study examining the microbiota marine octocorals, which grow at moderate depth (40 to 100 m) in the absence of direct sunlight.

Keywords: azooxanthellate octocoral — bacterial association — cultured bacterial isolates — FISH — 16S rRNA

Thomas B. Brück and Wolfram M. Brück have made equal contributions to this publication.

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Introduction

Sessile marine invertebrates, such as sponges, ascidians, and corals, are host to complex microbial assemblages including species-specific eukaryotic (fungi and dinoflagellates) and prokaryotic organisms (Santos et al. 2001; Knowlton and Rohwer 2003; Hill 2004; Gunasekera et al. 2005; Hentschel et al. 2006). Various combinations of culture-dependent and culture-independent techniques have been employed to describe marine invertebrate-microbe associations (Hentschel et al. 2003; Imhoff and Stöhr 2003). A comprehensive overview of the microbial diversity associated with marine invertebrates can be achieved by culture-independent methods, such as direct microbial counting using fluorescence in situ hybridization (FISH) and cloning of rRNA genes from total genomic DNA extracts (Webster and Hill 2001; Hentschel et al. 2003; Knowlton and Rohwer 2003; Lesser et al. 2004). On the other hand, the culture-dependent approach remains the method of choice to study the metabolic requirements and biochemical characteristics of isolated microbial strains associated with marine invertebrates (Unson et al. 1994; Gonzales et al. 1996; Faulkner et al. 1999; Schmidt et al. 2000; Fieseler et al. 2004; Lesser et al. 2004).

Screening the complexity of microbial communities not only leads to the definition of ecological networks (Santos et al. 2001; Hentschel et al. 2003; Knowlton and Rohwer 2003; Taylor et al. 2004; Fieseler et al. 2004; Harder et al. 2004); it is also a tool to uncover disease related processes (Cervino et al. 2004; Bourne and Munn 2005) and may even lead to the isolation of isolated strains that produce

commercially interesting biomolecules (Fenical 1993; Schmidt et al. 2000; Mincer et al. 2002, 2004; Piel et al. 2004). However, to define a particular heterotrophic microbe as the source of a bioactive natural product using techniques based on cell separation, it is often essential to culture invertebrate-associated microbes before the production of a particular compound can be unambiguously assigned (Faulkner et al. 1999; Webster and Hill 2001). This process in particular can be a very challenging task owing to the complexity of the microbial associations found in marine invertebrates and the limitations of current culturing methods (Hentschel et al. 2002, 2003).

It has been postulated that the biosynthesis of pseudopterosins, a class of diterpene glycosides with anti-inflammatory activity derived from the octocoral *Pseudoptergorgia elisabethae*, may be linked to the presence of a specific dinoflagellate symbiont (*Symbiodinium sp.*, Dinophyta) associated with the coral host (Mydlarz et al. 2003). As dinoflagellates themselves are host to a plethora of microbes (Ashton et al. 2003; Jasti et al. 2005), the source of these natural products remains elusive. Similarly, the lophotoxins, a group of diterpenes acting as potent neuromuscular toxins (Fenical et al. 1981; Abramson et al. 1988; Tornøe et al. 1995), have been isolated from various octocoral species of the genus *Leptogorgia sp.* (formerly *Lophogorgia sp.*), found in both the Atlantic and Pacific Ocean (Fenical et al. 1981; Epifanio et al. 2000). It is currently believed that the isolation of similar natural products from geographically distinct locations is a good indication that these compounds are produced by a microbe living in close association with a particular marine invertebrate (Hentschel et al. 2002). Interestingly, there is circumstantial evidence that *Leptogorgia* species do not harbor symbiotic dinoflagellates based on microscopic examination of these organisms (Bayer 1961). Therefore, the biosynthesis of diterpenes isolated from *Leptogorgia* species might be attributed to coral associated microbe(s) other than dinoflagellates. To test this hypothesis, however, requires a more detailed knowledge of the microbial assemblage associated with the host coral species under study.

As there is currently very limited information on the microbial communities associated with octocoral species, this study surveyed the predominant microbiota associated with West Atlantic octocorals (Bayer 1961) *Leptogorgia minimata* (LM, family Gorgoniidae), *Iciligorgia schrammi* (IS, family Anthothelidae), and *Swiftia exertia* (SE; family Plexauridae), which were collected at a depth of 45 m off the Florida coast. The dinoflagellate content of the selected coral species was examined using

dinoflagellate-specific primers and the spectrophotometric determination of the chlorophyll content.

To analyze the gross bacterial distribution in these three coral species, we used both culture-dependent and culture-independent techniques. In particular, we used FISH analysis with oligonucleotide probes specific to six of the most common marine bacterial genera to reveal the overall diversity of the coral associated microbiota (Sfanos et al. 2005; Penn et al. 2006; Wang 2006). We also constructed a strain collection of culturable bacterial isolates using diverse media, which encompassed a variety of growth conditions. The cultured bacterial strains were characterized using traditional microbiological techniques. Isolated bacterial strains were subsequently grouped and identified phylogenetically using restriction fragment length polymorphism (RFLP) pattern and 16S ribosomal DNA (rDNA) sequence analysis, respectively. This strain collection of cultured bacterial isolates will also allow us to examine the ability of individual bacterial species to produce natural products.

Materials and Methods

Materials. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO). DNA isolation DNeasy plant tissue kit and the Minelute gel elution kit were obtained from Qiagen (Valencia, CA). Polymerase chain reactions (PCR) (GoTaq Green Master Mix) and restriction enzymes were obtained from Promega (Madison, WI). Pre-formulated Nutrient Broth, Marine Broth 2216, YM Broth, Sabouraud Dextrose Broth, and their solid equivalents were supplied as powders and prepared according to the manufacturer's instructions (Difco Laboratories, Detroit, MI). DNA sequencing reagents were obtained from Applied Biosystems (Foster City, CA).

Organism Collection and Treatment. During collection and handling, all coral species were handled with latex gloves to avoid external bacterial and fungal contamination. Coral species were identified using gross morphology and by subsequent microscopic spicule analysis (Bayer 1961). LM, IS, and SE were collected by scuba in April 2005 at the Jim Atria wreck site off Pompano Beach, Florida at a depth of 45 m (27°C). All coral species were collected in cavities devoid of any direct sunlight. Samples of coral branches (approximately 10 to 12 g) taken for analysis were representative of the whole organism. Sterile seawater was prepared by filtering sea water

through 0.4- and 0.2- μm cartridge filters (Millipore, Maidstone, UK) and subsequent irradiation with UV light for 30 min (Breitbart et al. 2005).

In the laboratory, live coral species were placed in separate 10-l aquaria with sterile sea water (27°C) for 6 h under flow through conditions (4 l/h) to minimize the presence of marine bacterioplankton from the sea water column. About 10 g of each coral species was homogenized under sterile conditions for 3 min (25°C) in 100 ml of sterile phosphate-buffered saline (PBS, pH 7.2, Sigma) using a commercial Waring blender. This homogenate was then used to establish bacterial cultures on solid agar plates.

Isolation of Culturable Bacterial Symbionts.

Approximately 10 μl of each coral homogenate was spread out on each standard nutrient agar (NA), diluted marine agar 2216 (MA, 5 sea water:1 marine agar), YM, and Sabouraud dextrose agar (SB) plates, which were incubated aerobically at 27°C (Webster and Hill 2001). While MA targeted bacteria associated with the extracellular marine environment of the coral host, all other media mimic the possible physiological conditions inside living coral cells, which would allow growth of endosymbiotic bacteria. Bacterial growth was monitored for 4 weeks and individual colonies with unique morphotypes were serially streaked on their respective medium until pure cultures were obtained. Plates were retained for an additional 3 months to allow for the isolation of slow-growing bacteria. For long-term storage, purified bacterial clones were grown in 5 ml of either nutrient, marine, YM, or Sabouraud dextrose broth, and 1-ml aliquots containing 30% (vol/vol) glycerol were flash frozen with liquid nitrogen and stored at -80°C .

Gram Staining and Morphological Analysis of Cultured Bacterial Symbionts. Each purified bacterial strain was examined for its gross cell morphology and motility via light microscopic examination in combination with standard Gram staining procedures (Santavy et al. 1990; Murray et al. 1994). In addition, individual bacterial colonies isolated on solid agar media were described by morphology (Table 1). All isolated bacterial strains were deposited in the Harbor Branch Marine Microbial Culture Collection (http://www.hboi.edu/dbmr/dbmr_hbmmmd.html; Gunasekera et al. 2005).

Total DNA Extraction from Cultured Bacterial Clones and Coral Homogenate. Genomic DNA was extracted from isolated bacterial colonies on agar plates by touching the colony with a sterile needle that was

then placed in 10 μl of sterile distilled water. Genomic DNA from individual coral species was extracted from 100 μl of coral homogenate. As both coral homogenates and bacterial colonies showed the presence of small molecule contaminants, such as carotenoids and phenolics, that could interfere with the efficiency of DNA isolation and further enzymatic treatment, all genomic DNA extractions were performed with the DNeasy plant mini kit (Qiagen) according to the manufacturer's instructions. The isolated DNA was quantified and assessed for purity by measuring the absorbance at 260 and 280 nm and calculating the A_{260}/A_{280} ratio. The DNA was aliquoted at 50 ng/ μl and stored at -80°C until needed.

Amplification of dinoflagellate Internal Transcribed Spacer (ITS) Ribosomal DNA Region. To confirm that the octocorals LM, IS, and SE were devoid of symbiotic dinoflagellates (zooxanthellae, *Symbiodinium* sp., Dinophyta) a PCR-based approach was used with a dinoflagellate-specific primer set. The primer set ZITSUPM13 (5'-CAC GAC GTT GTA AAA CGA CCC GGT GAA TTA TTC GGA CTG ACG CAG TGCT-3') and ZITSDNM13 (5'-GGA TAA CAA TTT CAC ACA GGC TGT TTA GTT CCT TTT CCT CCG C-3') specifically amplified the ITS1 rDNA region of dinoflagellates, targeting the conserved regions between the 3' and 5' ends of the 18S and 28S-rRNA genes, respectively (Santos et al. 2001). As a positive control, we used DNA isolated from the dinoflagellate-containing coral *Pseudopterogorgia elisabethae* (Bayer 1961). All PCR reactions were performed on an ICycler (Bio-Rad, Hercules, CA). PCR conditions were as follows: 10 pmol of each primer, 12.5 μl of GoTaq Master Mix (Promega), and sterile H_2O to a final volume of 24 μl . The PCR mix was added to 1 μl (50 ng) of DNA template. PCR cycling conditions were as follows: initial denaturing period of 2 min at 94°C followed by 30 cycles of 94°C for 40 s, 53°C for 1 min, and 72°C for 1 min. A final extension of 30 min at 72°C was added. The presence of PCR amplicons was checked by electrophoresis in a 1% low melting agarose gel (LE Agarose, Fisher Scientific, Suwannee, GA) containing 0.001% ethidium bromide. PCR products were visualized via a Typhoon Fluorescence Imager (GE Life Sciences, Piscataway, NJ). The expected PCR product was approximately 750 bp.

Spectrophotometric Determination of Dinoflagellate Chlorophylls. The spectrophotometric quantification of the major dinoflagellate chlorophylls a and c_2 was used to determine the presence of dinoflagellates in

Table 1. Characterization of culturable coral microbiota

Isolate	GenBank no. of isolate	Cell morphology	Gram stain	Phylogenetic association	Closest GenBank Match	% Similarity	GenBank no. of closest relative
a. <i>Iciligorgia schrammi</i> (IS) isolates							
IS0105	DQ517183	Rods, single, pairs	+	Actinobacteria	<i>Streptomyces coelicolor</i>	100	DQ442496
IS0205	DQ517184	Cocci bacilli	+	Actinobacteria	Uncultured <i>Rhodococcus</i> sp. Clone	99	DQ066610
IS0305	DQ517185	Rods, single	-	Alphaproteobacteria	Bejerinckiacae bacterium J586	98	AY364592
IS0405	DQ517186	Rods, single	-	Alphaproteobacteria	<i>Pseudovibrio</i> sp. MKT84	98	AB112826
IS0505	DQ517187	Rods, single	-	Alphaproteobacteria	Uncultured bacterium PRD01b003B	92	AF289163
IS0605	DQ517188	Rods, single	-	Alphaproteobacteria	Uncultured marine bacterium Ch11.44	92	DQ071057
IS0705	DQ517189	Rods, single	-	Alphaproteobacteria	<i>Wolbachia</i> sp.	92	L02884
IS0805	DQ517190	Rods, single	-	Betaproteobacteria	<i>Ralstonia pickettii</i> strain 2000030635	99	AY268180
IS0905	DQ517191	Rods, single	-	Betaproteobacteria	Uncultured bacterium ZZ9C4	93	AY214174
IS1005	DQ517192	Rods, single	-	Gammaproteobacteria	<i>Pseudomonas</i> sp. HR 13	97	AY032725
IS1105	DQ517193	Rods, single	-	Gammaproteobacteria	<i>Shigella flexneri</i> 2a	96	AE005674
IS1205	DQ517194	Rods, single	-	Gammaproteobacteria	<i>Stenotrophomonas maltophilia</i>	94	AY445079
IS1305	DQ517195	Rods, single	-	Gammaproteobacteria	<i>Stenotrophomonas</i> sp.	99	AJ884482
IS1405	DQ517196	Rods, single	-	Gammaproteobacteria	<i>Stenotrophomonas</i> sp.	99	AJ884484
IS1505	DQ517197	Rods, single	-	Gammaproteobacteria	Uncultured bacterium clone 1982b-31	96	AY917978
IS1605	DQ517197	Rods, single	-	Gammaproteobacteria	Uncultured bacterium clone 1982b-31	99	AY917978
IS1805	DQ517198	Rods, single	+	Gammaproteobacteria	Uncultured bacterium clone TM15	100	AY838526
IS1905	DQ517199	Rods, single	+	Gammaproteobacteria	Uncultured gamma proteobacterium	96	AY590685
IS2005	DQ517200	Rods, single	+	Gammaproteobacteria	Uncultured gamma proteobacterium	93	AY627370
IS2105	DQ517201	Rods, single	-	Gammaproteobacteria	Unidentified bacterium Asp05	95	X95233
IS2205	DQ517202	Rods, single	-	Gammaproteobacteria	<i>Vibrio fischeri</i> ES114	96	EF187009
IS2305	DQ517203	Rods, single	-	Gammaproteobacteria	<i>Xanthomonas</i> group bacterium LA37	98	AF513452
IS2405	DQ517204	Rods, single, spores	+	Fimicutes	<i>Bacillus licheniformis</i>	100	CP000002
IS2505	DQ517205	Pleomorphic	-	Fimicutes	<i>Mycoplasma bovis</i> Donetta pMb	94	U02968
IS2605	DQ517206	Rods, single	+	Fimicutes	Uncultured bacterium clone LS8	98	AY559426
IS2705	DQ517207	Rods, single, spores	+	Firmicutes	<i>Bacillus algalicola</i> SB9	94	DQ001307
IS2805	DQ517208	Rods, single, spores	+	Firmicutes	<i>Bacillus</i> sp. MB-11	93	AF326360
IS2905	DQ517209	Rods, single, spores	+	Firmicutes	<i>Bacillus</i> sp. MB-11	98	AF326360

IS3005	DQ517209	Rods, single, spores	+	Firmicutes	<i>Bacillus</i> sp. PL-21	98	AF326368
IS3105	DQ517210	Rods, single	+	Firmicutes	Bacterium G100VIII	97	AJ786046
IS3205	DQ517211	Rods, single, spores	+	Firmicutes	<i>Desulfotomaculum kuznetsovii</i> 17	91	AY036904
IS3305	DQ517212	Rods, single	+	Firmicutes	<i>Phytoplasma</i> sp. ESFY	94	Y11933
IS3405	DQ517213	Cocci	+	Firmicutes	<i>Streptococcus</i> sp. LRC 0255	98	AF298198
IS3505	DQ517214	Rods, single, spores	+	Firmicutes	Uncultured <i>Clostridium</i> sp. MRE50b26	92	AY684082
b. <i>Leptogorgia minimata</i> (LM) isolates							
LM0105	DQ517215	Short rods	-	Gammaproteobacteria	<i>Acinetobacter junii</i> strain T3943D1	99	DQ298039
LM0205	DQ517216	Short rods	-	Gammaproteobacteria	<i>Acinetobacter</i> sp. 2002-2301217	99	AY244764
LM0305	DQ517217	Cocci	+	Actinobacteria	<i>Dietzia</i> sp. A14101	99	AY831396
LM0405	DQ517218	Cocci, pairs, clusters	+	Actinobacteria	<i>Micrococcus</i> sp. MSB2038	99	AY275512
LM0505	DQ517219	Rods, single	-	Alphaproteobacteria	<i>Sphingobacterium multivorum</i>	98	AB100739
LM0605	DQ517220	Rods, single	-	Betaproteobacteria	<i>Achromobacter xylosoxidans</i>	93	AJ560626
LM0705	DQ517221	Rods, single	-	Betaproteobacteria	<i>Burkholderia</i> sp. FL44	100	DQ091242
LM0805	DQ517222	Rods, single	-	Betaproteobacteria	<i>Ralstonia pickettii</i> strain 2000030635	99	AY268180
LM0905	DQ517223	Rods, single	-	Gammaproteobacteria	<i>Klebsiella milletis</i>	99	AY217656
LM1005	DQ517224	Rods, single	-	Gammaproteobacteria	<i>Klebsiella pneumoniae</i> isolate 1053	99	AY292866
LM1105	DQ517225	Rods, single	-	Gammaproteobacteria	<i>Klebsiella</i> sp. HPC61	99	AY996975
LM1205	DQ517226	Rods, single	-	Gammaproteobacteria	<i>Pseudalteromonas</i> sp. F46p0	100	AY451334
LM1305	DQ517227	Rods, single	-	Gammaproteobacteria	<i>Pseudomonas citronellolis</i>	97	AY559113
LM1405	DQ517228	Rods, single	-	Gammaproteobacteria	<i>Pseudomonas oryzihabitans</i>	99	AF501336
LM1505	DQ517229	Rods, single	-	Gammaproteobacteria	<i>Pseudomonas putida</i>	93	DQ229317
LM1605	DQ517230	Rods, single	-	Gammaproteobacteria	<i>Pseudomonas</i> sp. WAI-21	98	AY520572
LM1705	DQ517231	Rods, single	-	Gammaproteobacteria	<i>Stenotrophomonas maltophilia</i>	99	AJ011332
LM1805	DQ517232	Rods, single	-	Gammaproteobacteria	<i>Stenotrophomonas</i> sp.	99	AJ884480
LM1905	DQ517233	Rods, single	-	Gammaproteobacteria	<i>Stenotrophomonas</i> sp. 3A3C	99	AF368754
LM2005	DQ517234	Rods, single	-	Gammaproteobacteria	Uncultured gamma proteobacterium	99	AY580854
LM2105	DQ517235	Rods, single	-	Gammaproteobacteria	<i>Vibrio</i> sp. YBM23	90	AY770832
LM2205	DQ517236	Rods, single	-	Gammaproteobacteria	Uncultured bacterium clone Lan-75	95	AY741389
LM2305	DQ517237	short rods	-	Gammaproteobacteria	<i>Acinetobacter baumannii</i>	99	AY738399
LM2405	DQ517238	Rods, single	-	Gammaproteobacteria	Bacterium PT17	99	DQ136062
LM2505	DQ517239	Rods, single	-	Gammaproteobacteria	Gammaproteobacterium 10c-1	99	AY561547
LM2605	DQ517240	Rods, single	-	Gammaproteobacteria	<i>Pectobacterium atrosepticum</i> 9204	95	AF373181
LM2705	DQ517241	Rods, single	-	Gammaproteobacteria	<i>Pseudomonas aeruginosa</i>	95	AY771716
LM2805	DQ517242	Rods, single	-	Gammaproteobacteria	<i>Pseudomonas pachastrellae</i>	91	AB125367
LM2905	DQ517243	Rods, single	-	Gammaproteobacteria	<i>Pseudomonas pseudoalcaligenes</i>	99	AY880303
LM3005	DQ517244	Rods, single	-	Gammaproteobacteria	<i>Pseudomonas</i> sp. 679-2	95	AF005994
LM3105	DQ517245	Rods, single	-	Gammaproteobacteria	<i>Pseudomonas</i> sp. HR 13	98	AY032725
LM3205	DQ517261	Rods, single	-	Gammaproteobacteria	<i>Pseudomonas</i> sp. NPO-JL-67	98	AY745835
LM3305	DQ517246	Rods, single	-	Gammaproteobacteria	<i>Pseudomonas</i> sp. ps10-19	94	AY303259
LM3405	DQ517247	Rods, single	-	Gammaproteobacteria	<i>Psychrobacter alimentarius</i>	97	AY771725
LM3505	DQ517248	Rods, single	-	Gammaproteobacteria	<i>Psychrobacter celer</i>	99	AY842259
LM3605	DQ517249	Rods, single	-	Gammaproteobacteria	<i>Curcacobacter baltica</i> OS140	92	AJ002006
LM3705	DQ517250	Rods, single	-	Gammaproteobacteria	<i>Shewanella</i> algae	94	AF005250

Table 1. Continued

Isolate	GenBank no. of isolate	Cell morphology	Gram stain	Phylogenetic association	Closest GenBank Match	% Similarity	GenBank no. of closest relative
LM3805	DQ517251	Rods, single	-	Gammaproteobacteria	Uncultured bacterium clone D1-78	99	DQ113756
LM3905	DQ517252	Rods, single	-	Gammaproteobacteria	Uncultured bacterium clone D1-78 1	95	DQ113756
LM4005	DQ517253	Rods, single	-	Gammaproteobacteria	<i>Vibrio</i> sp. V615	99	DQ146987
LM4105	DQ517254	Rods, single	-	Deltaproteobacteria	<i>Desulfobrio desulfuricans</i> G20	96	AF000112
LM4205	DQ517255	Rods, single	+	Epsilonproteobacteria	Uncultured epsilon proteobacterium BD1-29	96	AB015529
LM4305	DQ517256	Rods, single	+	Firmicutes	<i>Listeria monocytogenes</i> str. 4b	100	AE017328
LM4405	DQ517257	Cocci, pairs, clusters	+	Firmicutes	<i>Staphylococcus pasteurii</i>	97	AJ717376
LM4505	DQ517258	Rods, single, spores	+	Firmicutes	<i>Bacillus pumilus</i> strain JH7	90	DQ232736
LM4605	DQ517259	Rods, single	+	Firmicutes	Bacterium M21-1	100	DQ235557
LM4705	DQ517260	Cocci, pairs, clusters	+	Firmicutes	<i>Staphylococcus</i> sp. R-23212	98	AJ969171
c. <i>Swifftia exertia</i> (SE) isolates							
SE0205	DQ517262	Short rods	-	Gammaproteobacteria	<i>Acinetobacter</i> sp. SYSN-1-1	94	DQ234063
SE0305	DQ517263	Cocci single, pairs	-	Gammaproteobacteria	<i>Acinetobacter tjernbergiae</i>	97	AF509826
SE0405	DQ517264	Short rods	+	Actinobacteria	<i>Arthrobacter</i> sp. S/4	91	AJ785568
SE0505	DQ517265	Rods, single	+	Alphaproteobacteria	Alphaproteobacterium CRA 15M	98	AY562566
SE0605	DQ517266	Rods, single	-	Alphaproteobacteria	Alphaproteobacterium MBIC3368	99	AF218241
SE0705	DQ517267	Rods, single	-	Alphaproteobacteria	<i>Sphingomonas</i> sp. CanClear1	98	AF395038
SE0805	DQ517268	Short rods	-	Betaproteobacteria	<i>Ralstonia pickettii</i> strain ATCC 27511	95	AY741342
SE0905	DQ517269	Rods, single	-	Gammaproteobacteria	<i>Acinetobacter johnsonii</i> strain CAI-27	98	DQ257430
SE1005	DQ517270	Rods, single	-	Gammaproteobacteria	<i>Pseudomonas aeruginosa</i>	96	AY771716
SE1105	DQ517271	Rods, single	-	Gammaproteobacteria	<i>Stenotrophomonas rhizophila</i>	94	AJ293462
SE1205	DQ517272	Rods, single	-	Gammaproteobacteria	<i>Stenotrophomonas</i> sp.	99	AJ884482
SE1305	DQ517273	Rods, single	-	Gammaproteobacteria	Uncultured bacterium clone OB3_172	94	AY133407
SE1405	DQ517274	Rods, single	-	Gammaproteobacteria	Uncultured bacterium clone TM15	94	AY838526
SE1505	DQ517275	Rods, single	-	Gammaproteobacteria	Uncultured bacterium clone TM15	99	AY838526
SE1605	DQ517275	Rods, single, spores	+	Gammaproteobacteria	<i>Xanthomonas</i> group bacterium LA37	99	AF513452
SE1705	DQ517276	Rods, single, spores	+	Firmicutes	<i>Bacillus</i> sp. MB-11	95	AF326360
SE1805	DQ517277	Rods, single, spores	+	Firmicutes	<i>Bacillus</i> sp. MB-11	99	AF326360
SE1905	DQ517277	Rods, single	+	Firmicutes	<i>Bacillus</i> sp. TA57	91	AY839813
SE2005	DQ517278	Rods, single	+	Firmicutes	Uncultured bacterium clone BT49MF4BA7	91	AF365547
SE2105	DQ517279	Rods, single	+	Firmicutes	Uncultured bacterium clone CD1E3	90	AY038540
SE2205	DQ517280	Bacilli	+	Firmicutes	Uncultured bacterium clone LS8	91	AY559426

each of the investigated coral species (Ritchie 2006). As a positive control, we used the dinoflagellate-containing coral *Pseudopterogorgia elisabethae*. Flash frozen coral tissue (0.5 g) was ground to a fine powder using motor and pestle under sequential addition of 50 ml of liquid nitrogen. Each tissue powder was re-suspended (10 ml) in a 1:1:1 mixture of acetone (90% vol/vol), methanol (100% vol/vol), and ethanol (99.5% vol/vol) to extract pigments. The slurry was neutralized with magnesium carbonate to prevent formation of chlorophyll allomerization products, which are spectrally distinct from native chlorophyll species. The tissue slurry was vortex-mixed vigorously for 3 min and then clarified by centrifugation at 2000 g for 30 min. Supernatants of each pigment extract were decanted and adjusted to a final volume of 12 ml. To avoid photobleaching, all pigment extracts were immediately processed in the dark. Each pigment extract (50 µl) was diluted 1:20 with acetone (90% vol/vol) to make 1 ml of assay solution. Absorbance spectra of each assay solution were recorded in triplicate between 400 and 750 nm (1 nm sampling intervals) using a HP8453 diode array spectrophotometer (Agilent Technologies, Santa Clara, CA). All of the spectrophotometric measurements were carried out in 1-ml quartz cuvettes (1-cm pathlength). To determine the concentration of the *Symbiodinium* chlorophyll species, Eq. 1 (Ritchie 2006) and absorbance values at 664 and 630 nm, representing the Soret absorption peaks (Q_y) of chlorophyll a and c_2 in acetone (90% vol/vol) respectively, were employed.

$$\begin{aligned} \text{Chlorophyll a } (\mu\text{g/ml}) &= -0.457A_{630} + 11.475A_{664} \\ \text{Chlorophyll } c_2 (\mu\text{g/ml}) &= 23.39A_{630} - 3.532A_{664} \end{aligned}$$

(1)

where A is the absorbance of the pigment extract at wavelength (λ) nm.

Amplification of Bacterial 16S Ribosomal RNA (rRNA)

Genes and Phylogenetic Analysis. Eubacterial universal primers FC27 (EB-R; 5'-AGAGTTTGATC CTGGCTCAG-3') and RC1492 (5'-TACGGCTAC CTTGTTACGACTT-3') were used to amplify the 16S rRNA gene sequence from bacterial isolated genomic DNA (McVeigh et al. 1996; Mincer et al. 2004). All PCR reactions were prepared as described in the preceding text. PCR cycling conditions were as follows: initial denaturing at 95°C for 5 min, 35 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 1.5 min. A final extension of 7 min at 72°C was added. Each PCR product was checked by gel electrophoresis. Expected PCR amplicons of the

correct size (approximately 1.5 kbp) were manually excised and gel purified using the Minelute gel extraction kit (Qiagen). The PCR products were screened and grouped by RFLP analysis employing *Hha*I restriction patterns. The PCR products of at least two cultures from each RFLP group and those cultures with unique morphotypes were directly sequenced using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), on an ABI 3100 automated sequencer (Applied Biosystems) using primers FC27 and RC1492. For the identification of cultured bacteria, the obtained 16S rRNA gene sequences were viewed in Chromas (Technelysium, Tewantin, Australia), edited in EditSeq (Lasergene 6.0, DNASTAR, Madison, WI), and aligned using the ClustalW Method (Thompson et al. 1994) in Megalign and SeqMan (Lasergene). Sequences were screened for similarity with other published bacterial 16S rRNA gene sequences at the NCBI database (<http://www.ncbi.nlm.nih.gov>) to determine their taxonomical associations (Altschul et al. 1997).

FISH Analysis of Coral Homogenates and Intact Coral Branchlets.

FISH with rRNA-targeted probes was carried out using a universal eubacterial probe (Amann et al. 1990) and 4',6-diamidino-2-phenylindole (DAPI) for positive controls and total counts. Total counts were based on the universal bacterial probe EUB 338, which is complementary to a conserved portion of the 16S rRNA gene in the domain *Bacteria*, as described previously (Amann et al. 1990; Sunde et al. 2003). To assess the predominant bacterial diversity of the three azooxanthellate soft corals, we employed previously described FISH probes specific to Alphaproteobacteria (Manz et al. 1992), Betaproteobacteria (Manz et al. 1992), Gammaproteobacteria (Manz et al. 1992), Cytophaga-Flavobacterium (CFB, Manz et al. 1992), Firmicutes (Meier et al. 1999), Chloroflexi (Fieseler et al. 2004), and Actinobacteria (Schuppeler et al. 1998). All probes were synthesized and monolabeled at the 5' end with Cy3 (Ex 552 nm, Em 568 nm) by MWG-Biotech (High Point, NC). Approximately 1 ml of coral homogenate of 1-cm cross sections of intact coral branchlets was fixed in 10 ml of either 100% vol/vol ethanol (for gram-positive bacteria) or 4% (wt/vol) paraformaldehyde in PBS (pH 7.2, for gram-negative bacteria) overnight. One-milliliter aliquots of fixed samples were washed three times in PBS and resuspended in a mixture of 150 µl of PBS and 150 µl of 96% (vol/vol) ethanol for storage at -20°C until further use. For hybridization, 200 µl of filtered buffer [40 mM Tris-HCl (pH 7.2), 1.8 M NaCl; 20 ml/l 10% (wt/vol) sodium dodecyl sulfate (SDS)] containing 30%, 35%, or 40% (vol/vol)

formamide (to probe requirements) and 64 μl of HPLC grade water was added to 16 μl of fixed cells and warmed to 46°C. Prewarmed hybridization solution (90 μl) was added to 10 μl of appropriate probe (50 ng/ μl), and the entire solution returned to the hybridization oven and left for 90 min followed by a stringency wash [20 mM Tris-HCl (pH 7.2), 0.9 M NaCl] containing 30%, 35%, or 40% (vol/vol) formamide (to probe requirements) at 48°C for 15 min. The washed sample was vacuum filtered onto a 0.2- μm pore-size polycarbonate filter (Millipore, Billerica, MA) and placed onto a microscope slide and mounted in one drop of SlowFade-Light Antifade Kit component A (Invitrogen, Carlsbad, CA). Cy3-stained bacteria were visualized using an epifluorescence microscope (Nikon, Melville, NY) with excitation set to 550 nm (emission 570 nm). Fifteen random fields with an average distribution of cells (10 to 100) were counted for each probe and sample to obtain mean counts. The additive composition of the bacterial groups as provided by the probes was calculated by summing up all probes applied and dividing by the total counts. Autofluorescence was assessed by applying the FISH protocol without probes.

Phylogenetic Reconstruction. All phylogenetic reconstruction for sequences obtained from bacterial culture were performed using ARB (Ludwig et al. 2004). The 16S rRNA gene sequence alignments were made with the ARB fast alignment tool and were checked manually before being added to the ARB data set using the Arb_Parsimony tool to select suitable outgroups. De novo trees were constructed using the ARB neighbor joining distance matrix with Felsenstein correction and termini (.-=0) and position variance (123456789.-=0) filters. The most appropriate substitution model for distance analyses was determined by MODELTEST (Posada and Crandall 1998).

Nucleotide Sequence Accession Number. The 16S rRNA gene sequences were deposited in GenBank and given accession numbers DQ517183 through to DQ517280 (Table 1).

Results.

Examination for the Presence of *Symbiodinium* sp. To test for the presence of *Symbiodinium* sp. in *L. minimata* (LM), *I. schrammi* (IS), and *S. exertia* (SE), we used a PCR-based approach with dinoflagellate-specific primers. On examination of the PCR products, we detected a band of the correct size (750 bp) in the positive control sample

(*P. elisabethae*, data not shown), while DNA isolated from LM, IS, and SE did not result in any amplification product, indicating the absence of dinoflagellate DNA in these samples. To ensure that the DNA extracts from each coral were of sufficient quality (positive control) for PCR analysis, aliquots of each sample were also amplified with 16S rRNA gene primers. This resulted in the expected 1.5-kB amplification product for all four samples, including *P. elisabethae*.

To confirm the absence of *Symbiodinium* sp. in LM, IS, and SE, we tested for the presence of dinophyte chlorophylls *a* and *c*₂ in organic extracts of coral tissue using an established spectrophotometric method (Ritchie 2006). The initial organic extract derived from our positive control sample *P. elisabethae*, showed an intense dark green color. The absorbance spectrum of the dilute organic extract in acetone showed several peak maxima between 400 and 500 nm and a prominent broad peak centered at 673 nm. The relative absorbance values for chlorophyll *a* ($Q_y=664$ nm) band *c*₂ ($Q_y=630$ nm) at their absorbance maxima were 0.437 and 0.108, respectively. Using Eq. 1, the dilution corrected concentrations of chlorophyll *a* and *c*₂ in extracts of *P. elisabethae* were determined to be 98 and 19.64 $\mu\text{g}/\text{ml}$, respectively. In contrast to the organic extract of *P. elisabethae*, the equivalent extracts of LM, IS, and SE were clear and showed a light pink color for LM, yellow/brown for IS, and brown/red for SE. Examination of the absorbance spectra of LM showed peaks centered at 443, 536, and 613 nm. While for the extract of IS distinct absorbance maxima at 427 and 478 nm were observed, the extract of SE had its most intense absorbance maximum at 585 nm. Whereas the observed absorbance spectra resembled those of carotenoids and other natural pigments, none of the organic extract showed significant absorbance in the region of the dinophyte chlorophylls. This spectroscopic observation, together with the molecular and microscopic evidence, suggests that the coral species LM, IS, and SE used in this study were devoid of symbiotic dinoflagellates.

These data were supported by careful microscopic examination of the homogenates of all three coral species. *Symbiodinium* sp. cells could not be detected in LM, IS, or SE, but were clearly visible in *P. elisabethae* samples.

FISH Analysis of Coral Homogenates and Intact Coral Branchlets. To assess the bacterial diversity associated with the corals LM, IS, and SE, FISH with specific oligonucleotide probes targeting the six common marine bacterial genera was used (Sfanos et al. 2005;

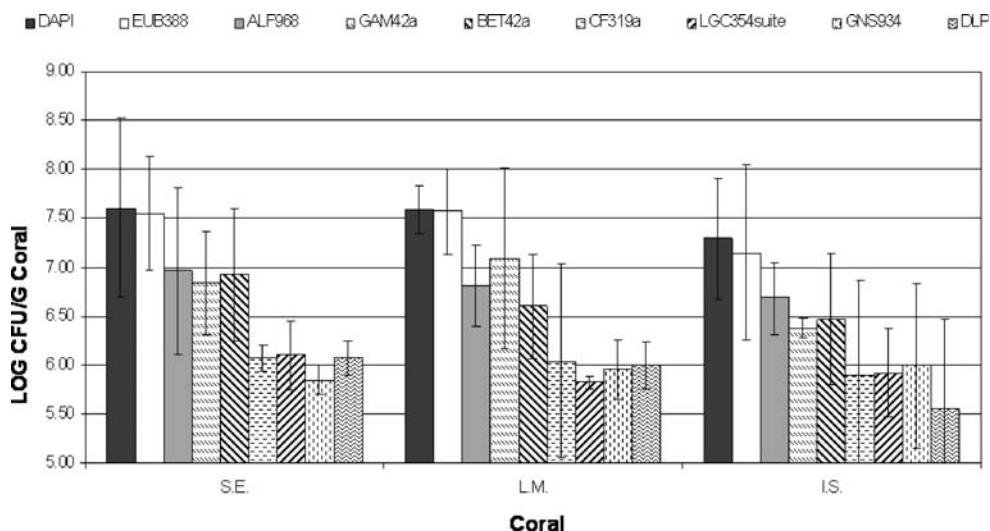


Figure 1. Log average populations of bacteria associated with *Iciligorgia schrammi* (IS), *Leptogorgia minimata* (LM), and *Swiftia exertia* (SE) estimated by means of FISH and DAPI counting. Error bars are ± 1 SD. LOG cfu/g coral is the logarithmic (base 10) count of colony forming units (bacteria) per gram of coral (wet weight). Probes stated in the legend are specific for the following organisms: DAPI, total counts (all DNA present in sample); EUB338, total Counts (most bacterial genera); ALF968, Alphaproteobacteria; BET42a, Betaproteobacteria; GAM42a, Gammaproteobacteria; CF319a, Cytophaga-Flavobacterium; LGC354suite, Firmicutes; GNS934, Chloroflexi; DLP, Actinobacteria.

Penn et al. 2006). Because the detection limit of FISH lies around 1000 cells/ml (Brow et al. 1996), bacterial populations below this detection limit were not included in this study. The total eubacterial population in each sample was determined with the FISH probe EUB338 (Amann et al. 1990; Sunde et al. 2003). To control for probe specificity, we compared the total bacterial counts estimated by FISH with those determined by a standard DAPI (DNA) staining protocol. An overview of average bacterial abundance using each of the described methods is given in Figure 1.

DAPI counts were only slightly higher than EUB338 counts, with Alpha-, Beta-, and Gammaproteobacteria making up the majority of the population of the overall FISH counts. Further examination of the FISH results (Figure 2) for whole coral in IS showed that the probes used in this study covered 49% of the total microbial diversity, while in LM and SE the percent distribution was 72% and 82%, respectively. FISH also showed that the phylum Proteobacteria, especially the class Gammaproteobacteria, dominated in all three coral species (up to 33% of the total bacterial population). By comparison, the remaining bacterial components as probed with FISH, Actinobacteria, Cytophaga, Chloroflexi, and Firmicutes were minor elements, comprising only 2% to 4% of the overall microbial diversity.

Bacterial Cultures. A total of 105 bacterial isolates were derived from the three corals using standard

aerobic bacterial culture techniques on five microbial media types, which varied in pH, nutrient content and ionic strength (salt content) to mimic different bacterial micro-environments (Figure 3; Table 2). These bacterial isolates were then analyzed via traditional microbiological methods, including Gram staining and cell morphology as determined by light microscopy. In addition, each bacterial species was identified based on its 16S rRNA gene sequence. It was found that the majority of the isolated bacteria were gram-negative (54% IS, 85% LM, and 67% SE). Although there was some overlap between bacterial species isolated from diluted marine and nutrient agar plates, most of the microbial isolates presented here, grew exclusively on one specific medium-type. Results are summarized in Table 1. During the optimization of the growth conditions, we also noticed an approximately 15% to 20% increase in bacterial morphotype diversity when diluted marine agar was used instead of the full-strength marine agar preparation recommended by the manufacturer. This phenomenon may in part be explained by the fact that many marine derived bacteria are oligotrophs, which are well adapted to nutrient poor environments (Hentschel et al. 2003; Suzuki et al. 1997). Therefore, certain bacterial populations may undergo shock when rapidly exposed to nutrient rich conditions, which may result in either cell death or inhibition of growth.

The phylum Proteobacteria dominated all cultures in all three corals, making up between 59%

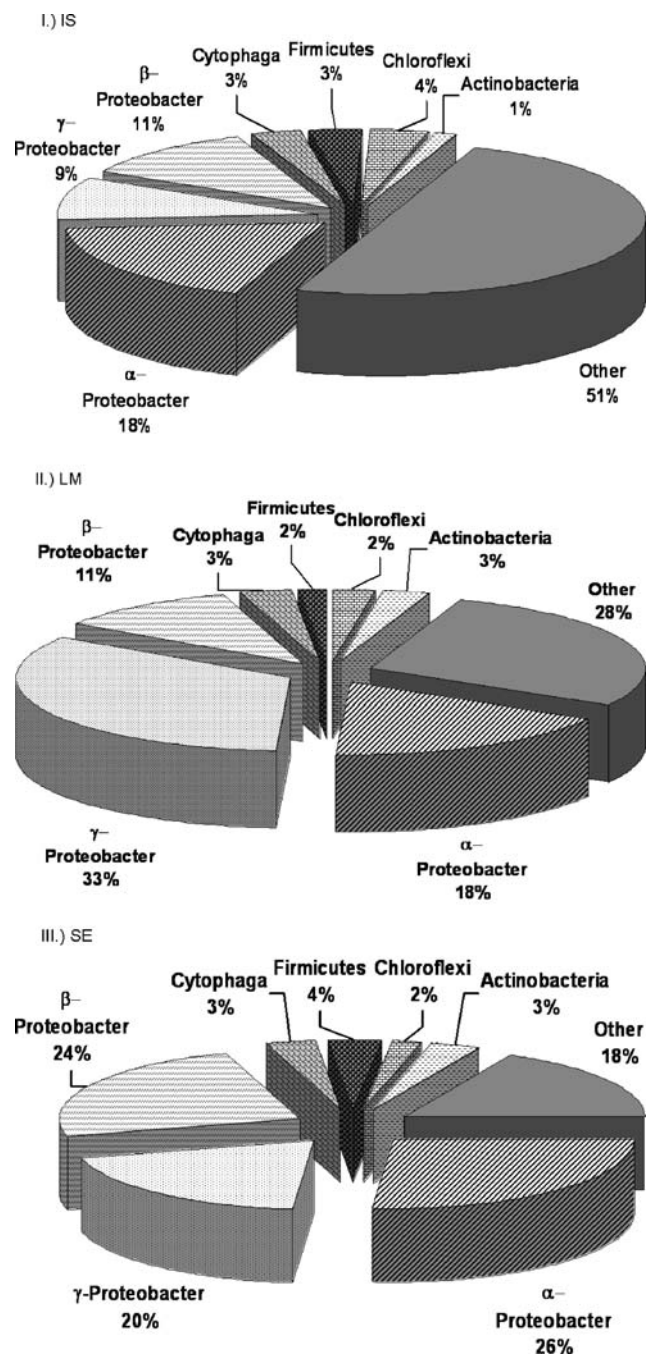


Figure 2. FISH analysis. Graphs were generated based on the analysis of the percentage distribution of the most common marine bacterial genera in the octocoral (I) *Iciligorgia schrammi*, (II) *Leptogorgia minimata*, and (III) *Swiftia exertia* as assessed by FISH analysis.

and 84% of all cultured organisms. Firmicutes represented the second most dominant bacterial grouping, comprising 10% to 33% of all bacterial isolates. Actinobacteria comprised 4% to 8% of the culturable bacterial diversity.

Cross-correlation of the bacterial isolates shows that bacterial strains with strong sequence similar-

ity to *Ralstonia pickettii* strain 2000030635 (accession number AY268180) and multiple species of *Stenotrophomonas* sp. (i.e., accession numbers AJ884482, AY445079) were associated with all three coral species examined in this study. Further, bacterial strains with close sequence similarity to *Pseudomonas* sp. HR 13 (accession number AY032725) and *Xanthomonas* group bacterium LA 37 (accession number AF513452) were allied with both IS and LM.

Examination of molecular identifications in Table 1 showed that the bacterial isolates IS2805, IS2905, SE1605, and SE1705 all displayed sequence similarity to *Bacillus MB-11* (accession number AF326360). Similarly, isolates IS1505 and IS1605 showed sequence similarity with the uncultured bacterial clone 1982b-31 (accession number AY917978), while both SE1405 and SE1505 showed similarity to the uncultured bacterium TM-15 (accession number AY838526). Although these isolates related to the same GenBank database entry, they differed in a combination of other parameters linked to their analysis set. Each culture was either isolated from a different growth medium and/or showed marked differences in gross colony morphology and color. Therefore, the bacterial isolates that fit the same GenBank entry may represent varying strains of the same species. Because different bacterial strains can have a divergent biochemical and genetic make-up, these are included in the data presented here.

Discussion.

The majority of shallow water coral species live in mutualistic associations with intracellular microalgae (dinoflagellates) symbionts of the genus *Symbiodinium* (Mitchellmore et al. 2002; Koike et al. 2004; Muscatine et al. 2005), which are thought to be involved in the biosynthesis of natural products previously attributed to the coral host (Mydlarz et al. 2003). It has been postulated that the exchange of nutrients between the algae symbiont and its coral host, is vital for maintaining the health of the coral reef ecosystem (Lesser et al. 2004; Knowlton and Rohwer 2003). The breakdown of this symbiotic relationship is associated with a marked discoloration of the coral host, commonly termed “coral bleaching.” The release of dinoflagellate symbionts

Figure 3. Distance based neighbor-joining phylogeny of 1-6S rRNA sequences obtained from cultured isolates from (I) *Iciligorgia schrammi*, (II) *Leptogorgia minimata*, and (III) *Swiftia exertia*. Bootstrap percentages after 1000 replications for the most significantly supported clades are shown in bold below the nodes.

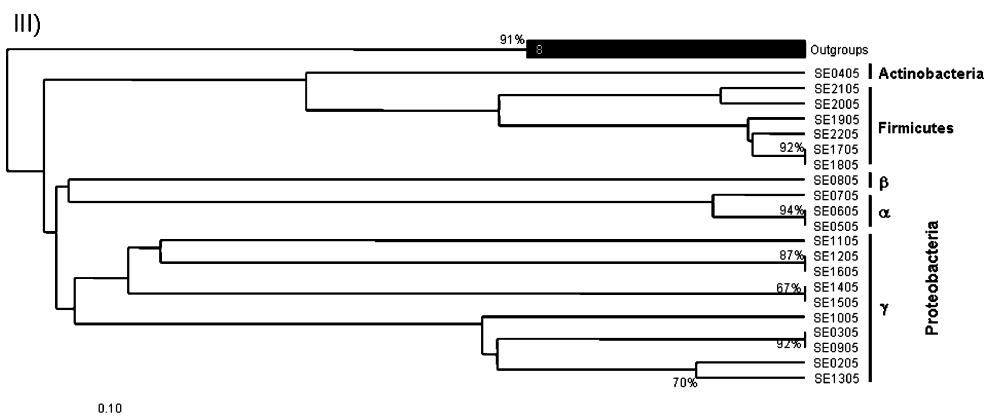
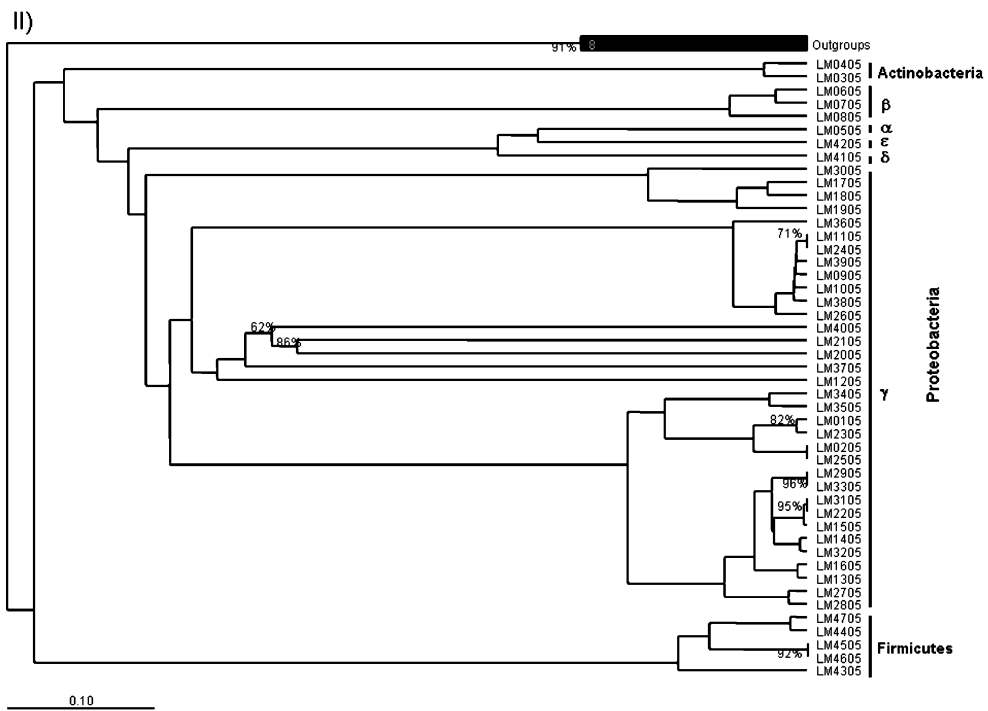
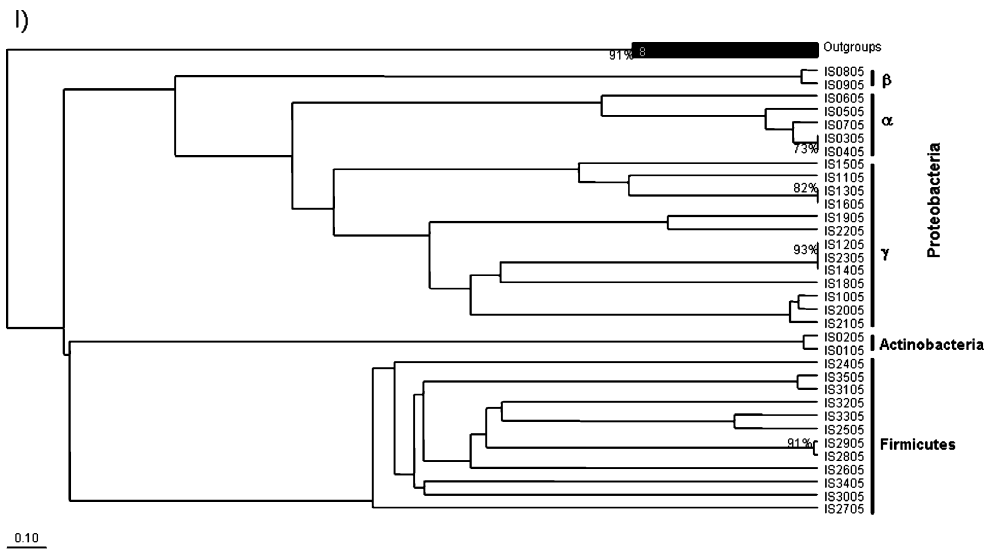


Table 2. Percentage distribution of bacterial genera in FISH counts and bacterial isolates

Organisms	FISH			Bacterial culture		
	<i>I. schrammi</i>	<i>L. minimata</i>	<i>S. exertia</i>	<i>I. schrammi</i>	<i>L. minimata</i>	<i>S. exertia</i>
Alphaproteobacteria	18	18	26	14	2	14
Betaproteobacteria	11	11	24	6	6	5
Deltaproteobacteria	0	0	0	0	2	0
Epsilonproteobacteria	0	0	0	0	2	0
Gammaproteobacteria	9	33	20	39	72	48
Actinobacteria	1	3	3	8	4	5
Firmicutes	3	2	4	33	11	28
Cytophaga	3	3	3	0	0	0
Chloroflexi	4	2	2	0	0	0
Other	51	28	18	0	0	0

may also result in an imbalance of the natural prokaryotic microbiota associated with the host coral (Bourne and Munn 2005). This microbial imbalance can ultimately lead to diseases and death of the coral host, threatening the existence of the entire shallow water reef community (Cervino et al. 2004).

In contrast to the scenario described for the shallow water coral species, deep water coral communities (>100 m), which occupy ecological niches of the ocean that are devoid of direct sunlight, do not require the symbiotic dinoflagellates for their long-term survival (Penn et al. 2006). However, coral species of the deep ocean also harbor a characteristic bacterial microbiota, which is distinct from the bacterial species associated with shallow water coral. It is currently unknown, whether changes in the prokaryotic microbiota of deep water coral species causes specific disease states.

In this study we have examined the symbiotic assemblages of three distinct coral species living at moderate depth (>40 m) in the absence of direct sunlight. In particular, we have examined their bacterial microbiota and the presence of symbiotic dinoflagellates to determine whether the symbiotic assemblages mimic those described in shallow or deep water corals.

Previous microscopic evidence suggests that coral of the genus *Leptogorgia*, which were also examined in this study, do not harbor symbiotic dinoflagellates (Bayer 1961). Therefore, the symbiotic assemblages of the octocoral species investigated in this study may resemble the symbiotic associations found in deep water (100 to 2000 m) coral species (Penn et al. 2006). To test this hypothesis we combined a PCR-based approach utilizing dinoflagellate-specific primers and a spectrophotometric method to test for the presence of dinophyte specific chlorophylls. In regards to the PCR results, while *P. elisabethae*, our positive control sample, resulted in an amplicon of

the correct size (750 bp), no amplification products were obtained for *L. minimata* (LM), *I. schrammi* (IS) and *S. exertia* (SE) samples. Similarly, the spectrophotometric method could detect dinophyte chlorophylls in the organic extract of *P. elisabethae* but none could be detected in either of the extracts prepared from tissue of LM, IS, or SE. The current data set suggests, that these coral species do not harbor symbiotic dinoflagellates. Therefore, it may be concluded, that the biosynthesis of diterpenes isolated from some of these coral species may be attributed to host associated microbe(s) other than dinoflagellates.

To describe further the microbiota of LM, IS, and SE we examined the prokaryotic diversity associated with these coral species. A common problem in the assessment of the bacterial diversity associated with marine organisms is a constant background of bacterioplankton, which is present in the water column (Hentschel et al. 2003; Breitbart et al. 2005). Previous studies have shown that the bacterial diversity of seawater differs from the microbial diversity associated with marine invertebrates (Bourne and Munn 2005). To minimize the effect of bacterioplankton on our study, we isolated the individual coral colonies immediately after harvest in a 10-l aquarium filled with sterile sea water. The same methodology has been employed previously to accurately assess the growth rates of microbial communities associated with skeletal tumors in the hard-coral *Porites compressa* (Breitbart et al. 2005).

In our experimental arrangement, the aquaria were set in flow through mode and coral colonies were washed with 2.4 aquaria volumes of sterile seawater. Although, the water flux rate was reasonably high (4 l/h), all coral colonies tolerated this treatment very well, showing extended polyps throughout the treatment period. Unlike some hard (Knowlton and Rohwer 2003; Bourne and Munn 2005) and soft coral species (Penn et al.

2006), the investigated organisms did not extrude exo-polysaccharide mucus, when stressed either in or out of the aquarium assembly. The absence of this mucus layer circumvented the formation of an extraskeletal biofilm (Bourne and Munn 2005; Penn et al. 2006), which would complicate the analysis of coral–microbe associations.

One way to assess the symbiotic microbiota of LM, IS, and SE is to use the culturable approach regardless of its inherent drawbacks. With the rise of bacterial cultivation studies, it became apparent that only a minority of the total microbiota of an environment could be recovered with conventional media and cultivation techniques (Hentschel et al. 2003). Several studies have estimated total recovery to be between 0.1% and 0.23% (Webster and Hill 2001) and 3% to 11% (Santavy et al. 1990), which is consistent with the phenomenon termed “the great plate anomaly” (Amann et al. 1990). However, at present the culture-dependent approach remains the method of choice to study the specific nutrient requirements and biochemical characteristics of microbial isolates. To compensate for the shortcomings of the culturable approach, FISH was also used in this study. This method allows the culture-independent microscopic detection of single bacterial cells based on their 16S rRNA gene identity. It is therefore possible to gain useful knowledge about their morphology and arrangement within the host tissues giving access to the remaining approximately 99% of EUB338-positive cells (Amann et al. 1990; Sunde et al. 2003) associated with the microbiota of LM, IS, and SE. In this study, we were able to characterize the majority of the whole symbiotic microbiota observed with FISH probe EUB338 of the azooxanthellate octocorals SE (82%), LM (72%), and IS (93%) through conventional plate culture and FISH methods while being able to preserve a selected microbiota in culture, which will allow detailed profiling of the individual metabolic requirements and biochemical characteristics for each bacterial isolate.

In this study, culture techniques selected for Gammaproteobacteria and Firmicutes. While Gammaproteobacteria were also the major bacterial genus observed in FISH experiments, the genus Firmicutes made up less than 6% of the total detectable microbiota approaching the limits of detection for this technique. This suggests that the culturing approach, using the media employed, is highly selective. Further evidence for this was previously reported by Imhoff and Stöhr (2003), showing that incubation temperatures influenced the proportions of major phylogenetic groups that could be isolated. At 22°C, low G+C gram-positive

bacteria, Actinobacteria and Gammaproteobacteria dominated cultures. By contrast, at 4°C numbers of low G+C gram-positive bacteria were significantly reduced and Actinobacteria as well as Gammaproteobacteria predominated. In this study, all organisms were incubated at ambient temperatures (27°C) which could be, along with the selection of general media, the cause for a possible overestimation of Gammaproteobacteria and Firmicutes, when compared to the FISH whole organism data. It is also interesting to note that these media allowed the isolation of a majority of gram-negative organisms, a phenomenon that has been discussed previously (Imhoff and Stöhr 2003). It is surprising that members of the Delta- and Epsilonproteobacteria (isolates LM 4105, LM4205) were isolated in culture from LM, as these bacterial strains represent only a very small subclass of the Proteobacteria and are not commonly isolated (Urakawa et al. 2005). In contrast to the Delta- and Epsilonproteobacteria, members of the Cytophaga and Chloroflexi were not observed in culture, even though they were observed by FISH analysis of the whole organism. The lack of these bacterial classes in culture could be due to the selection of culture media employed in this study or the lack of chemical/physical stimuli from the coral host (Gonzales et al. 1996; Taylor et al. 2004; Bourne and Munn 2005). Overall, the distribution of bacterial species identified in this study are in close agreement with the microbiota previously associated with other coral species, which have been identified using various culture-independent approaches (Bourne and Munn 2005; Penn et al. 2006).

Our screens of culturable bacterial species identified a group of bacterial strains with sequence similarity to *Ralstonia pickettii* and *Stenotrophomonas* sp. (Table 1), which were associated with more than one coral species. From these results, it could be argued that these bacteria are either of environmental origin or that they represent bacterial species that commonly associate with a variety of soft coral species. Recent evidence suggests that certain bacterial species form uniform communities when associated with different species of marine sponges (Hentschel et al. 2002; Imhoff and Stöhr 2003; Fieseler et al. 2004; Taylor et al. 2004). Similar bacterial associations may therefore be envisioned with other marine invertebrates, including coral. Utilizing aerobic culturing methodologies we isolated two bacterial species (isolate IS3205 and LM 4105), which were most closely related to marine heterotrophs that are involved in sulfur cycling (Penn et al. 2006). These facultative anaerobe species may have advantageous survival strategies,

when water temperatures rise in the summer month and oxygen is rapidly depleted either in the intra- or extracellular space of the coral colony.

We have also isolated several strains related to *Vibrio* sp. (isolates IS2205, LM2105, LM4005) from the octocorals IS and LM. The presence of *Vibrio* species has previously been linked to a variety of coral diseases (Knowlton and Rohwer 2003; Cervino et al. 2004; Bourne and Munn 2005). However, we could not detect any pathological changes in the octocorals studied when these organisms were observed in aquaria or in situ over a time period of several months. This may be an indication that species of *Vibrio* in these corals are a part of their natural microbiota. Further, additional environmental triggers such as elevated temperature, which result in an imbalance of the innate *Vibrio* population, may be necessary for progression to disease states such as yellow blotch disease (Cervino et al. 2004).

FISH analysis of coral homogenates indicated that Alpha-, Beta-, and Gammaproteobacteria were the most common bacterial genera observed. By contrast, the same method indicated that Actinobacteria, Chloroflexi, Cytophaga-Flavobacterium (CFB) group bacteria, and the low G+C gram-positive bacteria were only minor components of the total bacterial population. Literature evidence (Webster and Hill 2001) indicated that Gammaproteobacteria were prevalent throughout the mesohyl of the sponge *Rhopaloeides odorabile* and were especially prevalent in regions surrounding the choanocyte chambers. This suggests that Gammaproteobacteria may have a symbiotic function related to nutrient uptake in *R. odorabile* that may also be true in octocoral. This notion is further supported by the examination of the microbiota associated with the hard coral *Pocillopora damicornis* (Bourne and Munn 2005), where the most abundant group of associated bacteria was found to be the Gammaproteobacteria.

A future goal for this work will be to grow these bacterial isolates in liquid medium to test for the presence of secondary metabolites such as the lophotoxins in LM previously isolated from several *Leptogorgia* species found in the Pacific and Atlantic Oceans (Culver and Jacobs 1981; Fenical et al. 1981; Epifanio et al. 2000). The fact that lophotoxins are found in related coral species from different oceans may be a good indication that the natural product is produced by an associated microbe (Schmidt et al. 2000; Hentschel et al. 2003). Genera of bacteria belonging to the Proteobacteria, Actinobacteria, Cytophaga-Flavobacterium, and Firmicutes isolated from marine and terrestrial sources have previously

been associated with the production of bioactive metabolites (Fenical 1993; Mincer et al. 2002, 2004; Piel et al. 2004; Verburg et al. 2004). The isolation of the organisms presented here is the first step in the search for coral metabolites produced by associated microbes.

In conclusion, the data presented here show the differences in bacterial populations endogenous to octocorals that can be identified using direct observation and culturing techniques. The diverse microbiota associated with the examined host coral includes prokaryotic families which have been previously associated with other shallow and deep water coral species. The data presented in this study also suggest that the examined octocoral species are devoid of symbiotic dinoflagellates. In this respect, their symbiotic assemblies resemble those of deep water coral species. To the best of our knowledge, this is the first study examining the microbial associations of marine octocorals, which grow at moderate depth (40 to 100 m) in the absence of direct sunlight.

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