Coral Reefs (2013) 32:375–388
DOI 10.1007/s00338-012-0989-6

REPORT

A comparison of culture-dependent and culture-independent techniques used to characterize bacterial communities on healthy and white plague-diseased corals of the *Montastraea annularis* species complex

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Received: 30 December 2011 / Accepted: 28 November 2012 / Published online: 25 January 2013
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Abstract Diseases of hermatypic corals pose a global threat to coral reefs, and investigations of bacterial communities associated with healthy corals and those exhibiting signs of disease are necessary for proper diagnosis. One disease, commonly called white plague (WP), is characterized by acute tissue loss. This investigation compared the bacterial communities associated with healthy coral tissue ($N = 15$), apparently healthy tissue on WP-diseased colonies ($N = 15$), and WP-diseased tissues ($N = 15$) from *Montastraea annularis* (species complex) colonies inhabiting a Bahamian reef. Aliquots of sediment ($N = 15$) and water ($N = 15$) were also obtained from the proximity of each coral colony sampled. Samples for culture-dependent analyses were inoculated onto one-half strength Marine Agar (½ MA) and Thiosulfate Citrate Bile Salts Sucrose Agar to quantify the culturable communities. Length heterogeneity PCR (LH-PCR) of the 16S rRNA gene characterized the bacterial operational taxonomic units (OTU) associated with lesions on corals exhibiting signs of a white plague-like disease as well as apparently healthy tissue from diseased and non-diseased conspecifics. Analysis of Similarity was conducted on the LH-PCR fingerprints, which indicated no significant difference in the composition of bacterial communities associated with apparently healthy and diseased corals. Comparisons of the 16S rRNA gene amplicons from cultured bacterial colonies (½ MA; $N = 21$) with all amplicons obtained from the whole coral-associated bacterial community indicated ≥39 % of coral-associated bacterial taxa could be cultured. Amplicons from these bacterial cultures matched amplicons from the whole coral-associated bacterial community that, when combined, accounted for >70 % total bacterial abundance. An OTU with the same amplicon length as *Aurantimonas coralicida* (313.1 bp), the reported etiological agent of WPII, was detected in relatively low abundance (<0.1 %) on all tissue types. These findings suggest a coral disease resembling WP may result from multiple etiologies.

Keywords White plague type II · Coral disease · *Aurantimonas coralicida* · *Montastraea* · Bacterial community · Microbial dysbiosis

Communicated by Biology Editor Dr. Ruth Gates

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Introduction

The complex of diseases collectively known as white plague (WP) poses a major threat to reef-building stony corals throughout the Wider Caribbean Region (Richardson et al. 1998a; Green and Bruckner 2000; Sutherland et al. 2004; Weil 2004). In 1998, a bacterium isolated from a white plague type II (WPII)-affected elliptical star coral (*Dichocoenia stokesii*) was reported to be the causative agent of this disease (Richardson et al. 1998a, b). A 300-bp sequence of the 16S rRNA gene suggested that this isolate was a new

genus of Alphaproteobacteria and matched most closely to the *Erythromicrobium* (89 %), *Erythrobacter* (87 %), and *Sphingomonas* (86 %) groups (Richardson et al. 1998b). After being passed in culture for approximately 7 years, the isolate—coded WP1^{T}—was subjected to polyphasic taxonomic testing and determined to be the novel genus and species *Aurantimonas coralicida* (Denner et al. 2003).

If *A. coralicida* is the sole etiological agent of WPII, then it should be detectable throughout the reported geographic and biological range of all hosts exhibiting signs consistent with this disease (Sutherland et al. 2004; Weil 2004). However, multiple authors using various techniques have not found microscopic or molecular evidence of *A. coralicida* in samples of suspect WPII-diseased tissues from colonies of *Montastraea annularis* or *M. faveolata* inhabiting reefs in Barbados, St. Croix, Panama, and Puerto Rico (Bythell et al. 2002; Pantos et al. 2003; Sunagawa et al. 2009). Additionally, repeated sampling of WPII-affected corals inhabiting the same reefs in the Florida Keys on which the original WPII epizootic was documented has failed to detect *A. coralicida* (Richardson and Aronson 2000).

Both Bythell et al. (2002) and Pantos et al. (2003) acknowledged that the diseased colonies in their respective studies exhibited acute tissue loss initiating at the basal region. However, the lesions were small and circular—a gross morphology inconsistent with previous reports of WPII (Richardson et al. 1998a; Richardson and Aronson 2000). These authors concluded that they were not investigating the same disease from the original 1995 epizootic in the Florida Keys because *A. coralicida* was not detected and the lesions were uncharacteristic of WPII (Bythell et al. 2002, 2004; Pantos et al. 2003; Work and Aeby 2006). Nevertheless, these reports suggest the signs of rapid tissue loss among Caribbean scleractinians can appear similar, if not identical, to WPII. As such, the use of differential diagnostic approaches to discriminate WPII from other white plague-like (WPL) diseases should be of great importance to coral reef biologists and reef managers worldwide.

While some investigations of coral diseases have relied primarily on culture-dependent methods to assess the composition of coral tissue-associated bacterial communities, culture-independent approaches are becoming more common (Rowher et al. 2001; Wegley et al. 2007; Sato et al. 2010; Barott et al. 2011; Wilson et al. 2012). This transition appears to have been partially driven by the often repeated paradigm that <1 % of bacteria in some environmental samples, notably soil and water, are cultured using typical growth media (Rozsak and Colwell 1987; Amann et al. 1995; Fuhrman and Campbell 1998; Guthrie et al. 2000; Rowher et al. 2001). If this concept applies to coral tissues, then the composition of the associated microbial community would be severely misrepresented using only culture-dependent techniques (Rowher et al. 2001). This study used both culture-dependent and culture-independent methods to characterize bacterial communities associated with *M. annularis* species complex (*M. annularis*). To our knowledge, this is the first report to offer a direct comparison between these techniques to assess the microbial communities associated with any coral species exhibiting signs consistent with a WPL disease.

The goal of this project was to develop a clearer understanding of the coral-associated bacterial community and its relationship to a WPL disease. We established the following objectives to meet these aims: (1) characterize the relative size and composition of bacterial communities inhabiting coral tissue, skeleton, and the surface mucopolysaccharide layer (SML) from the edge of WPL lesions on *M. annularis* colonies, apparently healthy tissues from diseased colonies, and apparently healthy colonies exhibiting no signs of disease; (2) assess the similarity and diversity of these bacterial communities; (3) test for the presence of *A. coralicida*; and (4) examine the usefulness of a culture-dependent technique for bacterial community profiling.

Materials and methods

Sample site and collection

Healthy and WPL-diseased samples were collected in situ from five pairs of *M. annularis* colonies inhabiting offshore reefs at Lee Stocking Island, The Bahamas (Table 1). *M. annularis* was selected as the target species because it is an important massive framework-builder of reefs in the Wider Caribbean Region and is susceptible to all variants of WP (Sutherland et al. 2004). Sediment and seawater near each coral colony were also collected to enable comparisons among bacterial communities associated with surrounding environmental samples.

WPL lesions were identified following the guidelines of Work and Aeby (2006). In all cases, areas of acute, yet mild, tissue loss affecting the polyps and coenenchyme of *M. annularis* colonies exhibited the following signs: diffuse, peripherally and basally situated, small to medium sized, irregularly shaped, distinct edges, and areas of tissue loss revealing intact bare white skeleton. A light lawn of turf algae had begun developing on the recently denuded skeleton of colonies BC12-15, whereas colony BC11 presented a relatively denser community of epifaunal organisms. Due to temporal and funding limitations, rates of tissue loss were unable to be estimated.

Triplicate water samples (50 mL) were collected in sterilized syringes from approximately 10 cm above a colony's surface. Water was always obtained before

Table 1 Biological information associated with 45 coral cores collected from *M. annularis* colonies at Lee Stocking Island, The Bahamas

Date	Reef name	Sample ID	# of cores collected
10.10.2004	North Perry*	BC11D	3
10.10.2004	North Perry*	BC11HD	3
10.10.2004	North Perry*	BC16H	3
11.10.2004	North Perry*	BC12D	3
11.10.2004	North Perry*	BC12HD	3
11.10.2004	North Perry*	BC17H	3
11.10.2004	North Perry*	BC13D	3
11.10.2004	North Perry*	BC13HD	3
11.10.2004	North Perry*	BC18H	3
12.10.2004	Horseshoe†	BC14D	3
12.10.2004	Horseshoe†	BC14HD	3
12.10.2004	Horseshoe†	BC19H	3
12.10.2004	Horseshoe†	BC15D	3
12.10.2004	Horseshoe†	BC15HD	3
12.10.2004	Horseshoe†	BC20H	3

Sample identification denotes geographical location (B = Bahamas), type of environmental sample (C = coral), and whether the cores were obtained from the edge of white plague type II (WPII) lesions (D), apparently healthy tissue on the diseased coral (HD), or apparently healthy tissue on a coral exhibiting no signs of disease (H). Each coral pair was composed of 1 WPII-diseased colony and 1 apparently healthy colony (e.g., BC11/BC16, BC12/BC17, etc.)

* 23°46.97′N, 76°6.05′W

† 23°46.3′N, 76°5.33′W

sediment and coral to reduce the risk of contamination by these environmental samples. Water near the healthy colony ($N = 15$) in each coral pair was always sampled before the diseased colony ($N = 15$). Sediment samples, near both healthy ($N = 15$) and diseased colonies ($N = 15$), were collected in the same order using a sterile 20-cc syringe with the plunger temporarily removed.

We obtained triplicate 1.6-cm-diameter cores of coral sample (i.e., tissue, skeleton, and SML) from (1) the lesion edge of colonies exhibiting WPL signs (D); (2) apparently healthy tissue on the diseased colonies at least 5 cm from the lesion edge (HD); and (3) apparently healthy tissue on conspecifics exhibiting no observable signs of disease (H). The apparently healthy colony in each coral pair was located ≤10 m from the diseased colony. A total of 45 cores (D = 15, HD = 15, H = 15) were collected from the five pairs of *M. annularis* (Table 1). Duplicate 2.2-cm-diameter cores were also collected from the D, HD, and H tissues on each colony for histopathological examination.

Stainless steel corers (SS) were used to extract all coral samples. Prior to use, each SS was dipped in a 10 % bleach solution, heat-sterilized in freshwater, and transferred to 50-mL BD Falcon™ Conical Centrifuge Tubes (Becton, Dickinson and CO., Franklin Lakes, New Jersey) that were then filled with sterile water (Hawkins and Davies 2002; Kemp and Smith 2002; Whiteman et al. 2002). SS were hammered about 1.5 cm into a colony to excise cores of coral tissue, skeleton, and mucus containing approximately 10–15 (D) or 18–24 (HD and H) polyps. Each SS, now containing a single core, was withdrawn from the colony and returned to its centrifuge tube with ambient seawater. Wounds were patched with J-B Waterweld™ (J-B Weld Company, Sulphur Springs, TX). All samples were immediately returned to the surface.

The 1.6-cm-diameter cores were kept in darkness at ambient temperature during transport to the laboratory on Lee Stocking Island. Seawater in the tubes containing the 2.2-cm-diameter cores was carefully decanted and replaced with 30–40 mL of diluted zinc formaldehyde fixative comprised of 1 part buffered aqueous Z-Fix concentrate (Anatech, Ltd., Battle Creek, Michigan) mixed with 4 parts 0.2-µm-filtered seawater. All fixed samples were capped, stood upright, and kept in the shade during the return to the laboratory.

Sample processing

All 1.6-cm-diameter cores for bacterial community analyses were processed within 3 h of collection. Instruments were dipped in a 10 % bleach solution and rinsed with sterile 0.2-µm-filtered seawater before contacting any sample. Each coral core was weighed, crushed using needle-nosed pliers, and finally pulverized into a coarse homogenate with a pestle. Sterile 0.2-µm-filtered seawater (4 mL) was transferred to each centrifuge tube containing the homogenate. These slurries were vortexed (high speed, 2 min) and aliquoted (2 mL) to sterile cryovials (E & K Scientific, Santa Clara, CA) for culture-independent investigations. Samples were kept at −20 °C while in the field, shipped in a liquid nitrogen-chilled (−196 °C) cryoshipper (Cryodepot, Suwanee, Georgia) to the Microbiome Analysis Center (MAC) at George Mason University (Mason), and placed into long-term storage at −80 °C.

The 2.2-cm-diameter samples for histopathological examination were returned to the Histology Laboratory at George Mason University and processed using routine methods (Peters et al. 2005), except that tissue-loss margin samples were enrobed in 1.5 % agarose prior to decalcification. A solution of 10 % ethylenediaminetetraacetic acid (EDTA) adjusted to pH 7 with sodium hydroxide was used to decalcify the samples, changing the solution every 24–48 h until completion. Samples were trimmed into 2- to 3-mm-thick sections and placed in plastic cassettes for infiltration with paraffin. Embedded tissues were sectioned at 5 µm and stained with Harris's hematoxylin and eosin and special stains. Histoslides were examined using light microscopy.

Microbiological culturing

Growth plates containing one-half strength Difco™ Marine Agar 2216 (½ MA) (Becton, Dickenson, and Co., Sparks, MD) or Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS) (Becton, Dickenson, and Co., Sparks, MD) were prepared at ambient salinity (33 ‰) with a 2 % agar concentration. Serial dilutions (10^{-1}–10^{-6}) were created by mixing the remaining 2 mL of crushed coral, sediment, or water in each centrifuge tube with 5 mL of sterile 0.2-μm-filtered seawater to produce a nominal dilution of 10^{-1}.

Triplicate aliquots of 0.1 mL were taken from each tube dilution and spread on both types of media, thereby diluting each sample by yet another power of ten (e.g., 10^{-2} tube = 10^{-3} plate). All ½ MA and TCBS growth plates were cultured under light conditions in an aerobic environment at 26–28 °C for 24–48 h or until no new colonies developed and then stored at 4 °C in the dark. Digital images of each plate were taken to provide a permanent record of the cultured bacterial community.

Extraction of DNA from the coral-associated bacterial community

The BIO 101 FastDNA® SPIN® Kit for tissue (BIO 101 Systems, MP Biomedicals, Solon, Ohio) was used to extract whole bacterial community genomic DNA from coral slurries following manufacturer's instructions with slight modifications. Samples were mixed with cell lysing solution for tissue (CLS–TC) and then bead beaten twice for 30 s at the speed of 5.0 m s^{-1} in a Fastprep Instrument®. Whole community DNA extracts were eluted using 150 μl of DNAse/Pyrogen free water and stored at −20 °C until used for PCR.

Extraction of DNA from cultured bacteria

Samples of 24 bacterial isolates from the Bahamas Coral (BC) pair BC12/17 (8 from BC12D, 8 from BC12HD, and 8 from BC17H) were collected from ½ MA growth plates using sterile, flat-ended toothpicks and transferred to dedicated wells in a sterile 96-well microtiter plate containing 50-μL Tris–EDTA (TE) buffer. Isolates were selected based on observable differences in colony morphology and degree of isolation from other bacterial colonies. Cells were lysed at 96 °C for 10 min in a GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA) and then centrifuged at 2,000 rpm for 5 min to pellet debris.

Amplicon length heterogeneity PCR (LH-PCR) amplification

LH-PCRs were performed in triplicate on whole bacterial community DNA extracts from all cores of D, HD, and H tissues as well as the 24 picked bacterial colonies. Tenfold dilutions (1:1, 1:10, or 1:100) of each DNA extract were prepared to determine which concentration allowed for linear amplification (Suzuki et al. 1998). The first two hypervariable regions of the 16S rRNA gene were selectively amplified using the universal bacterial primers FAM-labeled 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 355R (5′-GCT GCC TCC CGT AGG AGT 3′) (Invitrogen™ Corporation, Carlsbad, CA) (Suzuki et al. 1998).

A 20-μl PCR mixture contained 0.5 units Ampli*Taq* Gold DNA polymerase (Applied Biosystems, Foster City, CA), 1X PCR Gold Buffer, 2.5 mM $MgCl_2$ solution, 0.01 % bovine serum albumin (BSA) (Promega, Madison, WI), 0.025 mM of each deoxynucleotide triphosphate (Promega, Madison, WI), 0.5 μM forward and reverse primers (Invitrogen™ Corporation, Carlsbad, CA), and approximately 10 ng of genomic DNA extract. An initial denaturation step at 95 °C for 11 min was followed by 35 cycles of denaturation (95 °C for 30 s), primer annealing (48 °C for 30 s), and primer extension (72 °C for 2 min+5 s $cycle^{-1}$). A final incubation at 72 °C for 30 min was done to ensure complete extension of all fragments. One percent agarose gel electrophoresis with ethidium bromide was used to visualize PCR products to determine appropriate dilutions for fingerprinting.

Amplicons were denatured at 96 °C for 3 min in HiDi Formamide™ (Applied Biosystems, Foster City, CA). Single-stranded DNA amplicons were analyzed using a 96-capillary Spectrumedix SCE9610 Genetic Analysis System (Spectrumedix LLC, State College, PA) using Internal Lane Standard (ILS) 600 (Promega Corp., Madison, WI). Data were processed with Genospectrum™ DNA Fragment Analysis Software v.2.2.1 (Spectrumedix LLC, State College, PA) to produce comparable electropherograms.

16S DNA sequencing of cultured bacteria

Universal bacterial primer 27F was also used to sequence the same variable regions of the 16S rRNA gene from the 24 bacterial isolates. A standard sequencing reaction was performed with Big Dye Terminator v3.1 (Applied Biosystems, Foster City, CA). Sequenced products were purified using Sephadex G-50 (Sigma-Aldrich, Natick, MA) according to the manufacturer's protocol, dried in a SpeedVac, and run on a Spectrumedix SCE9610 capillary sequencer (Spectrumedix LLC, State College, PA). The raw sequences were analyzed with BaseSpectrum v 2.0 software (Spectrumedix LLC, State College, PA) and imported to Sequencher software v 4.1 (Gene Codes Corp., Ann Arbor, Michigan) for manual base calling and editing. The 16S rRNA gene sequences were compared to GenBank using BLASTN 2.2.26 + (nr database) and the Ribosomal

Database Project (RDP) version 10 (Zhang et al. 2000; Cole et al. 2009).

Aurantimonas coralicida 16S rRNA gene amplicon length and sequence

Genomic DNA was extracted from pure cultures of *A. coralicida* and amplified as described above. *A. coralicida* was used as a positive control in every LH-PCR. This isolate returned 7 amplicons of different size (bp) and relative abundance: 310.0 (0.03 %), 311.0 (0.10 %), 311.8 (4.6 %), 312.6 (3.3 %), 313.1 (92 %), 314.9 (0.30 %), and 316.1 (0.03 %). Only the dominant amplicon (313.1 bp) was used to screen whole coral tissue-associated bacterial community fingerprints for the possible presence and relative abundance of the reported causal agent of WPII. Genomic DNA was also sequenced and confirmed the isolate's identity as *A. coralicida*.

Microbiological enumeration

Plate images were analyzed using BIOQUANT NOVA PRIME version 6.00.10 (Bioquant Image Analysis Corporation, Nashville, Tennessee). The average number of bacterial colony-forming units (CFU) was calculated from the triplicate ½ MA and TCBS plates for each dilution. Bacterial abundance per gram of coral sample (CFU g^{-1}) was tallied on the basis of the wet weight of coral tissue collected (0.20106 g) as determined by the volume of each 1.6-cm core assuming 1 mm thickness. Bacterial abundance in sediment (CFU g^{-1}) and water (CFU mL^{-1}) was determined on the basis of 1 g and 1 mL measured aliquots of each sample used to prepare serial dilutions.

Data analysis

LH-PCR. Average relative abundances for each operational taxonomic unit (OTU) were calculated from the triplicate LH-PCRs and presented in either graphical or tabular format to (1) compare the composition of whole bacterial communities associated with D, HD, and H samples; (2) identify the relative abundance of OTU(s) within each sample type; (3) determine which OTUs were shared among, or specific to, different coral samples and combinations of samples; (4) determine the number and amplicon size of OTUs associated with the cultured bacterial isolates; and (5) enable a direct comparison of OTUs from cultured bacteria with those from the whole coral-associated bacterial community. If the average normalized abundance of an OTU was $\leq$0.01 %, then it was removed from the analysis.

Nonmetric multidimensional scaling (NMDS) and hierarchical cluster analysis were performed on the LH-PCR Bray–Curtis similarity matrix (PRIMER v6). The minimum stress level for the analysis was 0.01 with 50 restarts. Analysis of Similarity (ANOSIM) was then performed on the Bray–Curtis resemblance matrix to determine the statistical significance ($\alpha \leq 0.05$) of compositional differences among bacterial communities associated with D, HD, and H coral samples (PRIMER v6). Exploration of the OTU composition in clusters generated by NMDS was conducted via the similarity percentages (SIMPER) routine (PRIMER v6). Shannon diversity indices ($\log_e$) were calculated for the suite of coral-associated bacterial OTUs (PRIMER v6). Indices were imported into SPSS 18.0 for analysis of variance (ANOVA). If F was significant ($\alpha \leq 0.05$), then either Tukey's HSD (equal variances assumed) or Tahmane's (equal variance not assumed) post hoc multiple comparison procedure was used to locate significant differences among D, HD, and H tissue groups.

Microbiological enumeration

SPSS 18.0 was used to analyze the bacterial enumeration dataset. Untransformed data and various transformations (e.g., natural log, square root, $\log_{10}$) were evaluated for fit to a normal distribution. Three categories of mean cultured bacterial abundances were compared among coral, sediment, and/or water: grand, combined, and individual. The grand means for each sample type were calculated from all samples collected, regardless of a coral's health status. The combined means for each sample type were calculated from all samples associated with D, HD, or H samples.

If the data were normally distributed, ANOVA was employed to test for equality among the means. Either Tukey's HSD or Tahmane's post hoc multiple comparison procedure was applied when F was significant ($\alpha \leq 0.05$). A Kruskal–Wallis (K–W) procedure was used for nonparametric data. If H was significant ($\alpha = 0.05$), then either multiple Mann–Whitney (M–W) U tests or nonparametric multiple comparison procedures were used.

Results

Whole bacterial community fingerprints

A total of 100 OTUs were associated with all coral samples. Thirty-three OTUs were removed for contributing $\leq$0.01 % average abundance to the total community on an individual basis. As such, 67 OTUs, each being $\geq$0.01 % average abundance, comprised the whole bacterial community associated with 45 coral cores (Fig. 1). All D, HD, and H samples shared 33 OTUs, accounting for 95.60 % of the total bacterial community abundance (Table 2). The remaining 34 OTUs constituted 3.72 % of community

abundance and were unique to either an individual type of coral sample or a pairing of coral sample types (Table 2). The 33 OTUs initially discarded accounted for a combined 0.68 % of the total community abundance.

The three most abundant OTUs associated with each tissue type had amplicon lengths 335.8, 342.1, and 342.8 bp. Their combined average relative abundances (mean ± SE) were 13 ± 2, 8.8 ± 2, and 11.7 ± 2 % in D samples; 24.8 ± 0.3, 8.3 ± 0.1, and 16.4 ± 0.2 % in HD samples; and 24.4 ± 5, 7.1 ± 2, and 11.4 ± 2 % in H samples, respectively (Fig. 1). SIMPER analysis confirmed these same three OTUs as having the highest average abundance in D, HD, and H samples. In H samples, the OTU with an amplicon of 343.3 bp had a higher percent contribution (10.13 %) than 342.1 bp (7.25 %) (Table 3). This can be attributed to the latter OTU having an average within-group similarity (2.24) lower than the former OTU. The average dissimilarity between the D-HD, D-H, and H-HD pairs was >65. Differences appear to be the result of the least abundant members of the respective bacterial communities, for the same six OTUs—amplicons 335.8, 342.8, 342.1, 352.4, 334.3, 344.4 bp—accounted for >50 % of the cumulative contribution in all coral samples (Fig. 1). ANOVA of the Shannon diversity indices (mean ± SD) for bacterial communities associated with D (2.28 ± 0.39), HD (1.85 ± 0.30), and H (2.00 ± 0.67) samples revealed no significant difference ($F = 3.08$; $p = 0.06$).

Two-dimensional NMDS of the LH-PCR Bray–Curtis similarity matrix indicated coral samples from different *M. annularis* colonies clustered together regardless of tissue type (Fig. 2; Table 1). Despite this apparent similarity, there was separation among samples that appeared to be based on coral pair. For example, all samples of D, HD, and H tissues from pairs BC14/19 and BC15/20 clustered together but were separate from BC11/16 (Fig. 2; Table 1). However, ANOSIM of the entire dataset confirmed no significant difference in bacterial community composition (Global $R = 0.02$, $p = 0.22$). Pairwise comparisons between coral samples supported this finding (D–HD: $R = 0.05$, $p = 0.12$; D–H: $R = 0.06$, $p = 0.11$; H–HD: $R = -0.03$, $p = 0.67$).

The molecular fingerprint of *A. coralicida* was found in very low abundance on all coral samples (i.e., D, HD, and H). Two of the ten OTUs only associated with diseased corals had amplicon lengths matching two of the seven amplicons associated with pure cultures of *A. coralicida* (314.9 and 316.1 bp). However, their combined relative

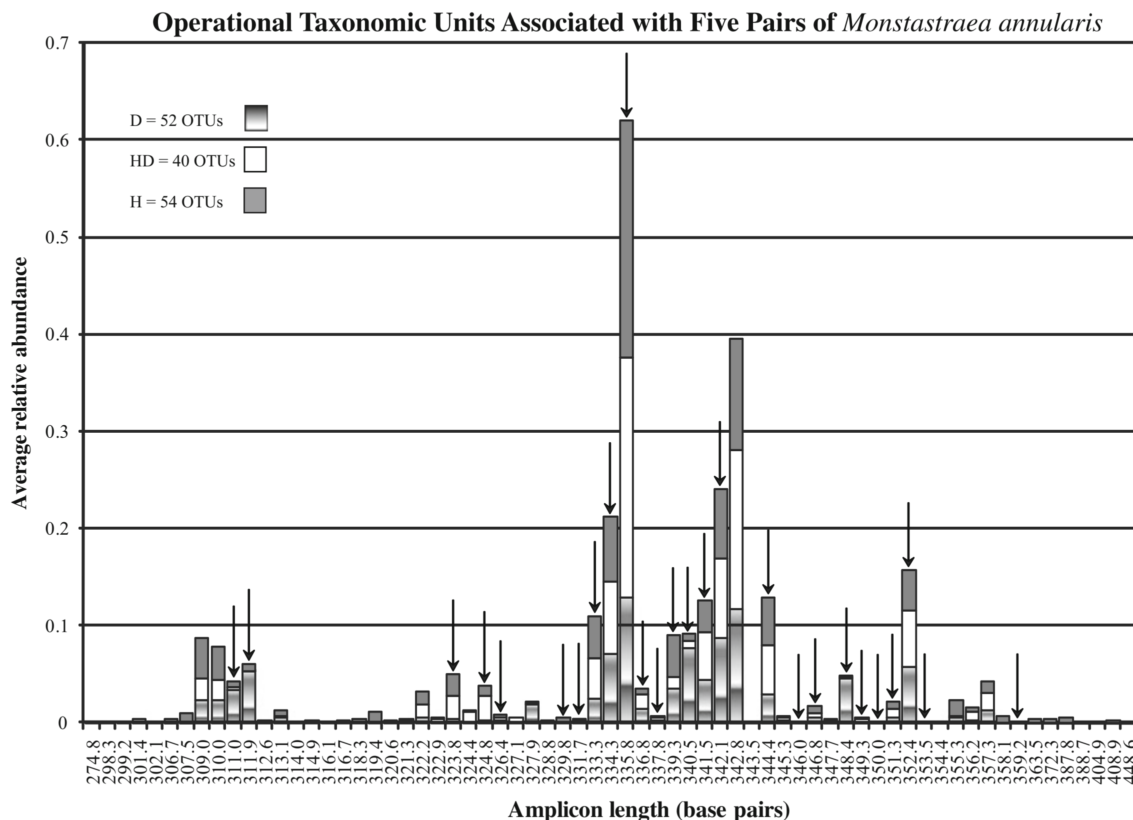

Fig. 1 Operational taxonomic units (OTUs) associated with coral samples from the white plague-like (WPL) lesion margin (D), apparently healthy areas on WPL-diseased colonies (HD), and healthy colonies of *M. annularis* exhibiting no signs of disease (H). Amplicons associated with bacterial isolates cultured from select coral samples are indicated by *arrows*

Table 2 The number and combined relative abundances of operational taxonomic units (OTUs) associated with a particular coral sample or combination of samples

Coral sample(s)	# of unique amplicons	Combined % of total abundance
HD	2	0.1
H + HD	2	0.3
D + HD	3	0.4
D	10	0.5
H	9	0.9
D + H	8	1.5
D + HD + H	33	95.6

Nearly 50 % of all described OTUs were shared among D, HD, and H samples and represented over 95 % of total bacterial community abundance

abundance accounted for less than 0.09 % of total community abundance. Fragments 314.9 and 316.1 bp were also two of the least abundant amplicons associated with pure cultures of the WP1^T pathogen (0.30 and 0.03 %, respectively). The 5 other amplicons associated with *A. coralicida* (310.0, 311.0, 311.9, 312.6 bp), including its dominant fragment length (313.1 bp), occurred at low average abundance (2.61, 1.44, 2.01, 0.06, and 0.44 %, respectively) among all coral samples.

Comparison between whole and cultured bacterial communities

Twenty-four bacterial isolates cultured on ½ MA from coral samples BC12D (A1-A8), BC12HD (B1–B8), and BC17H (C1–C8) tissues were sequenced; isolates A3, B5, and C1 were discarded because of either poor sequence quality or chimera formation. Isolates were also fingerprinted using LH-PCR, returning a total of 26 different amplicons that ranged in size from 311.0 to 359.2 bp (Table 4). Only one isolate, C8, was associated with a single amplicon (334.3 bp). All other isolates contained 2–6 amplicons at varying levels of relative abundance (Table 4). In isolate A5, two amplicons were approximately equally abundant; all others contained a single amplicon that was ≥52 % relative abundance. We identified the most abundant amplicon from each picked colony, thereby equating to 7 different OTUs associated with 21 bacterial colonies.

All amplicons identified from the cultured community ($N = 26$) matched amplicons from the whole coral-associated bacterial community fingerprint ($N = 67$; see arrows in Fig. 1). Thus, 39 % of bacterial OTUs associated with samples of *M. annularis*, as identified by LH-PCR, were cultured on ½ MA. Many of these cultured OTUs, such as 334.3, 335.8, 342.1, and 352.4 bp, corresponded to highly abundant members of the whole community (Fig. 1). Summing the relative abundances of OTUs from the whole coral-associated bacterial community that were matched by OTUs from bacterial isolates indicated 71.2 % of the coral-associated bacterial community abundance was represented by bacteria cultured on ½ MA. Comparing the most abundant amplicon identified from each bacterial colony ($N = 7$; Table 4) to the whole coral-associated bacterial community fingerprint suggests 10.4 % of the coral-associated bacterial OTUs, as identified by LH-PCR, can be cultured on ½ MA. Further, these cultured amplicons matched amplicons from the whole coral-associated bacterial community whose combined relative abundance totaled 36.4 %.

Ninety-five percent of the 16S rRNA gene sequences associated with these isolates were at least 94 % similar to previously identified, cultured, and uncultured bacteria in the NCBI and RDP 10 databases (Table 5); only colony B2 returned a sequence whose maximum identity was <94 %. Taxonomic assignments via BLAST and the RDP10 Classifier were in perfect agreement to the level of family. Isolates were assigned to the families *Alteromonadaceae, Bacillaceae, Pseudoalteromonadaceae, Sphingomonadaceae,* or *Vibrionaceae* (Table 5).

Cultured bacterial abundances

Enumeration of the bacterial communities cultured from *M. annularis* colonies, regardless of sample type, was larger than those cultured from either sediment or water (Fig. 3). Differences among the grand means (CFU g^{-1} or CFU mL^{-1} ± 95 % CI) from each environmental sample

Table 3 One-way similarity percentages (SIMPER) values determined from the Bray–Curtis resemblance matrix created from the normalized LH-PCR dataset of the whole coral-associated bacterial community

Coral sample	OTU 1 (% contribution)	OTU 2 (% contribution)	OTU 3 (% contribution)	Average similarity (S) or dissimilarity (D)
D	335.8 (15.5)	342.8 (13.6)	342.1 (12.4)	$S = 35.3$
HD	335.8 (28.6)	342.8 (21.8)	342.1 (9.8)	$S = 35.3$
H	335.8 (26.3)	342.8 (15.1)	334.3 (10.13)	$S = 30.9$
D + HD	335.8 (17.5)	342.8 (11.4)	342.1 (6.6)	$D = 67.6$
D + H	335.8 (18.7)	342.8 (9.3)	342.1 (6.6)	$D = 69.2$
H + HD	335.8 (22.6)	342.8 (11.5)	342.1 (6.8)	$D = 66.0$

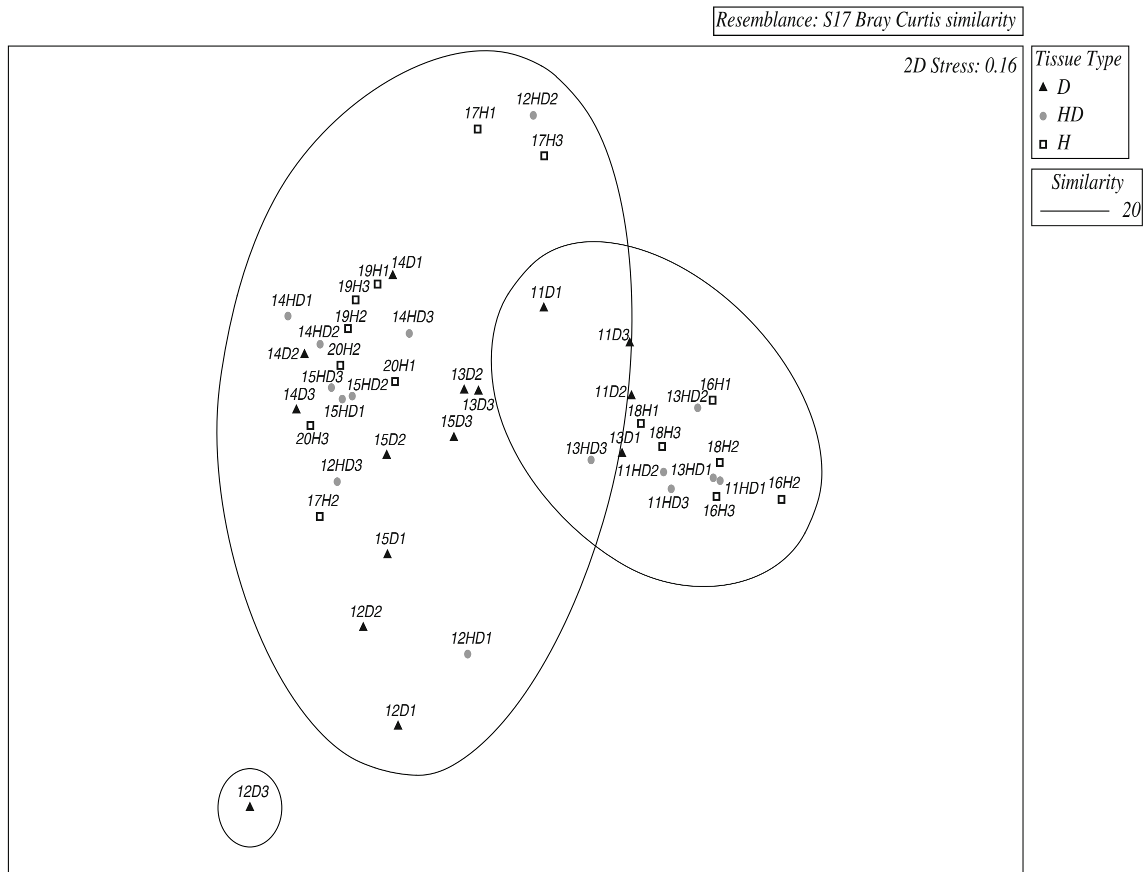

Fig. 2 Nonmetric multidimensional scaling (NMDS) plot of LH-PCR community fingerprints. Clusters imply compositional similarity among bacterial communities associated with D, HD, and H coral tissues yet suggest differences based on coral pair. Contours were generated by a hierarchical cluster analysis conducted on a Bray–Curtis similarity matrix for LH-PCR data

(e.g., all coral, all sediment, and all water) were statistically significant (½ MA: $H = 33.86$, $df = 2$, $p = 0.00$; TCBS: $H = 25.61$, $df = 2$, $p = 0.00$). M–W U tests indicate the bacterial community cultured from all corals (3.05E+08 ± 8.53E+07) is significantly larger than the cultured community associated with all sediment (3.08E+06 ± 1.66E+06) (Fig. 3). Furthermore, grand means from all coral and sediment samples were significantly larger than all water samples (6.89E+02 ± 5.52E+02). This pattern was repeated among the grand means of presumptive *Vibrio* spp. (TCBS), except no significant difference was detected between coral (4.40E+07 ± 4.54E+07) and sediment (5.53E+05 ± 9.47E+06) ($U = 183.00$, $z = -0.92$, $p = 0.36$).

There were no significant differences among the combined means of cultured bacterial abundances (½ MA) associated with all D (2.95E+08 ± 1.45E+08), all HD (3.80E+08 ± 1.85E+08), and all H (2.42E+08 ± 1.38E+08) coral samples ($H = 1.34$, $df = 2$, $p = 0.51$) (Fig. 3). Differences among the Log_{10}-transformed combined means of presumptive *Vibrio* spp. cultured from D (6.24E+00 ± 0.55), HD (6.48E+00 ± 0.91), and H (5.34E+00 ± 0.67) were significant ($F = 3.14$, $p = 0.05$). However, Tukey's HSD revealed the mean differences between HD and H (1.13, $p = 0.06$), D and HD (−0.24, $p = 0.87$), and D and H (0.89, $p = 0.16$) were not significant.

Histopathology

Results of these examinations will be reported fully in another paper, but three observations were made that are relevant to the interpretation of the microbiological results reported here.

First, obtaining the core from the coral colonies damaged the margin, leading to crushing of tissue and autolysis. A thick layer of mucus was also observed on the surface of the epidermis in many cores. In some cores, liquefaction and release of the gastrodermis from the mesoglea were present within the middle of the core. However, for each pair of cores from each colony, the reaction to sampling was usually similar.

Second, agarose enrobing successfully captured tissue remnants at the edge of the freshly denuded skeleton, including necrotic coral cells remaining deep within

Table 4 The number and length, in base pairs (bp), of amplicons associated with 21 bacterial isolates cultured from diseased (D), apparently healthy on diseased (HD), and apparently healthy (H) coral samples collected from *M. annularis* colony pair BC12/17

Colony	Sample type	Amplicon length (% relative abundance)					
A1	D	335.8* (0.59)	336.8 (0.09)	342.1 (0.07)	352.4 (0.24)		
A2	D	311.0 (0.14)	311.9* (0.86)				
A4	D	335.8* (0.77)	336.8 (0.20)	339.3 (0.01)	342.1 (0.01)	346.8 (0.01)	
A5	D	337.8 (0.01)	340.5 (0.01)	349.3 (0.47)	350.0* (0.50)		
A6	D	333.3 (0.11)	334.3* (0.81)	342.1 (0.04)	346.8 (0.02)	352.4 (0.01)	
A7	D	329.8 (0.13)	333.3 (0.17)	334.3 (0.06)	335.8* (0.57)	336.8 (0.06)	
A8	D	333.3 (0.10)	334.3* (0.66)	335.8 (0.24)			
B1	HD	334.3 (0.03)	335.8* (0.82)	336.8 (0.15)			
B2	HD	323.8 (0.03)	334.3 (0.04)	335.8 (0.34)	339.3 (0.01)	342.1 (0.06)	352.4* (0.52)
B3	HD	352.4* (0.85)	353.5 (0.12)				
B4	HD	342.1 (0.01)	352.4* (0.98)				
B6	HD	324.8* (0.77)	326.4 (0.18)	331.7 (0.03)			
B7	HD	335.8 (0.01)	341.5 (0.01)	348.4 (0.02)	349.3 (0.10)	350.0* (0.85)	
B8	HD	344.4 (0.03)	351.3 (0.16)	352.4* (0.80)			
C2	H	348.4 (0.01)	352.4* (0.87)	353.5 (0.10)			
C3	H	340.5 (0.02)	342.1 (0.03)	352.4 (0.01)	359.2* (0.93)		
C4	H	324.8 (0.01)	334.3* (0.98)	353.5 (0.01)			
C5	H	334.3* (0.95)	344.4 (0.05)				
C6	H	334.3 (0.03)	336.8 (0.03)	340.5 (0.01)	342.1 (0.03)	346.0 (0.02)	352.4* (0.89)
C7	H	333.3 (0.01)	334.3 (0.01)	335.8 (0.02)	337.8 (0.02)	342.1 (0.03)	359.2* (0.89)
C8	H	334.3* (1.00)					

* Amplicon with highest relative abundance

corallites, whereas the cells were no longer present in the shallower areas of corallites or on the coenosteal surfaces.

Third, the necrotic, lysed tissue deep within the corallites appeared to be covered with slightly curved, short, rod-shaped bacteria. These suspect bacterial cells were also present within the more intact tissues at the tissue-loss margin and into the gastrodermis of still intact polyps within the sampled cores from colonies BC12 and 14. More diverse populations of microalgae and bacteria were present at the lesion margins of BC11, 13, and 15; the microbial community of BC11 was visually most different from the others.

Discussion

Whole bacterial community fingerprints

It is recognized that diverse groups of bacteria inhabiting corals can have similar LH-PCR signatures (Mills et al. 2007). Consequently, the data reported here could underestimate the true diversity of bacterial communities associated with the samples of *M. annularis*. Conversely, it is also possible for a single organism to have different-sized amplicons due to multiple 16S rRNA gene operons (Crosby and Criddle 2003). This, in turn, could overestimate diversity. As such, subsequent interpretations of the LH-PCR dataset have been framed within these parameters. Despite these restrictions, LH-PCR has proven to be a profiling method that can be reproducibly used to discern shifts in the composition of bacterial communities (Mills et al. 2007).

Our results imply that the bacterial communities associated with all diseased (D), apparently healthy on diseased (HD), and apparently healthy (H) coral samples collected during this investigation were similar in composition. No single OTU dominated a tissue type. Rather, the same three OTUs were the most abundant in all coral samples, albeit at relatively low proportions. Subtle changes in bacterial community composition were detected but these differences existed primarily among the least abundant members and were determined not to be statistically significant.

These findings suggest it is possible for a bacterial consortium normally populating healthy *M. annularis* colonies to be altered and that such changes could generate lesions resembling WP, thereby making small compositional differences ecologically significant (Bourne et al. 2008). However, a paucity of baseline information about the composition and ecology of bacterial communities associated with apparently healthy corals complicates understanding and interpreting microbial dysbiosis in diseased corals (Rosenberg et al. 2007). Additional research has also revealed various etiologies of coral diseases, some

Table 5 BLASTN results (Megablast algorithm, nr/nt database) for 21 select bacterial isolates cultured from diseased (D), apparently healthy on diseased (HD), and apparently healthy (H) coral samples

Sample	Tissue type	Amplicon length	Taxonomic assignment (family)	Max. score	Accession #	E-value	Max. identity (%)
A1	D	335.8	Pseudoalteromonadaceae	440	JX284099	3.00E−120	94
A2	D	311.9	Sphingomonadaceae	488	JX284100	8.00E−135	99
A4	D	335.8	Pseudoalteromonadaceae	492	JX284101	7.00E−136	98
A5	D	350.0	Pseudoalteromonadaceae	516	JX284102	4.00E−143	97
A6	D	334.3	Alteromonadaceae	514	JX284103	2.00E−142	99
A7	D	335.8	Pseudoalteromonadaceae	470	JX284104	3.00E−129	98
A8	D	334.3	Pseudoalteromonadaceae	449	JX284105	4.00E−123	97
B1	HD	335.8	Pseudoalteromonadaceae	521	JX284106	9.00E−145	99
B2	HD	352.4	Vibrionaceae	316	JX284107	5.00E−83	85
B3	HD	352.4	Vibrionaceae	562	JX284108	6.00E−157	99
B4	HD	352.4	Vibrionaceae	549	JX284109	4.00E−153	99
B6	HD	324.8	Pseudoalteromonadaceae	523	JX284110	2.00E−145	99
B7	HD	350.0	Vibrionaceae	544	JX284111	2.00E−151	99
B8	HD	352.4	Vibrionaceae	538	JX284112	1.00E−149	99
C2	H	352.4	Vibrionaceae	553	JX284113	3.00E−154	100
C3	H	359.2	Bacillaceae	558	JX284114	8.00E−156	99
C4	H	334.3	Alteromonadaceae	505	JX284115	9.00E−140	99
C5	H	334.3	Alteromonadaceae	494	JX284116	2.00E−136	97
C6	H	352.4	Vibrionaceae	545	JX284117	5.00E−152	99
C7	H	359.2	Bacillaceae	510	JX284118	2.00E−141	99
C8	H	334.3	Alteromonadaceae	497	JX284119	1.00E−137	99

All samples were obtained from *M. annularis* colony pair BC12/17

pointing beyond bacteria as causative agents (Geiser et al. 1998; Ben-Haim et al. 2003; Ainsworth et al. 2008; Sussman et al. 2008; Toledo-Hernandez et al. 2008; Vega Thurber et al. 2009).

Our data also indicated that an OTU bearing the identical amplicon length as the $WP1^T$ pathogen, *A. coralicida*, did not dominate the diseased coral-associated bacterial community. Rather, an OTU or multiple OTUs bearing similar amplicon length profiles as *A. coralicida* were associated with WPL lesions as well as apparently healthy coral samples (Richardson et al. 2005). Given the inherent taxonomic limitations of LH-PCR, we cannot confirm whether these OTU(s) are *A. coralicida* or not. The application of other molecular techniques, such as next-generation sequencing, would prove valuable for resolving issues of taxonomic identity among the least abundant members of the coral-associated bacterial community.

Histopathology

Histopathological observations support LH-PCR results in which (1) most coral samples clustered together, (2) BC11D had a different microbial community than the other coral samples, and (3) one of the BC13D histopathology samples was different from the other (i.e., closer to BC11 in microbial community similarity). Light microscopy revealed the SML layer was trapped in all samples but some differences in mucocyte discharge and staining quality of the mucus were seen.

Culturing coral-associated bacteria

Thirty-nine percent of the bacterial taxa described using LH-PCR was cultured on ½ MA. This is of interest because some investigations have reported ≤1 % of bacteria from environmental samples, notably soil and water, are culturable (Amann et al. 1995; Koren and Rosenberg 2006). The levels of nutrients and labile organic compounds found in tropical sea water are known to be markedly lower than in coral tissue and mucus (Ducklow and Mitchell 1979; Mosley and Aalbersberg 2003; Ritchie and Smith 2004). The basic structure of the coral SML is secreted as an insoluble, hydrated glycoprotein composed of proteins, lipids, and polysaccharides (Ducklow and Mitchell 1979; Furnas 1992; Mosley and Aalbersberg 2003; Ritchie and Smith 2004). Such a relatively abundant and diverse food source is apt to attract and support larger, more diverse populations of microflora and may explain the high bacterial abundances reported here and previously (Koren and Rosenberg 2006). Therefore, our results seem reasonable

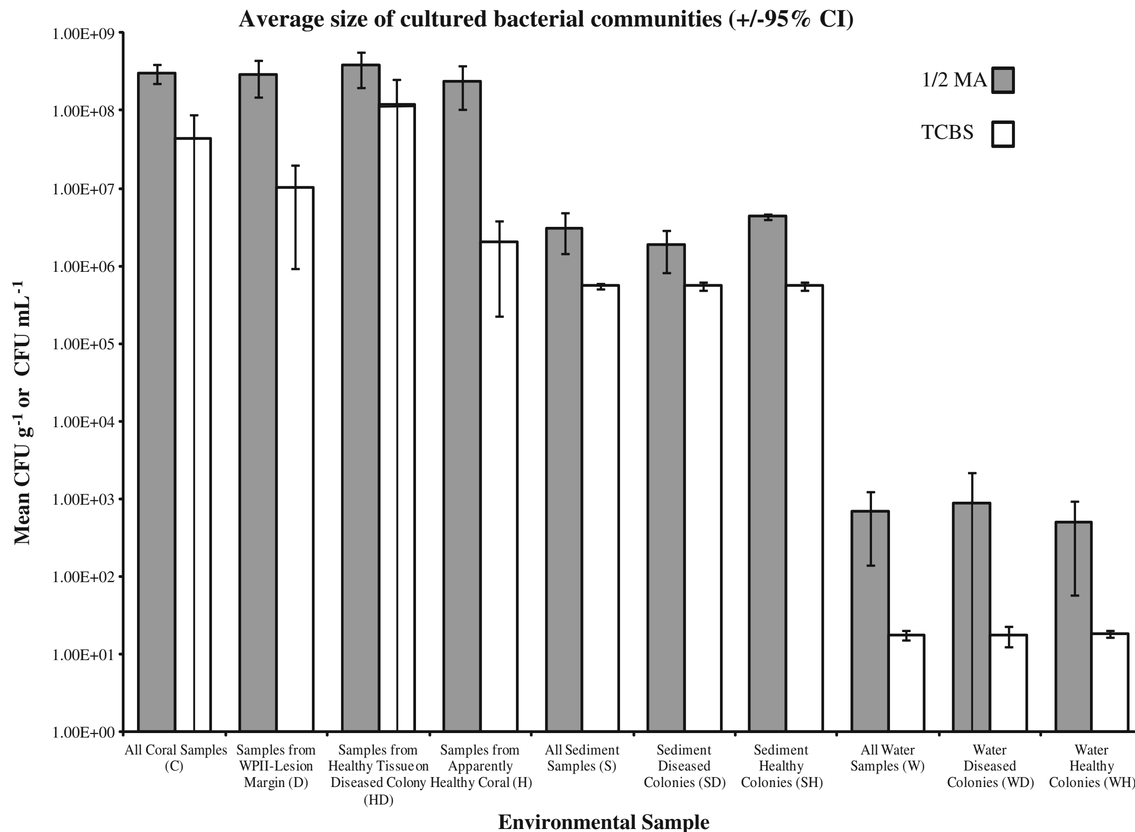

Fig. 3 Grand and combined mean comparisons of colony-forming units (CFU) from coral, sediment, and water samples cultured on one-half strength Marine Agar (½ MA) and Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS). Grand means were calculated from all samples collected in each environmental category. The combined means were tallied from all samples only associated with each coral tissue type (e.g., D, HD, or H)

given the greater organic content of a coral's tissues and SML as well as the possibility of dissolved labile organic carbon or other chemicals being leaked by endosymbiotic and/or adjacent macroalgae (Hamdan and Jonas 2006; Kline et al. 2006; Morrow et al. 2011).

Culturing on ½ MA growth plates also enabled taxonomic identification of select colonies. Our results suggest the cultured bacterial community may be dominated by two bacterial classes: *Alpha- and Gammaproteobacteria*, both of which contain known pathogens of corals and their endosymbiotic algae (Richardson et al. 1998b; Sussman et al. 2008). Colony A2, an *Alphaproteobacteria* in the genus *Erythrobacter*, is of particular interest. *Erythrobacter* was one of three genera originally assigned to the 16S rRNA gene sequence obtained from the disease isolate associated with the 1995 WPII outbreak among populations of *D. stokesii* in the Florida Keys (Richardson et al. 1998b).

Colony A2 exhibited a golden orange pigment and was cultured under light conditions in an aerobic environment. Previous reports have indicated the closest known species to the original WPII-disease isolate was *Erythrobacter litoralis* (Richardson et al. 2001). However, the reason cited for excluding both *Erythrobacter* and *Erythromicrobium* as possible candidates for $WP1^T$ pathogen was because the isolate did not express bacteriochlorophyll (BChl) *a* or *b* (Richardson et al. 1998b, 2001). Richardson et al. (2001) provided additional justification for excluding *Erythrobacter litoralis* as the WPII pathogen because the oxygen content at the lesion edge is fully aerobic and concluded that the WPII-disease isolate was not a member of the anaerobic *Erythrobacter*.

Yurkov et al. (1998) report that *E. litoralis* is an obligate aerobic phototroph. Furthermore, some of the main features distinguishing obligate aerobic bacteria containing BChl *a* from typical anoxygenic phototrophs are (1) the inability to grow photosynthetically under anaerobic conditions; (2) low level of photosynthetic units in cells; and 3) the strong inhibition of BChl synthesis by light (Harashima et al. 1987; Yurkov and Gemerden 1993; Shimada 1995; Yurkov and Beatty 1998; Yurkov et al. 1998). Bacterial isolates from the initial WPII outbreak were cultured on Marine Agar growth plates under light conditions (Richardson et al. 1998a, b). Therefore, it is possible that the WPII-disease isolate, $WP1^T$, did not produce BChl *a* or *b*. Further, the complete genome of *E. litoralis* HTCC2594 was recently sequenced and revealed this strain lacked genes for the production of BChl *a*, photosynthetic reaction

center proteins, and to be devoid of phototrophic potential (Oh et al. 2009).

In conclusion, outbreaks of lesions exhibiting signs consistent with variants of WP continue to occur on reefs around the Wider Caribbean Region, yet no recurrence of this disease as an epizootic in Florida has been reported since 1995 (Richardson and Aronson 2000; Croquer et al. 2003; Miller et al. 2003; Weil 2004; Brandt and McManus 2009). Nevertheless, lesions with a gross morphology resembling WPII have been consistently documented among Florida's coral populations (Santavy et al. 2006; Brandt 2007). Interestingly, *A. coralicida* has reportedly been isolated or identified from WPII-affected colonies of *D. stokesi* on reefs in Florida and The Bahamas as well as other coral species from St. John, USVI, and Curaçao (Richardson and Aronson 2000; Miller et al. 2003; Nugues et al. 2004; Richardson et al. 2005). However, presumably healthy corals inoculated with either the original laboratory or newly isolated stains of this pathogen have failed, and continue to fail, to develop signs of disease (Richardson and Aronson 2000; L. Richardson, pers comm).

Similarly, Rosenberg et al. (2009) noted the inability of a previously documented coral pathogen, *Vibrio shiloi*, to cause bleaching among colonies of the once susceptible coral *Oculina patagonica*. Evidence cited for the development of *O. patagonica*'s resistance includes the inability to isolate *V. shiloi* from bleached or bleaching corals, the inability of the once pathogenic laboratory strain of *V. shiloi* to cause bleaching in *O. patagonica* under laboratory conditions, and the intracellular destruction of *V. shiloi* cells after adhering to and penetrating the coral's epidermis (Reshef et al. 2006). Collectively, these findings, in conjunction with the results reported here, raise questions about *A. coralicida*'s virulence, its continued ability to cause disease, and host immunity (Richardson and Aronson 2000; Rosenberg et al. 2009).

Data from our investigation also provide evidence that supports the use of culture-dependent techniques as a valuable method for conducting research on coral-associated bacterial communities and should not be completely abandoned in favor of culture-independent methods. Further, our findings highlight a need for testing new hypotheses that aim to determine the etiology and pathogenesis of a suite of diseases producing lesions with a gross morphology resembling a variant of WP. If signs of rapid tissue loss resembling this disease are, in fact, the result of various causal mechanisms—as has been alluded to by Pantos et al. (2003), Bythell et al. (2004), Ainsworth et al. (2007), Sunagawa et al. (2009), and this study—then future investigations should consider the concurrent application of differential diagnostic techniques to discriminate among the growing list of coral diseases, ascertain causation, reveal drivers of microbial dysbiosis, and inform resource managers as part of a concerted effort to protect and manage coral reefs worldwide.

Acknowledgments This research was funded by the National Oceanic and Atmospheric Administration's, National Undersea Research Program (US Department of Commerce Award No. NA06RU0228 and No. NA16RU1496), the Perry Institute for Marine Science, Caribbean Marine Research Center (CMRC-03-NRTJ-01-04A), The Project AWARE Foundation, and The Explorers Club Washington Group, Inc. The authors wish to express their gratitude to the Microbiome Analysis Center at George Mason University for its support as well as the laboratory of Dr. L. Richardson for providing pure cultures of the WP1^T pathogen. Three anonymous reviewers and L.R. are also greatly thanked for providing valuable comments during the preparation of this manuscript. The findings and conclusions in this article are those of the author(s) and do not necessarily represent the views of the U.S. Fish and Wildlife Service. Any use of trade, product, website, or firm names in this publication is for descriptive purposes only and does not imply endorsement by the U.S. Government.

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