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ORIGINAL ARTICLE

Bacterial diversity and White Plague Disease-associated community changes in the Caribbean coral *Montastraea faveolata*

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Increasing evidence confirms the crucial role bacteria and archaea play within the coral holobiont, that is, the coral host and its associated microbial community. The bacterial component constitutes a community of high diversity, which appears to change in structure in response to disease events. In this study, we highlight the limitation of 16S rRNA gene (16S rDNA) clone library sequencing as the sole method to comprehensively describe coral-associated communities. This limitation was addressed by combining a high-density 16S rRNA gene microarray with, clone library sequencing as a novel approach to study bacterial communities in healthy versus diseased corals. We determined an increase in diversity as well as a significant shift in community structure in *Montastraea faveolata* colonies displaying phenotypic signs of White Plague Disease type II (WPD-II). An accumulation of species that belong to families that include known coral pathogens (Alteromonadaceae, Vibrionaceae), bacteria previously isolated from diseased, stressed or injured marine invertebrates (for example, Rhodobacteraceae), and other species (for example, Campylobacteraceae) was observed. Some of these species were also present in healthy tissue samples, but the putative primary pathogen, *Aurantimonas corallicida*, was not detected in any sample by either method. Although an ecological succession of bacteria during disease progression after causation by a primary agent represents a possible explanation for our observations, we also discuss the possibility that a disease of yet to be determined etiology may have affected *M. faveolata* colonies and resulted in (or be a result of) an increase in opportunistic pathogens.
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Introduction

Reef-building corals are associated with a dynamic, highly diverse consortium of microorganisms that includes protists, bacteria, archaea and endolithic algae and fungi (Shashar and Stambler, 1992; Bentis *et al.*, 2000; Rohwer *et al.*, 2002; Baker, 2003; Kellogg, 2004; Wegley *et al.*, 2004; Rosenberg *et al.*, 2007; Harel *et al.*, 2008). To study the phylogenetic diversity of the bacterial and archaeal components, sequencing the 16S rRNA gene (16S rDNA) is commonly used because of its ability to identify the species without the need for laboratory cultivation. Representative studies have uncovered that (i) different corals appear to harbor distinct and highly diverse bacterial communities (Rohwer *et al.*, 2002; Bourne and Munn, 2005), (ii) the overlap between bacteria inhabiting the water column and the coral host is small (Frias-Lopez *et al.*, 2002) and (iii) the community of coral-associated bacteria undergoes changes in response to stress or disease (Cooney *et al.*, 2002; Frias-Lopez *et al.*, 2002; Pantos *et al.*, 2003; Pantos and Bythell, 2006). Despite these advances, only a handful of primary coral pathogens have been identified to date (reviewed in Rosenberg *et al.*, 2007). Furthermore, the description of many coral diseases is often confounded by the lack of clear diagnostic criteria so that similar disease signs may emerge in multiple coral species, whereas a putative pathogen has only been verified for one or a subset of species (Richardson, 1998; Pantos *et al.*, 2003;

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Sutherland *et al*., 2004). For example, White Plague Disease type II (WPD-II) has been reported to affect more than 40 different coral species (Weil *et al*., 2006), whereas the bacterial pathogen *Aurantimonas corallicida* isolated from *Dichocoenia stokesi* (Richardson *et al*., 1998a, b; Denner *et al*., 2003) is the only example for which Koch's postulates have been fulfilled.

It is known that diseases may result from complex interactions between host, causative agent(s) and the environment (Martin *et al*., 1987) and it has been suggested that profiling the host microbiota will play an important role in better understanding coral diseases (Work *et al*., 2008). The technology of 16S rRNA gene microarrays performs massively parallel assays in a single experiment (Gentry *et al*., 2006), and thus represents a powerful tool to profile host microbiota at different health states. The most comprehensive 16S rRNA gene microarray to date (PhyloChip G2) consists of approximately 300 000 oligonucleotide probes assaying 8741 operational taxonomic units (Wilson *et al*., 2002; DeSantis *et al*., 2007). Although this technology may not be suitable for discovering novel taxa, recent studies have shown both its advantage over 16S rDNA library sequencing (DeSantis *et al*., 2007) and applicability to a variety of environmental samples including subsurface water, urban aerosols or uranium contaminated soil (Brodie *et al*., 2006, 2007; DeSantis *et al*., 2007). Introducing this cost-effective technology to the field of coral microbiology may not only help to better understand the diversity and community structure of coral-associated microbiota, but also to delineate various pathologies with similar disease signs but different etiologies.

In this study, PhyloChip hybridization data combined with 16S rDNA clone library sequences from a single colony of the Caribbean coral *Montastraea faveolata* suggests that corals represent an under-sampled environment, which can be expected to include a high level of novel bacterial species. A combined analysis of PhyloChip and 16S rDNA sequence data using *M. faveolata* samples that displayed phenotypic signs of WPD-II following a coral bleaching episode in 2005 revealed a significant shift in community structure in response to disease, with an accumulation of ribotypes that were similar to pathogens or bacteria previously isolated from diseased, injured or stressed marine invertebrates. The putative primary pathogen *A. corallicida*, however, was not identified. Based on our results, we discuss the possibility that *M. faveolata* colonies may have been affected by a disease of yet unknown etiology that may have resulted in (or be a result of) an increase in opportunistic pathogens.

Materials and methods

Sample collection

For in-depth sequencing of bacteria associated with *Montastraea faveolata*, we selected one coral fragment (collected near Isla San Cristobal in August 2006 at Bocas del Toro, Panamá) that was acclimated in a seawater tank for 23 days and shipped frozen to the University of California Merced (Merced, USA). *M. faveolata* colonies displaying visible signs of White Plague Disease type II (Richardson *et al*., 1998a), were sampled at two reefs, 'Turrumote' (17°56.097′ N, 67°01.130′ W) and 'The Buoy' (17°56.038′ N, 66°59.090′ W), off La Parguera on the southwest coast of Puerto Rico during disease outbreaks after the 2005 bleaching event in January (Turrumote: WP1-J, WP2-J) and May 2006 (The Buoy: WP3-M, WP4-M), respectively. In parallel, control samples from healthy looking colonies were collected (H1-J, H2-M, H3-M, H4-M). All samples were collected using a hammer and chisel at 10–20 m depth and immediately frozen in liquid nitrogen. Care was taken to keep seawater inclusion to a minimum.

DNA extraction and PCR amplification of 16S rDNA

On dry ice, coral tissue/skeleton was chiseled off the first 0.5–1 cm from the surface and ground to powder using a sterile mortar and pestle. The lesion boundary in diseased corals was sampled so that approximately equal amounts originated from healthy looking and white, tissue-devoid parts. For in-depth sequencing, about 125 mg of powder was added to 600 µl cell lysis buffer (100 mM NaCl, 100 mM Tris-Cl, 25 mM EDTA, 0.5% SDS, 500 µg ml^{-1} Proteinase K, pH 8.0) before incubation at 55 °C for 16 h. 8 µl RNAse A (1 mg ml^{-1}) was added before incubation at 37 °C for 60 min. Proteins and skeletal debris were removed after adding 1/3 vol. protein precipitation solution (4 M guanidine thiocyanate, 0.1 M Tris-Cl, pH 7.5) and centrifugation at 16 000 *g* for 5 min. The DNA was recovered after precipitation using 1 vol. isopropanol and two ethanol (70%) washes. PCR conditions were the same as described below with the exception of using the reverse primer 1492R(Y) (5′-CGGYTACCTTGTTACGACTT).

For healthy and diseased tissue samples, approximately 50 mg of powder was applied to the PowerPlant DNA extraction kit (MoBio Laboratories, Carlsbad, CA, USA), which removed PCR inhibitors more efficiently than other protocols (phenol/chloroform or proteinase K/guanidine thiocyanate-based protocols, UltraSoil kit (MoBio), CTAB/PVP-based protocols). We modified the manufacturer's instructions by: (a) adding lysozyme (Epicentre; final: 10 U µl^{-1}) to the Bead Solution/sample mixture, followed by an incubation of 10 min, (b) adding 25 µl proteinase K (20 mg ml^{-1}) to the lysozyme-treated mixture, followed by incubation at 65 °C for 10 min and (c) bead-beating on a Vortex Adapter (MoBio) for 15 instead of 10 min.

For the amplification of 16S rRNA genes, we used 50 ng DNA and universal bacteria-specific primers 27F (5′-AGAGTTTGATCCTGGCTCAG) and 1492R (5′-GGTTACCTTGTTACGACTT) in 25 µl PCRs containing 12.5 µl Buffer G (Epicentre), 1 µM of each

primer and 2.5 U REDgDNA Taq polymerase (Sigma-Aldrich, St Louis, MO, USA). We ran and pooled gradient PCRs (95 °C—3 min; 95 °C—1 min; 48–62 °C—1 min, 72 °C 2 min (25 ×); 72 °C 10 min) to increase the diversity of amplified 16S rRNA genes.

16S rDNA cloning, sequencing, assembly, classification and annotation

One 16S rDNA library ($N=943$) for in-depth sequencing of *M. faveolata*-associated bacteria and pooled libraries from four healthy (H) ($N=317$) and four diseased ($N=340$) samples were generated using the TOPO-TA cloning kit (pCR4-TOPO vector, Invitrogen). Selected clones were bi-directionally sequenced using the primers T3 (5′-ATTAACCCTC ACTAAAGGGA) and T7 (5′-TAATACGACTCACTAT AGGG) on an ABI3700 sequencer (Applied Biosystems, Foster City, CA, USA) at the DOE Joint Genome Institute (http://www.jgi.doe.gov/). Bases were called using Phred (Ewing and Green, 1998; Ewing *et al.*, 1998) and processed by Perl scripts that sequentially assembled the reads into 16S rDNA contigs, removed vector sequence, end-trimmed low quality bases and removed short as well as overall low quality sequences.

For the comparison of clone library and PhyloChip data, sequences were processed through tools available at http://greengenes.lbl.gov (DeSantis *et al.*, 2006b). Briefly, 16S rDNA sequences were aligned using NAST (DeSantis *et al.*, 2006a) and chimera-checked using Bellerophon (Huber *et al.*, 2004). Similarities to publicly available sequences were calculated using the DNADIST tool of the Phylip package (DNAML-F84 option; transition/transversion ratio: 2.0; A, C, C, G frequencies: 0.2537, 0.2317, 0.3167 and 0.1979, respectively; lane mask (Lane, 1991) used). For classification purposes, sequences were assigned to the taxonomic ranks 'Phylum', 'Class', 'Order', 'Family', 'Subfamily' and OTU (operational taxonomic unit), when the similarity to database records was equal to or higher than 80, 85, 90, 92, 94 and 97%, respectively (DeSantis *et al.*, 2007).

For class level comparison of clone library sequences from healthy and diseased tissues, sequences were classified as described above as well as according to nearly full-length 16S rDNA sequences using tools of the Ribosomal Database Project II (Cole *et al.*, 2007). Sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers: FJ202063–FJ203662.

Species richness estimates

A clone distance matrix was generated online at http://greengenes.lbl.gov and used to calculate non-parametric richness estimations Chao1 (Chao, 1984) and ACE (Chao and Lee, 1992) using the software DOTUR (Schloss and Handelsman, 2005) and furthest-neighbor as the clustering algorithm.

PhyloChip hybridizations

PCR amplicons from each of the four healthy and four diseased coral samples were hybridized on PhyloChips (G2). DNA quantification, fragmentation, addition of internal standards, labeling, PhyloChip hybridization, staining and scanning were performed as previously described (Brodie *et al.*, 2006). Detailed information on oligonucleotide probe selection and array design can be found in Brodie *et al.* (2006, 2007). Probe pairs scored as positive were those that met two criteria: (i) the intensity of fluorescence from the perfectly matching probe was greater than 1.3 times the intensity from the mismatching control and (ii) the difference in intensity, perfectly matching minus mismatching, was at least 130 times greater than the squared noise value ($>130\,N^2$). When summarizing PhyloChip results, all positive probe sets (pF $\geqslant 0.9$) at the OTU level were summarized to the bacterial family level (92% similarity).

Data analysis

Normalized intensity values were $\log_2$ transformed before PhyloChip hybridization data were analyzed using the TM4 software (Saeed *et al.*, 2003). Hierarchical clustering was done using the average linkage method and Euclidean distance matrix. The Kolmogorov–Smirnov test for equality of variances between healthy and diseased samples was performed in SigmaStat (Systat Software Inc.) and failed ($P<0.05$). Consequently, differentially abundant OTUs between healthy and diseased samples were tested using an unpaired *t*-test assuming unequal variance (Welch's approximation).

Results

Diversity and novelty of bacteria associated with Montastraea faveolata

The sequenced clone library ($N=943$) from a single *Montastraea faveolata* colony represented more than 10 times the data reported for libraries in previous coral microbiological studies (Frias-Lopez *et al.*, 2002; Rohwer *et al.*, 2002; Bourne and Munn, 2005; Pantos and Bythell, 2006; Barneah *et al.*, 2007). Here, 178 unique OTUs were detected and diversity estimates ranged from 307 to 329 ribotypes according to Chao1 and ACE, respectively. The rarefaction curve did not reach an asymptote (Figure 1) indicating insufficient sampling to capture the total diversity of the bacterial community.

Based on nearly full-length sequences, only 7.0% of the 16S rDNA sequences were classified at the OTU level (Table 1). At a decreasing taxonomic resolution, however, the number of classified sequences increased continuously to 99.7% at the phylum level. For example, more than 70% of the 16S rDNA sequences could be classified at the family level. When compared with clone library sequencing, PhyloChip hybridizations detected a

higher richness at all levels of taxonomic resolution. The taxonomic categories detected by cloning were generally found as a subset of those reported from the hybridization experiment (Table 1). As an exception to this trend, none of the 21 OTUs detected by clone library sequencing were reflected by the corresponding PhyloChip data indicating a high degree of yet uncharacterized species in coral-associated bacteria.

Comparison of bacterial diversity in healthy and diseased coral samples

PhyloChip data from healthy ($N=4$) and diseased ($N=4$) coral samples were pooled according to health state and analyzed for abundances of unique taxa in only healthy, or only diseased samples (Table 2). At all taxonomic levels, the majority of members could be found in both healthy and diseased coral samples. The relative abundance of taxon members in diseased corals was always higher than in healthy corals. For example, out of 8741 OTUs assayed by the current generation of the PhyloChip (G2), a total of 1702 were detected (pf $\geqslant 0.9$) across all arrays ($N=2\times4$). Approximately two-thirds of these OTUs were detected in both healthy and diseased states, 30.8% were unique to diseased corals and only 2.5% unique to healthy corals (Table 2). This trend suggests a higher level of diversity in bacterial community composition associated with the diseased samples.

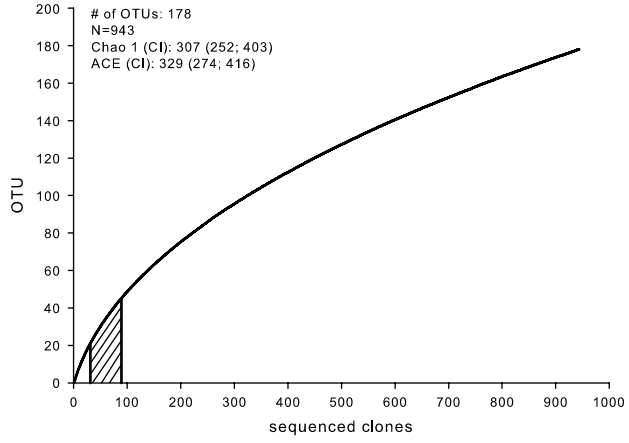

Figure 1 Rarefaction analysis for a recombinant 16SrDNA clone library ($n=943$) generated from a single *Montastraea faveolata* colony. Patterned area shows the range of typical clone library sizes from selected coral microbiological studies ((Frias-Lopez *et al.*, 2002; Rohwer *et al.*, 2002; Bourne and Munn, 2005; Pantos and Bythell, 2006; Barneah *et al.*, 2007)). Estimation of species diversity (Chao1) and abundance-based coverage estimation (ACE) are shown with 95% confidence intervals (CI). Distance matrix was generated online at http://greengenes.lbl.gov; cluster distance 0.03; rarefaction curve generated using the software DOTUR (Schloss and Handelsman, 2005).

Differentially abundant bacterial families in healthy versus diseased coral samples

Analysis of pooled clone libraries (healthy: $N=317$; diseased: $N=340$) suggested a significant difference ($P<0.05$) in the orders Rhodobacterales, Campylobacterales, Planctomycetales and Clostridiales (Table 3). PhyloChip hybridizations (healthy: $N=4$; diseased $N=4$) suggested that among a total of 275 families detected, 25 were found to contain at least one OTU that was significantly more abundant in samples from diseased corals (Table 4). Both methods agreed in finding the orders Rhodobacterales and Clostridiales as significantly more abundant in diseased samples, but an increase in Campylobacterales and a decrease in Planctomycetales as suggested by clone library data were not reflected in PhyloChip data. Although most of the sequences affiliated to Planctomycetaceae could not be classified at the genus level, all Campylobacterales belonged to the genus *Arcobacter*.

Classified 16S rDNA clone library sequences from healthy and diseased samples were mapped back to differentially abundant families and in addition compared to each other before and after filtering based on the PhyloChip results (Table 4). Twelve of the differentially abundant families contained at least one 16S rDNA representative among the combined clone libraries. With few exceptions, the

Table 1 Number of distinct taxonomic ranks identified by PhyloChip and/or clone library sequencing in a single *M. faveolata* sample

Taxonomic rank (% cutoff)	*Clones classified/not detected by PhyloChip (%)*	*Count of taxa detected by:*		
		Chip only (%)	*Chip and clone (%)*	*Clone only (%)*
Phylum ($\geqslant$80)	99.7[a]/0.5	27 (67.5)	13 (32.5)	0 (0)
Class ($\geqslant$85)	95.5/4.7	57 (72.1)	22 (27.9)	0 (0)
Order ($\geqslant$90)	79.5/20.7	113 (74.3)	37 (24.3)	2 (1.3)
Family ($\geqslant$92)	70.4/29.7	221 (83.4)	43 (16.2)	1 (0.4)
Subfamily ($\geqslant$94)	48.9/51.2	295 (90.8)	29 (8.9)	1 (0.3)
OTU ($\geqslant$97)	7.0/93.0	1441 (98.6)	0 (0)	21 (1.4)

The DNAML-F84 homology cutoff used for taxonomic rank assignment of 16S rDNA clones is shown in parentheses. Percentages of classifiable and not detected 16S rDNA clones are based on the total number of sequenced clones ($N=943$). Counts and percentages of distinct taxonomic rank members detected by PhyloChip hybridization, clone library sequencing and a combination of both methods are shown.
[a]Three sequenced clones (0.3%) could not be classified at a level of $\geqslant$80% to the reference set. (FJ202339, FJ202502, FJ202893).

Table 2 Number of taxonomic rank members detected in replicate hybridizations from healthy and diseased coral samples

Taxonomic rank (% cutoff)	*Healthy (abs.)*	*Diseased (abs.)*	*Increase (%)*	*Healthy only (%)*	*Diseased only (%)*	*Healthy and diseased (%)*	*Total*
OTUs (97)	1178	1659	40.8	43 (2.5)	524 (30.8)	1135 (66.7)	1702
Subfamilies (94)	306	342	11.8	7 (2.0)	43 (12.3)	299 (85.7)	349
Families (92)	244	269	10.2	6 (2.2)	31 (11.3)	238 (86.5)	275
Orders (90)	146	153	4.8	5 (3.2)	12 (7.6)	141 (89.2)	158
Classes (85)	77	79	2.6	3 (3.7)	5 (6.1)	74 (90.2)	82
Phyla (80)	41	42	2.4	3 (6.7)	4 (8.9)	38 (84.4)	45

The occurrence of taxonomic rank members in replicates from only healthy, healthy and diseased or only diseased samples is shown at various levels of taxonomic ranks.

Table 3 Order-level comparison of pooled 16SrDNA clone libraries from healthy ($N=4$) and diseased ($N=4$) *M. faveolata* samples

Taxonomic rank (Phylum; Class; Order)	*Healthy (%)*	*Diseased (%)*	P-*value*
Proteobacteria; Alphaproteobacteria; Rhodobacterales	19 (6.0)	89 (25.6)	1.05×10^{-11}
Proteobacteria; Epsilonproteobacteria; Campylobacterales	0 (0)	16 (4.6)	3.23×10^{-05}
Planctomycetes; Planctomycetacia; Planctomycetales	19 (6.0)	7 (2.0)	8.30×10^{-03}
Firmicutes;Clostridia; Clostridiales	2 (0.6)	10 (2.9)	3.38×10^{-03}

Taxa with significant differences ($P<0.05$) between libraries from healthy and diseased tissues are shown with the total number and percentages (%) of clones in the respective libraries. The 16SrDNA sequences were classified and compared using the LIBCOMPARE tool of the Ribosomal Database Project II (Cole *et al.*, 2007) at a confidence threshold of 95%.

significant increase in PhyloChip signal intensities was reflected in a greater number of 16S rDNA clones when libraries from healthy and diseased corals were compared (Table 4). Although three families (Alteromonadaceae, Enterobacteriaceae and Vibrionaceae) that are known to include coral pathogens were found to be more abundant in diseased coral samples, neither PhyloChip (similarity between assayed OTU and *Aurantimonas coralicida* is approximately 99.9%; Supplementary Table S1) nor clone library data ($N=340$ sequences from diseased libraries) provided any evidence for the presence of the WPD-II causing pathogen *A. coralicida*.

Similarity searches revealed that 4.1 and 18.2% of clone libraries from healthy and diseased coral samples, respectively, matched to sequences that were previously identified in diseased, stressed or injured marine invertebrates (for details see Supplementary Table S2). After integration of our PhyloChip results, that is, if only 16S rDNA clones that were classified according to the differentially abundant families were considered, the proportion of sequences that were previously identified in diseased, stressed or injured organisms increased from 18.2 to 42.9% (Table 4). Fifty-seven out of 62 clones (92%) that were similar to bacteria associated with stressed, diseased or injured marine invertebrates were affiliated to the statistically more abundant families.

Differentially abundant family members

Among the bacteria that were affiliated with the significantly more abundant families, we found close relatives to the putative pathogen of the Pacific white syndrome in the Red Sea, *Thalassomonas loyana* (Thompson *et al.*, 2006) and *Photobacterium eurosenbergii*, a *Vibrio*-like bacterium that has been associated with coral bleaching (Thompson *et al.*, 2005). In addition, we found close relatives to bacteria that had been isolated from corals with Black Band Disease, including members of Rhodobacteraceae that are known to associate with toxic dinoflagellates (Supplementary Table S2). Other 16S rDNA sequences that were classified to the family Peptostreptococcaceae had also been previously identified in Black Band Disease tissues (Supplementary Table S2).

Two 16S rDNA ribotypes had similarities to Rhodobacteraceae members that were found in body wall lesions of the sea urchin *Tripneustes gratilla*. Both of them were present in libraries from healthy as well as diseased corals. One of them (most similar to AM930419) was found only once in both libraries, whereas the other one (most similar to AM930434) was found two times in the library from healthy, but four times more often in the library from diseased coral samples (Supplementary Table S2). *Vibrio harveyi*-like (similarity $>99.7\%$) bacteria, previously shown to be associated with pathogenesis in the turbot *Scophthalmus maximus*, were identified in both healthy and diseased coral samples and bacteria associated with skin ulceration disease and viscera ejection syndrome of the sea cucumber *Apostichopus japonicus* were exclusively found in samples from diseased corals (Supplementary Table S2).

There were also a number of other differentially abundant families that contained at least one

Table 4 Differentially abundant bacterial families in healthy versus White Plague diseased *M. faveolata* samples are shown according to Phylochip G2 results (first column)

Differentially abundant families (Phylochip: >2.0 fold change)	*No. of clones healthy* ($N_{total} = 317$)	*No. of clones diseased* ($N_{total} = 340$)
Actinobacteria; Actinomycetales; Cellulomonadaceae*	0	0
Actinobacteria; Actinomycetales; Corynebacteriaceae*	0	0
Actinobacteria; Actinomycetales; Micrococcaceae*	0	0
Actinobacteria; Actinomycetales; Unclassified*	0	1
Bacteroidetes; Bacteroidales; Bacteroidaceae*	0	0
Flavobacteria; Flavobacteriales; Flavobacteriaceae*	9	10
Bacilli; Bacillales; Bacillaceae*	1	0
Clostridia; Clostridiales; Clostridiaceae*	1	0
Clostridia; Clostridiales; Lachnospiraceae*	0	0
Clostridia; Clostridiales; Peptostreptococcaceae*	1	10
Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae**	18	89
Alphaproteobacteria; Rhodobacterales; Unclassified*	0	0
Betaproteobacteria; Burkholderiales; Comamonadaceae*	0	0
Betaproteobacteria; Hydrogenophilales; Hydrogenophilaceae*	0	0
Betaproteobacteria; Rhodocyclales; Rhodocyclaceae*	0	0
Gammaproteobacteria; Alteromonadales; Alteromonadaceae**	3	11
Gammaproteobacteria; Alteromonadales; Pseudoalteromonadaceae**	0	0
Gammaproteobacteria; Alteromonadales; Shewanellaceae*	0	0
Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae*	0	0
Gammaproteobacteria; Oceanospirillales; Halomonadaceae*	1	0
Gammaproteobacteria; Oceanospirillales; Oceanospirillaceae**	0	1
Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae*	0	2
Gammaproteobacteria; Thiotrichales; Piscirickettsiaceae*	0	0
Gammaproteobacteria; Unclassified; Unclassified**	1	3
Gammaproteobacteria; Vibrionales; Vibrionaceae**	3	6
Vibrio_vulnificus_st**	0	0
No of clones previously found in health-impaired[a] marine invertebrates/total	9/38 (23.7%)	57/133 (42.9%)

The number of 16S rDNA clones assigned to each family (second and third column) and the sum of clones with similarities to bacteria previously isolated from health-impaired marine invertebrates (last row) are shown. Families that include known coral pathogens are boldfaced.
[a]Stressed, diseased or injured.
*$P<0.01$, **$P<0.001$).

classified 16S rDNA clone from healthy or diseased coral samples. These included Flavobacteriaceae, Bacillaceae, Clostridiaceae, Pseudomonadaceae, all of which include well-studied pathogenic members. According to PhyloChip data, there were additional families that were significantly increased in diseased coral samples for which no clone library support was available (Table 4). Finally, a probe set specifically designed to detect the marine pathogen *Vibrio vulnificus* suggested an increased abundance of this bacterium (or a close relative) in diseased coral samples (see Supplementary Information file).

Discussion

PhyloChip and clone library sequencing as a dual approach to study coral microbiology

The availability of universal 16S rRNA gene primers has made it possible to amplify a mixed population of 16S rDNA molecules and to characterize the phylogenetic diversity of coral-associated bacterial communities (Rohwer *et al.*, 2002; Kellogg, 2004; Wegley *et al.*, 2004; Bourne and Munn, 2005). Rohwer *et al.* (2002) estimated a richness of more than 6000 ribotypes in three different coral species based on 1178 sequenced 16S rDNA clones (Rohwer *et al.*, 2002). Although species richness may be predicted from only a few hundred sequences, it has been suggested that identifying 50% of a community of comparable composition would have required about 30 000 sequencing reactions (Dunbar *et al.*, 2002). Our rarefaction analysis on OTUs from a single *Montastraea faveolata* colony illustrates that a comprehensive description of the high bacterial diversity harbored in corals would easily be impeded by costs of sequencing at the required depth (Figure 1). This constitutes a major problem, as entire coral populations are increasingly afflicted by disease events (Sutherland *et al.*, 2004; Weil *et al.*, 2006), so that a rapid increase in our knowledge on both the diversity of and changes in bacterial communities is of critical importance. In this study, we show that the application of the PhyloChip was suitable to (a) detect the presence of sequenced 16S rDNA clones and categorize more than 70% at the family level, (b) distinguish healthy from diseased corals based on hybridization signal profiles and (c) enrich, after statistical analysis, 16S rDNA sequences from clone libraries with similarities to bacteria that were previously isolated from diseased/stressed marine invertebrates, including known pathogens.

Coral-associated bacteria detected by PhyloChip hybridization and clone library sequencing

The PhyloChip (G2) was able to detect the presence of every phylum and class that was identified in the corresponding clone library. The same concurrence was observed for nearly all orders, families and subfamilies. At the OTU level, however, there was no match between PhyloChip data and 16S rDNA sequencing results. Besides the novelty of sequences at the OTU level, the percentages of classifiable sequences at higher levels of taxonomic resolution indicates the existence of novel families, orders and classes that are harbored by corals (Table 1). Given that the classification was based on the availability of nearly full-length ($\geqslant$1250 bp) 16S rDNA sequences as of March 2004 (DeSantis *et al.*, 2006b), the high level of novelty was not surprising. Previous studies had also reported that coral-associated bacteria shared low sequence similarities (50% of over 1000 sequences of $\sim$500 bp shared $<$93% similarity) to public database entries (Rohwer *et al.*, 2002). Furthermore, 16S rDNA sequences from coral samples have rarely been sequenced to a length exceeding 1250 bp; however, the availability of nearly full-length sequences will be critical to increase the diversity assayed by future versions of 16S microarrays.

Comparison of healthy and diseased coral tissue samples

In addition to the adverse effects of rapid climate change and ocean acidification (Hoegh-Guldberg *et al.*, 2007), coral reefs are increasingly threatened by a number of diseases (Sutherland *et al.*, 2004; Weil *et al.*, 2006). In 1995, an unusually aggressive disease outbreak affected several different coral species in the Caribbean exhibiting similar signs, namely, a prominently sharp lesion line separating healthy looking tissues from white, tissue-devoid areas progressing at a rate of up to 2 cm per day (Richardson, 1998). The same signs were observed in 16 other coral species and coined 'Plague Type II' (Richardson *et al.*, 1998a). A bacterium isolated from *Dichocoenia stokesi*, later characterized as a novel species named *Aurantimonas coralicida* (Denner *et al.*, 2003), was identified as the causative agent of White Plague Disease type II. Subsequently, other sources (Richardson *et al.*, 2005; Weil *et al.*, 2006; Rosenberg *et al.*, 2007) emphasized the notion that *A. coralicida* was a broad host-range pathogen causing White Plague-like signs in many different coral species in both the Pacific and Atlantic regions.

In this study, neither PhyloChip hybridizations nor 16S rDNA clone library sequencing indicated that *A. coralicida* was present in putatively White Plague type II-diseased *M. faveolata* colonies. Instead, we observed an increase in diversity in samples from diseased tissues (Table 2), whereas there was no overwhelming dominance by a single bacterial species, which appears to be an unexpected result for a primary infection. It has been argued that opposed to the idea of a primary infection, coral diseases may also result from unchecked growth of otherwise harmless bacteria in compromised hosts and/or due to changes in the environment, for example, increased temperatures (Harvell *et al.*, 1999, 2007; Lesser *et al.*, 2007). The identification of bacteria in healthy corals that were similar to known pathogens or bacteria that were previously isolated from diseased, stressed or injured marine invertebrates may point toward a role of latent, usually non-pathogenic commensals. We also identified many bacteria closely related or identical to pathogens of other marine invertebrates in diseased corals, which may suggest a role of exogenous opportunistic pathogens of broad host-range. Nevertheless, it should be noted that we are not able to conclude from our results whether an increased population of different bacteria is the cause or rather the result of a disease. Alternatively, colonization by opportunistic pathogens or uncontrolled growth of commensals may have taken advantage of a compromised host immune system caused by a primary agent and/or unfavorable environmental conditions such as an increase in available nutrients or the preceding bleaching episode in 2005.

Bacterial family members with increased abundance in diseased coral samples

Bacterial families that we found at higher abundance in diseased coral samples and that had previously been associated with coral disease or bleaching included members of Vibrionaceae and Alteromonadaceae. *Vibrio* spp. have previously been reported to either cause or be associated with higher prevalence in a number of coral diseases (Kushmaro *et al.*, 1996; Ben-Haim and Rosenberg, 2002; Cervino *et al.*, 2004; Gil-Agudelo *et al.*, 2006, 2007; Sussman *et al.*, 2008). Furthermore, *Vibrio* spp. have been characterized as pathogens for a variety of other marine organisms and their zoonotic potential is well-known (Amaro and Biosca, 1996; Gonzalez *et al.*, 2004; Thompson *et al.*, 2004). As our data represent only a temporal snapshot, we are not able to determine how an increase in Vibronaceae is related to the development of the disease. Interestingly, it has been reported that an increase in *Vibro* spp. both reduces the protective properties of beneficial commensals in coral surface mucus layers during a bleaching event (Ritchie, 2006) and also precedes visible signs of bleaching (Bourne *et al.*, 2008).

The bacterium *Thalassomonas loyana* belongs to the order Alteromonadales and has been identified as a coral pathogen for a 'white plague-like disease' in the Red Sea (Thompson *et al.*, 2006). An increase of similar ribotypes in diseased samples in our study may be an indication for the presence of a closely

related species in Caribbean white plague-diseased corals. Other Alteromonadales that were detected in higher abundance included family members of Alteromonadaceae and Pseudoalteromonadaceae. Although we did not find support by clone library sequencing, it should be noted that some members of Pseudoalteromonadaceae have been shown to possess algicidal properties (Lovejoy *et al.*, 1998; Ivanova and Mikhailov, 2001; Mayali and Azam, 2004), which were proposed to play a role in the causation of Yellow Blotch Disease (Cervino *et al.*, 2004). The rapid progression of tissue whitening in white plague-like diseases could possibly be related to algicidal activities of bacteria that increase in abundance in diseased tissues.

A higher abundance of Rhodobacterales as indicated by PhyloChip results was highly consistent with the data obtained from clone library sequencing. The fact that many ribotypes belonging to Rhodobacterales were shared among healthy and diseased samples, but occurred at higher numbers in diseased tissues, could point towards an unchecked growth of opportunistic commensals as a response to disease. The significant increase in *Arcobacter* spp. (Supplementary Table S3) in diseased coral samples (only detected by clone library sequencing) is suggestive for a role of human and/or agricultural sewage in the development of the disease. The genus *Arcobacter* belongs to the Epsilonproteobacteria and comprises two species, *Arcobacter butzleri* and *Arcobacter cryaerophilus*, which can be found in animal livestock (Suarez *et al.*, 1997; Wesley *et al.*, 2000) and in association with human diarrheal illness (Kiehlbauch *et al.*, 1991). *A. butzleri* is also a close taxonomic relative of known human pathogens such as *Campylobacter jejuni* and *Helicobacter pylori* (Miller *et al.*, 2007). Other differentially abundant families included Flavobacteriaceae, Bacillaceae, Peptostreptococcaceae and Clostridiaceae, which include a multitude of examples of pathogenic species (Baron, 1996).

Conclusions and future outlook

Corals are simple organisms with limited phenotypic responses (disease signs), some of which may be similar for different infections. In this study, field collections were done after colonies recovered from intense and long-lasting thermal stress conditions, which presumably triggered the epizootic event observed in Puerto Rico and the Virgin Islands. It is possible that the bleaching event had already changed the microbiota, which may have contained bacteria that became pathogenic under repeated stressful conditions. Although we were not able to deduce more details about the etiology of the disease, we have shown that a combinatorial approach of PhyloChip hybridizations and clone library sequencing can substantiate a list of candidates that may play a significant role in disease development based on statistical support. Furthermore, the absence of *A. coralicida* suggest that 'White Plague Disease', in its current usage, refers to a group of distinct diseases with similar signs in different species, which necessitates further characterization at the pathological, cellular and molecular level at spatial and temporal scales.

With the advent of continued 16S rDNA sequencing efforts, it should be soon possible to design and implement high-density microarrays with higher sensitivity and specificity for coral-associated bacteria. We have demonstrated the ability of this technology to distinguish bacterial profiles of healthy and White Plague-diseased *M. faveolata* colonies, so that future work should be dedicated to extend the number of coral species and diseases investigated. Furthermore, temporal changes in bacterial community structures before, during and after a disease episode necessitates detailed investigation to address the question of whether changes in particular bacterial populations are an indication for the cause or rather the result of a disease. Future studies may also validate the application of PhyloChips as a versatile platform for monitoring and assessing reef water quality. This technology could also be coupled with other methods to assess the health state of corals including biochemical assays (Downs *et al.*, 2005) or gene expression microarrays (Morgan *et al.*, 2005; DeSalvo *et al.*, 2008). The generation of such data sets could guide the implementation of these technologies in effective management strategies to preserve the most diverse marine ecosystem: coral reefs.

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