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Shifts in bacterial communities of two caribbean reef-building coral species affected by white plague disease

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Coral reefs are deteriorating at an alarming rate mainly as a consequence of the emergence of coral diseases. The white plague disease (WPD) is the most prevalent coral disease in the southwestern Caribbean, affecting dozens of coral species. However, the identification of a single causal agent has proved problematic. This suggests more complex etiological scenarios involving alterations in the dynamic interaction between environmental factors, the coral immune system and the symbiotic microbial communities. Here we compare the microbiome of healthy and WPD-affected corals from the two reef-building species *Diploria strigosa* and *Siderastrea siderea* collected at the Tayrona National Park in the Caribbean of Colombia. Microbiomes were analyzed by combining culture-dependent methods and pyrosequencing of 16S ribosomal DNA (rDNA) V5-V6 hypervariable regions. A total of 20 410 classifiable 16S rDNA sequences reads were obtained including all samples. No significant differences in operational taxonomic unit diversity were found between healthy and affected tissues; however, a significant increase of Alphaproteobacteria and a concomitant decrease in the Beta- and Gammaproteobacteria was observed in WPD-affected corals of both species. Significant shifts were also observed in the orders Rhizobiales, Caulobacteriales, Burkholderiales, Rhodobacterales, Aleteromonadales and Xanthomonadales, although they were not consistent between the two coral species. These shifts in the microbiome structure of WPD-affected corals suggest a loss of community-mediated growth control mechanisms on bacterial populations specific for each holobiont system.
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Introduction

Coral reefs posses an immense biodiversity comparable only to that of the tropical rain forest (Mulhall, 2009). The structural, physiological and ecological bases of reefs are the scleractinian corals. They are in symbiotic relationship with a variety of bacteria and archaea as well as with microalgae (zooxanthellae), which are mainly responsible for the corals' high contribution to primary productivity of coral reefs (Rosenberg *et al.*, 2007). Modifications in the structure and relative density of symbiotic microbial communities might have a critical role in the coral adaptation to rapid environmental changes

(Reshef *et al.*, 2006). In the last three decades, local and global deterioration of environmental conditions have dramatically compromised the health of corals, which consequently affected the entire coral reef ecosystem. (Harvell *et al.*, 1999, 2007; Wilkinson, 1999; Green and Bruckner, 2000; Gardner *et al.*, 2003; Pandolfi *et al.*, 2003; Lesser *et al.*, 2007). Recent reports indicate that 58–70% of coral reefs worldwide are threatened by human activities, while more than 30% of the biota associated with Caribbean coral reefs have disappeared within the last 30 years (Hughes *et al.*, 2003; Weil *et al.*, 2006; Jackson, 2008). The dramatic situation of *Acropora palmata* and *A. cervicornis* clearly demonstrates the crisis. Despite of being the most important reef-building corals in the Caribbean in the last million years, they are currently listed as endangered (Pandolfi and Jackson, 2006).

The emergence of coral diseases is one of the most important causes for the reefs' global decline

(Wilkinson, 1999; Rosenberg and Ben-Haim, 2002; Harvell et al., 2007). Of more than 20 cataloged diseases affecting corals, three of them, the black band disease, the white band disease and the white plague disease (WPD) account for more than two thirds of cases reported in the Caribbean (Garzón-Ferreira et al., 2001; Sutherland et al., 2004; Weil et al., 2006). The WPD is the most common pathology in the southwestern Caribbean, affecting more than 50 species of scleractinian corals, with mortality rates reaching 40% in some species (Goreau et al., 1998; Garzón-Ferreira et al., 2001; Rosenberg and Ben-Haim, 2002; Weil et al., 2002; Sutherland et al., 2004). This disease is characterized by a sharp line (2–3 mm) of bleached tissue between apparently healthy coral tissue and the freshly exposed coral skeleton. The sharp line of tissue loss progresses across the colony leaving the coral skeleton exposed, eliminating the entire colony in approximately 10 weeks (Weil et al., 2006). Three types of WPD disease have been described, WPD-I, -II and –III, which are differentiated by the rate of tissue loss (Richardson, 1998; Gil-Agudelo et al., 2009).

Despite increasing efforts, the identification of etiological agents for coral diseases has proved problematic. Indeed, candidate causal microorganisms have been characterized in just a handful of diseases affecting reef-building corals. They include Serratia marcescens for white pox (Patterson et al., 2002), Vibrio shiloi and V. coralliilyticus for bacterial bleaching (Banin et al., 2000; Kushmaro et al., 2001; Ben-Haim et al., 2003; Rosenberg et al., 2009), Aurantimonas coralicida (Denner et al., 2003) and Thalassomonas loyana (Thompson et al., 2006) for WPD type-II in Dichocoenia stokesi from the Caribbean and in Favia favus from the Red Sea, respectively. However, a further attempt by Sunagawa et al., to identify A. coralicida (former Sphingomona sp.) in the Caribbean coral Montastraea faveolata with WPD using culture-independent methods failed. In addition, Pantos et al., showed that A. coralicida was present in both healthy and WPD-affected tissues in various species of reef-building corals. These and other studies have suggested that WPD represents a group of different diseases with similar signs in different species (Dustan, 1977; Rosenberg et al., 2007; Sunagawa et al., 2009). Furthermore, it has been suggested that some coral diseases have a polymicrobial origin involving an imbalance in the structure and diversity of microorganism communities symbiotically associated with their host (Harvell et al., 2007; Lesser et al., 2007). An example is the black band disease, which is associated to a bacterial consortium of cyanobacteria and other less abundant groups of sulfur-oxidizing and sulfur-reducing bacteria (Sekar et al., 2006; Barneah et al., 2007; Sato et al., 2010). Certainly, symbiotic bacteria have an important role in nutrition, respiration and defense, and deviations from a dynamic equilibrium might

lead to disease susceptibility and proliferation of opportunistic bacteria (Rohwer et al., 2001; Reshef et al., 2006; Rosenberg et al., 2007; Bourne et al., 2009). In this study we compared the microbiome of healthy and WPD-affected tissues from the two Caribbean coral species Siderastrea siderea and Diploria strigosa. The microbiomes were characterized by combining culture-dependent and culture-independent approaches on whole tissues, with the latter approach involving pyrosequencing of 16S ribosomal DNA (rDNA) amplicons. A consistent shift in the structure of bacterial communities associated to WPD-affected colonies was observed across the two coral species.

Materials and methods

Sample collection and preparation
Samples of the coral species S. siderea and D. strigosa were collected in 15 m depth at a reef off Aguja Island (11°18′57″N, 74°11′41″W) in the Tayrona National Park, Colombia, in October of 2009. Environmental conditions during sample collection were typical of a rainy season, with low salinity, low visibility, high nutrient concentrations and a surface seawater temperature of 28 °C. Five coral fragments with a size of 2×2 cm were collected with a hammer and chisel from each coral species and from each condition (healthy and diseased). Sampled colonies were at varying distances from one another, ranging from 10 to 25 m. Samples were transported immediately to the laboratory and washed with sterile seawater to minimize the inclusion of seawater. Each sample was washed five times with sterile seawater for 10 s with stirring, and water from the last wash was serially diluted and plated on Luria Bertani and marine agars. Only two colonies grew from the last undiluted wash of only one fragment, indicating that bacteria not associated to coral tissues were efficiently removed. There exists a natural variation in the structure of bacterial communities between colonies from the same coral species (Kvennefors et al., 2010) that might obscure true differences between healthy and diseased samples. To standardize for inter-colony bacterial variability, the five replicate samples from each coral species and from each condition (healthy and diseased) were pooled and macerated with a mortar in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 100 mM Na_2HPO_4 and 2 mM KH_2PO_4 at pH 7.4). One-half of each macerated pooled sample was used for bacteria isolation and the other half was conserved in phenol at −20 °C for DNA extraction.

Bacteria isolation and identification
The non-preserved samples were serially diluted in phosphate- buffered saline, plated on Luria Bertani and Marine agar (NaCl 20.8 g l^{-1}, KCl 0.6 g l^{-1}, $MgCl_2$

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$4.0 \mathrm{g\,l}^{-1}$, $MgSO_4$ $4.8 \mathrm{g\,l}^{-1}$, Rila salts $1.5 \mathrm{g\,l}^{-1}$, Tris-base $0.4 \mathrm{g\,l}^{-1}$, peptone $3.0 \mathrm{g\,l}^{-1}$, yeast extract $1.5 \mathrm{g\,l}^{-1}$), and incubated at $28\,°C$ for $48\,h$. Bacterial colonies were systematically purified and preserved in glycerol at $-80\,°C$. Each bacterial isolate was characterized morphologically by size, color, texture, borders and Gram staining, and biochemically by using the following standard bacteriological tests: catalase activity, oxidase activity, citrate utilization, indole production, urease activity, methyl red-Voges-Pros-kauer reaction, growth on triple sugar iron agar, growth on lysine iron agar, gelatin liquefaction, nitrates production and motility (Holt et al., 1994). Colonies displaying different morphologies, varying in abundance between healthy or affected corals, or present in one condition but absent in the other, were selected for species identification by sequencing the full-length 16S rRNA gene. DNA was extracted from 29 bacterial isolates (10 from D. strigosa and 19 from S. siderea) by boiling the colony in $100\,\mu l$ of sterile water for $10\,min$ at $94\,°C$ and immediately cooling the sample on ice for $5\,min$. The supernatant was used as a template to amplify the 16S rDNA using universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Lane, 1991) in a final volume of $25\,\mu l$ containing $5\,mM$ amplification buffer, $0.6\,mM$ $MgSO_4$, $0.4\,mM$ deoxyribonucleotide triphosphates, $0.4\,\mu M$ of each primer and $0.1\,U$ of Platinum Pfx Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). Amplification was carried out with an initial cycle of $94\,°C$ for $5\,min$, followed by 30 cycles of $94\,°C$ for $1\,min$, $52\,°C$ for $1\,min$ and $72\,°C$ for $1\,min$, with a final extension step of $72\,°C$ for $7\,min$. PCR products were sequenced at Macrogen (Seoul, Korea), and chromatograms were edited with the DNA BASER software (HeracleSoftware, http://www.dnabaser.com). Sequences were aligned using ClustalW (Thompson et al., 1994), and phylogenetic trees were constructed using the MEGA4 software (Tamura et al., 2007) with the Neighbor-joining method (Saitou and Nei, 1987) based on distances estimated by the Kimura two-parameter model (Kimura, 1980). The reliability of clustering pattern was evaluated by bootstrapping using 1000 replications. Sequence similarity values of 97–100% were used for species assignation (Stackebrandt and Goebel, 1994; Rosselló-Mora and Amann, 2001).

454 sequencing of 16S rDNA V5-V6 hypervariable regions

The phenol-preserved samples were mixed with $2\,ml$ of TE buffer ($10\,mM$ Tris-HCl and $1\,mM$ EDTA at pH 7.4) and $500\,\mu l$ SDS 10%, heated at $60\,°C$ for $5\,min$ and cooled on ice for $5\,min$. DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated with 95% ethanol and $1\,M$ ammonium acetate for $24\,h$ at $-20\,°C$. PCR amplification of 16S rDNA hypervariable regions V5–V6 (Youssef et al., 2009) was performed as a service provided by CorpoGen Corporation (Bogotá, Colombia) using degenerate primers derived from Escherichia coli that anneal at gene positions 807–1050, each of them having a unique barcode and a Roche 454 'A' and 'B' pyrosequencing adapter (Basel, Switzerland). Amplification products were verified in a 1% agarose gel electrophoresis at $80\,V$ for $40\,min$. PCR products were sent to the Environmental Genomics Core Facility at the University of South Carolina (Columbia, SC, USA), for pyrosequencing on a Roche FLX 454 instrument. Sequence reads of low quality, that is, those shorter than $50\,bp$ or with a quality score of 20 or less were removed from the analysis. Sequence richness and diversity were estimated by Chao1 and Shannon–Weaver indices, respectively (Shannon, 1948; Chao and Lee, 1992). Rarefaction curves and diversity analysis were performed on three subsamples of 800 sequences according to the methodology applied by Gaidos et al. (2011). The distance matrix and assignment of operational taxonomic units were defined at a similarity level of 97%, and rarefactions curves were calculated at 0%, 3% and 5% of dissimilarity using the software DOTUR (Schloss and Handelsman, 2005). Taxonomic assignment of sequence reads were done with the Ribosomal Database Project (RDP) Tool Classifier (Wang et al., 2007) at a confidence threshold of 80%. Standard errors of mean taxonomic group sequence abundances were calculated from the three subsamples of 800 reads.

PCR amplification of 16S rDNA from Aurantimonas coralicida

Specific PCR amplification of A. coralicida 16S rDNA was attempted using primers C-995F (TCGACGGGTATCCGGAGACGGAT) and UB-1492R (TACGGGTACCTTGTTACGACTT) reported by Polson et al. (2008) on DNA isolated from the phenol-preserved samples and from 16S rDNA generic amplification products. Amplification conditions were the same as described by Polson et al. (2008).

Results

Bacteria isolates associated to healthy and WPD-affected corals

A total of 71 bacteria isolates were obtained from the two coral species. In S. siderea, 22 bacteria colonies were isolated from the diseased sample and 14 from the healthy sample. In D. strigosa, 12 bacteria colonies were isolated from healthy and 23 colonies from affected samples. In all, 96% of isolates were Gram-negative rods, and most of the colonies were circular, shiny, cream-colored and with creamy texture. Colonies differing in morphology or abundance between healthy and affected corals were characterized biochemically and identified taxonomically by 16S rDNA sequencing. The standard biochemical tests revealed differences between

bacteria colonies isolated from healthy and WPD-affected corals. Among those differences were the use of citrate as sole carbon source in 29.2% of isolates from affected corals and 53.8% from healthy corals. Similarly, there was a higher usage of carbohydrates throughout by isolates from healthy compared with affected corals (glucose, 38.5% vs 12.5%; lactose, 23.1% vs 8.3%; sucrose, 23.1% vs 8.7%; mannitol, 60.0% vs 38.5%; and fructose 41.7% vs 31.8%).

In all, 29 bacterial isolates were selected for species identification by 16S rDNA sequencing. The taxonomic analysis based on full-length sequences in the context of reference strains showed that the isolates included representatives of Actinobacteria, Firmicutes and Proteobacteria phyla, with the latter accounting for 60% of all isolates (Figure 1). Among the phylum Proteobacteria there were representatives of the Alpha (two isolates, 10%), Beta (five isolates, 25%) and Gammaproteobacteria (13 isolates, 65%) classes. Only one Firmicutes species was found, whereas seven species of Actinobacteria were isolated, one derived from a healthy coral and six from WPD-affected corals. Cultured bacteria species were distributed unevenly between the two coral species
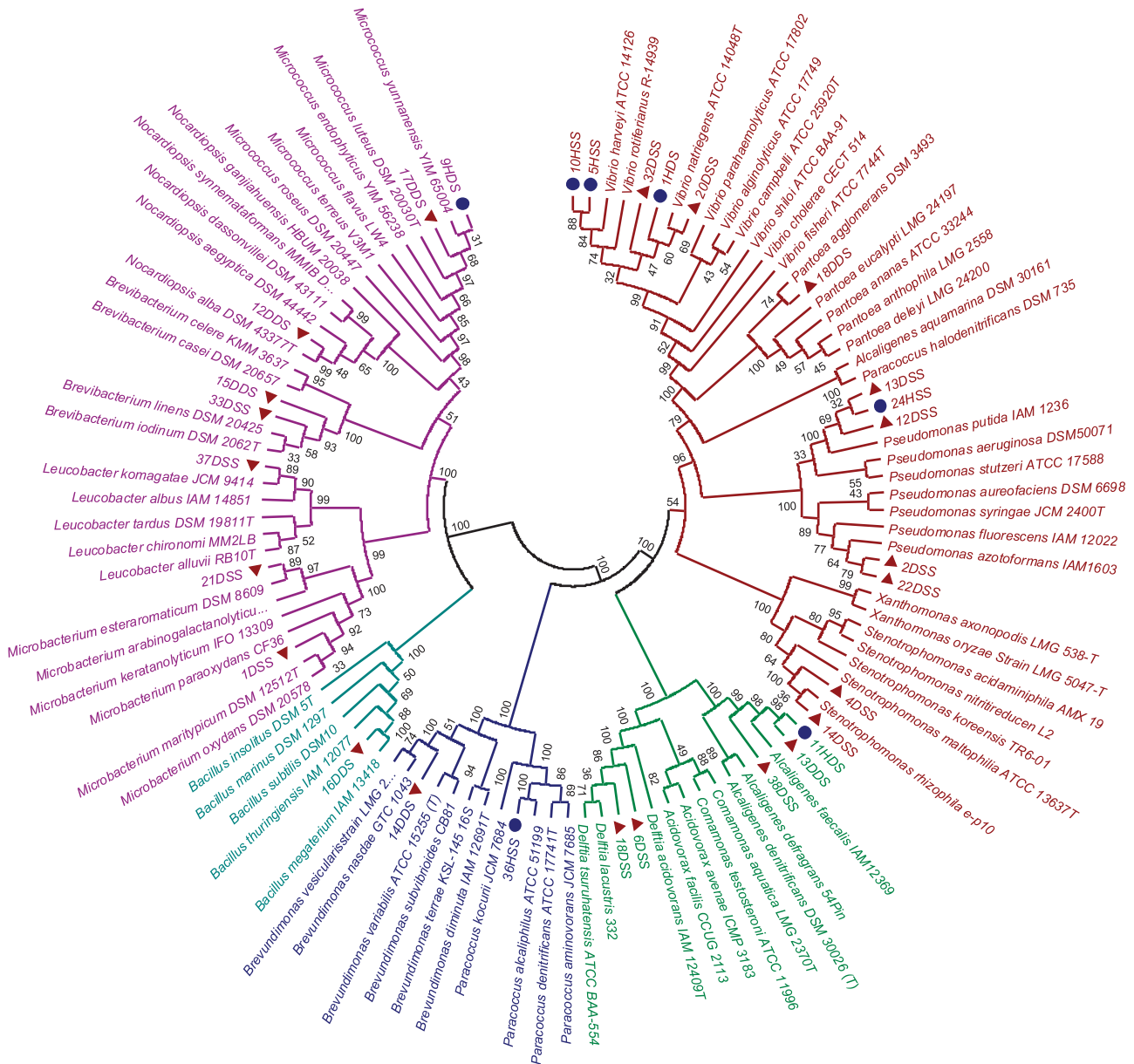

Figure 1 Taxonomic analysis of bacteria strains isolated from *S. siderea* and *D. strigosa* based on 16S rDNA sequences. Strains reported here are indicated by numbers followed by the letters H (healthy) or D (diseased) and the initials for each coral species (DS for *D. strigosa* or SS for *S. siderea*). Red diamonds preceding the code name indicate isolates from diseased corals and blue circles indicate isolates from healthy corals. All other strains are reference strains from culture collections with their respective GenBank accession numbers. Red branches are Gammaproteobacteria, green branches are Betaproteobacteria, dark blue branches are Alphaproteobacteria, light blue are Firmicutes and purple branches are Actinobacteria. Numbers on branches represent bootstrap percentages after of 1000 replicates.
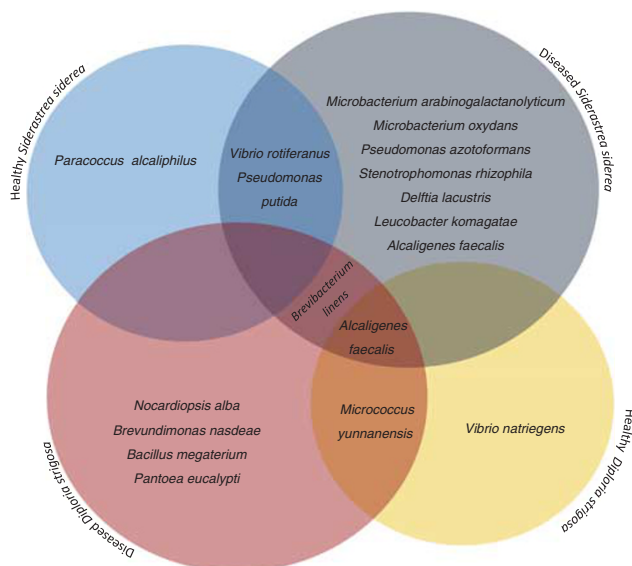
Figure 2 Venn diagram of cultured bacterium species showing their distribution in healthy and diseased corals. Circular areas represent HS (light blue), DS (dark blue), HD (orange) and DD (red).

Table 1 Number of OTUs obtained for each coral sample, and estimators of OTU richness and diversity

	Number of OTUs[a]	Chao1[b]	Shannon–Weaver[b]
Healthy S.siderea	378 (12.5)	800 (674.2–979.4)	5.4 (5.4–5.5)
Diseased S.siderea	319 (17.9)	523 (448.0–609.2)	5.3 (5.2–5.4)
Healthy D.strigosa	256 (6.8)	513 (427.2–672.5)	4.5 (4.4–4.6)
Diseased D.strigosa	372 (24.5)	828 (690.3–1025.9)	5.4 (5.3–5.5)

Abbreviation: OTUs, operational taxonomic units.
[a]OTUs obtained at 97% similarity using DOTUR.
[b]Parameters calculated for subsamples of 800 –sequences. Numbers within brackets indicate standard errors.

and between healthy and WPD-affected samples (Figure 2). There were no bacteria common to both healthy coral species, whereas there was a single bacterium, *Brevibacterium linens*, present in both WPD-affected coral species. It is, however, premature to derive any conclusion about the role of this bacterium in the pathogenesis of the disease. A formal demonstration of such causal association would require *in vitro* infection assays. In *S. siderea*, *Paracoccus alcaliphilus* was present only in healthy samples, while *Microbacterium arabinogalactanolyticum*, *M. oxydans*, *Pseudomonas azotoformans*, *Stenotrophomonas rhizophila*, *Delftia lacustris* and *Leucobacter komagatae* were found only in WPD-affected samples (Figure 2). In addition, *Vibrio rotiferanus* and *Pseudomonas putida* were isolated from both healthy and affected samples from this coral species. In *D. strigosa*, the only species isolated from healthy samples was *Vibrio natriegens*, while four species could be isolated from the affected samples: *Nocardiopsis alba*, *Brevundimonas nasdeae*, *Bacillus megaterium* and *Pantoea eucalypti*. *Micrococcus yunnanensis* was present in both healthy and affected samples of *D. strigosa*. Further, *Alcaligenes faecalis* was present in all coral samples except in healthy tissues from *S. siderea* (Figure 2). The 16S rDNA sequence data from these isolates have been submitted to the DDBJ/EMBL/ GenBank databases, under the accession numbers JF792066–JF792094. As the putative etiological agent for WPD in the Caribbean, *Aurantimonas coralicida* (Denner *et al.*, 2003), was not among the cultured bacteria, an attempt to amplify 16S rDNA from this species was done by using the specific PCR primers and conditions reported by Polson *et al.* (2008). No amplification was obtained from

either total DNA or 16S rDNA community PCR libraries derived from healthy or affected samples in any of the two corals species.

Bacteria community structure based on 454 sequencing of 16S rDNA
A total of 25 772 sequence reads were obtained from the four coral samples, healthy *S. siderea* (HS; 6162 reads), diseased *S. siderea* (DS; 857), healthy *D. strigosa* (HD, 14 169) and diseased *D. strigosa* (DD; 4584). After quality control, 20 410 sequence reads remained for further analyses, with an average length of 200 bp, ranging from 52 to 304 bp. Diversity analyses, number of observed operational taxonomic units at 97% similarity, and rarefaction curves were performed by averaging three subsamples of 800 sequences each, corresponding to the smallest number of sequences obtained from one of the coral samples (DS). From the three subsamples of 800 sequences, we obtained an average of 378 operational taxonomic units for HS, 319 for DS, 256 for HD and 372 for DD (Table 1). In *S. siderea*, the Chao1 index was higher in the healthy sample than in the affected sample, whereas in *D. strigosa* the opposite was observed, as the index was higher in the affected sample than in its healthy counterpart (Table 1). The Shannon–Weaver index, however, was comparable across samples indicating that bacterial community diversity is not significantly different between healthy and affected corals from both the species. Rarefaction curves based on the number of operational taxonomic units at 0%, 3% and 5% of dissimilarity showed that the 3% curves were approaching plateaus using subsamples of 800 sequence reads (Figure 3). Although the curves were not asymptotic, suggesting that deeper sequencing might be required, the fact that they were identical between subsamples indicates that the bacteria community was well represented in all samples.

In all, 99% of total classifiable sequence reads belonged to the domain Bacteria, 0.08% to Archaea and 0.07% were not classifiable. Sequences were classified into nine phyla, 18 classes and 39 orders of Bacteria, yet 6.7% of sequences were unclassifiable at the phylum level and only an average of 33.2% of sequences per sample reached the order
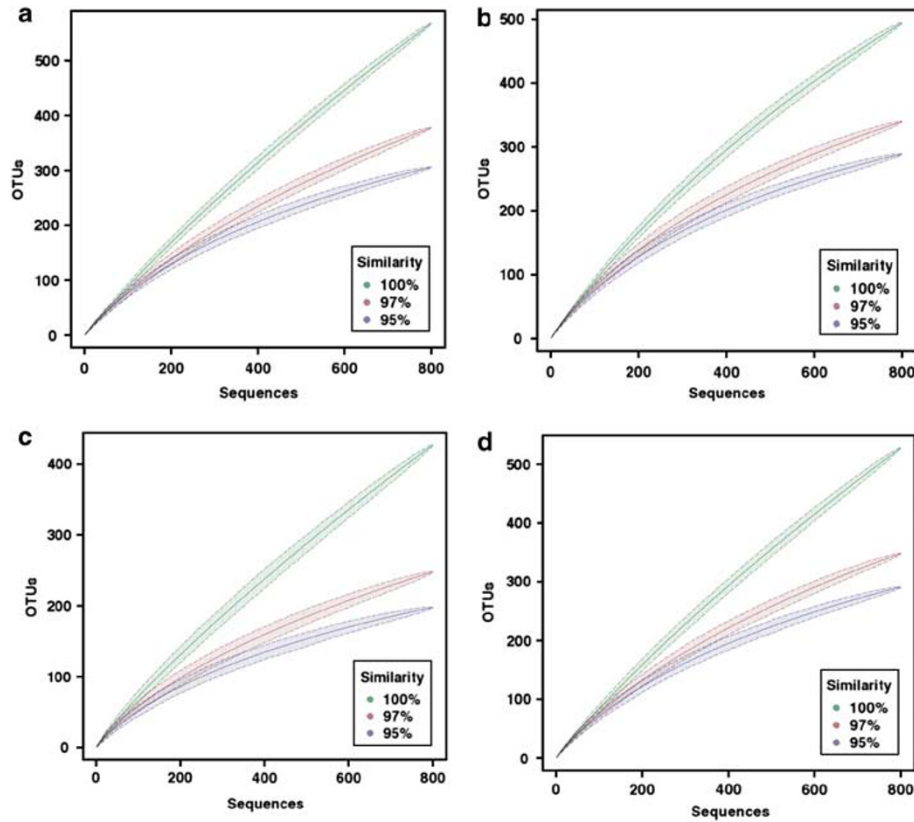
Figure 3 Rarefaction analysis of 16S rDNA sequence reads obtained by pyrosequencing from healthy and diseased coral samples. The analysis was done by subsampling 800 sequence reads from each coral sample using the software DOTUR with different percentages of similarity (100%, 97% and 95%). Confidence intervals for each curve are represented by dotted lines. (**a**) HS (**b**) DS (**c**) HD and (**d**) DD.
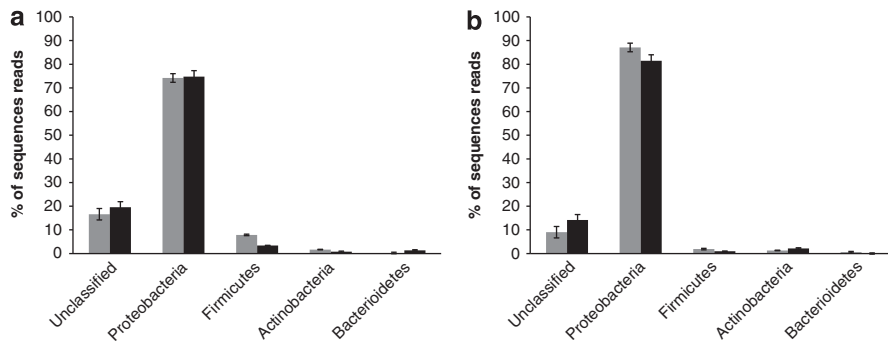

Figure 4 Relative abundance of 16S rDNA pyrosequencing reads from healthy and diseased coral samples classified by similarity at the phylum level. Each *bona fide* read obtained by pyrosequencing was compared with the RDP database at a confidence threshold of 80%. Bars denote the mean sequence abundance over three subsamples of 800 reads. Gray bars represent healthy corals and black bars represent diseased corals. (**a**) *S. siderea* and (**b**) *D. strigosa*.

level. The relationship between the percentage of sequences taxonomically assigned by RDP Classifier (Wang *et al.*, 2007) and the total number of sequences obtained from each coral sample showed that the phylum Proteobacteria contained most of the retrieved sequences, with $75.5 \pm 1.8\%$ in HS, $76.3 \pm 2.5\%$ in DS, $88.7 \pm 1.0\%$ in HD and $83.9 \pm 2.3\%$ in DD (Figure 4). Proteobacteria was followed in sequence abundance by Bacteroidetes, Chloroflexi, Fusobacteria, Verrucomicrobia,

Chlorobi, Tenericutes and Cyanobacteria at very low percentages. A detailed analysis of Proteobacteria showed a significant and consistent difference in the relative sequence abundance of Proteobacteria classes between healthy and diseased samples. Indeed, despite a higher sequence abundance of Gammaproteobacteria in healthy and diseased samples from both corals, WPD-affected tissues showed an increase in Alphaproteobacteria associated with a decrease in Beta and Gammaproteobacteria
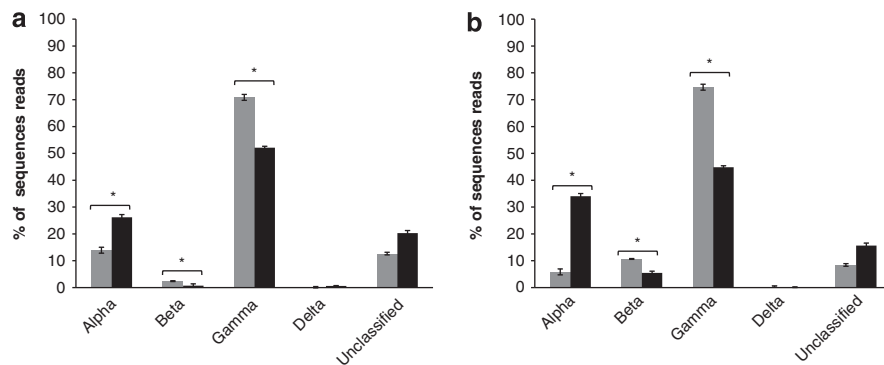
Figure 5 Relative abundance of 16S rDNA pyrosequencing reads classified at the class level from Proteobacteria only using the RDP classifier at a confidence threshold of 80%. Bars represent mean of sequence abundance over three subsamples of 800 reads from healthy (gray bars) and diseased (black bars) *S. siderea* (**a**) and *D. strigosa* (**b**) corals. *One-way analysis of variance $P < 0.02$.

in comparison with samples from healthy tissues (one-way analysis of variance, $P < 0.02$) (Figure 5).

From 39 bacteria orders identified, only 16 showed significant differences between healthy and affected corals, although these differences were not generally consistent in the two coral species (Figure 6). Two instances of consistent and significant shifts in the sequence abundance of bacterial orders between healthy and diseased samples in the two coral species were the Rhodobacterales (from 7% in HS to 19.4% in DS and from 0% in HD to 5.8% DD) and the Oceanospiralles (from 1% in HS to 2.4% in DS and from 1.3% in HD to 6% in DD). Remarkably, opposite shifts in sequence abundance between the two coral species were observed in Alpha, Beta and Gammaproteobacteria orders. The Alphaproteobacteria order Rhizobiales increased by 41% in DS and decreased by 72% in DD, while Caulobacteriales decreased by 33% in DS and increased by 52% in DD. Similarly, the Betaproteobacteria order Burkholderiales decreased by 22% in DS and increased by 20% in DD, and the Gammaproteobacteria order Xanthomonadales decreased by 16% in DS but increased by 39% in DD. Finally, the Enterobacteriales decreased significantly only in *D. strigosa,* from 26.5% in healthy to 1.7% in diseased samples, while the Aeromonadales and Neisseriales where unique of *S. siderea*, although they appeared at low frequencies and displayed no changes in sequence abundance between healthy and diseased samples.

Discussion

The dynamic interaction between environmental factors, the host immunity and the microbiome, maintains the coral homeostasis, and a modification of any of these components may condition the development of diseases. In turn, the disease may provoke a secondary change in the structure of associated microbial communities as well as in various immune parameters of the coral host. The

present work analyzed the microbiome structure of the two Caribbean coral species *D. strigosa* and *S. siderea* affected with WPD. In contrary to what was reported by Sunagawa *et al.*, (2009) for the coral *M. faveolata* affected with WPD, there were no differences in the bacterial diversity between healthy and affected samples from the two coral species. Significant changes, however, were observed in bacterial community richness between healthy and affected corals, although the direction of change was not consistent between the two coral species. In *S. siderea* the Chao1 index was higher in the healthy samples, whereas in *D. strigosa* the index was higher in the affected sample. This indicates that variations in those parameters are heavily dependent upon the basal community composition specific for each coral species, and are not a reflection of the disease condition.

The predominant Bacteria phylum in both healthy and affected corals was Proteobacteria, consistent with previous observations based on culture-independent methods in other reef-building corals (Santiago-Vazquez *et al.*, 2007; Kvennefors *et al.*, 2010). Yet, a shift in the relative sequence abundance of the Proteobacteria classes was strongly associated to the disease in the two coral species. Although Gammaproteobacteria were the most abundant class in both healthy and affected corals from the two species, Alphaproteobacteria increased significantly and the Beta and Gammaproteobacteria classes decreased significantly in the samples affected with WPD compared with healthy samples in both corals. The consistency of such changes across coral species from different suborders suggests that this pattern is indeed an indicator of disease, although no causality explanations can be derived from this observation. Such a shift in the abundance of Proteobacteria classes was not observed in the bacterial isolates from diseased corals. An increase in Alphaproteobacteria abundance has also been observed using culture-independent methods in other coral species affected with black band disease (Sekar *et al.*, 2006; Barneah *et al.*, 2007;
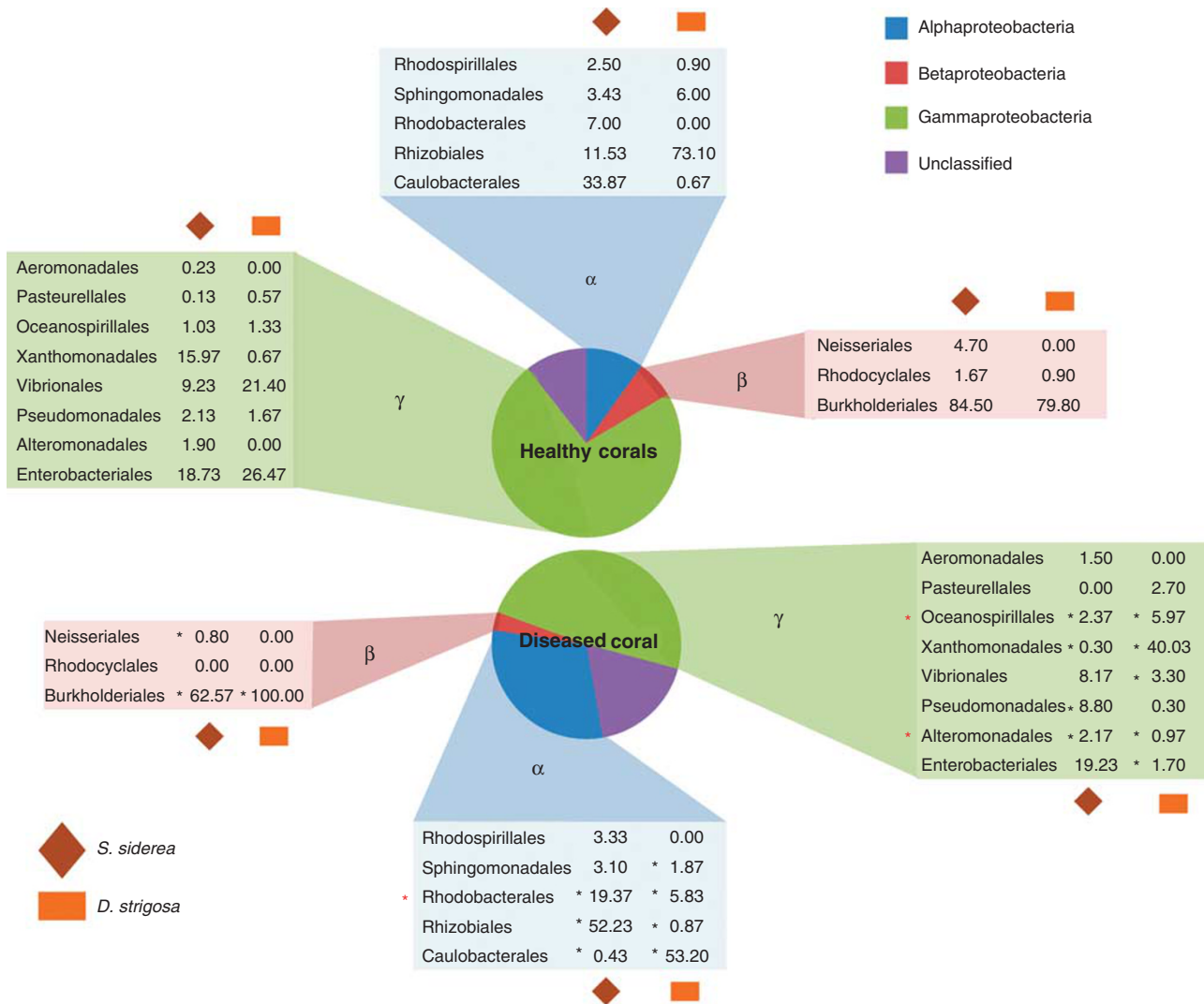
Figure 6 (α — Healthy corals)

	S. siderea	D. strigosa
Rhodospirillales	2.50	0.90
Sphingomonadales	3.43	6.00
Rhodobacterales	7.00	0.00
Rhizobiales	11.53	73.10
Caulobacterales	33.87	0.67

Legend:
- Alphaproteobacteria
- Betaproteobacteria
- Gammaproteobacteria
- Unclassified

(γ — Healthy corals)

	S. siderea	D. strigosa
Aeromonadales	0.23	0.00
Pasteurellales	0.13	0.57
Oceanospirillales	1.03	1.33
Xanthomonadales	15.97	0.67
Vibrionales	9.23	21.40
Pseudomonadales	2.13	1.67
Alteromonadales	1.90	0.00
Enterobacteriales	18.73	26.47

(β — Healthy corals)

	S. siderea	D. strigosa
Neisseriales	4.70	0.00
Rhodocyclales	1.67	0.90
Burkholderiales	84.50	79.80

Healthy corals

Diseased coral

(β — Diseased coral)

	S. siderea	D. strigosa
Neisseriales	* 0.80	0.00
Rhodocyclales	0.00	0.00
Burkholderiales	* 62.57	* 100.00

(γ — Diseased coral)

	S. siderea	D. strigosa
Aeromonadales	1.50	0.00
Pasteurellales	0.00	2.70
* Oceanospirillales	* 2.37	* 5.97
Xanthomonadales	* 0.30	* 40.03
Vibrionales	8.17	* 3.30
Pseudomonadales	* 8.80	0.30
* Alteromonadales	* 2.17	* 0.97
Enterobacteriales	19.23	* 1.70

(α — Diseased coral)

	S. siderea	D. strigosa
Rhodospirillales	3.33	0.00
Sphingomonadales	3.10	* 1.87
* Rhodobacterales	* 19.37	* 5.83
Rhizobiales	* 52.23	* 0.87
Caulobacterales	* 0.43	* 53.20

◆ S. siderea
■ D. strigosa

Figure 6 Relative sequence abundance of Alpha-, Beta- and Gammaproteobacteria orders in healthy and diseased corals classified using the RDP database. An average of 235 sequences from HS, 220 from DS, 336 from HD and 272 from DD reached order level from the three subsamples of 800 pyrosequencing reads at a confidence threshold of 80%. Populations showing significant changes are marked with an asterisk (*one-way analysis of variance P < 0.05). Black asterisks denote significant changes between healthy and diseased corals. Red asterisks denote common changes between both coral species.

Sato *et al.*, 2010), white band disease (Pantos and Bythell, 2006) and WPD (Pantos *et al.*, 2003; Sunagawa *et al.*, 2009), yet, a concomitant decrease in Beta- and Gammaproteobacteria has not been reported before. The increase in Alphaproteobacteria associated to disease might represent an opportunistic colonization of a variety of bacterial communities on damaged coral tissues rather than the effects of a single pathogenic agent. The reciprocal decrease of Beta- and Gammaproteobacteria might favor the increase of Alphaproteobacteria by a loss of population-based growth control mechanisms in the normal microbiota, such as antagonism, *Quorum Sensing* inhibition or antibiotic production.

Variation in Bacteria orders between healthy and diseased tissues was observed in 41% of cases, yet this variation was not consistent between the two coral species. On the contrary, orders displaying the most important changes in sequence abundance, that is, Rhizobiales, Caulobacteriales, Burkholderiales and Xanthomonadales, shifted in opposite directions in *S. siderea* and *D. strigosa* as consequence of the disease. We must highlight the change in the order Vibrionales in both corals, because they are often related with marine diseases and here we show a common decrease during the disease condition. Bacterial community shifts associated to WPD were consistent across coral species when the more inclusive (higher) taxonomic levels are considered, but this consistency disappears at lower taxonomic levels. The nature of the disease-induced shift at the most discriminatory levels is likely to be heavily dependent upon the specific bacterial communities associated to a given coral host. An analogous pattern is seen in mammals when comparing the gut microbiome of healthy unrelated individuals,

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where despite of a high conservation at higher taxonomic levels, diversity and population structure of bacteria at the species level varies extensively (Stecher *et al.*, 2010; Spor *et al.*, 2011). In addition, shifts in the abundance of bacteria communities at higher taxonomical levels have been well documented for various diseases in humans. For example, Proteobacteria have displaced Bacteroidetes in inflammatory bowel disease, Bacteroidetes have displaced Firmicutes in type-II diabetes and Proteobacteria have displaced Firmicutes in necrotizing enterocolitis (Spor *et al.*, 2011). Furthermore, differences between healthy and affected corals rarely involved gaining or eliminating bacteria orders, supporting the idea that the imbalance resulting from the disease is likely due to an alteration in the community-based mechanisms controlling population growth. Experimental approaches that could be used to test this hypothesis include searching for *Quorum Sensing* circuit components differentially present in bacterial communities from healthy and diseased tissues, comparisons of syntrophy patterns by metagenomics methods, evaluation of antibiotic production and antagonistic behaviors or inhibition of *Quorum Sensing* between isolates.

Results from isolated bacteria showed that a higher fraction of strains from healthy corals were able to use the carbon sources tested than strains from diseased corals. This finding needs further experimental investigation to confirm that it relates to a specific pathogenic feature. Different species of bacteria that have been associated to other organisms were also characterized, including *Vibrio rotiferianus* associated with normal microbiota of rotifers (Gomez-Gil *et al.*, 2003), and *Microbacterium oxydans* and *Nocardiopsis alba* associated with normal microbiota of different insects (Pidiyar *et al.*, 2004; Rudolf *et al.*, 2009; Patil *et al.*, 2010). Other bacterial species involved in nosocomial infections were isolated, such as *Stenotrophomonas rhizophila* (normally associated with plants) (Wolf *et al.*, 2002) and *Stenotrophomonas maltophilia* (Waters *et al.*, 2007). *Brevibacterium linens* was isolated exclusively from WPD-affected corals. The genus and family of this species were not found among the sequencing reads, but its suborder, Micrococcineae, was represented in both healthy and disease corals from both species. *B. linens* is ubiquitously distributed in the environment and normal microbiota of different organisms and most likely represents an opportunistic colonizer of pathological tissues. The present attempt to identify the putative causal agent for WPD *A. coralicida* has failed as it has in other studies (Pantos *et al.*, 2003; Sunagawa *et al.*, 2009). This, together with the identification of *A. coralicida* in healthy tissues from *Acropora* species and from tissues affected with other coral diseases (Polson *et al.*, 2008) argues against simple etiological scenarios for WPD.

This work supports the notion that WPD is associated to an imbalance of resident bacterial populations, characterized by an increase in the relative abundance of Alphaproteobacteria with a parallel decrement of Beta- and Gammaproteobacteria. This pattern could be a potential biomarker for coral disease, yet it is still necessary to consider additional factors such as environmental conditions and coral immunity to understand the etiology of this devastating coral disease.

Acknowledgements

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