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Microbial diversity associated with four functional groups of benthic reef algae and the reef-building coral *Montastraea annularis*

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Summary

The coral reef benthos is primarily colonized by corals and algae, which are often in direct competition with one another for space. Numerous studies have shown that coral-associated *Bacteria* are different from the surrounding seawater and are at least partially species specific (i.e. the same bacterial species on the same coral species). Here we extend these microbial studies to four of the major ecological functional groups of algae found on coral reefs: upright and encrusting calcifying algae, fleshy algae, and turf algae, and compare the results to the communities found on the reef-building coral *Montastraea annularis*. It was found using 16S rDNA tag pyrosequencing that the different algal genera harbour characteristic bacterial communities, and these communities were generally more diverse than those found on corals. While the majority of coral-associated *Bacteria* were related to known heterotrophs, primarily consuming carbon-rich coral mucus, algal-associated communities harboured a high percentage of autotrophs. The majority of algal-associated autotrophic *Bacteria* were *Cyanobacteria* and may be important for nitrogen cycling on the algae. There was also a rich diversity of photosynthetic eukaryotes associated with the algae, including protists, diatoms, and other groups of microalgae. Together, these observations support the hypothesis that coral reefs are a vast landscape of distinctive microbial communities and extend the holobiont concept to benthic algae.

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Introduction

Microbes are associated with a wide variety of organisms, and are increasingly recognized to play an important role in host health and metabolism (Gill *et al*., 2006; Taylor *et al*., 2007; Ley *et al*., 2008; Turnbaugh *et al*., 2008). Corals, for example, are inhabited by a diverse and abundant array of microbes that are distinct from the surrounding seawater (Ritchie and Smith, 1997; Rohwer *et al*., 2001; 2002; Frias-Lopez *et al*., 2002; Wegley *et al*., 2004; Bourne and Munn, 2005; Koren and Rosenberg, 2006; Sunagawa *et al*., 2010). These microbes produce antibiotics (Ritchie, 2006; Nissimov *et al*., 2009) and are involved in the biogeochemical cycling of nitrogen, carbon and sulfur on the holobiont (Lesser *et al*., 2004; Wegley *et al*., 2007; Siboni *et al*., 2008; Raina *et al*., 2009; Fiore *et al*., 2010; Kimes *et al*., 2010). Despite the known and potential roles of microbes in coral health and metabolism, identification of the primary factors controlling the types of microbes associated with corals is an ongoing question. There is evidence that coral-associated microbial communities are species specific (Rohwer *et al*., 2002; Sunagawa *et al*., 2010), but it has also been shown that the same species from different locations harbour distinct microbial communities (Littman *et al*., 2009). In addition, the composition of coral-associated microbial communities has been found to change when corals undergo bleaching (Bourne *et al*., 2007), are housed in aquaria versus natural environments (Kooperman *et al*., 2007), and when they are exposed to stressors (Thurber *et al*., 2009). These observations are intriguing because they open the possibility that the coral holobiont may adapt to changing conditions by changing microbial associates in a manner similar to adaptive bleaching (Rohwer *et al*., 2002; Reshef *et al*., 2006; Rosenberg *et al*., 2007). Furthermore, the microbial communities themselves play a role in determining the types of microbes that colonize the coral surface through niche occupation and

antagonism towards other bacteria (Ritchie, 2006; Rypien *et al*., 2010). This suggests that a combination of host factors, microbial associates and environmental conditions plays an important role in shaping microbial associations, which then play a role in the health and function of the host coral.

Benthic algae are a major component of coral reef ecosystems and are increasingly abundant on coral reefs around the world (McCook, 1999; Hughes *et al*., 2003; 2007). However, despite the significance of microbial associations recognized with other benthic reef organisms (e.g. corals and sponges), our knowledge of algal-associated microbial communities is limited. Benthic algae are often grouped together by form and ecological function (Littler *et al*., 1983). Turf algae, for example, are small filamentous algae and are among the most productive groups on the reef benthos (Hatcher, 1988), and this success has been attributed in part to the contribution of high nitrogen-fixation rates due to *Cyanobacteria* present among the turf community (Wilkinson *et al*., 1984; Shashar *et al*., 1994; Williams and Carpenter, 1998). In addition, many species of crustose coralline algae (CCA) promote settlement of coral and other invertebrate larvae (Sebens, 1983; Morse and Morse, 1984; Morse *et al*., 1988). This effect is due primarily or in part to *Bacteria* associated with the CCA (Johnson and Sutton, 1994; Negri *et al*., 2001; Huggett *et al*., 2006); however, little is know about the composition of these microbial communities as a whole or their interactions with the host CCA. Recently, it has been shown that elevated temperature alters the composition of CCA-associated *Bacteria*, which in turn negatively affects the recruitment of coral larvae (Webster *et al*., 2010). A few studies of cultivable bacteria found that CCA harbour some unique isolates compared with other reef substrates (Lewis *et al*., 1985; Johnson *et al*., 1991), but cultivation techniques notoriously miss the vast majority of environmental microbes (Fuhrman and Campbell, 1998). Finally, despite these hints at their significance, very little is known about microbial associations with the diverse suite of macroalgal species on coral reefs.

Field observations have shown that benthic algae affect microbial communities in the surrounding seawater. For example, algal-dominated patches of reefs have been found to have lower levels of oxygen in the overlying seawater, indicating that microbial activity is higher in these areas (Haas *et al*., 2010). Furthermore, Dinsdale and colleagues found that reefs dominated by algae had higher abundances of heterotrophic bacteria and potential pathogens than coral-dominated reefs (Dinsdale *et al*., 2008). It has been proposed that these changes are the result of labile organic carbon released by benthic algae that is stimulating microbial activity, and indeed it has been found that microbes rapidly consume algal-derived organic matter (Haas *et al*., 2010). Increases in the release of algal exudates on a reef as a result of increased algal abundance on the benthos may be affecting reef health by altering the production, abundance and function of the surrounding microbial communities, thus potentially leading to increases in coral disease, coral death and increased algal proliferation on the reef. Understanding the diversity and function of microbes associated with benthic reef algae will further our understanding of potential relationships between these two groups, as well as provide insight into how benthic algae interact with the microbial world, including on their surfaces, the surrounding water column, and organisms with which they come into contact (e.g. corals, herbivores, etc.).

Here we describe the composition of bacterial communities associated with four major ecological functional groups of benthic algae: encrusting calcifying algae (CCA), upright calcareous algae (*Halimeda opuntia*), fleshy macroalgae (*Dictyota bartayresiana*) and turf algae. For comparison, the bacterial communities associated with the common reef-building coral *Montastraea annularis* were also analysed using the same approach: high-throughput sequencing of the V1–V3 region of the 16S rRNA gene. We show that the algae host characteristic bacterial communities that are more diverse than those associated with corals.

Results

Each library contained between 33 321 and 107 917 reads, with an average read length between 305 and 439 base pairs (bp) after primer and barcode removal (Table S2). Algal libraries had the highest abundance of chloroplast contamination, which ranged from 12–86% of the sequences. The total number of bacterial sequences per library after chloroplast removal is listed in Table S2, and ranged from 9503–104 364 sequences. All sequences were submitted to the NCBI Sequence Read Archive (SRA023821.1).

Diversity of bacteria associated with corals and algae

The values of the alpha diversity metrics [i.e. observed operational taxonomic units (OTUs), predicted OTUs (Chao1) and Shannon-Weiner Diversity (H′)] varied depending on whether the RDP or QIIME pipeline was used and whether or not the data was error corrected by denoising; however, the relative differences between samples were consistent regardless of the method used (Table S3). For ease of interpretation, only the QIIME analyses of the denoised sequences are discussed.

A range of 163–259 different OTUs were observed associated with coral tissue from Site 1, with a predicted

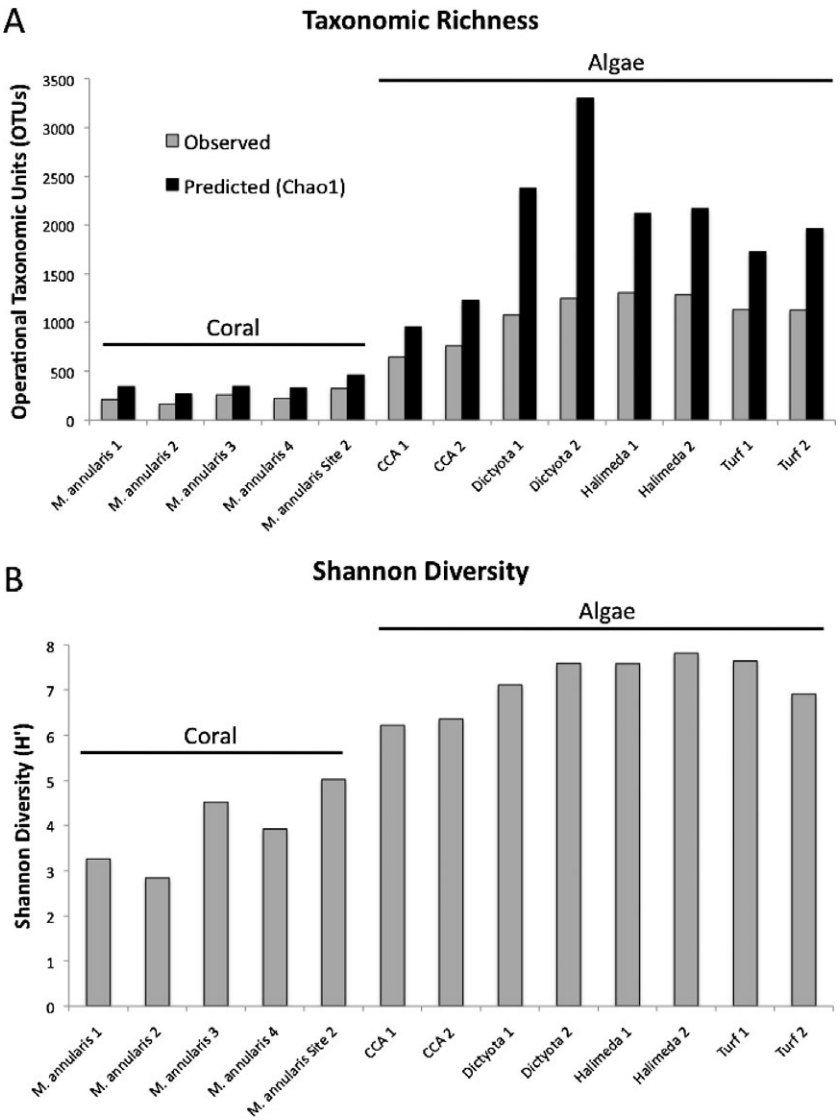

Fig. 1. Alpha diversity of bacteria associated with the coral *M. annularis* and benthic algae. (A) Number of OTUs observed and predicted (Chao1) and (B) Shannon-Weiner diversity of bacteria from corals and algae. OTUs were grouped at 97% similarity.

range of 266–346 OTUs in the community (Chao1, Fig. 1A). Richness was higher on corals at Site 2 (323 and 461 OTUs observed and predicted respectively; Fig. 1A). Bacterial diversity as determined by the Shannon–Weiner index (H′) ranged from 2.84–4.51 at Site 1, and was highest at Site 2 (5.03, Fig. 1B). The richness associated with each of the different types of algae was higher than that observed on any of the coral samples. Of the different algae, *D. bartayresiana* had the highest number of predicted OTUs (2375–3300) followed by *H. opuntia* (2119–2167), turf algae (1725–1961) and CCA (953–1232; Fig. 1A). Bacterial diversity (H′) associated with algal tissue was also higher than that observed on corals, with H′ ranging from 6.22–7.82 (Fig. 1B). The highest observed diversity was associated with *H. opuntia*, but overall, bacterial diversity was similar for all algae except CCA, which was lower than the other three algal types (6.22–6.36 versus 6.91–7.82 respectively; Fig. 1B).

Composition of bacteria associated with corals and algae

A range of 14–21 unique phyla were found associated with corals. Coral-associated bacterial communities were dominated by sequences related to *Proteobacteria* (~75%), followed by *Bacteroidetes*, *Firmicutes* and *Actinobacteria* (Fig. 2). Coral from Site 2 had a greater abundance of sequences related to *Actinobacteria* than corals from Site 1 (23% versus 2.1–5.8%; Fig. 2). The most abundant genus from all coral samples was *Acidovorax* (43%, Table 1), a member of the *Comamonadaceae* family. Other members of this family were also common on corals [e.g. *Diaphorobacter* (5.3%), *Delftia* (2.3%) and *Curvibacter* (3.6%)]. Other genera common on coral tissue included *Lactobacillus* (6.6%), *Aquabacterium* (8.2%), *Cloacibacterium* (8.2%) and *Propionibacterium* (3.7%, Table 1).

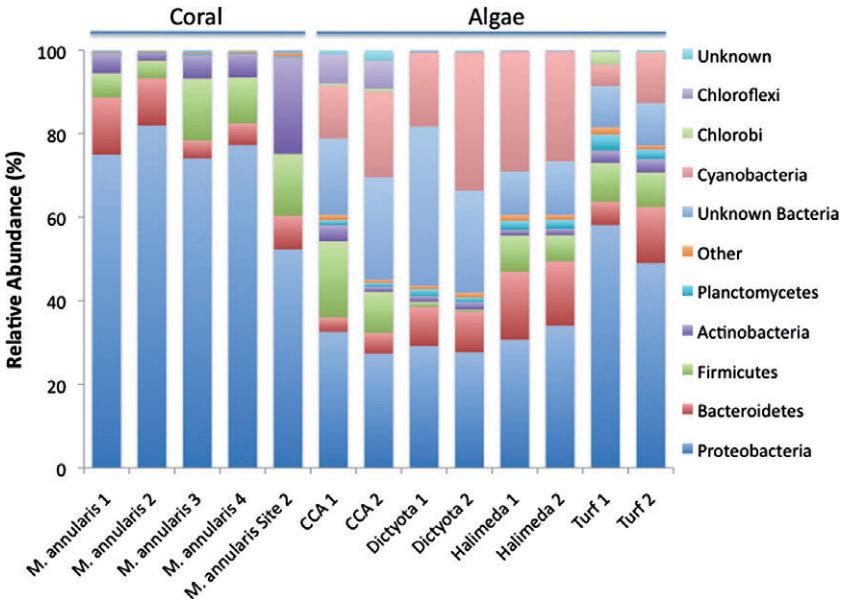

Fig. 2. Relative abundance of phyla associated with the coral *M. annularis* and benthic algae. 'Unknown' sequences could not be classified into any known group. 'Unknown *Bacteria*' sequences classified as *Bacteria* but could not be further identified.

Between 18–22 unique phyla were associated with the different types of algae. Sequences similar to *Proteobacteria* and *Cyanobacteria* were abundant across all types of algae (Fig. 2). Of the different algae, *D. bartayresiana* and *H. opuntia* had the highest abundance of sequences related to *Cyanobacteria* (33% and 27% respectively; Fig. 2). CCA communities also included an abundance of sequences similar to *Firmicutes* (18%) and *Chloroflexi* (7%), while *D. bartayresiana*, *H. opuntia* and turf algae included an abundance of sequences related to *Bacteroidetes* (10–15%). A large proportion of both the CCA and *D. bartayresiana* libraries could not be classified beyond *Bacteria* (18–38%; Fig. 2). The most abundant OTU (97% similarity) associated with algae varied by functional group. CCA libraries were dominated by sequences most closely related to various *Cyanobacteria* (5.1–5.6%), *Lactobacillus* (8.0%), *Chloroflecaceae* (6.5%), *Curvibacter* (4.2%), *Pseudomonas* (3.9%) and *Delftia* (3.8%, Table 1). The ten most abundant OTU associated with *D. bartayresiana* included groups related to various unclassified *Bacteria* and Cyanobactera (1.5–5.0%), while *H. opuntia* libraries were dominated by sequences related to *Cyanobacteria* Group I (8.0%), *Lactobacillus* (5.1%), *Curvibacter* (2.7%), *Silicibacter* (2.0%) and *Rhodobacteraceae* (2.0%, Table 1). Turf algae shared several OTU in common with corals, including those most similar to *Acidovorax* (11%), *Lactobacillus* (5.3%), *Cloacibacterium* (4.9%) and *Curvibacter* (3.8%, Table 1). The most abundant OTU associated with turf algae also included sequences most similar to an unknown *Alphaproteobacteria* (2.4%), *Rhodobacteraceae* (2.0%), *Rhizobiales* (1.3%) and *Prosthecochloris* (1.1%).

All of the algal-associated communities contained a low abundance of sequences similar to several genera of *Cyanobacteria* previously found associated with coral black band disease (BBD), including *Leptolyngbya*, *Geitlerinema*, *Oscillatoria*, *Phormidium* and *Cyanobacterium* SC-1 and OSC (Myers *et al.*, 2007) (Table 2). None of

Table 1. Classification of the 10 most abundant bacterial OTUs associated with the coral *Montastraea annularis* and benthic algae, listed from most to least abundant.

M. annularis	Coralline crustose algae (CCA)	*D. bartayresiana*	*Halimeda opuntia*	Turf algae
Acidovorax (43)	*Bacteria* (11)	*Cyanobacteria* (5.0)	*Cyanobacteria* Group I (8.0)	*Acidovorax* (11)
Cloacibacterium (8.2)	*Lactobacillus* (8.0)	*Bacteria* (4.2)	*Cyanobacteria* (6.0)	*Lactobacillus* (5.3)
Aquabacterium (8.2)	*Chloroflexaceae* (6.5)	*Acidovorax* (3.7)	*Lactobacillus* (5.0)	*Cloacibacterium* (4.9)
Lactobacillus (6.6)	*Cyanobacteria* Group I (5.6)	*Cyanobacteria* (2.8)	*Cyanobacteria* (4.9)	*Curvibacter* (3.8)
Diaphorobacter (5.3)	*Cyanobacteria* (5.1)	*Bacteria* (2.7)	*Curvibacter* (2.7)	*Alphaproteobacteria* (2.4)
Propionibacteria (3.7)	*Curvibacter* (4.2)	*Cyanobacteria* (2.1)	*Silicibacter* (2.0)	*Cyanobacteria* (2.1)
Curvibacter (3.6)	*Pseudomonas* (3.9)	*Bacteria* (2.0)	*Rhodobacteraceae* (2.0)	*Rhodobacteraceae* (2.0)
Pseudomonas (2.9)	*Delftia* (3.8)	*Cyanobacteria* (2.0)	*Delftia* (2.0)	*Silicibacter* (1.6)
Methylobacterium (2.3)	*Bacteria* (3.2)	*Bacteria* (1.7)	*Psuedomonas* (1.5)	*Rhizobiales* (1.3)
Novosphingobium (2.3)	*Cyanobacteria* Group VIII (2.0)	*Cyanobacteria* (1.5)	*Cyanobacteria* (1.2)	*Prosthecochloris* (1.1)

Relative abundance (%) of each OTU is included in parentheses.

Table 2. Relative abundance (%) of potential pathogens associated with corals and algae.

Library	White plague (*A. coralicida*)	Black band disease	Coral disease associated	Potential pathogens
Coral 1	0.04	0	12.4	4.7
Coral 2	0.01	0	12.2	3.5
Coral 3	**0.05**	0	22.3	**7.3**
Coral 4	**0.10**	0	26.0	5.3
Coral Site 2	**0.08**	0	29.1	**14.6**
CCA 1	0.01	0.08	22.6	6.0
CCA 2	0.02	0.18	20.1	4.6
Dictyota 1	0.003	0.37	**39.6**	4.6
Dictyota 2	0.01	**1.7**	**40.8**	4.1
Halimeda 1	0.004	**1.2**	30.2	4.9
Halimeda 2	0.01	**0.87**	33.4	5.2
Turf 1	0.04	0.05	**51.1**	**9.3**
Turf 2	0.04	0.10	25.3	7.1

Libraries were analysed by BLASTn. Values listed are the percentage of the sequences in each library that were similar to the listed coral diseases. The top three most abundant of each pathogen are in bold. The list of coral disease-associated bacteria was obtained from Mouchka and colleagues (2010).

these genera was found associated with any of the coral tissue samples. In addition, analysis of the libraries by BLASTn found a low abundance of hits to *Aurantimonas coralicida* 16S rDNA, the only known coral pathogen previously found associated with algae (Nugues *et al*., 2004), in every library (Table 2). The abundance of sequences similar to coral disease associated bacteria was generally higher in the algal libraries with the exception of CCA (Table 2). Conversely, the abundance of sequences similar to general potential pathogens was highest on coral from Site 2 and turf algae (Table 2).

Phylogenetic distance between coral and algal-associated bacterial communities

Principal component analysis (PCA) of the weighted UniFrac distance showed that corals and algae harbour characteristic communities of *Bacteria*. All of the coral tissue samples clustered to the right of the graph along the primary axis (65% of the variability) and away from the algal samples, but did not cluster in the second dimension (12% of the variability, Fig. 3). The bacterial communities associated with the corals from Site 2 did not fall within the loose cluster of communities from Site 1, indicating that there may possibly be some differences in communities due to location. Algal-associated bacterial communities clustered separately from the corals and by the type of alga. *Halimeda opuntia* communities were the most similar to each other, clustering tightly (Fig. 3). The *Dictyota bartayresiana* libraries also clustered together, although not as closely as the *H. opuntia* libraries. Turf algal communities had the greatest separation between the two libraries, and one turf library most closely clustered with one of the CCA libraries (Fig. 3). Overall, the two PCA axes explained 76.8% of the variation between the different communities.

General metabolic composition of coral and algal-associated communities

Coral-associated bacterial libraries were dominated by sequences most similar to facultative anaerobes (55–83%, Fig. 4A). In contrast, the majority of classifiable *Bacteria* associated with the various algal tissues were most similar to obligate aerobes (54–93%, Fig. 4A). Coral bacterial communities were dominated by groups most closely related to known heterotrophs (> 99% for coral tissue; Fig. 4B), while algal-associated bacterial communities contained more groups related to photoautotrophs, varying between 32% of the community for *H. opuntia* to 5% for turf algae (Fig. 4B). Algal-associated communities also harboured a greater abundance of sequences that could not be classified to family or genus, and thus had more unknown metabolisms. The majority of the sequences were most closely related to Gram-negative *Bacteria* for all coral and algal- associated bacterial libraries (data not shown).

Chloroplast diversity and taxonomy associated with benthic reef algae

The number of OTUs similar to chloroplasts was highest in the *H. opuntia* samples (115–123), followed by *D. bartayre-*

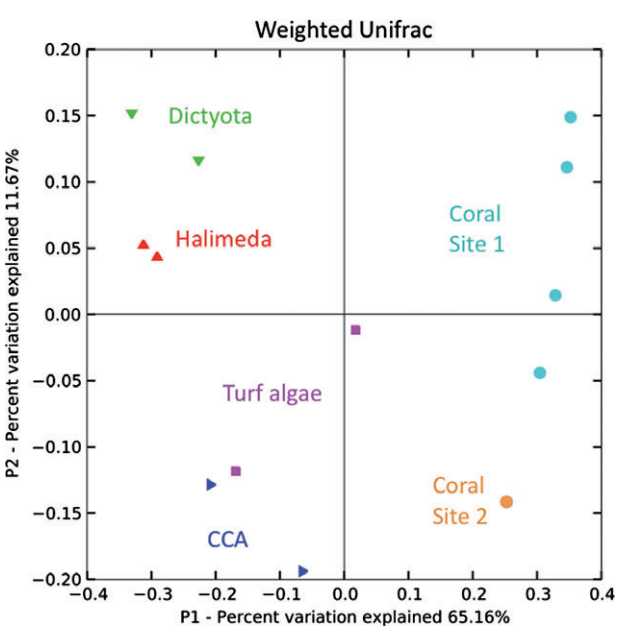

Fig. 3. Principal component analysis of weighted UniFrac distance. Light blue = *M. annularis* from Site 1, orange = *M. annularis* from Site 2, dark blue = CCA, green = *Dictyota bartayresiana*, purple = turf algae, red = *Halimeda opuntia*.

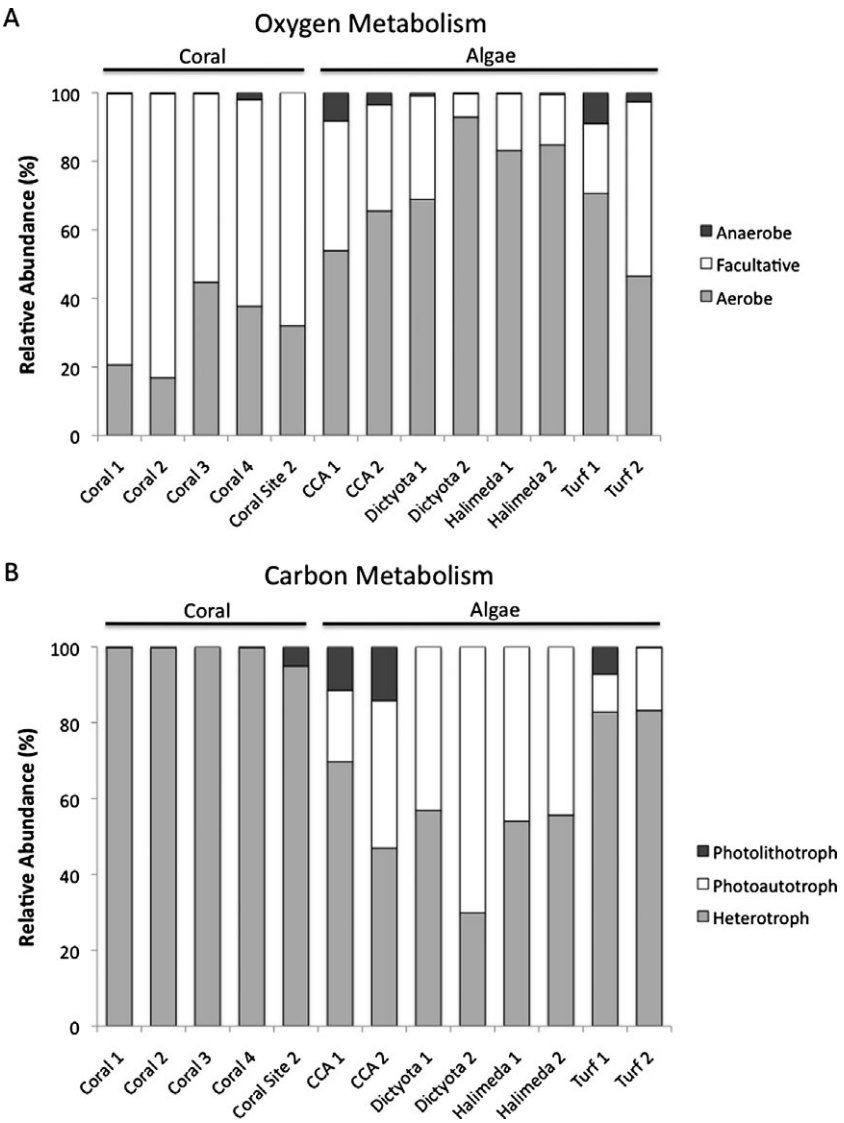

Fig. 4. Relative abundance of classifiable bacterial metabolisms associated with the coral *M. annularis* and benthic algae. (A) Oxygen metabolism and (B) carbon metabolism.

siana and turf algae (76–100), and was lowest associated with CCA (43–58; Fig. 5A). There was little difference between the number of observed and predicted OTUs (Fig. 5A), indicating high coverage of the communities. Again, the highest predicted richness was observed on *H. opuntia*, followed by *D. bartayresiana* and turf algae, and lastly CCA (Fig. 5A). Shannon–Weiner diversity of algal-associated chloroplasts followed a different pattern than richness. *Halimeda opuntia* and turf algae had the highest diversity (4.04–4.68), followed by CCA (2.56–3.10) and lastly *D. bartayresiana* (1.67–2.26) (Fig. 5B).

The majority of chloroplast sequences associated with each algal library were likely from the host [*Florideophyceae* (red algae) for CCA, *Phaeophyta* (brown algae) for *D. bartayresiana*, and both *Phaeophyta* and *Florideophyceae* for turf algae]. The exception was the *H. opuntia* libraries, which were dominated by sequences most closely related to the diatom family *Bacillariophyceae* (40–70%), followed by *Florideophyceae* (13–35%; Fig. 6). *Florideophyceae* were also present on *D. bartayresiana* (4.3–8.1%; Fig. 6). CCA libraries included sequences similar to both green and brown algae [*Ulvophyceae* (2.2–11%) and *Phaeophyta* (6.8–21%) respectively; Fig. 6]. All libraries contained a low abundance of sequences similar to a wide variety of unicellular algae (Fig. 6).

Discussion

Expanding the holobiont concept to algae

The holobiont concept was first applied to corals after they were found to host abundant and species-specific microbial communities, and these microbes were hypothesized to provide some benefit to their host (Rohwer *et al*., 2002;

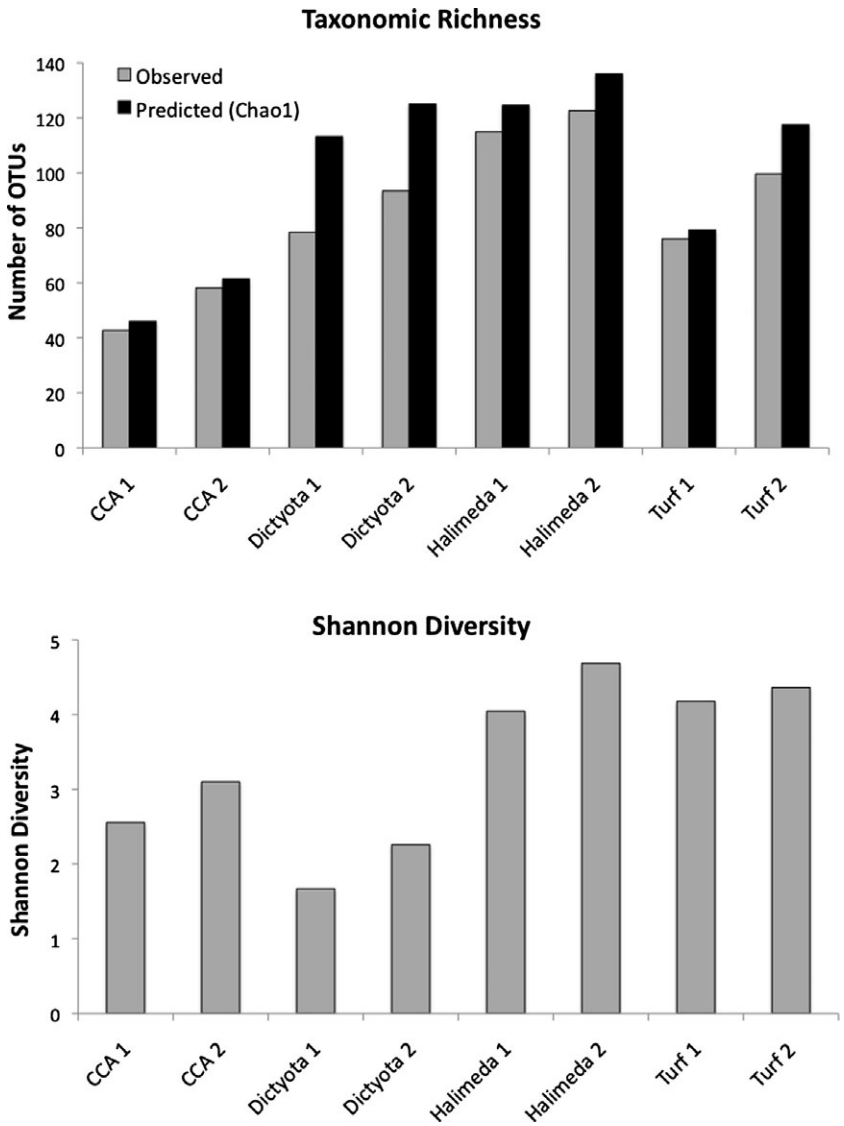

Fig. 5. Diversity of chloroplasts associated with benthic algae. OTUs were grouped at 97% similarity.

Reshef *et al*., 2006; Rosenberg *et al*., 2007). Our results demonstrate that the holobiont concept may also be applicable to benthic algae. *Bacteria* are abundant on algal surfaces, ranging from 1×10^6–3×10^7 per cm^2 on various macroalgae (Maximilien *et al*., 1998) and 1.6×10^7 per gram wet weight on CCA (Lewis *et al*., 1985). Here we have found that, much like coral-associated *Bacteria*, algal-associated bacterial communities are specific to the type of algae examined. In addition, these characteristic bacterial communities associated with each of the different types of algae were more diverse than those found on corals, including up to 10 times more types of taxa. The data from each library in this study represent a pool of five different individuals, thereby providing an in-depth snapshot of the characteristic types of *Bacteria* that typically colonize these organisms. There is some variability between the two pools from the same species, indicating that there is variability between individuals; however we cannot assess the magnitude of this individual variation. Our data do suggest that *H. opuntia*, for example, has the least variability and hence likely the greatest host specificity. Conversely, turf-associated *Bacteria* showed the most variability between libraries. It is likely that the diverse heterogeneous assemblage of algae that makes up turf algal communities increases the heterogeneity of the associated bacterial communities, leading to the differences observed in this study.

The characteristic nature of some of the algal-associated *Bacteria* support the hypothesis that algae influence the types of *Bacteria* that can survive on the algal surface, and there are a variety of mechanisms algae may employ to achieve this. For example, some algae produce secondary metabolites that are directly toxic to *Bacteria* (Gross, 2003; Lam *et al*., 2008a) or

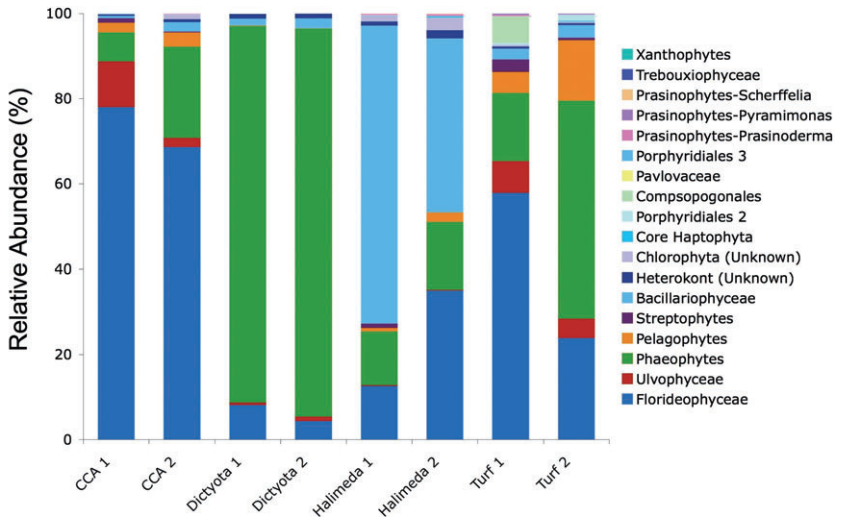

Fig. 6. Relative abundance of chloroplast phyla associated with benthic algae. Taxa listed fall into the following groups: green algae – *Prasinophytes-Prasinoderma*, *Prasinophytes-Scherffelia*, *Prasinophytes-Pyramimonas*, *Streptophytes*, *Trebouxiophyceae*, *Ulvophyceae* and *Chlorophyta*; *Haptophytes* (unicellular algae) – Core *Haptophytes* and *Pavlovaceae*; heterokonts – *Bacillariophyta* (diatoms), *Pelagophytes* (algae), *Phaeophytes* (brown algae) and *Xanthophytes* (yellow-green algae); and Red algae – *Compsopogonales*, *Florideophyceae*, *Porphyridiales* 2 and *Porphyridiales* 3.

inhibit quorum sensing (Givskov *et al*., 1996; Steinberg *et al*., 1997), and physical mechanisms like mucus release and tissue sloughing likely affect the types of bacteria that survive on the algal surface (Keats *et al*., 1997; Gross, 2003). Furthermore, release of organic compounds by algae may selectively promote growth of certain groups of bacteria, and it has been shown that algal DOM differentially stimulates bacterial growth based on the type of alga from which it originated (Haas *et al*., 2010).

Bacteria associated with algae are also likely providing some benefits to their algal host. Similar to coral-associated microbes, studies have found that *Bacteria* isolated from algae are antagonistic towards some types of fouling *Bacteria* (Boyd *et al*., 1999), and in addition some isolates help prevent fouling by invertebrate larvae (Holmström *et al*., 1996; Egan *et al*., 2000). It is possible that resident *Bacteria* on algal surfaces are excluding algal pathogens and resource competitors (i.e. fouling invertebrates and photosynthetic eukaryotes), thus indirectly promoting the health of the host. In addition, some groups of *Bacteria* associated with algae may be fulfilling multiple beneficial roles for the host. *Cyanobacteria*, for example, which were abundant on the various benthic algae, have been shown to protect algae from herbivory (Fong *et al*., 2006), and nitrogen fixed by this group may serve as an important nutrient source for the host alga (Wilkinson *et al*., 1984; Shashar *et al*., 1994).

Finally, the types of microbes associated with algae may have implications in coral reef health. First, all of the algal libraries examined contained sequences related to *Bacteria* found associated with coral disease states. While coral disease associated *Bacteria* are not necessarily pathogens and may be opportunistic colonizers of degraded coral tissue, the presence of these types of *Bacteria* suggest that benthic algae may be a potential source of coral pathogens and/or opportunistic colonizers that may lead to coral death in the wake of stress or disease. Second, *Cyanobacteria* most similar to those associated with coral black band disease were observed on all four groups of algae examined. *Halimeda opuntia* has previously been found to harbour and transmit the coral pathogen *A. coralicida* (Nugues *et al*., 2004). Given that the present study found bacteria closely related to *A. coralicida* as well as BBD, it is possible that various benthic reef algae serve as reservoirs for a variety of potential coral pathogens. It remains to be determined if the presence of these bacteria associated with CCA, *D. bartayresiana*, or turf algae can lead to transmission of coral disease.

Benthic algae harbour diverse communities of photosynthetic eukaryotes

Abundant sequences related to chloroplasts in the libraries showed that there is a large diversity of photosynthetic eukaryotes associated with benthic reef algae. Over a 100 different OTUs were observed, most of which were novel. The presence of a high diversity of photosynthetic eukaryotes suggests that there may be intense competition on the surface of the algae for nutrients and light, a phenomenon that has been hypothesized with interactions between algae and benthic diatoms (Gross, 2003). In fact, many types of macroalgae release allelochemicals that target photosynthetic eukaryotes such as diatoms (Keats *et al*., 1997; Gross, 2003; Lam *et al*., 2008b), yet despite these defences it is clear that a wide variety of microalgae are capable of colonizing algal surfaces.

Coral-associated bacteria are primarily facultative anaerobes and show site specificity

The diversity of the coral-associated bacterial communities in this study is similar to the diversity observed from

previous 16S rDNA and metagenomic studies of coral-associated microbes (Rohwer *et al*., 2002; Bourne and Munn, 2005; Bourne *et al*., 2007; Wegley *et al*., 2007; Thurber *et al*., 2009; Sunagawa *et al*., 2010). These communities were dominated by sequences similar to known facultative anaerobes (60–80% total), with the remainder comprised of sequences similar to strict aerobes. The capacity of the microbial community for oxygen-dependent and anaerobic respiration presumably reflects adaptations to the variations in oxygen saturation of coral tissues and the surrounding boundary layer, which range from superoxic during the day to anoxic at night (Shashar *et al*., 1993). Previous work has demonstrated that the heterotrophic communities associated with corals are more productive than the surrounding seawater communities (Paul *et al*., 1986), and the dominance of heterotrophs and relatively rapid production rates of coral-associated microbes reflect the lability of the carbon-rich habitat of the coral mucus (Brown and Bythell, 2005).

The composition of the coral-associated bacterial communities showed differences between the two sites studied, indicating that location plays a role in shaping coral–bacterial associations. While the phylogenetic composition of coral-associated bacteria from the two different sites, located approximately 40 km apart, was similar, the relative abundances of each of the different taxa present were different. This suggests that while the bacterial communities associated with *M. annularis* vary significantly between sites, likely due to environmental factors, the species of coral likely shapes the phylogenetic composition of the associated bacterial community regardless of location.

Microbial diversity associated with coral reefs

Benthic organisms are proving to be an enormous reservoir of microbial diversity. Given an average of ~ 300 bacterial OTUs estimated to be associated with any given coral species and that there are ~ 50 coral species in the Caribbean, there are an estimated 15 000 different types of bacteria associated with Caribbean corals. If we now include the four different groups of algae in this study (fleshy macroalgae, encrusting calcareous algae, upright calcareous algae and turf algae), there are an estimated 7700 OTUs associated with this very small sample of algal diversity in the Caribbean (out of ~ 500 spp.). If bacterial taxa associated with different species of algae within these four functional groups are as species specific as coral-associated bacteria appear to be, there are potentially tens of thousands of unique bacterial taxa associated with the reef benthos. On Curacao, for example, there are as many as 142 different algal species along one 115 m reef transect, and an average of 54 unique algal species per 25 m^2 (Van Den Hoek *et al*., 1975). If each algal species has a characteristic bacterial community as diverse as those identified here, there are potentially 135 326–468 600 different bacterial taxa along one 115 m transect from the shoreline down the reef slope. Additionally, within one 25 m^2 reef plot, there are between 51 462 and 178 200 bacterial taxa associated with the algae alone. Given that a large proportion of all the algal-associated bacterial libraries were unclassifiable beyond *Bacteria*, this represents an underexplored reservoir of microbial diversity in the world.

Conclusion

Benthic reef algae have characteristic microbial communities associated with their tissue. Very little is known about the role that this diversity plays in reef ecology, but there are likely both positive or facilitative interactions as well as negative or antagonistic interactions between the microbiota and macrobiota. These microbial assemblages likely contribute to nutrient cycling and gas exchange and subsequent growth and abundance of corals and algae but may also include several potential pathogens. The specific interactions between algae and the microbial world have important implications for reef health. As reservoirs of coral pathogens, they have the potential to transmit disease across the reef, and as algae become increasingly abundant on coral reefs around the world, this may create a positive feedback loop whereby the more algae that are present, the greater the potential to transfer pathogens. In addition to their affects on corals, bacteria associated with benthic algae likely play a role in the proliferation of algae by fixing nitrogen, preventing herbivory, and possibly by exclusion of algal pathogens and competing primary producers. Photosynthetic eukaryotes associated with algae, on the other hand, may be competing with the host alga for nutrients, light and inorganic carbon. It remains to be seen how changes in environmental conditions such as reduced herbivory, increased eutrophication and elevated sea surface temperature influence the microbial communities associated with benthic reef algae and how these changes affect the physiology and success of algae on coral reefs around the world.

Experimental procedures

Sample collection

Samples were collected from the island of Curacao, Netherlands Antilles, with permission from the CARMABI research station. All algal samples and the majority of the coral samples were collected 8–10 m deep at Site 1 (Water Factory) on the southern side of the island (12°06′35.10″N, 68°57′22.91″W). An additional set of coral samples was collected from a similar depth at a distant site on the far western point of the island (Site 2; 12°22′36.40″N, 69°09′39.51″W).

Tissue samples were collected from the coral *M. annularis* and four different types of algae: (i) crustose coralline algae (CCA), (ii) *Halimeda opuntia*, (iii) *Dictyota bartayresiana* and (iv) turf algae. Tissue punches were collected underwater using a hollow punch and hammer (diameter = 0.64 cm), with the exception of the *H. opuntia*, which was collected by hand. Each tissue sample was placed in an individual sterile whirl-pack underwater. Two tissue samples were taken from five different individuals for each of the four types of algae, 20 different coral colonies of *M. annularis* were sampled at Site 1, and five different colonies of *M. annularis* were sampled at Site 2. Samples were returned to the lab within 30–60 min, placed in a solution of 25 mM sodium citrate, 10 mM ethylenediaminetetraacetic acid (EDTA), and 10 mM ammonium sulfate to preserve nucleic acids, and frozen at −20°C. All reagents were from Fisher Scientific unless otherwise noted.

DNA extraction

Coral tissue was removed from the skeleton using an airbrush with 0.2 μm filter-sterilized TE buffer [10 mM Tris(hydroxymethyl)aminomethane hydrochloride (pH 8)/1 mM EDTA]. An aliquot of the tissue slurry (500 μl) was centrifuged for 20 min at 14 000 *g* and resuspended in lysis buffer (50 mM Tris-HCl, pH 8.3, Sigma-Aldrich; 40 mM EDTA, pH 8; 0.75 M sucrose, Sigma-Aldrich). A lysozyme digestion (5 mg ml^{-1}, Sigma-Aldrich) was performed for 30 min at 37°C. This was followed by a second lysis with proteinase K (0.5 mg ml^{-1}) and sodium dodecyl sulfate (SDS, 1%) at 55°C overnight. Following lysis the sample was incubated at 70°C for 10 min to inactivate the enzyme, and DNA was precipitated by adding sodium acetate (0.3 M final concentration, Sigma-Aldrich) and an equal volume of isopropanol. This was then incubated at −20°C for 4–5 h. The DNA was then pelleted by centrifugation at 14 000 *g* for 20 min at 4°C and resuspended in TE buffer. At this point a cetyltrimethylammonium bromide (CTAB, Sigma-Aldrich) extraction was performed (1% SDS, 0.7 M NaCl, 0.27 mM CTAB). The sample was incubated at 65°C for 10 min. The sample was then extracted with phenol : chloroform : isoamyl alcohol (25:24:1; Sigma-Aldrich). The DNA was precipitated by adding 0.7 volume isopropanol and incubating at −20°C overnight. DNA was pelleted by centrifugation at 14 000 *g* for 15 min at 4°C. The pellet was washed with cold 70% ethanol, dried and resuspended in 10 mM Tris (pH 8, Sigma-Aldrich). Algal tissue was homogenized with an epimortar. An aliquot of homogenate (250 μl) was used for DNA extraction with the Mo Bio UltraClean Soil Kit (Solana Beach, CA, USA) according to the manufacturer's instructions. All DNA extracts were stored at −20°C.

PCR and sequencing preparation

A 526 bp region of the 16S rRNA gene (16S rDNA) including the variable regions 1–3 was selected for tag pyrosequencing. This region was amplified using the bacterial forward primer 27F, which also included the primer B adaptor for pyrosequencing on the 5′ end (5′-GCCTTGCCAGCCCG CTCAGTCAGAGTTTGATCCTGGCTCAG-3′). The bacterial reverse primer 534R was also used, and included the sequencing primer A and a unique 8 bp barcode on the 5′ end (5′-GCCTCCCTCGCGCCATCAGNNNNNNNNCAATTACCG CGGCTGCTGG-3′). Barcodes were error-correcting Hamming sequences (Hamady *et al.*, 2008), and can be found in Table S1. The length of the amplicon including barcode and 454 primers was 578 bp. Amplifications were run under the following conditions: 94°C for 5 min; 29 cycles of 94°C for 1 min, 60°C - 0.5°C/cycle for 1 min, and 72°C for 1 min; followed by 72°C for 10 min. Each DNA extract was amplified by four replicate PCR reactions, which were then combined. These PCR products were then purified using the Bioneer AccuPrep PCR Purification Kit (Alameda, CA, USA) and the amount of DNA in each sample was quantified using the Quant-iT PicoGreen assay (Invitrogen, Carlsbad, CA, USA). The algal PCR products were then pooled such that two pools were generated per algal type. To generate these pools, PCR amplicons from five different individuals of the same type of alga were combined in equimolar amounts. The same barcode was used for each of the five algal samples within one pool. Coral samples were also pooled in groups of five individuals for a total of four pools from Site 1 and one pool from Site 2. Each sample within a pool was amplified independently but with the same barcode, as done with the algal samples. The barcode used for each library is listed in Table S2. Once this was complete, sample pools were combined together in equimolar amounts and sequenced using the 454 Titanium platform at Engencore (University of South Carolina).

Sequence analysis

Sequences were first screened for quality using the following parameters: minimum quality score of 25, minimum sequence length of 200 bp, maximum length of 1000 bp, and no ambiguous bases in the entire sequence or mismatches in the primer sequence. Any sequences not meeting these parameters were excluded from downstream analyses. Sequences were then sorted by barcode into their respective samples and the barcode and primer sequences were removed. Sequences were then denoised (i.e. error corrected) (Reeder and Knight, 2010). For comparison, denoised and non-denoised sequences were analysed for diversity by two different methods: the Ribosomal Database Project (RDP) pyrosequencing pipeline (http://pyro.cme.msu.edu) and the Quantitative Insights into Microbial Ecology (QIIME) pipeline (Caporaso *et al.*, 2010). Sequences were first grouped into OTUs with a 97% identity threshold. Using the RDP pipeline, sequences were aligned with the Infernal aligner and then grouped using a complete linkage clustering method. Using QIIME the sequences were clustered by CD-HIT (Li and Godzik, 2006). Once clustered, a representative sequence from each OTU was selected and taxonomic identity was assigned to each representative sequence using the RDP taxonomic classifier at 80% confidence (Wang *et al.*, 2007). Sequences classified as chloroplasts by the RDP were removed from the bacterial libraries and analysed separately. Sequences classified as unknown *Cyanobacteria* were suspected to include chloroplasts, and were further screened by BLASTn against the Silva SSU rRNA database (*E*-value = 10–20, minimum alignment length = 151 bp). Sequences with a best match to eukaryotes (i.e. chloroplasts) were separated from the bacterial libraries and analysed with the other chloroplasts.

Chloroplast taxonomy was determined by aligning the representative sequences from each OTU to 185 organism-specific plastid and bacterial 16S rRNA genes downloaded from GenBank using MAFFT (Katoh *et al*., 2005). A PHYML maximum likelihood (ML) tree (GTR + I + gamma 4) with aLRT branch supports was calculated from the total dataset (590 sequences; Anisimova and Gascuel, 2006). Short, low quality or divergent sequences were separated at this point and their phylogenetic affiliations to the organism specific sequences were determined individually. The remaining sequences from the total dataset were processed through multiple rounds of ML tree drawing, and sequences with significant (> 0.95 aLRT supports) to known plastids were progressively excluded from the dataset. Narrowing the total dataset using this procedure allowed for determination of phylogenetic affiliations of most of the sequences to eukaryotic phyla or families. Where the phylogenetic position was not significantly resolved or the sampling of plastid lineages was not sufficient the classification was designated as such (e.g. unidentified heterokont).

In order to analyse alpha diversity, bacterial and chloroplast libraries were randomly sub-sampled using QIIME so that sequencing effort (i.e. the number of sequences in each library) did not affect diversity comparisons. Bacterial libraries were sub-sampled at a step size of 90 sequences from 1–9500 sequences a total of 10 times. Chloroplast libraries were sub-sampled at a step size of 50 sequences from 1–5500 sequences a total of 10 times. Once the libraries were rarified, the following alpha-diversity metrics were determined: total observed species (OTUs), predicted species (Chao1) and Shannon–Weiner diversity (H′). The same alpha diversity metrics were also determined using the RDP pyrosequencing pipeline. Beta-diversity of the bacterial communities was analysed in QIIME using a weighted UniFrac analysis. Principal components for each sample library were generated from the UniFrac distances and plotted in two dimensions. Finally, individual bacterial libraries were analysed by BLASTn against two different databases of 16S rDNA sequences of (i) potential pathogens and (ii) coral disease-associated bacteria (Mouchka *et al*., 2010). BLASTn parameters for a significant hit required over 150 bp alignment, *E*-value less than 1×10^{-10} and greater than 95% identity, and the number of sequences that hit each database was tallied.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. List of barcode and primer sequences used for multiplex tag sequencing.
Table S2. Summary of the number of reads and average read length per library following quality screen and primer removal. Reads analysed refers to the number of reads following removal of chloroplast contamination. Barcode number corresponds to the barcode sequences found in Table S1.
Table S3. Comparison of diversity results [Chao1 (H′)] of bacterial 16S rDNA sequences. Libraries were analysed for diversity before and after denoising using either the QIIME or RDP pyrosequencing pipeline with 97% similarity clustering.

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