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ORIGINAL ARTICLE

Crustose coralline algal species host distinct bacterial assemblages on their surfaces

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Crustose coralline algae (CCA) are important components of many marine ecosystems. They aid in reef accretion and stabilization, create habitat for other organisms, contribute to carbon sequestration and are important settlement substrata for a number of marine invertebrates. Despite their ecological importance, little is known about the bacterial communities associated with CCA or whether differences in bacterial assemblages may have ecological implications. This study examined the bacterial communities on four different species of CCA collected in Belize using bacterial tag-encoded FLX amplicon pyrosequencing of the V1–V3 region of the 16S rDNA. CCA were dominated by Alphaproteobacteria, Gammaproteobacteria and Actinomycetes. At the operational taxonomic unit (OTU) level, each CCA species had a unique bacterial community that was significantly different from all other CCA species. *Hydrolithon boergesenii* and *Titanoderma prototypum,* CCA species that facilitate larval settlement in multiple corals, had higher abundances of OTUs related to bacteria that inhibit the growth and/or biofilm formation of coral pathogens. Fewer coral larvae settle on the surfaces of *Paragoniolithon solubile* and *Porolithon pachydermum.* These CCA species had higher abundances of OTUs related to known coral pathogens and cyanobacteria. Coral larvae may be able to use the observed differences in bacterial community composition on CCA species to assess the suitability of these substrata for settlement and selectively settle on CCA species that contain beneficial bacteria.
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Introduction

Crustose coralline algae (CCA) are widely distributed and can be found in virtually all marine habitats. They are important calcifying species and contribute greatly to both reef accretion and stabilization (Littler and Littler, 2013). CCA have long been recognized as important settlement substrata for a variety of marine invertebrate larvae including commercially important species of abalone and sea urchins (Pawlik, 1992; Hadfield and Paul, 2001). CCA also induce larval settlement in many coral species, and this interaction has received much attention in light of the ecological importance of coral reefs and their drastic declines in recent years. Although several studies have demonstrated the ability of CCA to induce the settlement of coral larvae, the effects are not ubiquitous among CCA species. Important reef-building acroporid species, in both the Indo-Pacific and Caribbean, exhibit settlement preferences for certain CCA species (Harrington *et al.*, 2004; Ritson-Williams *et al.*, 2010, 2014). On the basis of these preferences, it is clear that some coral larvae are capable of recognizing and discriminating among CCA species, however, the mechanism by which they are able to differentiate among CCA is unknown. There is growing evidence that bacteria associated with potential settlement substrata induce settlement activity in many invertebrates including corals (Webster *et al.*, 2004; reviewed in Hadfield, 2011), and that in some cases, they respond to specific bacterial strains within the bacterial community (Negri *et al.*, 2001; Tebben *et al.*, 2011; Tran and Hadfield, 2011; Sneed *et al.*, 2014). It is therefore likely that larvae may use bacteria to recognize appropriate settlement substrata.

Several studies have demonstrated that different species of macroalgae harbor unique bacterial communities (Longford *et al.*, 2007; Lachnit *et al.*, 2009, 2011; Sneed and Pohnert, 2011). Within a given geographical area, bacterial communities are often more similar on individuals from the same algal species than they are to individuals from other algal species (Sneed and Pohnert, 2011). In some cases, algal species host specific bacterial assemblages across large geographic distances (Lachnit *et al.*, 2009). To date, most studies on algal associated bacterial communities have focused on

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temperate, fleshy algal species (Lachnit *et al*., 2011) with the exception of a few studies of tropical fleshy algae (Barott *et al*., 2011; Sneed and Pohnert, 2011). Although CCA are known to be important settlement substrata for a number of invertebrate species and bacteria are known to produce chemical cues that induce invertebrate larval settlement, little is known about the bacterial communities associated with CCA. A limited number of studies have characterized CCA-associated bacteria using molecular techniques; however, these studies did not compare bacterial communities among different CCA species (Barott *et al*., 2011; Webster *et al*., 2011, 2013). In the early 1990s, Johnson *et al.* (1991) characterized the culturable bacteria associated with two species of South African CCA based on morphological and physiological characteristics and suggested that some bacterial strains may have a specialized association with their CCA host. Although this work suggests that, like other macroalgae, CCA may also harbor specific bacterial communities, culture-independent techniques are necessary to more fully characterize these microbial communities.

In this study, we examined the bacterial communities on four different species of CCA collected in Belize using bacterial tag-encoded FLX amplicon pyrosequencing of the V1–V3 region of the 16S rDNA. We chose CCA species that had previously been tested for their ability to facilitate coral recruitment. The CCA *Titanoderma prototypum* and *Hydrolithon boergesenii* facilitate larval settlement and post-settlement survival of two important reef-building acroporid species in the Caribbean (*Acropora palmata* and *Acropora cervicornis*) (Ritson-Williams *et al*., 2010, 2014). These corals are of special interest because they have declined so drastically that they are now listed as threatened on the endangered species list. Two co-occurring CCA species, *Paragoniolithon solubile* and *Porolithon pachydermum*, are common members of the reef community but do not directly facilitate recruitment in these acroporid corals (Ritson-Williams *et al*., 2010, 2014). We compared the bacterial communities associated with these four CCA species to determine whether these algae harbor species-specific bacterial communities that may have implications for coral larval settlement preferences.

Materials and methods

Sample collection

CCA were collected on small pieces of coral rubble from a variety of locations along the Meso-American Barrier Reef at Carrie Bow Cay (CBC), Belize in July 2011. *P. solubile* ($n=5$) and *H. boergesenii* ($n=3$) were collected at 1 m depth in coral rubble found on the reef flat directly adjacent to the Carrie Bow Cay Field Station (Site 1). *T. prototypum* ($n=5$) was collected from 5 m depth from the bottom of the spur and groove habitat in front of South Water Cay (Site 2). *P. pachydermum* ($n=5$) was collected at 0.5 m depth from the top of spurs located just over the reef crest at CBC (Site 3). Additional *H. beorgesenii* ($n=2$) samples were collected from 10 to 15 m depth in a rubble field along the barrier reef (Site 4). CCA were identified to species based on morphological characteristics (Ritson-Williams *et al*., 2014).

Individual pieces of CCA were collected in individual sterile Whirl-Pak bags or 50 ml Falcon tubes. Samples were kept in the shade in a 5-gallon bucket with seawater until processing (<3 h). CCA were rinsed under a stream of 30 ml 0.22 μm-filtered seawater and the surfaces (3–14 cm^2) were swabbed vigorously with a sterile swab. Swabs were placed in 1 ml filtered seawater and vortexed for 1 min on high. Swabs were removed with sterile forceps, 200 μl was removed for use in other assays and the remaining 800 μl suspension was centrifuged for 25 min at 13 000 r.c.f. The supernatant was removed and the resulting pellet was dissolved in 175 μl of lysis buffer (400 mM NaCl, 750 mM sucrose, 20 mM ethylenediaminetetraacetic acid, 50 mM Tris-HCl pH 9.0) and stored at −20 °C.

Water samples ($n=5$) were taken from each CCA collection site within Belize in 2 ml sterile microcentrifuge tubes by opening the tubes underwater, allowing them to fill, and then closing them before removing them from the water column. Using aseptic technique, 100 μl were removed from each sample for other assays and the remaining volume was centrifuged at 13 000 r.c.f. for 25 min. The supernatant was removed and 175 μl of lysis buffer was added. Samples were stored at −20 °C.

Sequencing

Samples were sent to Molecular Research LP (MR DNA, Shallowater, TX, USA) for DNA extraction and Bacterial Tag Encoded FLX Amplicon Pyrosequencing of the V1–V3 region of the 16S rDNA (Dowd *et al*., 2008). 16S rDNA was amplified using universal bacterial primers 27Fmod (AGRGTTTGAT CMTGGCTCAG) and 519Rmodbio (GWATTACCGC GGCKGCTG). These primers span the V1 to V3 regions of the 16S rRNA gene, which allows for coverage of multiple hypervariable regions that have been commonly used as taxonomic marker regions for bacteria in marine environments (for example, Barott *et al*., 2011; Morrow *et al*., 2012). Amplification was performed in a single step 30 cycle PCR using HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA, USA) starting with an initial denaturation step (3 min at 94 °C), followed by 28 cycles (30 s at 94 °C, 40 s at 53 °C, 1 min at 72 °C) and a final elongation step (5 min at 72 °C). Amplicon products were mixed in equal concentrations, purified using Agencourt Ampure beads (Beckman Coulter, Inc., Danvers, MA, USA), and sequenced with Roche 454 FLX titanium instruments (Roche Diagnostics, Basel, Switzerland) and reagents following the manufacturer's guidelines. The resulting sequence

data were processed using a proprietary analysis pipeline (MR DNA, Shallowater, TX, USA). Barcodes and primers were removed and sequences that had less than 200 bp remaining, contained ambiguous base calls or had homopolymer runs exceeding 6 bp were excluded from analysis. The read length of the remaining sequences ranged from 200 to 597 bp with an average read length of 373 bp. These sequences were denoised and chimeras were removed using UCLUST and UCHIME. Sequences that clustered together (UCLUST) with a 97% similarity were defined as an operational taxonomic unit (OTU) and the centroid of each cluster was assigned as the representative sequence. OTUs were taxonomically classified using the curated Greengenes database (DeSantis *et al.*, 2006). Sequences of OTUs that were identified as contributing $>0.5\%$ to the dissimilarity among CCA species were also subjected to a BLAST search of the NCBI database. Sequences were submitted to the NCBI SRA database under the study accession number SRP056487. Rarefaction curves for CCA samples (Supplementary Figure S1) and seawater samples (Supplementary Figure S2) can be found in the supplementary information.

Statistical analysis

All statistical analyses were performed based on relative abundances of OTUs within samples. Bacterial community composition of CCA surface and seawater samples were compared using a two-way analysis of similarity (ANOSIM, 9999 permutations) based on the Bray-Curtis distance measure with collection site and sample source (CCA or seawater) as factors. Subsequently, one-way ANOSIMs (9999 permutations) were performed to determine differences in bacterial communities among CCA species and in seawater among collection sites. Similarity percentage analyses based on the Bray-Curtis distance measure were performed to determine which taxa contributed the most to the differentiation among CCA-associated bacterial communities. Data were also subjected to nonmetric multidimensional scaling using the Bray-Curtis measure of similarity for graphical representation. The Chao1 index was used to estimate richness and the Buzas and Gibson evenness index (e^{H}/S, where H = Shannon Diversity Index and S = total number of OTUs) was used to measure evenness. Evenness was compared among CCA species using a one-way analysis of variance (ANOVA). Chao 1 richness estimates failed to meet the assumptions of the one-way ANOVA, and therefore, bacterial richness among CCA species was compared using the Kruskal–Wallis one-way ANOVA followed by the Dunn's Method for multiple comparisons. Statistical analyses were performed using PAST v. 2.17b (Paleontological Statistics) (Hammer *et al.*, 2001) and SigmaPlot 11. Percent abundance data of taxonomic groups within the CCA-associated bacterial communities are presented as the mean ± s.d. among replicate individuals within each CCA species unless otherwise stated.

Results and discussion

Surfaces of CCA collected in Belize were dominated by Proteobacteria, which made up an average of $27 \pm 14\%$ to $61 \pm 12\%$ of the total bacterial abundance (Figure 1). Of these, most OTUs belonged to the Alphaproteobacteria with average relative abundances ranging from $15 \pm 16\%$ on samples of *H. boergesenii* to $37 \pm 23\%$ on samples of *T. prototypum* (Figure 1). Gammaproteobacteria were also present in high abundances and accounted for $7 \pm 7\%$ (*H. boergesenii*) to $27 \pm 21\%$ (*P. pachydermum*) of the total relative abundance (Figure 1). Firmicutes were abundant on *H. boergesenii* ($23 \pm 11\%$) and on *P. solubile* ($20 \pm 27\%$), but were not major contributors to the bacterial communities on other samples. These results were similar to those found associated with two species of CCA (*Neogoniolithon fosliei* and *Hydrolithon onkodes*) from Australia and unspecified CCA species from Curacao. CCA species from Australia were dominated by Alphaproteobacteria, Gammaproteobacteria and Bacteriodetes (Webster *et al.*, 2011; Webster *et al.*, 2013), and whole tissues of unspecified CCA from Curacao were dominated by Proteobacteria, Firmicutes and unknown bacteria (Barott *et al.*, 2011). Contrary to the results of studies of *N. fosliei* and *H. onkodes* from Australia, Bacteriodetes were not among the most dominant bacterial phyla found on the surfaces of CCA from Belize. The highest average relative abundance of Bacteroidetes was found on *H. boergesenii* ($7 \pm 6\%$, Figure 1). In addition to the major phyla found associated with CCA in previous studies (Barott *et al.*, 2011; Webster *et al.*, 2011; Webster *et al.*, 2013), CCA samples from Belize had relatively high numbers of Actinomycetes, ranging from $8 \pm 17\%$ on *T. prototypum* to $37 \pm 14\%$ on *H. boergesenii* (Figure 1).

Interspecies variability: diversity

There was a significant difference in the species richness of bacteria associated with different species of CCA as estimated by the Chao1 richness estimator (Kruskal–Wallis one-way ANOVA, $P = 0.034$, Figure 2). Pairwise comparisons revealed that *T. prototypum* samples had significantly higher estimated species richness (1026.58 ± 610.00 OTUs) compared with *H. boergesenii* (196.08 ± 74.96 OTUs, Dunn's Method, $P < 0.05$, Figure 2). Bacterial species richness increases with algal age for some algal species (Bengtsson *et al.*, 2012). The high level of species richness found on the surface of *T. prototypum* may be related to the fact that this CCA species, unlike the others tested here, does not slough its surface cells (see supplementary material in Ritson-Williams *et al.*, 2014) allowing for the

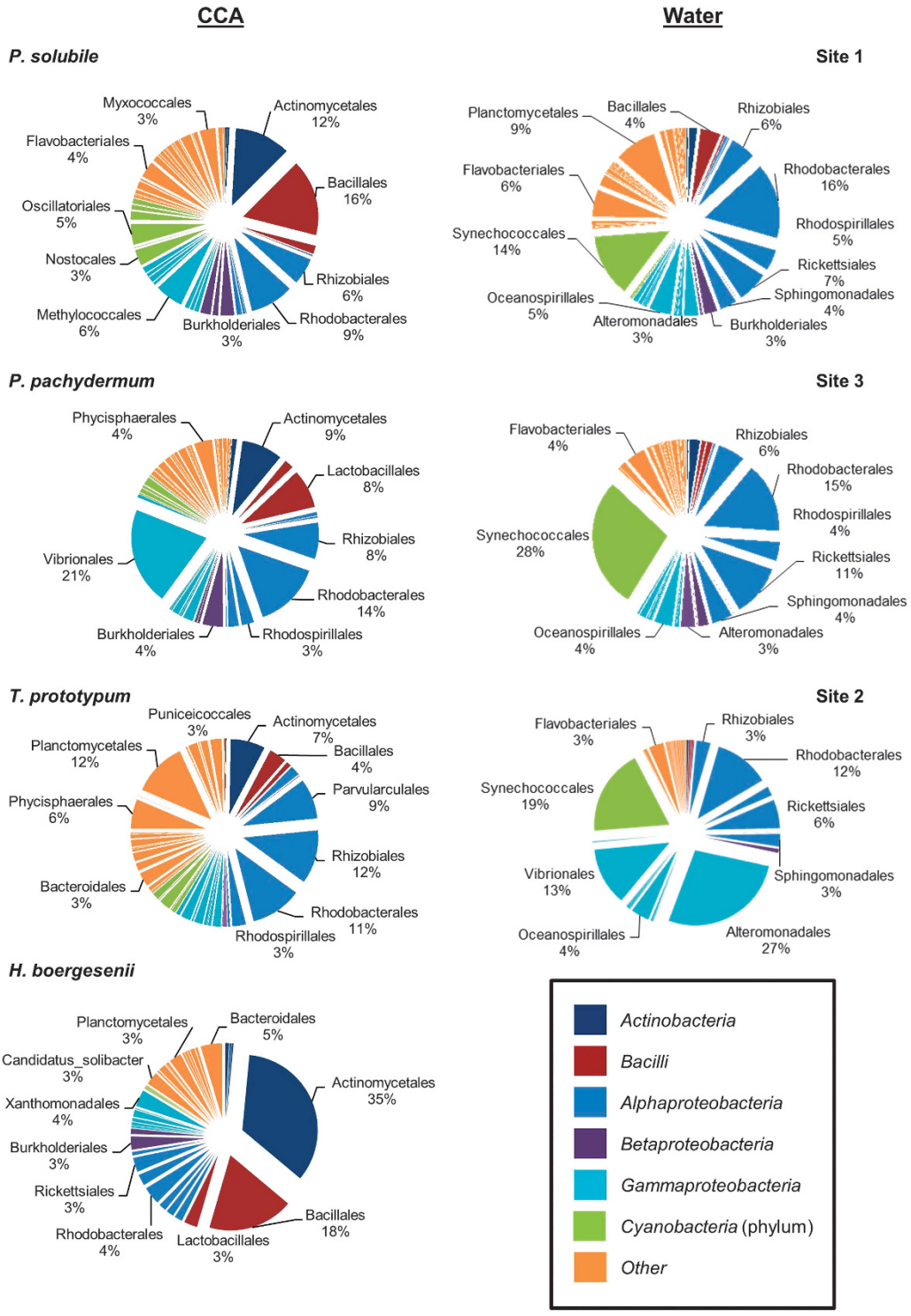

Figure 1 Relative abundances of OTUs found on the surfaces of four CCA species and in seawater samples from their corresponding collection sites. *H. boergesenii* was collected at sites 1 and 4 (no seawater samples). Pie wedges represent order level classification and colors indicate class. Orders making up ⩾3% of the relative abundance are labeled.

development of a more mature bacterial community. Estimated species richness of neither *P. solubile* (288.06 ± 76.87 OTUs) nor *P. pachydermum* (419.04 ± 104.90 OTUs) was significantly different from any of the other CCA species tested. There was no difference in the evenness of bacterial communities among CCA species (one-way ANOVA, $P = 0.852$). There was no significant difference in species richness among water samples (777.24 ± 217.82–822.48 ± 261.67 OTUs) collected from the three collection sites (one-way ANOVA, $P = 0.945$) suggesting that variations in richness among CCA

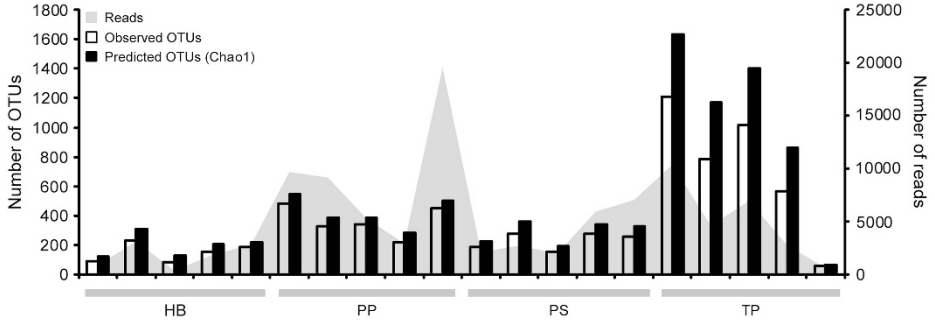

Figure 2 Number of observed and predicted OTUs and number of total reads in samples of CCA surface-associated bacteria. PS, *P. solubile*; HB, *H. boergesenii*; TP, *T. prototypum*; PP, *P. pachydermum*. Predicted OTUs were calculated using the Chao1 index.

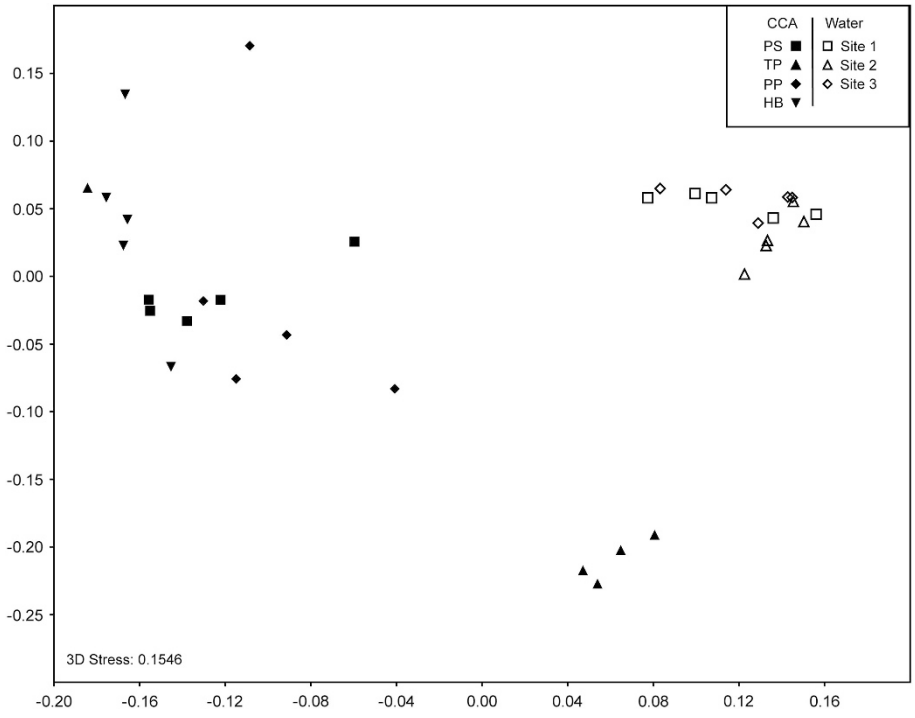

Figure 3 Nonmetric multidimensional scaling plot of bacterial communities on CCA surfaces and in seawater using the Bray-Curtis measure of similarity. Water was collected from the same sites that the CCA were collected. PS and HB were collected from Site 1. TP was collected from Site 2. PP was collected from Site 3. PS, *P. solubile*; HB, *H. boergesenii*; TP, *T. prototypum*; PP, *P. pachydermum*.

species is not determined by the available pool of bacteria in the water column.

Interspecies variability: composition

The two CCA species known to facilitate coral larval settlement represent the extremes in bacterial species richness, which suggests that the composition may be more important than diversity in terms of providing a bacterial community conducive to larval settlement. What is known to date about the role of bacteria in the induction of coral larval settlement has focused on the role of individual bacterial strains. Several strains have been shown to induce settlement, suggesting that the presence of specific inductive species is more important than the overall diversity of the bacterial community (Negri *et al.*, 2001; Tebben *et al.*, 2011; Tran and Hadfield, 2011; Sneed *et al.*, 2014). However, little work has been carried out to examine the importance of multi-species conglomerations of bacteria on larval settlement preferences.

Bacterial community composition on CCA surfaces was significantly different among different CCA species (one-way ANOSIM, $R = 0.5308$, $P < 0.0001$, Figure 3). Pairwise comparisons showed that each CCA species had a significantly different bacterial community composition compared with every other CCA species (Figure 3, Table 1). According to similarity percentage analyses, 14 OTUs contributed $> 0.5\%$ to the dissimilarity among CCA species (Table 2). The percent similarity for many of

Table 1 Pairwise comparisons of bacterial communities associated with CCA species *Hydrolithon boergesenii* (HB), *Titanoderma prototypum* (TP), *Paragoniolithon solubile* (PS) and *Porolithon pachydermum* (PP) following one-way ANOSIM

	R-*values*			
	HB	*TP*	*PS*	*PP*
P-*values*				
HB	—	*0.458*	*0.384*	*0.528*
TP	**0.0492**	—	*0.532*	*0.492*
PS	**0.0086**	**0.0459**	—	*0.508*
PP	**0.0063**	**0.0468**	**0.0078**	—

Abbreviations: ANOSIM, analysis of similarity; CCA, crustose coralline algae.
R values are given above the diagonal and *P* values beneath the diagonal. *P* values <0.05 are indicated in bold.

these sequences to their closest match is low ($<96\%$) even within the NCBI database. This highlights that the diversity of marine bacteria has much still to be discovered.

Surfaces of *H. boergesenii* had higher abundances of Actinomycetes compared with the other three CCA species (Figure 1, Table 2). The OTU (1504) that contributed the most to the dissimilarity between CCA species aligns most closely (96.5%) with an Actinomycete in the genus *Propionibacterium* that was broadly present across CCA species, but made up a large proportion ($12.10 \pm 8.25\%$) of the sequences found on the surfaces of *H. boergesenii* and was less common ($2.61 \pm 1.69\%$ to $4.06 \pm 9.09\%$) on the other three species (Table 2). Another OTU (1778) aligning most closely (99.2%) to *Propionibacterium acne* was also present in higher abundances on *H. boergesenii* ($3.64 \pm 3.33\%$) compared with the other CCA species (0.98 ± 2.19–$1.48 \pm 0.48\%$, Table 2). Propionibacteria are known mostly for their role as commensal microbes or opportunistic pathogens on human skin and as fermenters in food making (Grice and Segre, 2011; Thierry *et al*., 2011). They have been found in marine samples (La Rivière *et al*., 2013; Li *et al*., 2014), and a BLAST search of these two OTUs reveals close matches (98% and 99%, respectively) to uncultured bacteria from deep-sea sediments and ocean drilling cores (Table 2). Although their ecology in this environment remains largely unknown, two strains of *Propionibacterium* isolated from the coral *Acropora digitifera* exhibit broad-spectrum antibiotic activity (Nithyanand *et al*., 2011). Two OTUs (198 and 1555) aligning most closely (95.7% and 96.8%, respectively) to *Planomicrobium* spp. (order Bacillales) were found associated with *H. boergesenii* ($2.20 \pm 3.32\%$ and $1.19 \pm 2.35\%$). The same two OTUs were found in high abundances on one of the five *T. prototypum* samples (4.90% and 2.63%) but were not found on either of the other two CCA species (Table 2). *Planomicrobium* spp. have been isolated from diverse marine environments and exhibit algicidal and antimicrobial activity (Skerratt *et al*., 2002). Members of this genus isolated from coral mucus reduced growth and biofilm formation of coral pathogens (Skerratt *et al*., 2002; Shnit-Orland and Kushmaro, 2009; Alagely *et al*., 2011). *T. prototypum* also had higher abundances than other CCA of an OTU (6908) in the order Rhodobacterales ($2.26 \pm 3.67\%$) that aligned weakly (88.6%) to species of Rhodobacteraceae and an OTU (8276) in the order Planctomycetales ($1.93 \pm 1.47\%$) that aligned weakly (87.7%) to *Blastopirellula* spp. Rhodobacterales have often been associated with coral disease (Mouchka *et al*., 2010); however, the low percent similarity of OTU 6908 to its closest match in the curated Greengenes database makes it difficult to attribute taxonomic identification. A BLAST search reveals that this OTU is most closely related (92–94% similarity) to uncultured bacteria associated with a variety of corals including early life history stages of *Porites astreoides*. The closest match (99%) for OTU 8276 within the NCBI database is that of an uncultured bacterium from a biofilm within a nuclear plant (Table 2). The closest match to a marine source was a 96% match to an uncultured bacterium associated with a marine sponge (Table 2).

P. solubile had higher numbers of *Staphylococcus epidermidis* and cyanobacteria (Oscillatoriales and Nostocales). Three OTUs (4092, 2743 and 1706) aligning with *S. epidermidis* contributed greater than 0.5% to the differences among CCA species and were found in higher relative abundances on *P. solubile* ($3.89 \pm 7.41\%$, $1.82 \pm 3.36\%$ and $1.66 \pm 3.12\%$) compared with the other three CCA species. Additionally, two OTUs that align with cyanobacteria in the orders Oscillatoriales and Nostocales were found only on *P. solubile* ($2.41 \pm 5.36\%$ and $2.31 \pm 5.16\%$, respectively) and not on any of the other species. Although these OTUs were not found on every *P. solubile* sample, they constituted a relatively high percentage of the total reads among *P. solubile* samples. *Staphylococcus epidermidis* is most often associated with human skin; however two of the three *S. epidermidis* OTUs found on *P. solubile* had close matches within the NCBI database to uncultured bacteria from marine environments (Table 2). *Staphylococcus* spp. have been isolated from algal biofilms and coral mucus (de Castro *et al*., 2010; Horta *et al*., 2014), and there is some evidence linking *Staphylococcus* strains to coral disease (Kellogg *et al*., 2013). Cyanobacteria, especially those in the order Oscillatoriales, inhibit settlement of coral larvae and have been implicated as causative agents of black band disease (Frias-Lopez *et al*., 2003; Kuffner and Paul, 2004; Kuffner *et al*., 2006; Casamatta *et al*., 2012). Larvae of multiple coral species avoid settling on the surface of *P. solubile* (Ritson-Williams *et al*., 2010; Ritson-Williams *et al*., 2014), and this may be due to inhibitory microbial OTUs that cause larval avoidance of this species of CCA. Interestingly, two methanotrophic OTUs (1796 and 378) were found exclusively on *P. solubile* and

Table 2 SIMPER analyses, OTUs contributing >0.5% to the dissimilarity among CCA species *Hydrolithon boergesenii* (HB), *Titanoderma prototypum* (TP), *Paragoniolithon solubile* (PS) and *Porolithon pachydermum* (PP)

OTU #	Mean % abundance (# of samples containing OTU)				% contr.to dissim.	Order	Closest taxonomic match—Greengenes	% ID	Closest match source—NCBI (accession #)	% ID
	HB	*TP*	*PS*	*PP*						
1504	**12.10 (5)**	4.06 (1)	3.64 (5)	2.61 (5)	3.90	Actinomycetales	*Propionibacterium acnes*	96.5	Deep-sea sediment (JN914883.1)	98
4606	0.00 (0)	0.26 (4)	0.05 (1)	**5.87 (4)**	1.59	Vibrionales	*Vibrio harveyi*	97.5	Coral-associated (KC545338.1)	97
4092	0.90 (4)	0.00 (0)	**3.89 (3)**	0.23 (5)	1.22	Bacillales	*Staphylococcus epidermidis*	96.1	Deep-sea sediment (JN913945.1)	96
1778	**3.64 (5)**	0.98 (1)	1.48 (5)	1.29 (5)	1.17	Actinomycetales	*Propionibacterium acnes*	99.2	Ocean drilling core (AB803950.1)	99
198	**2.20 (3)**	0.53 (1)	0.00 (0)	0.00 (0)	0.69	Bacillales	*Planomicrobium* spp.	95.7	River sediment (HF545332.1)	98
2743	0.93 (4)	0.00 (0)	**1.82 (2)**	0.11 (5)	0.68	Bacillales	*Staphylococcus epidermidis*	97.3	Coral-associated (FJ809136.1)	99
911	0.00 (0)	0.00 (0)	**2.41 (2)**	0.00 (0)	0.64	Oscillatoriales	*Oscillatoria spongeliae*	85.5	Coral-associated (JQ347397.1)	94
5734	0.00 (0)	0.00 (0)	**2.31 (1)**	0.00 (0)	0.61	Nostocales	*Anabaena cylindrica*	85.8	Coral-associated (JQ347397.2)	93
6908	0.00 (0)	**2.26 (4)**	0.00 (0)	0.00 (0)	0.60	Rhodobacterales	*Rhodobacteraceae* spp.	88.6	Coral-associated (GU119607.1)	94
635	0.00 (0)	0.06 (2)	0.01 (1)	**2.06 (4)**	0.56	Vibrionales	*Vibrio harveyi*	97.1	*V. harveyi*/*V. corallilyticus* genome (CP009467.2/CP009617.1)	97/97
1555	**1.19 (2)**	**0.98 (1)**	0.00 (0)	0.00 (0)	0.54	Bacillales	*Planomicrobium* spp.	96.8	Bovine rumen (EU845583.1)	98
1706	0.49 (4)	0.04 (1)	**1.66 (3)**	0.13 (5)	0.53	Bacillales	*Staphylococcus epidermidis*	96.1	Human skin (JF030251.1)	96
8276	0.00 (0)	**1.93 (4)**	0.00 (0)	0.00 (0)	0.51	Planctomycetales	*Blastopirellula* spp.	87.7	Nuclear plant biofilm/marine sponge (HM596372.1/FJ652502.1)	99/96
580	0.00 (0)	0.00 (0)	0.07 (1)	**1.85 (1)**	0.51	Rhodobacterales	*Roseovarius* spp.	94.0	Coral reef microbial mat (AB294940.1)	95

Abbreviations: CCA, crustose coralline algae; OTU, operational taxonomic unit; SIMPER, similarity percentage.
Closest taxonomic match based on percent similarity to sequences in the Greengenes database. Closest match source based on a BLAST search of the NCBI database. The highest mean % abundances are highlighted in bold for each OTU.

were found on every *P. solubile* sample. A BLAST search of both OTUs revealed that the closest matches (93%) are uncultured bacteria associated with the coral *Montastraea* (=*Orbicella*) *faveolata* (Sunagawa *et al*., 2009). The presence of methanotrophs within marine algal biofilms has rarely been reported and is more common on freshwater plants (Yoshida *et al*., 2014). The presence of these bacteria on the surfaces of *P. solubile* and their relatedness to OTUs found in coral mucus makes further exploration of these bacteria and their ecological roles an interesting direction for future research.

Porolithon pachydermum samples had high abundances of Vibrios with sequences in the order Vibrionales making up 0–63.1% (21.0±25.0%) of the total sequences found on the surfaces of individuals (Figure 1). No other CCA species had an average abundance of Vibrionales greater than 3% (Figure 1). Of these sequences, two OTUs (4606 and 635) aligning with *V. harveyi* were found in high abundances on *P. pachydermum* (5.87±7.60% and 2.06±2.76%) and contributed greater than 0.5% to the differences between CCA species. Additionally, an OTU (580) aligning (94%) with *Roseovarius* spp. in the family Rhodobacteraceae was found in higher abundances on *P. pachydermum* (1.85±4.14%) compared with other CCA species. Both *V. harveyi* and members of the Rhodobacteraceae have been associated with invertebrate diseases including coral disease (Godwin *et al*., 2012; Roder *et al*., 2014).

Water samples collected from the CCA collection sites had significantly different bacterial communities compared with CCA surfaces (two-way ANOSIM, $R=0.7506$, $P<0.001$, Figure 3). Bacterial communities (including those associated with CCA and seawater) also differed by collection site (two-way ANOSIM, $R=0.4214$, $P<0.001$), indicating that collection site may be impacting the bacteria associated with CCA. However, *H. boergesenii* samples were collected from two different sites and showed no significant difference in bacterial communities across sites (one-way ANOSIM, $R=0$, $P=0.5034$, Figure 4). Coral larvae will settle on individuals of *H. boergesenii* collected from both sites (Ritson-Williams, personal observation) providing further support that properties specific to the species and not the collection site are responsible for the settlement inductive activity of this CCA.

Implications

CCA are important components of marine environments, and CCA species are often grouped together in terms of their functioning in ecosystems. This study demonstrates that different CCA species host different bacterial communities, highlighting the importance of assessing the ecological roles of individual CCA species. Here, we examined CCA species known to elicit different settlement responses in coral larvae. Identifying the bacterial OTUs that contribute to the dissimilarity among CCA

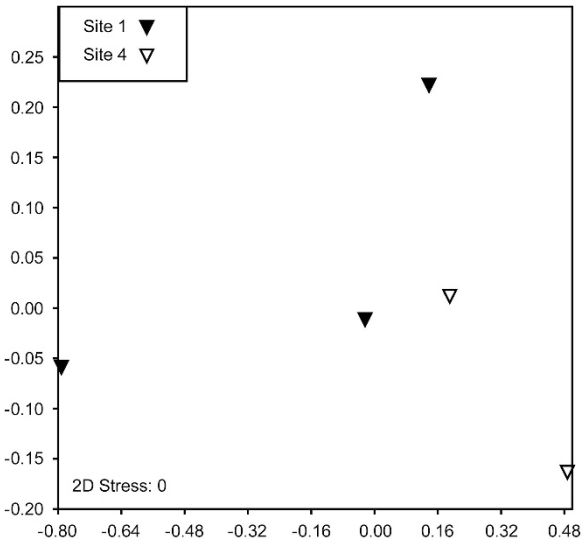

Figure 4 Nonmetric multidimensional scaling plot of bacterial communities on the surface *H. boergesenii* samples collected from two different sites based on the Bray-Curtis measure of similarity.

species provides insights into the microbial ecology of these algae and allows us to form hypotheses about the complex interactions between CCA and invertebrate larvae. Corals may have evolved to select settlement substrata that contain beneficial bacteria on their surfaces. For example, *Planomicrobium* spp. that were found on CCA species that facilitate coral larval settlement (*T. prototypum* and *H. boergesenii*) may enhance the survival of new recruits by inhibiting the growth of coral pathogens. Additionally, larvae may avoid certain substrata owing to the presence of pathogenic or allelopathic bacteria. Both of the CCA species that have been shown to be avoided by coral larvae, *P. solubile* and *P. pachydermum*, have high abundances of bacteria that are closely related to known coral pathogens (Vibrios and Rhodobacteraceae) or cyanobacteria that are known to produce allelopathic compounds (Oscillatoriales). Coral larvae respond to a variety of physical and chemical cues (Gleason and Hofmann, 2011) during the settlement process including the presence of bacterial biofilms and specific bacterial strains within biofilms (Negri *et al.*, 2001; Tran and Hadfield, 2011; Sneed *et al.*, 2014). Gleason and Hofmann (2011) proposed a 'hierarchy of action' in which coral larvae hone in on a settlement location through a series of cues that function at increasingly smaller spatial scales. It is possible that coral larvae may be able to assess the suitability of substrata for settlement at a fine scale (within a reef) based on the bacterial communities on the surface of the preferred CCA. Corals are especially vulnerable during their early life history stages and recruiting into a favorable environment is critical for their survival and persistence (Ritson-Williams *et al.*, 2009). Some corals acquire their internal bacterial communities only after settlement and metamorphosis (Sharp *et al.*, 2010). It is therefore likely that the bacterial communities associated with settlement substrata are important to coral recruits not only in terms of direct impacts on their health, but also as a reservoir for the development of the coral's microbiome. The findings presented here demonstrate that different species of CCA have different assemblages of bacteria associated with them and provide a basis for future research into the role of microbial community composition on the selection of settlement substrata by invertebrate larvae.

Conflict of Interest

The authors declare no conflict of interest.

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