Environmental Microbiology (2004) 6(11), 1137–1148 doi:10.1111/j.1462-2920.2004.00647.x

Widespread association of a *Rickettsiales*-like bacterium with reef-building corals

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Summary

White band disease type I (WBD I) has been a major cause of the dramatic decline of Acroporid coral populations throughout the Caribbean during the last two decades, yet the aetiological agent of this disease is unknown. In this study, the bacterial communities associated with both healthy and diseased *Acropora* species were compared by 16S rDNA analyses. The bacterial communities of both healthy and diseased *Acropora* spp. were dominated by a single ribotype with 90% identity to a bacterium in the order *Rickettsiales*. Screening by nested PCR specific to the coral-associated *Rickettsiales* 1 (CAR1) bacterium showed that this microbe was widespread in both healthy and diseased *A. cervicornis* and *A. palmata* corals from 'healthy' (i.e. low WBD I incidence) and 'stressed' reefs (i.e. high WBD I incidence). These results indicate that there were no dramatic changes in the composition of the microbial community associated with WBD I. CAR1 was also associated with non-Acroporid corals of the Caribbean, as well as with two Acroporid corals native to the Pacific. CAR1 was not present in the water column. This bacterium was also absent from preserved Caribbean Acroporid samples collected between 1937 and 1980 before the outbreak of WBD I. These results suggest CAR1 is a relatively new bacterial associate of Acroporids and that a non-bacterial pathogen might be the cause of WBD I.

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Introduction

Coral disease incidences are on the rise worldwide (Goreau *et al*., 1998; Hayes and Goreau, 1998; Garzon-Ferreira *et al*., 2001). In the Caribbean, Acroporid corals are afflicted by white band disease type I and II (WBD I, WBD II) and white pox (Peters *et al*., 1983; Aronson and Precht, 2001; Patterson *et al*., 2002). These diseases, along with other factors such as overfishing and hurricane damage, have led to a severe decline in the Acroporid coral population of the Caribbean (Knowlton *et al*., 1990; Hughes, 1994). White band disease type I has only been reported in Acroporid corals where a white band of necrotic tissue moves from the base of the coral to the tip, leaving behind a bare skeleton which is rapidly colonized by algae (Aronson and Precht, 2001). This band advances up the coral branch at a rate of a few millimetres per day (Antonious, 1981b). The loss of zooxanthellae adjacent to the dying band of tissue in WBD II distinguishes WBD I from WBD II (Ritchie and Smith, 1995a). The causative agent of WBD I has not been identified (Antonious, 1981a,b; Gladfelter, 1982; Peters, 1993; Richardson *et al*., 2001).

Acroporids were the dominant shallow water corals in the Caribbean, but since the appearance of WBD I in the mid-1980s, up to 95% of the Acroporid population has been destroyed (Hughes, 1994; Aronson and Precht, 2001). Recovery of Caribbean Acroporid populations has been slow (Hughes, 1994). New recruitment of affected species does occur, but the new recruits are often rapidly infected by WBD I. This limits the coral population to levels below those present before the disease outbreak (Goreau *et al*., 1998).

Another factor impacting *Acropora* spp. recovery is the emergence of a landscape dominated by algae. The sea urchin *Diadema antillarum* was a major consumer of macro algae until a mass mortality event in 1983–84 dramatically reduced this species throughout the Caribbean (Lessios *et al*., 1984). The loss of *D. antillarum* led to a phase shift from coral- to algal-dominated reefs (Hughes *et al*., 1987; Hughes, 1994). This phase shift decreased recovery of diseased corals such as *A. cervicornis*, *A.*

palmata and *A. prolifera* due to the inability of coral larvae to settle on macro algal-dominated surfaces (Sammarco, 1985; Hughes, 1994; Kojis and Quinn, 1994).

Bacteria have been observed in the WBD I zone of coral tissue degradation through microscopic examination; however, attempts at culturing and isolating the pathogen have failed (Peters *et al.*, 1983; Goreau *et al.*, 1998; Richardson *et al.*, 2001; Bythell *et al.*, 2002). Peters *et al.* (1983) showed, through histopathological examinations, that Gram-negative rod-shaped bacteria were contained within basophilic ovoid bodies of *A. palmata* and *A. cervicornis* tissues (Peters *et al.*, 1983). These bacteria were found in healthy tissue and WBD I tissues of *A. palmata* and *A. cervicornis*. Bythell *et al.* (2002) used fluorescence *in situ* hybridization (FISH) to investigate WBD I. This analysis showed that the WBD I lesions did have tissue fragmentation, but were relatively free of microbes. The inability to culture the bacteria associated with WBD I has made it impossible to fulfill Koch's postulates (Koch, 1884). At this point it is not clear whether or not WBD I is caused by a bacterial pathogen.

Culture-independent techniques have been used to analyse the species composition of bacterial communities associated with healthy corals (Rohwer *et al.*, 2001; 2002). An uncultured bacterial community of *Montastraea franksi* was shown to have greater diversity and more species than the cultured bacterial community from the same coral species (Rohwer *et al.*, 2001). Bacterial communities of healthy *M. franksi*, *Diploria strigosa* and *Porites astreoides* were highly diverse, composed mostly of novel species, and showed a species–specific association of some bacteria with corals (e.g. PA1 with *P. astreoides*). Rohwer *et al.* (2002) proposed that these species–specific associations were important to the health of the coral animal because they were part of the holobiont community which includes zooxanthellae (Toller *et al.*, 2001), fungi (Bentis *et al.*, 2000), and endolithic algae (Odum and Odum, 1955; Shashar *et al.*, 1997; Bentis *et al.*, 2000).

Culture-independent techniques have also been used to characterize bacterial communities associated with diseased corals (Bythell *et al.*, 2002; Cooney *et al.*, 2002; Frias-Lopez *et al.*, 2002). Frias-Lopez *et al.* (2002) compared the bacterial communities of seawater, healthy corals, black band disease (BBD)-infected corals, and dead coral surfaces by restriction fragment length polymorphism (RFLP) and sequencing of 16S rDNAs. This study found a distinct partitioning of the coral microbiota between the overlying seawater, healthy, BBD-infected and dead coral surfaces (Frias-Lopez *et al.*, 2002). Microbes previously believed to be associated with BBD, *Phormidium corallyticum* and *Beggiatoa* spp., were absent from the BBD communities of *M. annularis*, *M. cavernosa* and *D. strigosa* (Garret and Ducklow, 1971; Antonious, 1981a,b; Rutzler and Santavy, 1983; Frias-Lopez *et al.*, 2002). The species composition of BBD on *D. strigosa*, *M. annularis* and *Colpophyllia natans* was also investigated by amplified 16S rDNA restriction analysis (ARDRA) and denaturing gradient gel electrophoresis (DGGE) to analyse the 16S rDNAs of the bacterial communities (Cooney *et al.*, 2002). The libraries from diseased samples all contained a *Cyanobacterium* sp., *Cytophaga* sp., and an α-proteobacterium identified as the aetiological agent of juvenile oyster disease (Cooney *et al.*, 2002). The results from Cooney *et al.* (2002) differed from those of Frias-Lopez *et al.* (2002) in the types of bacteria found. Cooney *et al.* (2002) did not detect *P. corallyticum* in their BBD samples, however, they did detect *Desulfovibrio* spp. previously observed with BBD lesions (Garret and Ducklow, 1971).

This study characterized the bacterial communities associated with healthy and WBD I Acroporid corals using culture-independent techniques. Our results showed that there were no bacterial species found exclusively on WBD I Acroporids. Both healthy and WBD I Acroporid bacterial communities were dominated by a coral-associated *Rickettsiales* 1 (CAR1) bacterium.

Results

A coral-associated Rickettsiales 1 (CAR1) bacterium was present in bacterial communities from healthy and white band type I diseased (WBD I) Caribbean Acroporid corals

The bacterial communities associated with healthy and white band type I diseased (WBD I) Acroporid corals were evaluated by cloning and sequencing of bacterial 16S rDNAs. Following dereplication of the 501 16S rDNA sequences with FastGroup (Seguritan and Rohwer, 2001), 93 different ribotypes were identified among the 29 healthy and diseased *Acropora cervicornis*, *A. prolifera* and *A. palmata* samples. There were no 16S rDNA sequences exclusively associated with diseased corals. Figure 1A shows the taxonomical composition, as identified by ARB alignment, of the bacterial community associated with healthy and WBD I Acroporid corals. Proteobacteria comprised 85% of the 501 16S rDNA sequences. The second most abundant group, *Spirochaetes*, only comprised 4% of the sequences. The proteobacteria community itself consisted mainly of α-proteobacteria (85%), the majority of which were a single bacterial species, which was named coral-associated *Rickettsiales* 1 (CAR1; Fig. 1B).

The bacterial communities from the three regions represented in the Acroporid clone libraries (Key West and Key Largo, Florida; Puerto Rico) showed significant overlap (data not shown). More importantly, CAR1 was iden-

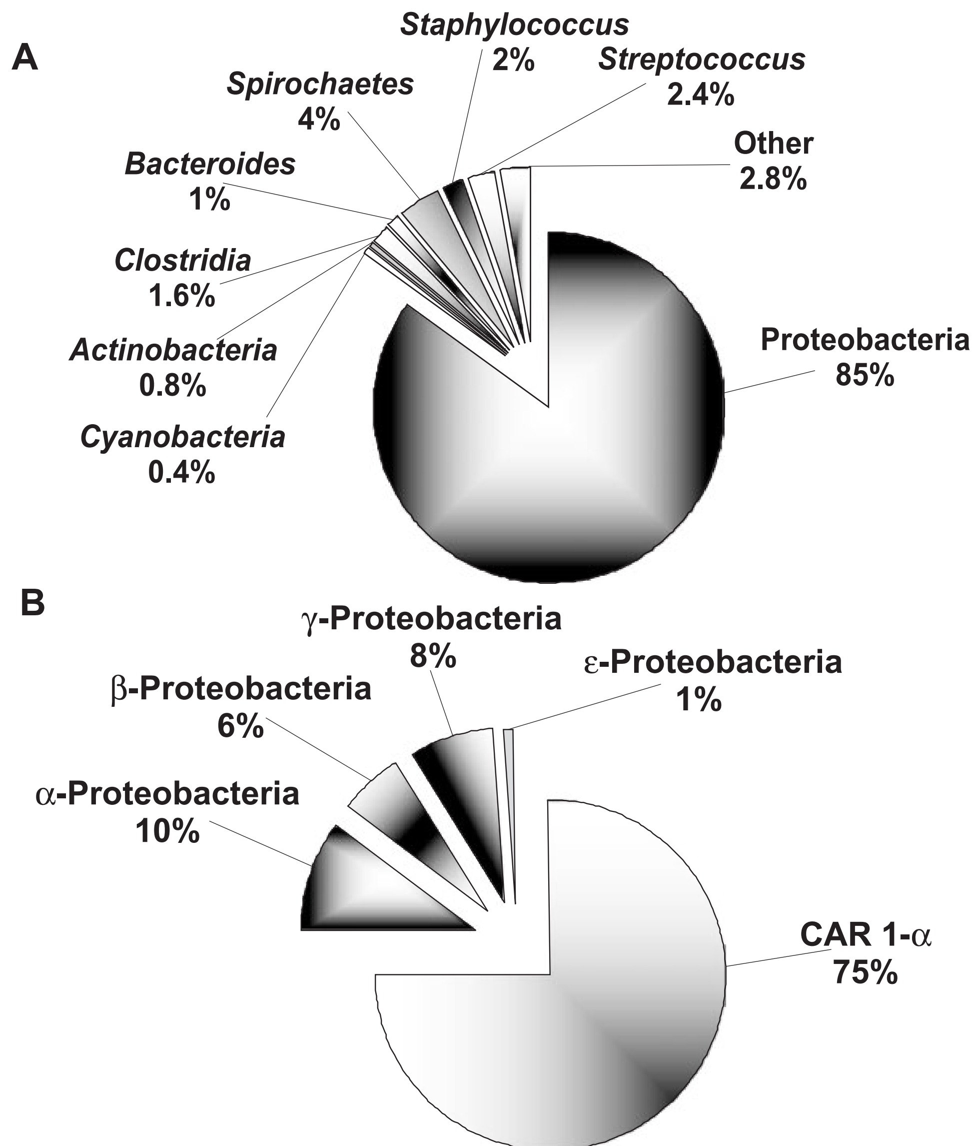

Fig. 1. Identities of Acroporid-associated bacteria.
A. Identity of the bacterial ribotypes in the Caribbean Acroporid partial 16S rDNA libraries. Following dereplication by FastGroup, representative 16S rDNA sequences were identified by an ARB alignment.
B. The Acroporid-associated bacterial community was dominated by α-proteobacteria. The coral-associated *Rickettsiales* 1 (CAR1) bacterium made up the majority of the α-proteobacteria.

tified in each of these regions and all three regions were dominated by proteobacteria.

CAR1 was found to be only 90% identical to its top BLASTN hit – an uncultured *Rickettsiales* bacterium. Figure 2 shows a tree generated by ARB alignment of CAR1, five members of the *Rickettsiales* order and four other proteobacteria as reference organisms. CAR1 grouped with the *Rickettsiales*.

The bacterial 16S rDNA sequences obtained from healthy and WBD I *A. cervicornis* and *A. prolifera* were dominated by CAR1. Overall, CAR1 was present in 64% of the clones. CAR1 was present in *A. cervicornis* and *A. prolifera*, but was absent in *A. palmata* (Table 1). The absence of CAR1 in *A. palmata* samples was most likely due to a low abundance of CAR1 in these samples. As described below, specific PCR did identify CAR1 on *A. palmata* samples.

Species richness predictions of the Acroporid-associated microbial community

The bacterial community of healthy and diseased Caribbean Acroporids was dominated by α-proteobacteria,

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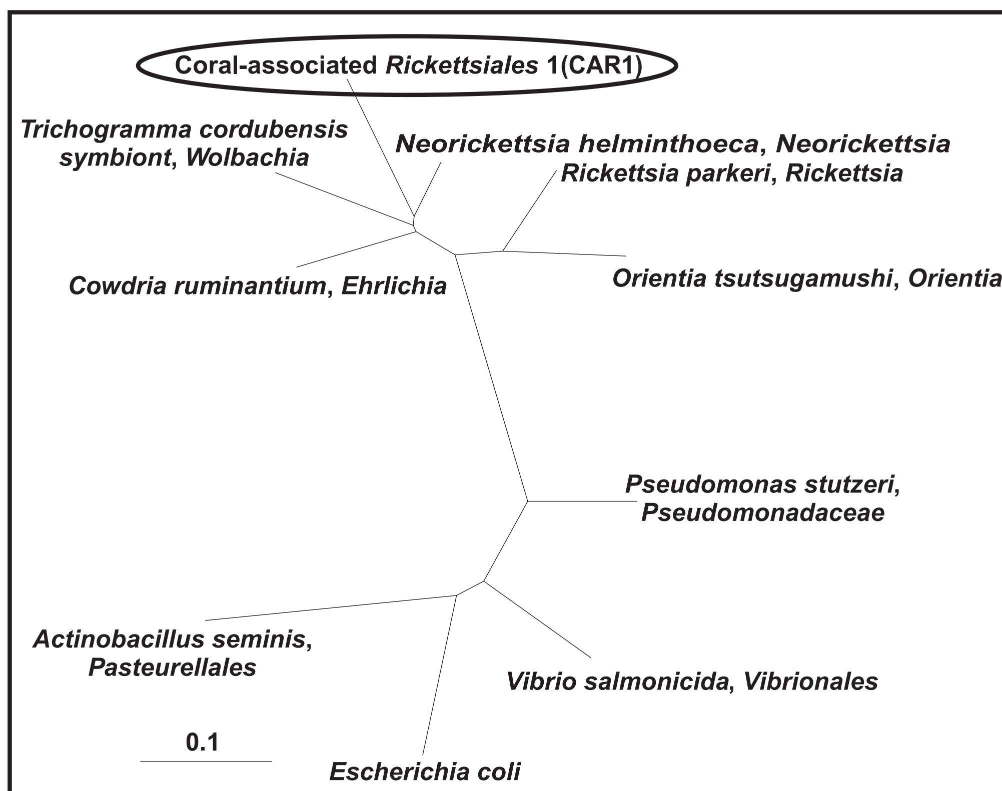


Fig. 2. The coral-associated *Rickettsiales* (CAR1) bacterium is distant from its nearest relative. Following ARB alignment analysis, a tree diagram was constructed of CAR1 and five of its nearest relatives. CAR 1 is an α-proteobacteria most closely related to *Rickettsiales*, however, it probably forms a novel genera.

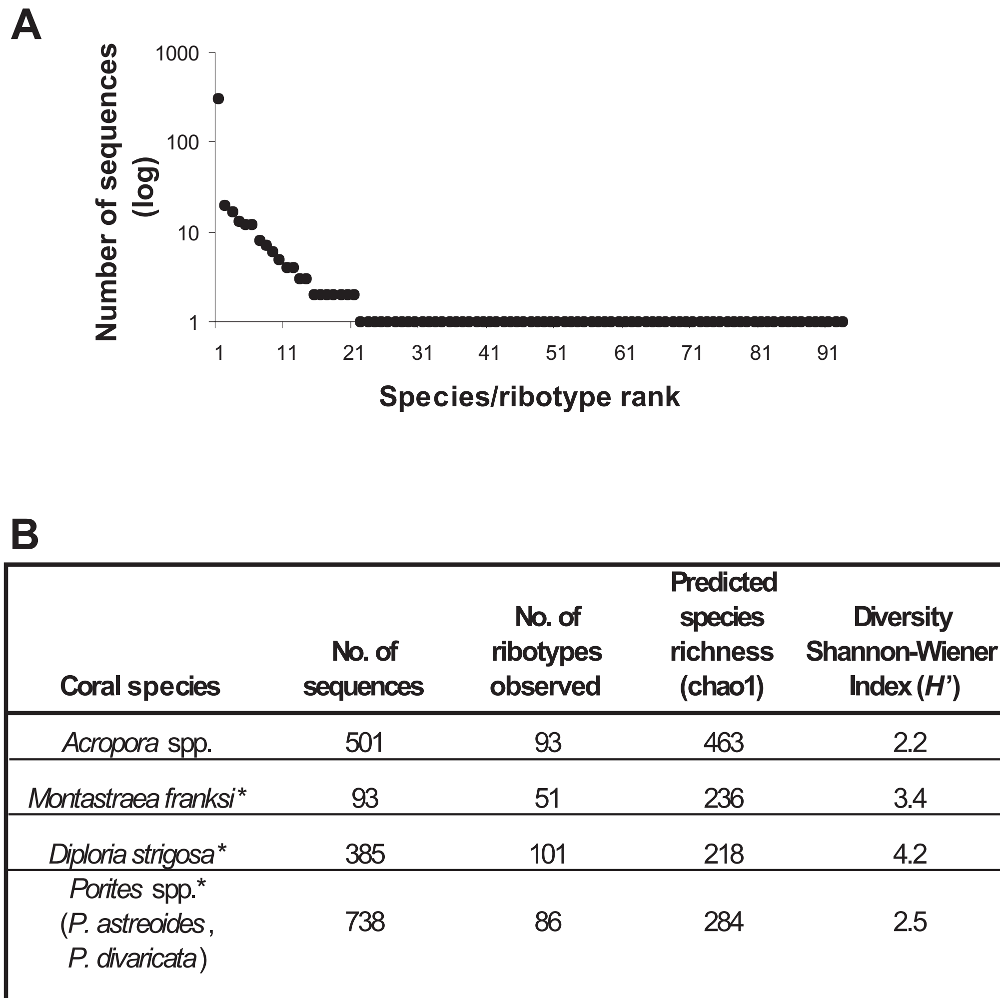
particularly CAR1. Most of the other ribotypes were rare and observed only once in the Acroporid libraries – as depicted in the rank abundance curve (Fig. 3A). The total number of species (richness) of the Acroporid library was calculated using the Chao1 estimator (Chao, 1984; Colwell and Coddington, 1994). Species richness was also estimated for the bacterial communities from *Montastraea franksi*, *Diploria strigosa* and *Porites* spp. based on previously collected data (Rohwer *et al*., 2001; 2002; Fig. 3B). The Acroporid bacterial community had the highest species richness prediction (463 species), whereas the other three coral-associated bacterial communities had richness predictions ranging from 218 to 284 species.

Diversity predictions of the Acroporid-associated microbial community

Diversity was estimated using the Shannon-Wiener Index (H') to determine if the diversity of the Acroporid-associated bacterial community was similar to other coral-associated bacterial communities (Shannon and Weaver, 1949; Rohwer *et al*., 2001; 2002). The Shannon-Wiener Index is a diversity index which considers both the total number of species and the relative abundances of these species. The *Acropora* spp.-associated bacterial community ($H' = 2.2$) was less diverse than the communities associated with *M. franksi* ($H' = 3.4$), *D. strigosa* ($H' = 4.2$) and *Porites* spp. ($H' = 2.5$; Fig. 3B).

Table 1. Distribution of the coral-associated *Rickettsiales* 1 (CAR1) sequence in the partial 16S rDNA clone libraries from different *Acropora* spp. CAR1 dominated the bacterial communities from *A. cervicornis* and *A. prolifera*, but was absent from the *A. palmata* library. The number of healthy and diseased samples from which these sequences were obtained is indicated in parentheses.

Species	Total no. of sequences	Total no. of CAR1 sequences	% CAR1 sequences
Acropora cervicornis	410 (14 healthy; 2 diseased corals)	300 (6 healthy; 2 diseased)	73
Acropora prolifera	24 (1 healthy; 2 diseased corals)	21 (1 healthy; 2 diseased)	88
Acropora palmata	67 (9 healthy; 1 diseased corals)	0	0



Coral species	No. of sequences	No. of ribotypes observed	Predicted species richness (chao1)	Diversity Shannon-Wiener Index (*H*')
Acropora spp.	501	93	463	2.2
*Montastraea franksi**	93	51	236	3.4
*Diploria strigosa**	385	101	218	4.2
Porites spp.* (*P. astreoides*, *P. divaricata*)	738	86	284	2.5

Fig. 3. Species richness and diversity predictions for Acroporid-associated bacteria.
A. The rank abundance curve of the 93 ribotypes observed in the Acroporid-associated bacterial 16S rDNA libraries. Most of the ribotypes were rare and only observed once.
B. Predicted species richness (Chao1) and predicted diversity (*H*') of the bacterial communities associated with the Acroporid coral (this study) compared with those of *Montastraea franksi*, *Diploria strigosa* and *Porites* spp. (* indicates data taken from Rohwer *et al*., 2002).

The CAR1 bacterium was present in Acroporids from 'healthy' and 'stressed' Caribbean reefs

Certain members of the order *Rickettsiales* are known to cause disease in humans and invertebrates. For example, the withering syndrome of black abalone, *Haliotis cracherodii*, has been reported to be caused by a *Rickettsiales*-like prokaryote (RLP) (Gardner *et al*., 1995; Friedman *et al*., 1997; Antonio *et al*., 2000). To specifically target the *Rickettsiales* found in the Acroporid clone libraries, a nested PCR assay was developed. The Acroporid samples from which the clone libraries were made had all been collected from reefs where WBD I incidence was high. It was postulated that the 'healthy' samples containing the CAR1 bacterium might actually be 'diseased' samples which had yet to show symptoms of WBD I. To test this hypothesis, Acroporids from 'healthy' (Punta Vieja, Panama; low incidence of WBD I) and 'stressed' (Casa Blanca, Panama; high incidence of WBD I) reefs were screened using the CAR1-specific nested PCR. Screening was performed on healthy and diseased *A. cervicornis* samples from each reef, as well as on healthy and diseased samples of *A. palmata* from Punta Vieja. A total of 166 healthy and diseased samples were screened and CAR1 was found on 84% of the coral samples (Table 2). CAR1 was present on 79% of the healthy and 91% of the diseased samples. At Punta Vieja, the 'healthy' reef, CAR1 was detected on 79% of the healthy corals and 85% of the diseased corals. At the 'stressed' Casa Blanca reef, CAR1 was detected in 79% of the healthy samples and 94% of the diseased samples.

Table 2. Nested PCR screen for the coral-associated *Rickettsiales* 1 (CAR1) bacterium on Acroporids from Punta Vieja ('healthy' reef) and Casa Blanca ('stressed' reef) in Bocas del Toro, Panama. CAR 1 was found on *Acropora cervicornis* samples from both healthy and stressed reefs. The 'healthy' reef contained a low incidence of WBD I, whereas the 'stressed' reef contained a high incidence of WBD I. CAR1 was found on *A. palmata* samples using this approach. The number of CAR1 positives over the total number of samples screened is shown and the percentage is indicated in parentheses. DNA was confirmed to be PCR quality DNA through 16S rDNA amplification with bacterial-specific primers. The limit of detection of the CAR1 nested PCR was 1000 copies. NA = samples not available

	Punta Vieja ('healthy')		Casa Blanca ('stressed')	
Species	Healthy	Diseased	Healthy	Diseased
Acropora cervicornis	21/24 (88%)	21/25 (84%)	38/48 (79%)	47/50 (94%)
Acropora palmata	12/18 (67%)	1/1 (100%)	NA	
Both species	33/42 (79%)	22/26 (85%)	38/48 (79%)	47/50 (94%)
All samples	140/166 (84%)			

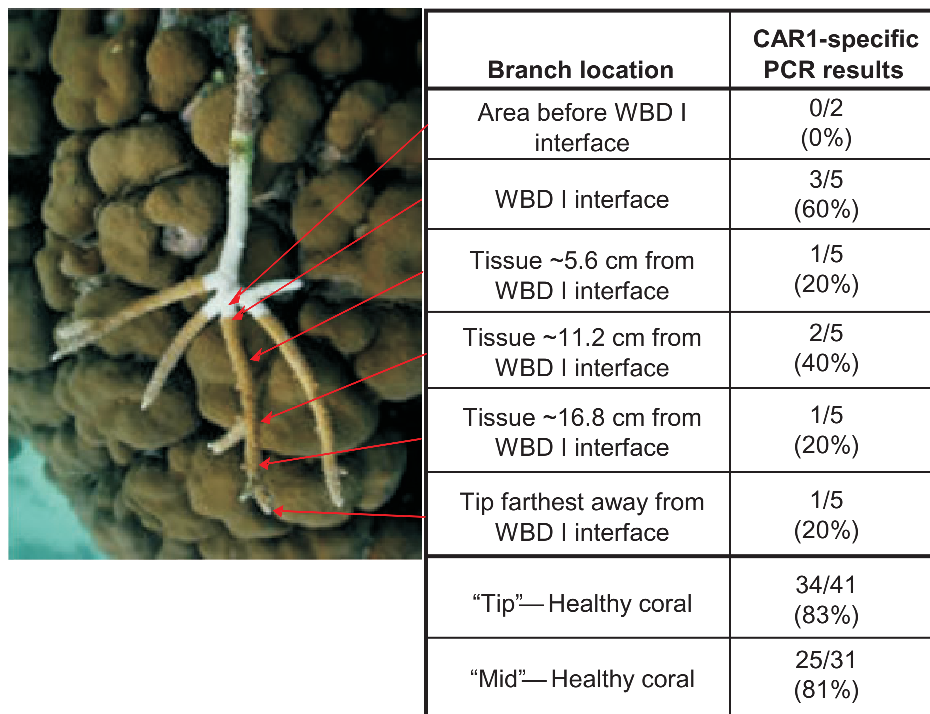
The CAR1 bacterium was distributed throughout the coral branches

One of the characteristics of WBD I is the progression of the disease band from the base of the coral branch to the tip, destroying the coral tissue along the way and leaving behind bare skeleton (Gladfelter, 1982; Peters *et al*., 1983; Aronson and Precht, 2001). To test whether the presence of the CAR1 bacterium reflected the progression of the disease, sections of diseased branches were tested using the nested PCR. CAR1 was found throughout the diseased branch (Fig. 4). The tissue at the WBD I interface had the highest CAR1 frequency, with 60% of the samples testing positive for CAR1. CAR1 was not detected in the area just before the disease interface.

A larger sample set of healthy *A. cervicornis* 'tip' and 'mid' sections was also screened. In these samples, 83% of the 'tip' and 81% of the 'mid' sections were positive for the CAR1 bacterium (Fig. 4).

The CAR1 bacterium was present on non-Acroporid Caribbean corals

To determine if CAR1 was exclusively associated with Acroporids, six species of non-Acroporid corals (*M. franksi*, *M. annularis*, *D. strigosa*, *P. astreoides*, *P. furcata* and *Colpophyllia natans*) were tested for the presence of CAR1 using the nested PCR. The CAR1 bacterium was present on all the non-Acroporid species tested (Table 3),



Branch location	CAR1-specific PCR results
Area before WBD I interface	0/2 (0%)
WBD I interface	3/5 (60%)
Tissue ~5.6 cm from WBD I interface	1/5 (20%)
Tissue ~11.2 cm from WBD I interface	2/5 (40%)
Tissue ~16.8 cm from WBD I interface	1/5 (20%)
Tip farthest away from WBD I interface	1/5 (20%)
"Tip"— Healthy coral	34/41 (83%)
"Mid"— Healthy coral	25/31 (81%)

Fig. 4. Nested PCR screen for the coral-associated *Rickettsiales* 1 (CAR1) bacterium on sections of healthy and WBD I *A. cervicornis* branches. CAR1 was detected throughout both diseased and healthy coral branches. Tissue samples from along a white band I diseased *A. cervicornis* branch were screened with the CAR1-specific primers. Arrows point to the approximate sections of the branch from which tissue samples were collected. 'Tip' and 'Mid' samples from healthy *A. cervicornis* were also screened. These samples were collected in Casa Blanca and Punta Vieja reefs from Bocas del Toro, Panama. The number of CAR1 positives over the total number of samples screened is shown with the percentage indicated in parentheses. The limit of detection of the CAR1-specific nested PCR was 1000 copies.

Table 3. Nested PCR screen for the coral-associated *Rickettsiales* 1 (CAR1) bacterium in non-Acroporid corals. CAR1 was also detected on non-Acroporid corals from both Panama and Bermuda. The number of CAR1 positives over the total number of samples screened is shown with the percentage indicated in parentheses. The limit of detection of the CAR1 nested PCR was 1000 copies. NA = samples not available

Species	Bocas del Toro, Panama (April 1999, June 2000)	Whale Bone Bay, Bermuda (August 1999)
Montastraea franksi	5/6 (83%)	3/7 (43%)
Montastraea annularis	0/3 (0%)	NA
Diploria strigosa	2/3 (67%)	4/6 (67%)
Porites astreoides	19/26 (73%)	2/6 (33%)
Porites furcata	9/10 (90%)	NA
Colpophyllia natans	10/16 (63%)	NA

except on *M. annularis*, for which only three samples were screened. CAR1 was present in samples collected from Bocas del Toro, Panama and Bermuda. The samples tested were those from which 16S rDNA clone libraries had previously been sequenced and CAR1 had not been identified (Rohwer *et al*., 2001; 2002). To confirm that the PCR products from this screen were the CAR1 sequence, some PCR products from each species were sequenced. The PCR products were aligned against the CAR1 positive control with CLUSTALX and on average displayed 94% similarity to CAR1.

CAR1 was not found in the water column

Most *Rickettsia* species are obligate intracellular organisms (Walker, 1999). Six water samples from Casa Blanca and Isla Solarte reefs, Bocas del Toro, Panama and from the Smithsonian Tropical Research Institute (STRI) field station were tested for CAR1. The samples were obtained near reefs of *A. cervicornis*, *M. annularis*, *M. cavernosa, Agaricia tenufolia* and *P. furcata* at various depths (1.5 m to 15 m). CAR1 was absent from all six water column samples (Table 4).

The CAR1 bacterium was found in Acroporids from the Pacific

To determine whether or not CAR1 was limited to the Caribbean, two Pacific Acroporid species, *A. formosa* and *A. hummilus*, were screened with the CAR1-specific PCR (Table 5). Nested PCR and sequencing results showed that CAR1 was present in both *A. formosa* and *A. hummilus*.

The CAR1 bacterium was not found in pre mid-1980s Acroporid samples

WBD I has been responsible for the dramatic decline of the Caribbean Acroporid population in the last two decades (Peters *et al*., 1983; Aronson and Precht, 2001; Patterson *et al*., 2002). To test if CAR1 was present in Caribbean Acroporids prior to the major WBD I outbreak, preserved samples of *A. cervicornis* and *A. palmata* collected between 1937 and 1980 were screened using CAR1-specific nested PCR. The four Acroporid samples

Table 4. Nested PCR screen for the coral-associated *Rickettsiales* 1 (CAR1) bacterium in water column samples. CAR1 was not found in the water column samples. The limit of detection of the CAR1 nested PCR was 1000 copies. The negative results indicate that a PCR product was not detected in these samples. DNA was confirmed to be of PCR quality through 16S rDNA PCR. STRI = Smithsonian Tropical Research Institute (Bocas del Toro, Panama)

Water sample	Water depth (m)	Location	CAR1-specific PCR
Near *Acropora cervicornis*	1.5	Casa Blanca, Panama	Negative
Near *Acropora cervicornis*	2.3	Casa Blanca, Panama	Negative
Near *Montastraea annularis*	4–5	Isla Solarte, Panama	Negative
Near *Montastraea annularis*	11.5	Isla Solarte, Panama	Negative
Near *Montastraea cavernosa*	15	Isla Solarte, Panama	Negative
Near *Agaricia tenufolia* and *Porites furcata*	9 (~10 m away from reef)	STRI; Bocas del Toro, Panama	Negative

Table 5. Nested PCR screen for the coral-associated *Rickettsiales* 1 (CAR1) bacterium in Pacific *Acroporids*. CAR1 was found on Pacific Acroporid samples collected in May 2003. The limit of detection of the CAR1 nested PCR was 1000 copies. Samples were also tested with 16S rDNA PCR.

Species	Date collected	Location	CAR1-specific PCR	Bacterial-specific 16S rDNA PCR
Acropora hummilus	May 2003	Pacific Ocean	Positive	Positive
Acropora formosa	May 2003	Pacific Ocean	Positive	Positive

Table 6. Nested PCR screen for the coral-associated *Rickettsiales* 1 (CAR1) bacterium in preserved Acroporids. CAR1 was not detected in Acroporids harvested before the mid-1980s. The four preserved Acroporid samples were collected between 1937 and 1980 and preserved in ethanol. The limit of detection of the CAR1 nested PCR was 1000 copies. The negative results indicate that a PCR product was not detected in these samples. DNA was confirmed to be of PCR quality through 16S rDNA PCR.

Species	Date collected	Location	CAR1-specific PCR	Bacterial-specific 16S rDNA PCR
Acropora sp.	1937	Virgin Islands	Negative	Positive
Acropora cervicornis	1978	Belize	Negative	Positive
Acropora cervicornis	1978	Belize	Negative	Positive
Acropora palmata	July 1980	Puerto Rico	Negative	Positive

were all negative for CAR1 (Table 6). Polymerase chain reaction of the 16S rDNA of these samples showed that PCR-quality bacterial DNA was present in all samples.

Discussion

Caribbean Acroporid corals have declined dramatically over the last two decades, largely the result of white band disease type I (WBD I; Aronson and Precht, 2001). Acroporids were the major shallow water reef builders in the Caribbean and the death of these corals by WBD I has major implications for the future of Caribbean coral reefs. In the present study, culture-independent techniques were used to determine if a bacterial pathogen was associated with WBD I. Sequencing of bacterial 16S rDNAs from healthy and WBD I Acroporid samples showed that no specific bacterial species was exclusively associated with WBD I samples. However, there was a coral-associated *Rickettsiales* 1 (CAR1) bacterium which dominated both healthy and diseased Acroporid clone libraries (Fig. 1).

The Acroporid bacterial community was different from other coral-associated bacterial communities

There was little overlap between the Acroporid-associated bacterial community and other coral-associated bacterial communities (Rohwer *et al*., 2001; 2002; Cooney *et al*., 2002; Frias-Lopez *et al*., 2002). No bacterial species previously linked with other coral diseases – such as *Phormidium corallyticum* (black band disease, BBD), *Vibrio carchiariae* (WBD II), *Serratia marcescens* (white pox), *P. valderianum* (pink line syndrome) or the juvenile oyster disease pathogen (BBD, WP)—were identified in the Acroporid bacterial community (Rutzler and Santavy, 1983; Ritchie and Smith, 1995b; Richardson, 1998; Richardson *et al.*, 1998; Cooney *et al*., 2002; Patterson *et al*., 2002; Ravindran and Raghukumar, 2002). Most notable, the dominant individual in the Acroporid bacterial community, CAR1, has not been previously identified in other coral bacterial 16S rDNA libraries. Its nearest relative, a member of the order *Rickettsiales*, was only 90% similar, suggesting it is a novel genus (Fig. 2). The lack of overlap between the Acroporid bacterial community and other coral-associated bacterial communities suggested that Acroporid corals, like other corals, may harbour unique and specific microbial communities (Rohwer *et al*., 2002).

Coral-associated bacterial diversity

The Acroporid bacterial community had a Shannon-Wiener Index (H') of 2.2, which was similar to that measured on *Porites* spp. ($H' = 2.5$) and less than the calculated diversity of the communities associated with *M. franksi* ($H' = 3.4$) and *D. strigosa* ($H' = 4.2$; Fig. 3; Rohwer *et al*., 2001; 2002). The Shannon-Wiener Index, which takes into account the evenness (i.e. relative abundance of each species) of a community, was relatively low in *Porites* spp. and *Acropora* spp. corals because their microbial communities were dominated by one type of bacteria (e.g. PA1 and CAR1 respectively). If these dominant bacteria are removed from the data set, the Shannon-Wiener Index increases to 3.9 for the *Acropora* spp. and 4.2 for the *Porites* spp. CAR1 and PA1 are most likely located within the coral tissue (Peters *et al*., 1983; Rutzler and Santavy, 1983), whereas the rest of the microbes and higher Shannon-Wiener Indices probably reflect the high diversity of the skeleton and mucus-associated bacteria.

Coral-associated Rickettsiales 1 (CAR1) – pathogen, symbiont or none of the above?

The initial goal of the present study was to determine if there was a bacterial causative agent of WBD I. Comparison of the bacterial communities associated with healthy and WBD I corals did not clearly indicate that a bacterial pathogen was responsible for WBD I. Instead, CAR1 was found in abundance in both healthy and diseased Acroporid clone libraries and subsequently in samples tested with CAR1-specific nested PCR. This CAR1 abundance raises interesting questions about the possible roles, if any, CAR1 plays in the health of corals with which it is associated.

Rickettsiales are Gram-negative microbes which have been shown to cause disease in invertebrates (Gardner *et al*., 1995; Friedman *et al*., 1997; Antonio *et al*., 2000). CAR1 was abundant in the Acroporid clone libraries and

in the samples tested specifically for CAR1 by nested PCR (Fig. 1 and Tables 1 and 2). CAR1 was also not detected in pre mid-1980s Acroporid samples, suggesting that CAR1 was not a member of the coral microbiota before the 1980s (Table 6) and may have been the WBD I pathogen of the 1980s. However, only four pre mid-1980s samples were available for testing and it is not known if CAR1 DNA would have survived the ethanol preservation.

CAR1 is a member of the post-epidemic coral microbiota. CAR1 was present in other Caribbean corals, including *Montastraea franksi*, *Diploria strigosa*, *Porites astreoides*, *P. furcata* and *Colpophyllia natans*, as well as two Acroporid species from the Pacific (*A. hummilus* and *A. formosa*). CAR1 is therefore a component of the microbiota of multiple genera of corals from the Caribbean and the Pacific. Is CAR1 in a symbiotic relationship with these corals? Rohwer *et al*. (2002) proposed a model of the coral colony as a holobiont composed of the coral polyp, the zooxanthellae and prokaryotes. Disruption of this holobiont, due to stresses such as temperature changes, sedimentation, or other anthropogenic factors, could result in death or disease for the coral (Rohwer *et al*., 2002). One of the main differences between the 'healthy' (low WBD I incidence) and 'stressed' (high WBD I incidence) reefs in this study was their proximity to human activities. Whereas the occurrence of CAR1 in the 'healthy' and 'stressed' reef samples were relatively equal (Table 2), the 'stressed' reef, which was closer to human activities, showed a higher incidence of WBD I. It may also be possible that WBD I is not caused by a bacterial pathogen and could be a result of attacks by viruses or some other biological entity.

Experimental procedures

Sampling of Acroporid corals

A total of 31 *Acropora cervicornis* (21 healthy, 10 diseased), 11 *A. palmata* (10 healthy, 1 diseased), and 4 *A. prolifera* (two healthy, two diseased) corals were sampled from La Parguerra, Puerto Rico in April 2001 and January 2002; and from the Florida Keys in June 2000 (Key Largo) and June 2001 (Key West). Diseased samples were identified by the characteristic white band of diseased tissue proceeded by bare skeleton. 'Healthy' samples were those that did not have apparent signs of white band disease type I (WBD I) or other diseases. Healthy and diseased samples were removed from the same coral patches and different coral patches were sampled to account for local spatial variations. *Acropora cervicornis* and *A. palmata* were also sampled from Punta Vieja and Casa Blanca reefs in Bocas del Toro, Panama in November and December 2002. For the samples from Casa Blanca and Punta Vieja, whole branches from healthy Acroporid corals were collected and a subset was sectioned into 'tip' and 'middle' before further processing. Acroporid corals were collected underwater using bone clippers. Corals were then placed in sealable plastic bags. At the surface excess water was removed from the bags and the corals were placed on ice. Upon returning to the laboratory the fragments were either immediately airbrushed or frozen at −20°C.

Pacific Acroporid samples (*A. formosa* and *A. hummilus*) were obtained from a local aquarium company (Octopuss Gardens; San Diego, CA). The samples were sectioned into smaller pieces, rinsed with sterile seawater and stored at −20°C.

Acroporid samples originally collected in 1980 or earlier and since preserved in ethanol were obtained from the invertebrate collection at the Smithsonian Institution National Museum of Natural History (Washington, DC). There were two *A. cervicornis* samples collected from Belize in 1978, one *A. palmata* collected from Puerto Rico in July 1980, and one *Acropora* sp. collected from the Virgin Islands in 1937.

Sampling of non-Acroporid corals

For the non-Acroporid corals, 13 *Montastraea franksi*, three *M. annularis*, 32 *Porites astreoides*, 15 *P. furcata*, nine *Diploria strigosa* and 16 *Colpophyllia natans* coral pieces were collected from Whale Bone Bay, Bermuda in August 1999 and Bocas del Toro, Panama in April 1999 and June 2000. These corals were collected as described above.

Sampling of the water column

Six water column samples were obtained from Casa Blanca and Isla Solarte reefs in Bocas del Toro, Panama, as well as at the Smithsonian Tropical Research Institute's (STRI) Caribbean field station in Bocas del Toro. The Casa Blanca water samples were obtained near *A. cervicornis* reefs (~1.5 m and ~2.3 m depth) whereas the Isla Solarte samples were taken near two *M. annularis* colonies and one *M. cavernosa* colony at depths of ~4–5, 11.5 and 15 m respectively. The STRI point sample was collected at a depth of ~9 m approximately 10 m away from a reef dominated by *Agaricia tenufolia* and *P. furcata*. These water samples were collected at depth using SCUBA with 38 litre polystyrene sampling bags which were opened at the sampling depth and then capped. Approximately 15 l of the seawater was filtered through a 0.2 μm Sterivex™ filter (Millipore; Billerica, MA). The filters were then broken open and the DNA was isolated from the membranes using an UltraClean Soil DNA Kit (MoBio; Carlsbad, CA).

Sample processing

Tissue from all coral samples and the associated microbes were removed from the skeleton by airbrushing (< 40 p.s.i.) with 10X TE buffer (100 mM Tris [hydroxymethyl]-aminomethane hydrochloride pH 8.0, 10.0 mM EDTA; Sigma-Aldrich; St Louis, MO). The coral tissue/TE slurry was then aliquoted into microfuge tubes and frozen at −20°C. To isolate total DNA, an aliquot of the coral tissue/TE slurry was first thawed and then centrifuged in a refrigerated microfuge at maximum speed (~27 000 *g*) for 15–30 min. The supernatant was decanted and DNA was extracted from the pellet using the UltraClean Soil DNA Kit (MoBio; Carlsbad, CA).

Amplification and sequencing

Using a bacterial-specific primer 27F (5′-AGAGTTTGATC MTGGCTCAG-3′) and the universal 1492R primer (5′-TACGGYTACCTTGTTACGACTT-3′; Amann *et al*., 1995) each sample was screened for the presence of bacterial 16S rDNA by PCR. In each 50 μl PCR the following concentrations of reagents were used: 1.0 Unit *Taq* DNA polymerase, 200 μM dNTPs, 1.0 μM each primer, 1 × *Taq* buffer (70 mM Tris-HCL pH 8.8, Tween 20, 16 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$) and 1.0 μl of target DNA. The PCR protocol consisted of 5 min at 94°C, 30 cycles of (1 min at 94°C, 1 min at 65°C, – 0.5°C/cycle, 3 min at 72°C), and 10 min at 72°C. The detection limit of the PCR was 1000 targets, as determined by using an *Escherichia coli* DNA standard curve.

Those samples which displayed a PCR product ~1500 bp on a 1% agarose ethidium bromide gel were selected for cloning into a pCR®4-TOPO® vector as recommended by the manufacturer (Invitrogen; Carlsbad, CA). Selection of transformants was carried out on 50 μg ml^{-1} ampicillin/Luria–Bertani (LB) agar plates with X-gal (5-bromo-4-chloro-indoly β-D-galactopyranoside). Transformants producing white colonies were picked with a sterile toothpick, transferred to 96-well plates containing 50 μl of LB with 50 μg ml^{-1} ampicillin and grown overnight at 37°C with shaking. Plasmid DNA isolations and sequencing with the 27F primer were performed at the San Diego State University Microchemical Core Facility (SDSU-MCF; San Diego, CA).

In some cases, transformants were first checked for presence of the correct sized insert by PCR with M13F (–20) (5′-GTAAAACGACGGCCAGT-3′) and M13R (5′-CAGGAAACAGCTATGAC-3′) primers. Each 50 μl PCR mixture was set-up with the reagent concentrations as described above. The PCR protocol was 10 min at 94°C, 30 cycles of (1 min at 94°C, 1 min at 55°C, 2 min at 72°C), and 10 min at 72°C. The PCR products of the correct size were purified using a Qiagen 96-well PCR clean-up kit (Valencia, CA) or a MoBio PCR clean-up kit (Carlsbad, CA). Clones were sequenced with the 27F primer by the SDSU-MCF.

Analysis of 16S rDNA sequences

FastGroup was used to dereplicate the libraries of 16S rDNA sequences (Seguritan and Rohwer, 2001). FastGroup dereplicates 16S rDNA libraries by comparing all the sequences in a data set with each other, grouping similar sequences together, and outputting a representative sequence from each group. For this study, ambiguous bases (N) were removed at the 5′ end, sequences were trimmed to the bacterial 534 conserved site from the 3′ end, and sequences >97% identical were placed in the same group (Seguritan and Rohwer, 2001). Sequences with many N′s or those less than 300 bp in length were also removed by FastGroup. BLASTN (http://www.ncbi.nlm.nih.gov/BLAST/BLAST.cgi; Altschul *et al*., 1990) was then used to characterize each sequence group. Those groups which were identified as cloning vector sequences were removed from the data set. The identity of each sequence group was then determined using ARB (http://www.arb-home.de/).

Richness and microbial diversity predictions

Chao1 was used to estimate the number of species (i.e. species richness) in the bacterial communities associated with *Acropora* spp. samples as well as *M. franksi*, *D. strigosa* and *Porites* spp. (Chao, 1984; Colwell and Coddington, 1994; Rohwer *et al*., 2002).

$$S_{chao1} = S_{obs} + n_1^2/2n_2$$

S_{chao1} is the species richness calculated using the occurrence of singlets (n_1) and doublets (n_2) and the number of different groups observed in the library (S_{obs}).

Diversity of the microbial populations associated with these same corals was characterized using the Shannon-Wiener Index (*H*'); (Shannon and Weaver, 1949), where P_i is the per cent of the total number of sequences for one group.

$$H' = -\Sigma[P_i(\ln P_i)]$$

The Shannon-Wiener Index considers both the total number of species and the relative abundance of these species.

Specific PCR for the coral-associated Rickettsiales *1 (CAR1) sequence*

To screen coral samples for the coral-associated *Rickettsiales* 1 (CAR1) sequence, two sets of primers for nested PCR were designed by aligning the CAR1 16S rDNA representative sequence from FastGroup to its top 10 BLASTN hits using CLUSTALX (Thompson *et al*., 1997). The two sets of primers selected included a set of outside primers – Ricket87F2 (5′-AAATAAAGTTAGTGGCAAACGGGTG-3′) and Ricket447R2 (5′- GTGGAGAAGATAATGACGGTAT-3′) and a set of inside primers—Ricket207F (5′-GCAAGATAAGCCCATGCAAG-3′) – and Ricket386R (5′-GGCGAAAGCCTGATCCAGCG ACGTCA-3′). This technique allows for both increased sensitivity and specificity. Two rounds of PCR were performed for the screen. For the second round of PCR, 1.0 μl of products from the first PCR were used as the target DNA. Each round of PCR was performed in a total volume of 50 μl with the following concentrations of reagents: 1.0 unit *Taq* DNA polymerase, 200 μM dNTPs, 1.0 μM each primer, 1× *Taq* buffer (70 mM Tris-HCL pH 8.8, Tween 20, 16 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$), and 1.0 μl target DNA. The PCR protocol was 5 min at 94°C, 30 cycles of (1 min at 94°C, 1 min at 65°C, – 0.5°C/cycle, 3 min at 72°C), and 10 min at 72°C. The positive control was a 16S rDNA PCR product from a healthy *A. cervicornis* from Key West, Florida which was known, by sequence data, to contain the CAR1 sequence. The sensitivity of the nested PCR was 1000 copies, as determined by a standard curve of the positive control. Several CAR1 PCR positives were picked and sequenced to ensure that they were the correct sequence.

Acknowledgements

This research was supported by a grant from the National Science Foundation Biological Oceanography (OCEO1-37748) and San Diego State University Minority Biomedical Research Support program (NIH/NIGMS SDSU MBRS Program, 5 R25 G58906-05). David Kline was supported by a

Department of Defense NDSEG fellowship, a Smithsonian predoctoral fellowship and a Tinker UCSD Latin American Studies Fellowship. Mya Breitbart was supported by an Environmental Protection Agency (EPA) STAR fellowship. The authors thank the Smithsonian Tropical Research Institute field station in Panama for the use of their facilities and Dr Stephen Cairns, curator of the invertebrate collection at the Smithsonian Institution National Museum of Natural History, for his generosity and efforts to help us attain the preserved Acroporid samples. The authors also thank the Republic of Panama (Autoridad Nacional del Ambiente, Departamento de Cuarentena Agropecuaria del Ministerio de Desarollo Agropecuaria) and the Fish and Wildlife Department of the Florida Keys for permission to collect corals. Thank you to the SDSU-MCF for sequencing of the 16S rDNAs.

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