The ISME Journal (2011) 5, 51–60

www.nature.com/ismej

ORIGINAL ARTICLE

The impact of reduced pH on the microbial community of the coral *Acropora eurystoma*

Dalit Meron[1,2], Elinor Atias[1,2], Lilach Iasur Kruh[3], Hila Elifantz[3], Dror Minz[3], Maoz Fine[1,4,5] and Ehud Banin[1,2,5]

[1]*The Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat Gan, Israel;* [2]*The Institute for Nanotechnology and Advanced Materials, Bar-Ilan University, Ramat Gan, Israel;* [3]*Institute of Soil, Water, and Environmental Sciences, Agricultural Research Organization, Bet Dagan, Israel and* [4]*The Interuniversity Institute for Marine Science in Eilat, Eilat, Israel*

Rising concentrations of atmospheric carbon dioxide are acidifying the world's oceans. Surface seawater pH is 0.1 units lower than pre-industrial values and is predicted to decrease by up to 0.4 units by the end of the century. This change in pH may result in changes in the physiology of ocean organisms, in particular, organisms that build their skeletons/shells from calcium carbonate, such as corals. This physiological change may also affect other members of the coral holobiont, for example, the microbial communities associated with the coral, which in turn may affect the coral physiology and health. In the present study, we examined changes in bacterial communities in the coral mucus, tissue and skeleton following exposure of the coral *Acropora eurystoma* to two different pH conditions: 7.3 and 8.2 (ambient seawater). The microbial community was different at the two pH values, as determined by denaturing gradient gel electrophoresis and 16S rRNA gene sequence analysis. Further analysis of the community in the corals maintained at the lower pH revealed an increase in bacteria associated with diseased and stressed corals, such as *Vibrionaceae* and *Alteromonadaceae*. In addition, an increase in the number of potential antibacterial activity was recorded among the bacteria isolated from the coral maintained at pH 7.3. Taken together, our findings highlight the impact that changes in the pH may have on the coral-associated bacterial community and their potential contribution to the coral host.

The ISME Journal (2011) **5,** 51–60; doi:10.1038/ismej.2010.102; published online 29 July 2010
Subject Category: microbial population and community ecology
Keywords: coral; pH; bacteria; holobiont

Introduction

Coral reefs are magnificent massive structures covering $\sim 620\,000\,km^2$ of the earth (Farber *et al.*, 2002) and hosting an enormous diversity of organisms (Sebens, 1994). Scleractinian corals, the primary framework builders of coral reefs, comprise a mutualistic interaction between the coral and a dinoflagellate of the genus *Symbiodinium*, and also contain diverse populations of microorganisms (*Bacteria*, *Archaea, Eucarya* and viruses) (Rohwer *et al.*, 2001, 2002; Pantos *et al.*, 2003; Kellogg, 2004; Ritchie and Smith, 2004; Bourne and Munn, 2005; Koren and Rosenberg, 2006) that have apparently co-evolved with the corals (Ritchie and Smith, 2004; Rohwer and Kelly, 2004). This has recently led to the idea that the host (coral animal) and its associated microorganisms should be considered as a holobiont (Rohwer *et al.*, 2002; Rosenberg *et al.*, 2007).

The interaction between corals and their symbiotic microbial community can have a dramatic influence on coral physiology and health. To date, most reports on coral–bacterial interactions have focused on pathogens (reviewed in Harvell *et al.*, 2007). However most bacterial–coral associations are likely to be advantageous, providing essential nutrients for the coral host (for example, nitrogen) (Wegley *et al.*, 2007; Siboni *et al.*, 2008; Rypien *et al.*, 2009; Vega-Thurber *et al.*, 2009) or protecting the coral from infection by producing antimicrobial agents that restrict the growth of potential pathogens (Loya *et al.*, 2001; Ritchie, 2006; Shnit-Orland and Kushmaro, 2009). An interesting hypothesis that was recently presented, and that took into consideration the dynamic relationship between symbiotic microorganisms and corals under different environmental conditions, is the coral probiotic hypothesis (Reshef *et al.*, 2006). This hypothesis suggests that

Correspondence: E Banin, The Mina and Everard Goodman Faculty of Life Sciences, Bar Ilan University, Ramat Gan 52900, Israel.
E-mail: banine@mail.biu.ac.il
[5]These authors contributed equally to the work.
Received 5 February 2010; revised 21 May 2010; accepted 8 June 2010; published online 29 July 2010

by altering the structure of coral-resident microbial community, the holobiont can adapt to the changing environmental conditions more rapidly and with greater versatility than a process that is dependent on genetic mutation and selection of the coral host (Rosenberg *et al.*, 2007, 2009). This hypothesis is supported by the large diversity in microbial populations associated with corals, the benefit of the coral-associated microbes to the host and the ability to rapidly change the coral-associated microbial population in response to changing environmental conditions.

Industrial revolution, increase in human population and human activities, especially over-fishing and pollution have brought about dramatic and rapid environmental changes that have had substantial influence on the world's oceans. In the last two decades we have experienced an increase in both seawater temperature and carbon dioxide (CO_2) concentrations, which are projected to continue to increase over the next 100 years (Hoegh-Guldberg, 1999; Hoegh-Guldberg *et al.*, 2007; Rahmstorf *et al.*, 2007). Climate and environmental changes are having a devastating effect on coral reefs. Reports on coral bleaching and disease outbreaks have been on the rise and several reefs around the world are in danger of extinction (Carpenter *et al.*, 2008). An emerging environmental stress to coral reefs integrity is ocean acidification, a result of increasing atmospheric CO_2 concentration. Atmospheric CO_2 now exceeds 380 p.p.m., which is more than 80 p.p.m., above the maximum values of the past 740 000 years (Petit *et al.*, 1999). This increase has already resulted in a change in seawater pH (decreased by 0.1 pH unit) since pre-industrial times and is predicted to decrease by up to 0.4 units by the end of the twenty-first century (IPCC, 2007). The ocean absorption of anthropogenic CO_2 also reduces the saturation state of seawater with respect to calcite and aragonite, two forms of calcium carbonate secreted by calcifying marine biota. Calcification is strongly dependent on the carbonate saturation state of seawater (Caldeira, 2007). Thus the drastic change in pH may dramatically influence the physiology of corals and other oceanic organisms, in particular organisms that deposit calcium carbonate to build their skeletons/shells (Gattuso *et al.*, 1998; Orr *et al.*, 2005).

Recent studies have characterized the influence of pH decrease on marine ecosystems (Ishimatsu *et al.*, 2005; Bibby *et al.*, 2007; Fine and Tchernov, 2007; Hoegh-Guldberg *et al.*, 2007; Kuffner *et al.*, 2007). These studies presented several effects, such as decreased abundance of crustose coralline algae (Kuffner *et al.*, 2007), shell dissolution in living pteropods (Orr *et al.*, 2005) and chronic effects on fish physiology (Ishimatsu *et al.*, 2005). Fine and Tchernov (2007) reported that a decrease in pH (from 8.2 to 7.4) led to physiology alterations in the coral *Oculina patagonica* and *Madracis pharensis*, leading to complete dissolution of their skeleton. It is interesting that the corals returned to calcify when returned to ambient pH after a year in low pH (Fine and Tchernov, 2007).

The change in pH, most likely, does not solely affect the coral host but can also influence the associated microbial community (Vega-Thurber *et al.*, 2009). In this study, we characterized the microbial community associated with the scleractinian coral *Acropora eurystoma* and the changes in the microbial community following exposure to decreased pH.

Materials and methods

Coral collection and maintenance

The corals used in this study were collected from the reef off the Interuniversity Institute for Marine Sciences in Eilat (IUI, 29°30′N; 34°55′E, Gulf of Aqaba) Red Sea. Twenty colonies of the scleractinian coral *Acropora eurystoma* were collected at 10–15 m depth and fragmented to 5 cm long fragments. The fragments were immediately placed in running seawater (temperature of 26 °C, salinity 3.9%, irradiance of 250 $\mu mol\, m^{-2}\, s^{-1}$ and photoperiod of 10 L:14D and pH 8.2). Following an acclimation period of 2 weeks and full recovery of the tissue, these mini-colonies (obtained from the 20 colonies) were distributed between the two pH treatments: 7.3 and 8.2 and maintained under these conditions for 10 weeks. The distribution of the fragments was carried out systematically such that fragments from the same colony were evenly distributed between the treatments to avoid any possible bias because of sample collection.

To reach the desired pH, water from the Red Sea, pumped from 30 m depth (salinity 39% and temperature 26 °C), was continuously supplied to two 1000 l tanks. pH was manipulated by bubbling pure CO_2 into seawater in the tanks and controlled by using a pH controller (Aquastar, IKS Computer-Systeme GmbH, Karlsbad, Germany) connected to pH electrodes (S-200C, Sensorex, Garden Grove, CA, USA). A detailed description of the pH system is provided in the Supplementary Information. All mini-colonies of *Acropora eurystoma* in the two pH treatments survived to the end of the experiment and did not show any signs of stress.

Coral fractionation, bacteria isolation and DNA extraction

At the end of the experiment (10 weeks) the corals were fractionated to mucus, tissue and skeleton fractions, and samples were taken for isolation of bacteria and DNA extraction. To remove the mucus, the corals were placed in 50-ml centrifuge tubes, centrifuged for 3 min at 2675 *g*, and the mucus collected. The coral was then washed several times in seawater and the tissue was then extracted from the coral using an airbrush. To minimize any cross contamination from the tissue to the skeleton, the

skeleton was thoroughly cleaned from remaining tissue residues by airbrush, followed by several washes with sterile seawater, only then the skeleton was crushed with a mortar and pestle. To isolate bacteria from these fractions, 10-fold serial dilutions of coral mucus, tissue, skeleton and seawater were plated on Marine Agar (Difco, Detroit, MI, USA) and incubated at 26 °C. To obtain bacteria from the seawater, 1 l of seawater from each pH treatment was filtered through polycarbonate filters (0.22 μm). The filters were homogenized in 50 ml of filtered sterile seawater and serial dilutions were plated on Marine Agar. To extract DNA from coral mucus, crushed tissue, skeleton and seawater, samples were centrifuged at 9300 *g* for 15 min. DNA was extracted from the pellets using the UltraClean Soil DNA kit according to the manufactures guidelines (MoBio, Carlsbad, CA, USA). DNA from cultured bacteria was extracted from individual colonies using a DNA Purification kit (Promega, Madison, WI, USA).

PCR amplification of 16S rRNA gene and clone library construction

Bacterial primers 8F and 1492R (Lane, 1991) were used for amplification of the 16S rRNA gene from the various fractions and isolated colonies. 16S rRNA genes were amplified in a 25 μl reaction mixture consisting of 2.5 μl of 10 × buffer, 2.5 μl of a 2.5 mM total dNTP mixture, each primer at 5 μM, 10 ng of template DNA, and 2.5 U of Ex *Taq* DNA polymerase (TaKaRa, Bio, Shiga, Japan). Amplification conditions for the PCR included an initial denaturation step of 94 °C for 3 min, followed by 29 cycles of 94 °C for 35 s, 56 °C for 35 s and 72 °C for 1.5 min and a final extension step of 72 °C for 7 min. Reaction products were checked for size and purity on 1% agarose gel. PCR products obtained from bacterial isolates were sequenced. Amplified DNA from seawater, mucus, tissue and skeleton samples was ligated into the pGEM-T Easy vector according to the manufacturer instructions (Promega) and transformed into competent *Escherichia coli* DH5α. For each fraction, 2–3 libraries were constructed. The clones obtained were amplified by colony PCR with pGEM primers (M13F and M13R). Amplification conditions for the colony PCR included an initial denaturation step of 95 °C for 4.5 min, followed by 29 cycles of 95 °C for 30 s, 59.5 °C for 30 s and 72 °C for 1 min and a final extension step of 72 °C for 10 min. Reaction products were checked for size and purity on 1% agarose gel. Before sequencing, the PCR products were enzymatically purified using ExoSAP-IT (USB, Cleveland, OH, USA). Sequencing was carried in Macrogen, Korea, using the 8F forward primer. The nucleotide sequence data for all of the clones and colonies reported in this paper were deposited in the GenBank nucleotide sequence database under accession numbers GU319121–GU319777.

Phylogenetic analysis

For initial identification the obtained sequences were compared with the NCBI database (National Center for Biotechnology Information; www.ncbi.nlm.nih.gov) using the BLASTn algorithm. Phylogenetic trees were constructed and checked by bootstrap analysis using the ARB phylogenetic program package software (Ludwig *et al*., 2004). Sequences (650 bp) were aligned to the ARB gene database with the ARB automated alignment tool, and refined manually by visual inspection and secondary-structure analysis. Phylogenetic affiliation of the clone sequences was determined according to both ARB and NCBI-BLAST results. The similarity of the clone libraries from the different fractions was compared by Unifrac Metric analysis using the Jackknife algorithm where 100 replicates of trees were examined (Lozupone *et al*., 2006). In each replicate tree the different samples were represented by 30 clone sequences each. Diversity indices and operational taxonomic units were determined using DOTUR (Schloss and Handelsman, 2005).

Denaturing gradient gel electrophoresis (DGGE) analysis

PCR amplifications for DGGE were performed as previously described using bacterial primer pair 341F (with a GC clamp at its 5′ end) and 907R (Muyzer and Smalla, 1998). Amplification conditions for the PCR included an initial denaturation step of 95 °C for 4 min, followed by 34 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s and a final extension step of 72 °C for 10 min. Products were resolved on 6% (w/v) polyacrylamide gels containing a linear urea-formamide gradient ranging from 20 to 60% denaturant (with 100% defined as 7 M urea and 40% formamide). Gels were run for 17 h at 60 °C at a constant voltage of 100 V using the Dcode Universal Mutation System (Bio-Rad Laboratories, Hercules, CA, USA). Community patterns were visualized after staining with Gelstar (Invitrogen, Carlsbad, CA, USA) by UV transillumination (302 nm) and were photographed with a Kodak KDS digital camera (Kodak, New Haven, CT, USA).

Cluster analysis of DGGE community fingerprints

DNA fingerprints obtained from the 16S rRNA gene banding patterns on the DGGE gels were analyzed using FingerprintingII Informatix Software (Bio-Rad Laboratories). The lanes were normalized to contain the same amount of total signal after background subtraction. The gel images were straightened, aligned and analyzed to give a densitometric curve for each gel. Band positions were converted to Rf values between 0 and 1, and profile similarity was calculated by the unweighted pair-group method using arithmetic average algorithm, for the total number of lane patterns. Cluster analyses of the lane patterns were constructed by FingerprintingII software.

Antibacterial screening
Screening the cultured bacteria for antibacterial activity was performed as described previously (Shnit-Orland and Kushmaro, 2009) against common indicator bacteria, including *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa, Staphylococcus aureus*, as well as the coral pathogen, *Vibrio coralliilyticus* (Ben-Haim *et al*., 2003). We used a drop technique for the screening process. Isolated marine bacteria, from the mucus of the corals, were grown on Marine Broth (Difco) for 48 h at 26 °C (ambient seawater temperature). After sufficient growth, a 10 μl drop of the cell suspension (1.0 $OD_{600\,nm}$) was placed on agar plates freshly covered with an overnight culture of a specific indicator bacterium (100 μl of 1.0 $OD_{600\,nm}$ culture). Activity was measured after 24 h by the formation of an inhibition zone where the drop had been placed. Each analysis was performed four times. All indicator bacteria were cultured on Luria-Bertani Broth agar (Difco) plates, except for *V. coralliilyticus*, grown on Marine Broth agar, at their optimal growth temperatures (30 °C for *V. coralliilyticus* and *B. subtilis* and 37 °C for the others).

Results

Changes in the microbial community following exposure to decreasing pH
DGGE was initially used to examine the bacterial community composition of the corals exposed to reduced and ambient pH treatments. Pattern comparison (Figure 1) showed that the bacterial communities in all coral fractions (mucus, tissue and skeleton) were influenced by the pH. The clusters were divided according to the pH level that the coral was exposed to (that is, pH 8.2 and pH 7.3) and coral fractions were distributed within the pH clusters, indicating that the bacterial community had shifted in response to the environmental change.

16S rRNA gene clone libraries of *A. eurystoma* microbial community revealed changes in the community composition following exposure to reduced pH. To further analyze the community composition, 16S rRNA gene clone libraries of *A. eurystoma* microbial community were constructed and sequenced ($n = 415$). Rarefaction analyses were performed using DOTUR (Schloss and Handelsman, 2005) on the clone libraries to determine the number of unique bacterial clones as a proportion of the estimated total diversity within each library. The rarefaction curves did not reach an asymptote (Supplementary Figure S1), indicating insufficient sampling to capture the total diversity of the bacterial community. Overall we identified 103 operational taxonomic units from the corals maintained at pH 7.3 versus 74 operational taxonomic units in the corals maintained at pH 8.2 (Table 1). To determine the abundance and richness of the bacterial communities associated with the coral, the Shannon–Wiener index was calculated to quantify species diversity (Table 1). The community from pH 7.3 was more diverse than that at pH 8.2, with

Table 1 Number of OTUs and richness estimation of bacterial 16S rRNA gene libraries from corals maintained at pH 8.2 and 7.3

	pH 7.3	*pH 8.2*
OTUs (97)	103	74
Families (92)	90	65
Orders (90)	76	58
Class (85)	55	43
Phyla (80)	35	29
Shannon–Wiener index (97)	4.34 (± 0.15)	3.94 (± 0.16)
Coverage[a]	0.62	0.69

Abbreviation: OTU, operational taxonomic unit.
[a]Coverage was calculated as 1-(n/N) and where *n* is the number of singletons and *N* is the total number of clones.
% Cutoff is presented in parentheses.

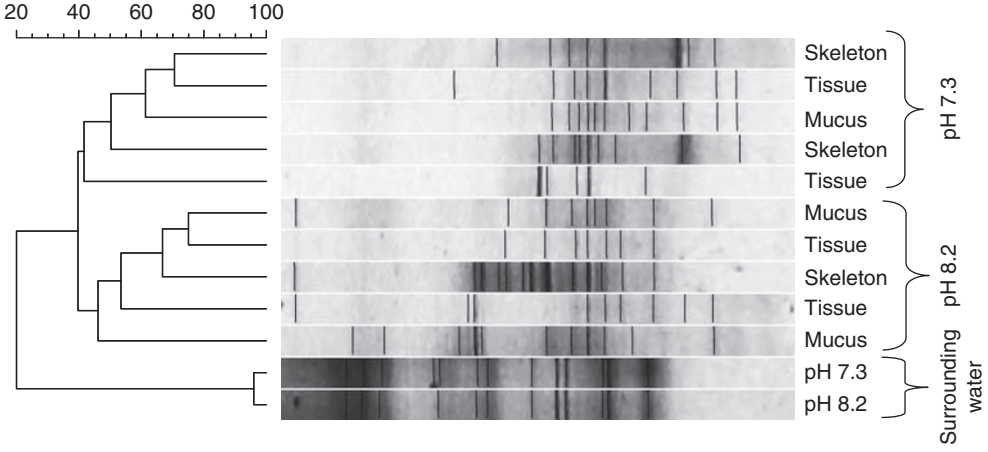

Figure 1 Cluster analysis of DGGE patterns of bacterial communities in *Acropora eurystoma* and seawater exposed to pH 7.3 and 8.2. The cluster analysis was performed using the Fingeprint II software. The unweighted pair-group method using arithmetic average tree was constructed on the basis of Jaccard similarity matrix between the different DGGE patterns and presented in each lane.

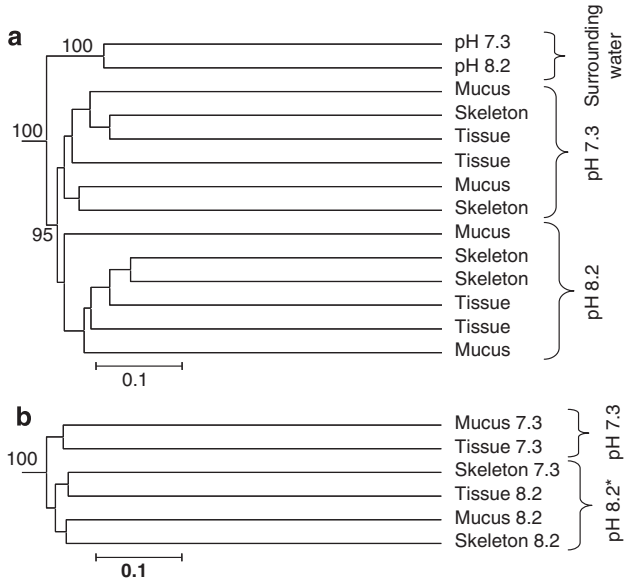

Figure 2 Cluster analysis of sequences obtained from microbial community 16S rRNA clone libraries from *A. eurystoma* (**a**) uncultured 16S rRNA clones ($n=415$). (**b**) Cultured isolates ($n=263$). *Note that the isolates from skeletons of corals maintained at pH 7.3 are clustered with isolates from the corals maintained at pH 8.2 (see text). Cluster analysis was performed using *Jackknife Environment Clusters* with 100 permutations and minimum sampling size of 30 sequences per environment (Unifrac Metric analysis) (Lozupone and Knight, 2005).

a Shannon–Wiener index of 4.34 compared with 3.94 at pH 7.3.

A phylogenetic comparison between the different treated samples showed considerable differences in the microbial community composition (Figure 2a) that closely reflected the clustering observed with DGGE analysis. The clusters were mainly divided by the pH treatments and not by the coral fractions (that is, mucus, tissue and skeleton). Furthermore, there was a clear difference between the bacterial community in the coral and that of the surrounding water.

Diversity of bacterial groups and their distribution

Sequences obtained from the 16S rRNA gene libraries were used to analyze the diversity of bacterial communities associated with *A. eurystoma* exposed to pH 8.2 and 7.3 (Figure 3 and Supplementary Table S1 showing the percent distribution between fraction in each treatment, Supplementary Table S2 providing the complete list of clones analyzed in the study and Supplementary Tables S3–S4 providing the most abundant clones in each treatment). The two dominant bacterial groups *Gammaproteobacteria* (26 and 27% of the total clones at pH 8.2 and 7.3, respectively) and *Cyanobacteria* (23 and 38%, respectively) remained dominant in the corals incubated at the two pH treatments (Figures 3a and b). Furthermore in several instances a close homolog of the same strain was present at both pH treatments, for example: *Leptolyngbya* sp. (EU249128), *Phormidium persicinum* (EF654085) and *Thalassolituus* sp. (GQ426888) (Supplementary Tables S2–S4). A significant change in gene frequency was observed in the following bacterial groups at the lower pH. The *Alphaproteobacteria* increased in their overall presence from 11 to 20% and the dominant clones belonged to the *Rhodobacteraceae*. Two other groups in which their gene frequency had changed were *Deltaproteobacteria* and *Bacteroidetes*. The gene frequency of *Deltaproteobacteria* was reduced from 24 to 5% when shifted to pH 7.3. This group was especially dominant in the skeleton fraction (see Supplementary Tables S1–S4) and most bacteria in this group (more than 60%) showed homology to *Desulfobacter* species that belong to the sulfate-reducing bacteria. The gene frequency of the *Bacteroidetes* decreased from 6 to 2%. Examination of the community structure within the fractions (mucus, tissue and skeleton) further reveals these trends (Supplementary Tables S1 and S2).

Changes were also detected in the less abundant bacterial groups. Of those, noteworthy are the changes in *Verruncomicrobiae* and *Gammatimondetes*, which were present only at pH 7.3, whereas other groups, such as *Epsilon* and *Beta Proteobacteria*, were only present in the corals maintained at ambient pH (Figure 3 and Supplementary Tables S1 and S2). The coral microbial community showed a clear difference from the microbial community of the surrounding water (Figures 2a and 3 and Supplementary Table S2). Furthermore, no major difference in diversity was evident between the surrounding water maintained at the two pH levels (Figures 2a and 3c, d).

Diversity of the culturable bacterial communities and their antibacterial activities

The diversity of bacteria isolated from the coral fractions exposed to the two pH conditions was examined. Analysis of the 16S rRNA gene sequences obtained from isolates ($n=263$) showed that the clustering into two different communities correlated, to some extent, with pH treatment (Figure 2b). One cluster contained isolates obtained from mucus and tissue fractions from the coral maintained at pH 7.3. The other contained the bacteria isolated from the mucus, tissue and skeleton at pH 8.2. It is interesting that the skeleton bacterial community isolated from corals at pH 7.3 clustered with the bacterial communities fractions isolated from corals exposed to pH 8.2 (Figure 2b). As expected, the diversities of the culturable isolated bacteria from both pH treatments differed from those of the culture-independent groups (Figures 3 and 4 and Supplementary Tables S1–S4). For example, *Bacillus* sp. (*Bacillus jeotgali* (GU397390) and *Bacillus litoralis* (FJ188307)) were dominant in the culturable fraction and rarely detected in the culture-independent

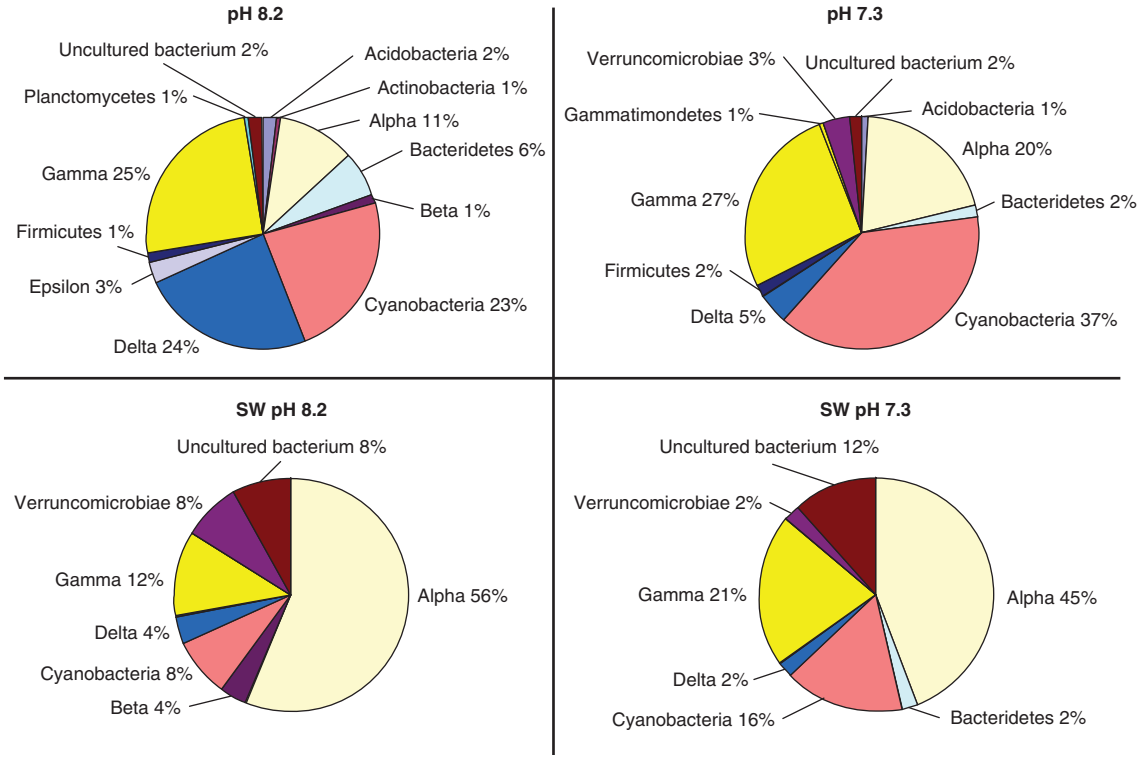

Figure 3 The diversity of the bacterial groups found in *A. eurystoma* and seawater exposed to pH 7.3 and pH 8.2. The chart was constructed on the basis of 16S rRNA gene sequences derived from clone libraries obtained from the coral *A. eurystoma* maintained at pH 7.3 ($n=173$) or 8.2 ($n=159$) and from the surrounding water ''sw'' maintained at pH 7.3 ($n=43$) and 8.2 ($n=24$). The *alpha, beta, gamma, delta* and *epsilon* belong to the *Proteobacteria* phylum.

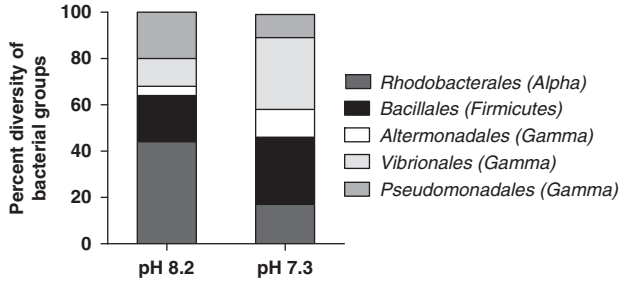

Figure 4 The diversity of the bacterial groups isolated from *A. eurystoma* exposed to pH 7.3 and pH 8.2. The chart was constructed on the basis of 16S rRNA gene sequences derived from bacterial isolates that were associated with *A. eurystoma* maintained at pH 7.3 ($n=150$) and 8.2 ($n=108$).

group. Similarly, *Rohodobacteraceae* were dominant at pH 8.2 in the culturable fraction and decreased in pH 7.3. This was opposite to the trend that was evident for this family in the culture-independent group.

An important difference apparent in the isolated community at the lower pH treatment was the increase in *Vibrionaceae* (for example, *Vibrio sp.* GQ406613 and uncultured *Vibrio* sp. AM941184) and *Alteromonadaceae* (*Alteromonas* sp. GU726843) (Tables S2–S4). In a few cases the isolates were detected in corals maintained at pH 8.2, but increased in pH 7.3, for example *Vibrio* sp. (FJ161306) and *Alteromonas* sp. (GU726843) (see Supplementary Table S2). This change is especially interesting as species that belong to *Vibrionaceae* and *Alteromonadaceae* were previously associated with diseased or stressed corals (Ritchie, 2006; Bourne *et al.*, 2007; Arboleda and Reichardt, 2009; Sunagawa *et al.*, 2009).

To examine the antibacterial activity of isolated bacteria from each pH, a recently established screening method was used (Shnit-Orland and Kushmaro, 2009). The antibacterial activity of 165 isolates was tested against five indicator strains (*Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa, Staphylococcus aureus*, as well as the coral pathogen *Vibrio coralliilyticus*). The screen was carried out with bacteria isolated from the mucus fraction, as we expected these bacteria to most likely serve as the first line of defense. Corals maintained at pH 7.3 showed a marked increase in the isolated bacteria, producing antibacterial activity (42%) as compared with isolates obtained from corals maintained at the ambient pH (17%). It is interesting that most of the bacteria that showed antibacterial activity belong to the *Vibrionaceae* and *Rhodobacteraceae*, 50 and 29%, respectively. The complete list of bacteria showing antimicrobial activity and against which indicator strains are summarized in Supplementary Table S5.

Discussion

Coral colonies represent a complex landscape, comprising the coral animal, a community of symbiotic dinoflagellates and associated bacterial biota that can thrive in fascinating harmony. Many environmental factors can influence this delicate balance and affect coral health and disease (Bruno *et al.*, 2007; Rosenberg *et al.*, 2007; Garren *et al.*, 2009). This complexity also provides means for coral resistance and persistence. One of the major environmental factors that may directly affect coral health and this delicate symbiosis is an increase in CO_2 concentration, which is projected to result in ocean acidification (Anthony *et al.*, 2008). Extensive research is currently on-going to examine the impact of changes in pH on the physiology of corals, but little is known about the impact of reduced pH on coral-associated microbial communities (Vega-Thurber *et al.*, 2009). The current study addresses this question by examining whether or not reduced pH can influence the microbial community structure and ability to provide ecological services to the coral host (for example, antibacterial activity). It should be emphasized that the pH used in this study is lower than what is expected in the next 100 years.

The results show that the coral *A. eurystoma* harbors a specific microbial community that is distinct from the surrounding water, as reported in other corals from various locations (Bourne and Munn, 2005; Guppy and Bythell, 2006; Ritchie, 2006; Kooperman *et al.*, 2007; Lampert *et al.*, 2008; Arboleda and Reichardt, 2009). The coral–bacterial community structure was influenced by seawater pH, and the communities were clustered according to the pH treatment, more so than the coral microenvironment, for example, mucus, tissue and skeleton. A higher Shannon–Wiener index, indicating an increase in coral-associated microbial diversity, was recorded at the lower pH treatment. This could be the result of an intermediate disturbance (Connell, 1978), leading to an increase in biodiversity. We speculate that reduced pH triggers this disturbance. When characterizing the microbial community of the *A. eurystoma* using 16S rRNA clone libraries, the major bacterial groups found were similar to those found in other studies (Sekar *et al.*, 2006; Garren *et al.*, 2009; Nithyanand and Pandian, 2009; Rypien *et al.*, 2009; Vega-Thurber *et al.*, 2009). These included mainly the *Gammaproteobacteria* and *Cyanobacteria*, which composed approximately 50% of the bacterial community and remained dominant in the corals incubated at the two pH treatments. However, several interesting shifts in the microbial community were found following the decrease in pH. The *Alphaproteobacteria*, dominated by *Rhodobacteraceae*, increased in their overall presence from 11 to 20% (pH 8.2 and 7.3, respectively). Sequences found from this group were closely related to sequences that were previously described in studies examining the microbial community associated with diseased corals, such as white plague disease in *Montastraea faveolata* (GenBank accession numbers FJ403086, FJ202287, FJ202842, FJ203583, FJ202745 and FJ203565 all showing 97–98% sequence identity), white syndrome in *Turbinaria mesenterina* (GenBank accession number EU780231, 85–91% identity) and *Muricea elongate* in bleached corals (GenBank accession number DQ917824, 98% identity). In addition, clones reported to be associated with *Oculina patagonica* (Koren and Rosenberg, 2006) and sponge-associated bacteria isolated from *Axinella corrugate* (GenBank accession number EF092256, 99% identity) and *Haliclona (gellius)* sp. (GenBank accession number U346416, 92% identity) were also detected.

The *Deltaproteobacteria*, which contained sequences primarily homologous to species belonging to the sulfate-reducing bacteria *Desulfobacter*, had a lower frequency (5%) at pH 7.3 as compared with pH 8.2 (24%). sulfate-reducing bacteria have been implicated as part of the microbial consortium that induces black band disease in corals (Schnell *et al.*, 1996; Richardson *et al.*, 1997) or to increase in frequency in corals exposed to pollution (Garren *et al.*, 2009). No signs of disease were detected in the corals (at either pH), and it is important to note that sulfate-reducing bacteria are also present in healthy corals (Arboleda and Reichardt, 2009).

The abundance of the *Bacteroidetes* decreased from 6% (pH 8.2) to 2% (pH 7.3). In this group, clones were found related to coral bacterial symbionts previously found in the Caribbean coral *Montastraea faveolata* (Sunagawa *et al.*, 2009), *Siderastrea siderea* (Sekar *et al.*, 2008) and the Mediterranean coral *Oculina patagonica* (Koren and Rosenberg, 2006). Although *Bacteroidetes* have been previously detected in healthy corals (Sekar *et al.*, 2008), an increase in *Bacteroidetes* was associated with Black Band Disease (Cooney *et al.*, 2002; Frias-Lopez *et al.*, 2002, 2004; Barneah *et al.*, 2007). *Bacteroidetes* were also reported to increase in corals exposed to various stress conditions (Vega-Thurber *et al.*, 2009).

Another interesting finding we observed when examining the community in the corals exposed to pH 7.3 was the increase in species showing close homology to families that include known coral pathogens, such as *Vibrionaceae* and *Alteromonadaceae* (Ritchie, 2006; Bourne *et al.*, 2007; Arboleda and Reichardt, 2009; Sunagawa *et al.*, 2009), as well as *Rhodobacteraceae* (*Alphaproteobacteria*), previously isolated from diseased, injured or stressed marine invertebrates (Sekar *et al.*, 2006; Sunagawa *et al.*, 2009).

Anthony *et al.* reported an adverse effect of reduced pH on corals health and function (Anthony *et al.*, 2008). In the present study, we did not observe signs of bleaching or disease and did not detect identical sequences of known coral pathogens. Close homologous of bacteria reported to be associated

with diseased or stressed corals were detected in the lower pH treatment. For example, bacteria isolated from a diseased gorgonian coral showing homology to the coral pathogen *V. coralliilyticus* were identified (GQ406701, 93% identity and GQ406613, 98% identity). In addition, close homologous of bacteria associated with diseased marine organisms were also identified (for example, *Vibrio pectenicida* a pathogen of scallop (Y13830, 98% identity) and bacteria isolated from diseased fish (FN432989, 99% identity)). Part of this change in the bacterial community was markedly detected only in the culturable bacteria (*Vibrionaceae and Alteromonadaceae*), yet we believe this trend represents an important phase-shift in the community, probably a result of environmental change and/or changes in the physiological state of the holobiont. Several studies have reported an increase in similar bacterial families under various stress conditions. For example, Ritchie examined mucus from healthy *Acropora palmata* during high temperatures and reported that *Vibrio* sp. increased and replaced the community of beneficial bacteria (Ritchie, 2006). Garren *et al*. (2009) also observed an increase in the number of *Vibrios* following exposure of corals to yet another common stressor increased pollution as a result of intensive fish farming (Garren *et al*., 2009). Similar results were also reported in a recent study examining the metagenomic profile of coral holobionts exposed to various stressors (Vega-Thurber *et al*., 2009). It is interesting that one of the stress conditions tested in that study was pH. Although the coral species was different (*Porites compressa*), the pH was lower (6.7 versus 7.3) and the exposure time shorter (64 h versus 10 weeks in our study), the authors also reported that decreased pH resulted in a shift from a bacterial community associated with healthy corals towards a community often associated with diseased corals. *Vibrio* sp., for example, increased at the lower pH treatment and were found to significantly alter the microbiome metabolism (Vega-Thurber *et al*., 2009). On the basis of this, the authors hypothesized that the contribution of just a few members of a community can profoundly shift the health status of the coral holobiont (Vega-Thurber *et al*., 2009). It is hard to conclude from our study whether the corals indeed experienced environmental stress due to the decrease in pH. However, the shift to opportunistic bacterial species that are known to be associated with diseased or stressed corals is in our opinion an important biomarker that may suggest this is indeed the case. Our study opens the path for future work that can address this important hypothesis.

Changes in bacterial communities usually provide limited information on the direct impact the microbial community may have on the coral host. One of the important traits that is thought to be contributed by the microbial community and can influence the coral host is the production of antibacterial agents (Kelman *et al*., 2006; Nithyanand and Pandian, 2009; Rypien *et al*., 2009; Shnit-Orland and Kushmaro, 2009). We examined the ability of the isolated bacterial community to produce antibacterial activity against five bacterial species (including a coral bleaching pathogen) and detected an increase from 17 to 42% in the percent of coral bacteria showing antibacterial activity following a decrease in pH. This increase in antibacterial activity may impact the dynamics of the microbial community and highlights the forces that take place in structuring its diversity. In fact, most of the antibacterial activity was produced by isolates belonging to the *Vibrio* sp. (50%), suggesting that this may be one of the mechanisms by which these bacteria may colonize the coral host. These results further support the metagenomic study by Vega-Thurber *et al*. (2009) who reported that under low pH, in addition to the increase in pathogens, there was an increase in secondary metabolites and stress resistance (for example, antibiotics and toxins) (Vega-Thurber *et al*., 2009).

In conclusion, the present study emphasizes the complexity of the coral holobiont and its response to changes in environmental conditions. The microbial community may be a key factor in determining holobiont's health and function. It is, therefore, imperative to further explore interactions between the microbial community and its hosting coral under environmental change, such as pH.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work was partially supported by a grant (no. 2006318) from the United States-Israel Binational Science Foundation to EB.

References

Anthony KR, Kline DI, Diaz-Pulido G, Dove S, Hoegh-Guldberg O. (2008). Ocean acidification causes bleaching and productivity loss in coral reef builders. *Proc Natl Acad Sci USA* **105**: 17442–17446.

Arboleda M, Reichardt W. (2009). Epizoic communities of prokaryotes on healthy and diseased scleractinian corals in Lingayen Gulf, Philippines. *Microb Ecol* **57**: 117–128.

Barneah O, Ben-Dov E, Kramarsky-Winter E, Kushmaro A. (2007). Characterization of black band disease in Red Sea stony corals. *Environ Microbiol* **9**: 1995–2006.

Ben-Haim Y, Thompson FL, Thompson CC, Cnockaert MC, Hoste B, Swings J *et al*. (2003). *Vibrio coralliilyticus sp. nov.*, a temperature-dependent pathogen of the coral *Pocillopora damicornis*. *Int J Syst Evol Microbiol* **53**: 309–315.

Bibby R, Cleall-Harding P, Rundle S, Widdicombe S, Spicer J. (2007). Ocean acidification disrupts induced

defenses in the intertidal gastropod *Littorina littorea*. *Biol Lett* **3**: 699–701.

Bourne D, Iida Y, Uthicke S, Smith-Keune C. (2007). Changes in coral-associated microbial communities during a bleaching event. *ISME J* **2**: 350–363.

Bourne DG, Munn CB. (2005). Diversity of bacteria associated with the coral *Pocillopora damicornis* from the Great Barrier Reef. *Appl Environ Micro* **7**: 1162–1174.

Bruno JF, Selig ER, Casey KS, Page CA, Willis BL, Harvell CD *et al.* (2007). Thermal stress and coral cover as drivers of coral disease outbreaks. *Plos Biology* **5**: 1220–1227.

Caldeira K. (2007). What corals are dying to tell us about CO_2 and ocean acidification. *Oceangraphy* **20**: 188–195.

Carpenter KE, Abrar M, Aeby G, Aronson RB, Banks S, Bruckner A *et al.* (2008). One-third of reef-building corals face elevated extinction risk from climate change and local impacts. *Science* **321**: 560–563.

Connell H. (1978). Diversity in tropical rain forests and coral reefs. *Science* **199**: 1302–13100.

Cooney RP, Pantos O, Le Tissier MD, Barer MR, O'Donnell AG, Bythell JC. (2002). Characterization of the bacterial consortium associated with black band disease in coral using molecular microbiological techniques. *Environ Microbiol* **4**: 401–413.

Farber SC, Costanza R, Wilson MA. (2002). Economic and ecological concepts for valuing ecosystem services. *Ecol Econ* **41**: 375–392.

Fine M, Tchernov D. (2007). Scleractinian coral species survive and recover from decalcification. *Science* **315**: 1811.

Frias-Lopez J, Klaus JS, Bonheyo GT, Fouke BW. (2004). Bacterial community associated with black band disease in corals. *Appl Environ Microbiol* **70**: 5955–5962.

Frias-Lopez J, Zerkle AL, Bonheyo GT, Fouke BW. (2002). Partitioning of bacterial communities between seawater and healthy, black band diseased, and dead coral surfaces. *Appl Environ Microbiol* **68**: 2214–2228.

Garren M, Raymundo L, Guest J, Harvell CD, Azam F. (2009). Resilience of coral-associated bacterial communities exposed to fish farm effluent. *PLoS One* **4**: e7319.

Gattuso JP, Frankignoulle M, Bourge I, Romaine S, Buddemeier RW. (1998). Effect of calcium carbonate saturation of seawater on coral calcification. *Global and Planetary Change* **18**: 37–46.

Guppy R, Bythell JC. (2006). Environmental effects on bacterial diversity in surface mucus layer of the reef coral *Montastraea faveolata*. *Mar Ecol Prog Ser* **328**: 133–142.

Harvell CD, Jordan-Dahlgren E, Merkel S, Rosenberg E, Raymundo L, Smith GW *et al.* (2007). Coral disease, environmental drivers, and the balance between coaral and microbial associated. *Oceangraphy* **20**: 172–195.

Hoegh-Guldberg O. (1999). Climate change, coral bleaching and the future of the world's coral reefs. *Marine and Freshwater Research* **50**: 839–866.

Hoegh-Guldberg O, Mumby PJ, Hooten AJ, Steneck RS, Green P, Gomez E *et al.* (2007). Coral reefs under rapid climate change and ocean acidification. *Science* **318**: 1737–1742.

The International Panel on Climate Change (2007) (Report). http://www.ipcc.ch.

Ishimatsu A, Hayashi M, Lee KS, Kikkawa T, Kita J. (2005). Physiological effects on fishes in a high-CO2 world. *Journal of Geophysical Research-Oceans* **110**: C09S09.

Kellogg CA. (2004). Tropical Archaea: diversity associated with the surface microlayer of corals. *Mar Ecol Prog Ser* **273**: 81–88.

Kelman D, Kashman Y, Rosenberg E, Kushmaro A, Loya Y. (2006). Antimicrobial activity of Red Sea corals. *Mar Biol* **149**: 357–363.

Kooperman N, Ben-Dov E, Kramarsky-Winter E, Barak Z, Kushmaro A. (2007). Coral mucus-associated bacterial communities from natural and aquarium environments. *FEMS Microbiol Lett* **276**: 106–113.

Koren O, Rosenberg E. (2006). Bacteria associated with mucus and tissues of the coral *Oculina patagonica* in summer and winter. *Appl Environ Microbiol* **72**: 5254–5259.

Kuffner BI, Andersson JA, Jokiel LP, Rodgers SK, Mackenize TF. (2007). Decreases abundance of crustose coralline algae due to ocean acidification. *Nature* **1**: 114–117.

Lampert Y, Kalman D, Nitzan Y, Dubinsky Z, Behar A, Hill RT. (2008). Phylogenetic diversity of bacteria associated with the mucus of Red Sea corals. *FEMS Microbiol Ecol* **64**: 187–198.

Lane DJ. (1991). *16S/23S rRNA Sequencing*. John Wiley & Sons: Chichester, United Kingdom.

Loya Y, Sakai K, Nakano Y, Sambali H, Van Woesik R. (2001). Coral bleaching: the winners and the losers. *Ecol Lett* **4**: 122–131.

Lozupone C, Hamady M, Knight R. (2006). UniFrac—An online tool for comparing microbial community diversity in a phylogenetic context. *BMC Bioinformatics* **7**: 371.

Lozupone C, Knight R. (2005). UniFrac: a new phylogenetic method for comparing microbial communities. *Appl Environ Microbiol* **71**: 8228–8235.

Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar *et al.* (2004). ARB: a software environment for sequence data. *Nucleic Acids Research* **32**: 1363–1371.

Muyzer M, Smalla K. (1998). Application of denaturing gradient gel electophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek* **73**: 127–141.

Nithyanand P, Pandian SK. (2009). Phylogenetic characterization of culturable bacterial diversity associated with the mucus and tissue of the coral *Acropora digitifera* from the Gulf of Mannar. *FEMS Microbiol Ecol* **69**: 384–394.

Orr JC, Fabry VJ, Aumont O, Bopp L, Doney SC, Feely RA *et al.* (2005). Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. *Nature* **437**: 681–686.

Pantos O, Cooney RP, Le Tissier MDA, Barer MR, O'Donnell AG, Bythell JC. (2003). The bacterial ecology of a plague-like disease affecting the Caribbean coral *Montastrea annularis*. *Appl Environ Microbiol* **5**: 370–382.

Petit JR, Jouzel J, Raynaud D, Barkov NI, Barnola JM, Basile I *et al.* (1999). Climate and atmospheric history of the past 420 000 years from the Vostok ice core, Antarctica. *Nature* **399**: 429–436.

Rahmstorf S, Cazenave A, Church JC, Keeling RF, Parker DE, Somerville CJ. (2007). Recent climate observations compared to projections. *Science* **316**: 709.

Reshef L, Koren O, Loya Y, Zilber-Rosenberg I, Rosenberg E. (2006). The coral probiotic hypothesis. *Appl Environ Microbiol* **8**: 2067–2073.

Richardson LL, Kuta KG, Schnell S, Carlton RG. (1997). Ecology of the black band disease microbial consortium.

In: Lessios HA, Macintyre IG (eds). *Proceedings of the Eighth International Coral Reef Symposium* vol 1 Smithsonian Tropical Research Institute: Balboa, Panama, pp 597–600.

Ritchie KB. (2006). Regulation of microbial population by coral surface mucus and mucus-associated bacteria. *Mar Ecol Prog Ser* **322**: 1–14.

Ritchie KB, Smith GW. (2004). *Coral Health and Disease*. Springer Press: New York/Berlin.

Rohwer F, Breitbart M, Jara J, Azam F, Knowlton N. (2001). Diversity of bacteria associated with the Caribbean coral *Montastraea franksi*. *Coral Reefs* **20**: 85–91.

Rohwer F, Kelly S. (2004). *Coral Health and Disease*. Springer Press: New York/Berlin.

Rohwer F, Seguritan V, Azam F, Knowlton N. (2002). Diversity and distribution of coral-associated bacteria. *Mar Ecol Prog Ser* **243**: 1–10.

Rosenberg E, Koren O, Reshef L, Efrony R, Zilber-Rosenberg I. (2007). The role of microorganisms in coral health, disease and evolution. *Nature Reviews Microbiology* **5**: 355–362.

Rosenberg E, Kushmaro A, Kramarsky-Winter E, Banin E, Yossi L. (2009). The role of microorganisms in coral bleaching. *ISME J* **3**: 139–146.

Rypien KL, Ward JR, Azam F. (2009). Antagonistic interactions among coral-associated bacteria. *Environ Microbiol* **12**: 28–39.

Schloss PD, Handelsman J. (2005). Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. *Appl Environ Microbiol* **71**: 1501–1506.

Schnell S, Assmus S, Richardson LLR. (1996). Role of sulfate reducing bacteria in the black band disease of corals. *Abstr Annu Meet VAAM (Ver Allg Angew Mikrobiol) GBCH (Ges Biol Chem) Elsevier, Cologne, Germany*, **116**.

Sebens K. (1994). Biodiversity of coral reefs: what are we losing and why? *Amer Zool* **34**: 115–133.

Sekar R, Kaczmarsky L, Richardson LL. (2008). Microbial community composition of black band disease on the coral host *Siderastrea siderea* from three regions of the wider Caribbean. *Mar Ecol Prog Ser* **362**: 38–98.

Sekar R, Mills DK, Remily ER, Voss JD, Richardson LL. (2006). Microbial communities in the surface mucopolysaccharide layer and the black band microbial mat of black band-diseased *Siderastrea siderea*. *Appl Environ Microbiol* **72**: 5963–5973.

Shnit-Orland M, Kushmaro A. (2009). Coral mucus-associated bacteria: a possible first line of defense. *FEMS Microbiol Ecol* **67**: 371–380.

Siboni N, Ben-Dov E, Sivan A, Kushmaro A. (2008). Global distribution and diversity of coral-associated Archaea and their possible role in the coral holobiont nitrogen cycle. *Environ Microbiol* **10**: 2979–2990.

Sunagawa S, DeSantis TZ, Piceno YM, Brodie EL, DeSalvo MK, Voolstra CR *et al*. (2009). Bacterial diversity and White Plague Disease-associated community changes in the Caribbean coral *Montastraea faveolata*. *ISME J* **3**: 512–521.

Vega-Thurber R, Willner-Hall D, Rodriguez-Mueller B, Desnues C, Edwards RA, Angly F *et al*. (2009). Metagenomic analysis of stressed coral holobionts. *Environ Microbiol* **11**: 2148–2163.

Wegley L, Edwards R, Rodriguez-Brito B, Liu H, Rohwer F. (2007). Metagenomic analysis of the microbial community associated with the coral *Porites astreoides*. *Environ Microbiol* **9**: 2707–2719.

Supplementary Information accompanies the paper on The ISME Journal website (http://www.nature.com/ismej)