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Bacteria appear to play important roles in both causing and preventing the bleaching of the coral *Oculina patagonica*

Elad Mills[1], Karina Shechtman[1], Yossi Loya[2], Eugene Rosenberg[3,*]

[1]Porter School of Environmental Studies, Tel Aviv University, Tel Aviv 69978, Israel
[2]Department of Zoology, Tel Aviv University, Tel Aviv 69978, Israel
[3]Department of Molecular Microbiology and Biotechnology, Tel Aviv University, Tel Aviv 69978, Israel

ABSTRACT: *Vibrio shiloi* was reported in 1996 to be the causative agent of bleaching of the coral *Oculina patagonica* in the eastern Mediterranean Sea, and the mechanisms of infection and bleaching were studied intensively from 1996 to 2002. Sometime between 2002 and 2004, *O. patagonica* became resistant to *V. shiloi* yet continued to bleach seasonally. These findings raised 2 questions: (1) What is the current cause of the bleaching? (2) How did the coral become resistant to the pathogen *V. shiloi*? Data presented here indicate that a bacterium, or bacteria, other than *V. shiloi* is currently responsible for the bleaching, because the antibiotic nalidixic acid inhibited heat-induced bleaching in aquaria. Raising the temperature of *O. patagonica* in aquaria to 31°C caused 24 of 28 coral fragments to bleach. When the *O. patagonica* was exposed to the same temperature increase but treated with nalidixic acid for 24 h when the temperature reached 28°C, only 10 of 34 fragments bleached. Antibiotic-treated corals had 5.3-fold more zooxanthellae at the end of the heating experiment than the no-antibiotic control, supporting the visual bleaching observations. *V. coralliilyticus* strain EM1 is a candidate bleaching pathogen because it was isolated from bleached corals, caused bleaching in 5 of 6 antibiotic-treated corals and could be recovered from the experimentally bleached corals. In support of the coral probiotic hypothesis, antibiotic-treated corals became sensitive to *V. shiloi* infection and bleaching (10 of 12 fragments), presumably by killing beneficial bacteria that inhibited *V. shiloi* growth. Bacterial strain EM3, isolated from *O. patagonica* fragments, may play a role in preventing *V. shiloi* infection of corals because it releases a diffusible material that specifically inhibited the growth of *V. shiloi in vitro*.

KEY WORDS: Probiotic hypothesis · Coral bleaching · *Oculina patagonica* · *Vibrio shiloi* · Temperature-induced bleaching · Holobiont

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INTRODUCTION

The zooxanthellaete scleractinian coral *Oculina patagonica* was first observed in the Mediterranean Sea in 1966 (Zibrowius 1974). Bleaching of *O. patagonica*, first recorded along the Israeli Mediterranean shoreline in the summer of 1993 (Fine et al. 2001), has been monitored periodically since that time (Israely et al. 2001, Shenkar et al. 2005). Bleaching begins to increase late in the spring and rises in the summer, reaching approximately 80% in September, and the corals then slowly recover in the autumn and winter. By early spring, most of the corals have completely recovered.

Vibrio shiloi was reported to be the causative agent of the temperature-induced bleaching disease (loss of the endosymbiotic zooxanthellae) of the coral *Oculina patagonica* (Kushmaro et al. 1996, 1997). The *V. shiloi*/*O. patagonica* model system of coral bleaching was studied intensively from 1996 to 2002

*Corresponding author. Email: eros@post.tau.ac.il

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(reviewed by Rosenberg & Falkowitz 2004). The bacteria are chemotactic to the coral mucus (Banin et al. 2001a), adhere to a β-D-galactopyranoside-containing receptor on the coral surface (Toren et al. 1998), penetrate the epidermal layer and multiply intracellularly, reaching 10^9 cells cm^{-3} (Banin et al. 2000). The intracellular *V. shiloi* produces an extracellular peptide toxin (PYPVYPPPVVP) that inhibits algal photosynthesis and causes bleaching (Ben-Haim et al. 1999, Banin et al. 2001b).

Sometime between 2002 and 2004, *Oculina patagonica* became resistant to the pathogen yet continued to bleach seasonally. Since 2004, researchers have been unable to isolate *Vibrio shiloi* from harvested bleached corals (Koren & Rosenberg 2006, Ainsworth et al. 2008), and *V. shiloi* stock strains that previously successfully bleached *O. patagonica* in the laboratory no longer bleach the coral (Reshef et al. 2006). Interestingly, *V. shiloi* still adheres to the coral and penetrates the tissue, but it is then rapidly killed (Reshef et al. 2006).

The development of coral resistance to *Vibrio shiloi* was surprising, because corals do not produce antibodies and are considered to lack an adaptive immune system (Nair et al. 2005). The coral probiotic hypothesis (Reshef et al. 2006) was thus presented to explain the data. This hypothesis posits that the coral acquired beneficial bacteria that inhibit infection and prevent bleaching. The hypothesis stems from the concept of the coral holobiont, where the coral functions as the sum of the coral host and all of its symbiotic microorganisms (Rohwer et al. 2002). During changing environmental conditions, the dynamic properties of the microbiota lead to a natural selection for the most advantageous coral holobiont.

The data presented in this article provide support for the coral probiotic hypothesis, showing that treatment of corals in aquaria with antibiotics both inhibits temperature-induced bleaching and returns the corals to their sensitivity to bleaching by *Vibrio shiloi*.

MATERIALS AND METHODS

Bleaching survey

The bleaching survey was performed monthly from April 2011 to April 2012 at Sdot Yam (Mediterranean coast of Israel, 32° 29.77′ N, 34° 53.23′ E) using SCUBA equipment at depths ranging from 0.5 to 4 m. A 10 m long, 1 m wide belt transect was used to estimate the number of apparently healthy and bleached corals. Coral bleaching was scored as positive only if it was observed to be above 10% of the healthy (pigmented) coral tissue. The main platform is 100 m from the shoreline and contains abundant coral colonies because of its complex structure with numerous notches and crevices (Fig. 1). The site has been well studied and documented over the years with regard to *Oculina patagonica* temperature-dependent bleaching (e.g. Shenkar et al. 2005). Monthly seawater temperature means were obtained from the Israel Meteorological Service (www.israelweather.co.il/).

Aquaria experiments

Collection and maintenance of the corals

Apparently healthy, intact colonies of *Oculina patagonica* (10 to 50 cm^2) were collected from the Sdot Yam site between March 2011 and May 2012 at depths of 1 to 3 m under permit no. 2012/38476. Corals were harvested using a hammer and chisel and immediately brought back to the laboratory (within 2 h), where they underwent fragmentation, removing all rock attachments. They were placed in aerated aquaria containing sea water from the study site adjusted to 22°C and a salinity of 40 ppt. Aquaria were exposed to a diurnal (12 h light:12 h dark) light cycle. Water in the aquaria was gradually raised to 25°C and shifted to 0.45 µm filtered artificial sea water (ASW; Reef Salt, Aqua Medic). Water was filtered using a sterile cellulose nitrate membrane filter in a 2 l vacuum-filtering apparatus. Coral fragments were kept under these conditions for 1.5 to 2 wk

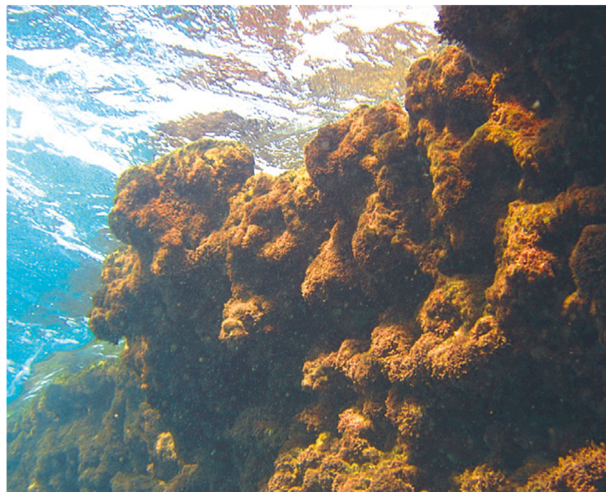

Fig. 1. Underwater view of the survey site. Many notches and crevices create a suitable habitat for the settlement and growth of *Oculina patagonica*

before the start of each experiment. If any piece failed to heal (complete cover of damaged skeleton by new tissue), it was discarded and not used in any experiment.

Heat-induced bleaching experiments

Healthy fragments were placed into sterilized aquaria filled with 1.5 l of 0.45 µm filtered ASW. Each aquarium in the experimental system harbored 2 to 5 fragments; 8 aquaria were used for the no-antibiotic control, and 11 aquaria were used for antibiotic-treated experiments Temperature was raised daily by 1°C until it reached 31°C and then was left for 2 to 3 wk. Temperature monitoring and manipulation was regulated by an Aqua Medic AT Control computer programmed to ±0.2°C. Salinity was checked daily using a refractometer, and any increase in salinity due to evaporation was compensated with 0.45 µm filtered deionized water. Along the time course of the experiment, corals were visually inspected for bleaching and photographed. Similar to field surveys, only corals showing above 10% bleaching were considered bleached.

Zooxanthellae counts were determined at the end of the bleaching experiments. Coral fragments were disrupted by a water jet stream using a Waterpik (Johannes & Wiebe 1970) with 50 to 200 ml of ASW. The skeletons were photographed and surface area calculated using ImageJ software. The tissue suspension was centrifuged at 2000 × *g* for 30 min at 20°C and the pellet suspended in 1 ml of ASW. After transferring the suspension to a 1.5 ml Eppendorf tube, it was again centrifuged (4 min at 9300 × *g*). The resulting pellet was resuspended in 1 ml of ASW and centrifuged for 20 min at 1000 × *g*. The pellet was then resuspended in ASW to a final concentration of ca. 10^6 zooxanthellae ml^{-1} and counted using an improved Neubauer hemocytometer under a light microscope at 400× magnification. Three fields, each 1 mm^2, were counted, and the number of zooxanthellae cells per milliliter was determined and subsequently converted to cells per square centimeter of coral surface area.

For the antibiotic-treated coral experiments, a 10 mg ml^{-1} stock solution of nalidixic acid (Sigma-Aldrich) was prepared by dissolving 800 mg in 1 ml 0.1 N NaOH and then adding 79 ml of distilled water. The antibiotic solution was then filtered using a 0.22 µm sterile syringe filter (Millex, Millipore) into sterile Falcon tubes and kept at 4°C. When the temperature in the aquarium reached 28°C, antibiotic was added to a final concentration of 50 µg ml^{-1}. After 24 h, the antibiotic was removed by thoroughly rinsing the corals from the antibiotic and placing them in fresh aquaria containing filtered ASW. The temperature was then allowed to gradually increase (1°C d^{-1}) until it reached 31°C.

Infections with *Vibrio shiloi* and strain EM1 were performed after the antibiotic had been removed and the corals had reached 29°C. Overnight cultures of *V. shiloi* AK1 (Banin et al. 2001b) and the freshly isolated strain EM1, each grown at 30°C in MBT medium (1.8% Marine Broth plus 0.9% NaCl, supplemented with 0.45% Tryptone), were centrifuged (10 min at 6800 × *g*), and the pellets were suspended in sterile ASW to a concentration of 10^9 cells ml^{-1}; 1.5 ml of each suspension was then inoculated into separate aquaria containing the antibiotic-treated coral fragments to give a final concentration of 10^6 bacteria ml^{-1}. The temperature was then allowed to gradually increase (1°C d^{-1}) until it reached 31°C.

Isolation and characterization of strains EM1 and EM3

Strains EM1 and EM3 were isolated from crushed fragments of *Oculina patagonica* by plating diluted samples on marine agar (1.8% marine broth plus 0.9% NaCl solidified with 1.8% Bacto Agar) and incubating the plates at 30°C. Isolated colonies were then streaked on thiosulfate-citrate-bile-sucrose (TCBS) Agar (Difco) to obtain pure cultures.

DNA was extracted from each strain using the UltraClean Soil DNA Kit (MO BIO Laboratories) and read in a NanoDrop 1000 spectrophotometer to estimate concentrations. 16S rRNA genes were amplified by PCR using primers 8F and 1492R (Lane 1991) in a 25 µl reaction mixture consisting of 2.5 µl 10× buffer, 1 µl of a 2.5 mM total deoxyribonucleoside triphosphate mixture, 5 µM of each primer, 100 ng template DNA and 2.5 units Ex Taq DNA polymerase (Takara Bio). Amplification conditions for the PCR included an initial denaturation step at 94°C for 3 min, followed by 30 cycles of 94°C for 35 s, 55°C for 35 s and 72°C for 45 s and a final extension step at 72°C for 3 min. Reaction products were checked for size and purity on 1% agarose gel and then cleaned using ExoSAP-IT (USB Corporation). DNA sequencing was performed using the chain-termination method in an ABI PRISM (Model 377, version 2.1.1) automated sequencer. Primers used for the sequencing reaction were complementary to the conserved regions of the 16S rRNA genes. Sequences were first

edited using ApE: A Plasmid Editor software, version 2.0.44, and then aligned using ClustalW (BioEdit, version 7.0.9.0), generating a sequence identity matrix to search for identities between sequences. BLASTn (Altschul et al. 1990; www.ncbi.nlm.nih.gov/BLAST/BLAST.cgi) was then used to identify each sequence, and results were supported by the EzTaxon-e website (Chun et al. 2007; http://eztaxon-e.ezbiocloud.net).

Inhibition of *Vibrio shiloi* growth by strain EM3

Cross-streaking experiments were performed by placing various bacteria, previously isolated from healthy *Oculina patagonica*, in a line at the center of Marine Agar plates using a sterile Quadloop. After incubating the plates for 24 h at 30°C, *Vibrio shiloi*, *V. coralliilyticus* and several other bacteria were streaked perpendicular to the line of growth without touching it and incubated for another 48 h. At the end of the second incubation period, plates were inspected and marked for zones of inhibition, indicating the diffusion of an antibacterial compound produced by the initially inoculated bacterium.

Inhibition of *Vibrio shiloi* growth by diffusible compounds produced by strain EM3 was further tested in liquid culture. The cell-free extracellular fluid from the growth medium of strain EM3 on marine broth for 24 h at 30°C was obtained by removing the cells by centrifugation and then filtering the supernatant through a 0.45 µm filter (EM3-sup). An overnight culture of *V. shiloi* (0.1 ml) was inoculated into 125 ml flasks containing 20 ml of marine broth (Culture 1) and 20 ml of EM3-sup (Culture 2). The flasks were incubated with shaking at 30°C. At 0, 2, 4 and 8 h, samples were removed for determination of turbidity (A_{600nm}).

RESULTS

Bleaching of *Oculina patagonica* colonies in the Mediterranean Sea

The monthly data collected at the Sdot Yam site from May 2011 to May 2012 showed a clear seasonal bleaching profile, with a strong correlation between the frequency of bleached colonies and seawater temperature change (Fig. 2, r^2 = 0.80), as previously reported at this site from 1995 to 2000 (Fine et al. 2001, Israely et al. 2001). At the peak of the bleaching period, when summer temperatures reached approximately 30°C, 79.5% of the coral colonies showed bleaching. The recovery period started in November and continued until May, when the percentage of bleached colonies decreased to a low of 15%. Bleaching was typically patchy, i.e. bleached colonies were often found next to or surrounded by apparently healthy colonies. As previously reported

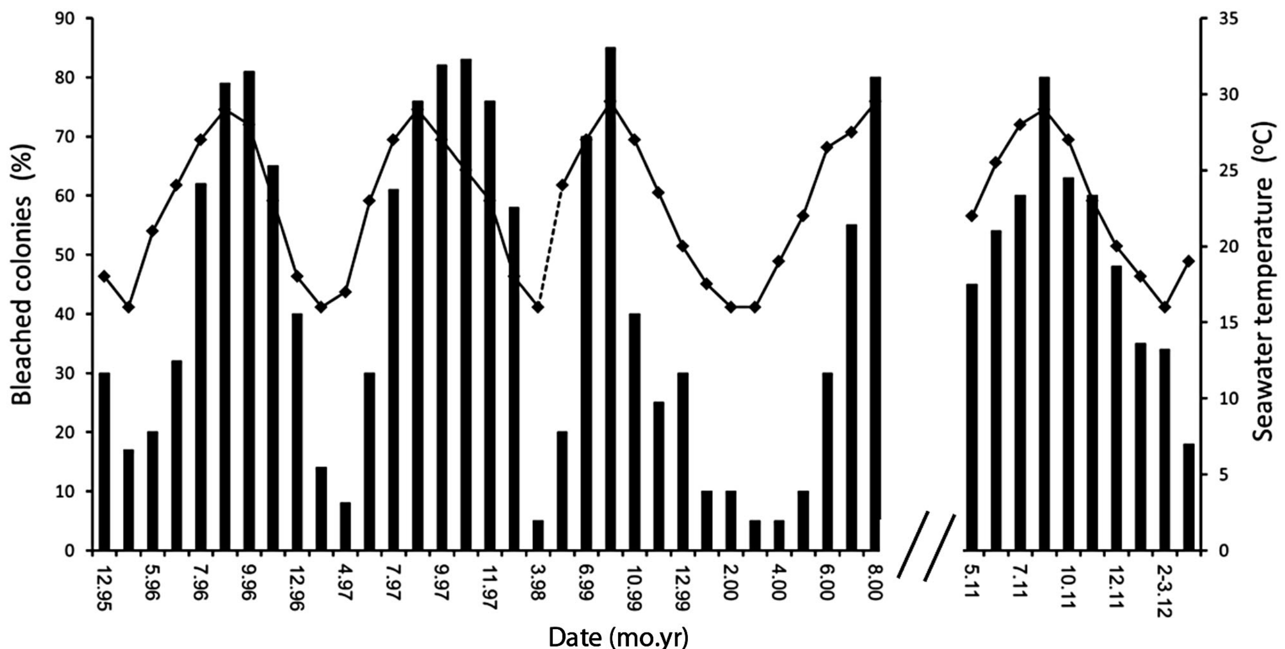

Fig. 2. *Oculina patagonica*. Seasonal bleaching (bars) at the Sdot Yam field site from May 2011 to May 2012 compared to published data from 1995 to 2000 (Fine et al. 2001, Israely et al. 2001), and average monthly seawater temperatures (diamonds)

(Shenkar et al. 2005), bleached corals were on average (±SE) larger than unbleached corals: 52 ± 4 cm^2 compared to 39 ± 3 cm^2, respectively.

Temperature-induced bleaching of *Oculina patagonica* in aquaria

Slowly raising the temperature of *Oculina patagonica* in aquaria to 31°C caused 86% of the coral fragments to bleach (Table 1). This value is similar to that obtained in the field at the peak of bleaching (Fig. 1). When the *O. patagonica* were exposed to the same temperature increase but treated with nalidixic acid for 24 h when they reached 28°C, only 29% of the fragments bleached (Table 1). Many of the antibiotic-treated fragments did not bleach for several weeks at 31°C. Even amongst the 29% that showed bleaching after antibiotic treatment, it was less extensive than when the temperature was raised without addition of nalidixic acid. The visibly observed inhibition of bleaching by the antibiotic treatment was confirmed by zooxanthellae counts, where the antibiotic-treated corals had 5.3-fold more algae at the end of the heating experiments than the untreated control. Nalidixic acid was chosen as the antibiotic of choice after preliminary experiments indicated that it was efficient at killing coral-associated bacteria and had no apparent deleterious effect on the corals.

Nalidixic acid reduced the *Vibrio* count 670-fold in aquaria water (1.2×10^5 to 1.8×10^2 CFU ml^{-1}). When the antibiotic-treated corals were infected with *V. shiloi*, the percentage of bleached fragments increased to 83% and the zooxanthellae counts decreased by 53 ± 6% (Table 1) compared to those fragments treated only with antibiotic, suggesting that the beneficial bacteria that had caused the corals to become resistant to *V. shiloi* infection and bleaching were killed by nalidixic acid.

Table 1. *Oculina patagonica*. Temperature-induced bleaching of fragments in aquaria. Temperature was raised 1°C d^{-1} until it reached 31°C and then was maintained at this temperature for 2 wk. Antibiotic (nalidixic acid, 50 mg l^{-1}) was added for 24 h when the aquarium reached 28°C. *Vibrio shiloi* and strain EM1 were added to a final concentration of 10^6 ml^{-1} after the antibiotic was removed. Algae (zooxanthellae) counts are means ± SE at the end of the experiment. n = sample size (in parentheses). See 'Materials and methods' section for details

Treatment	% Bleached corals (n)	Algae (10^6 cells cm^{-2})
31°C only	86 (28)	0.37 ± 0.17
31°C + antibiotic	29 (34)	1.95 ± 0.25
31°C + antibiotic + *V. shiloi*	83 (12)	0.93 ± 0.10
31°C + antibiotic + EM1	83 (6)	0.51 ± 0.05

Isolation and characterization of strains EM1 and EM3

To test the hypotheses that corals now contained a bleaching bacterium other than *Vibrio shiloi* and acquired one or more strains of bacteria which inhibit the growth of *V. shiloi*, we isolated several bacterial strains from crushed *Oculina patagonica* and checked their ability to bleach and/or their anti-*V. shiloi* activity. Out of 5 isolates tested, only 1 strain, referred to as EM1, bleached antibiotic-treated corals and reduced the zooxanthellae count by 74% (Table 1). Strain EM1 could be recovered from the laboratory-bleached corals at the end of the experiment by plating on TCBS Agar. Based on its 16S rRNA gene sequence (652 bp length), strain EM1 is a strain of *V. coralliilyticus*, showing 99.8% identity to *V. coralliilyticus* YB1 (GenBank accession no. NR028014.1).

One strain, referred to as EM3, consistently inhibited the growth of *Vibrio shiloi*. In cross-streaking experiments between strain EM3 and *V. shiloi*, there was a clear zone of inhibition around the growth of strain EM3, suggesting that strain EM3 releases a diffusible inhibitor of *V. shiloi* growth. *V. shiloi* is not particularly sensitive to bacterial-produced antibiotics (Nissimov et al. 2009). The inhibition by EM3 appeared to be specific, since several other bacteria, including strain EM1 and the coral pathogen *V. coralliilyticus* YB1 (Ben-Haim et al. 2003), were not inhibited by strain EM3. The inhibition of *V. shiloi* growth by an extracellular compound(s) produced by strain EM3 was further demonstrated in liquid culture (Table 2). *V. shiloi* grew in marine broth at 30°C,

Table 2. *Vibrio shiloi*. Inhibition of growth by the cell-free supernatant fluid of strain EM3. An overnight culture was inoculated (0.1 ml) into 20 ml of marine broth (Culture 1) and 20 ml of the cell-free supernatant fluid of strain EM3 (Culture 2). The flasks were incubated with shaking at 30°C. At 0, 2, 4 and 8 h, samples were removed for determination of turbidity (A_{600nm})

Time (h)	Culture 1	Culture 2
0	0.07	0.07
2	0.30	0.11
4	1.20	0.19
8	4.64	0.28

with a doubling time of ca. 1 h, and reached an A_{600} of 4.6 after 8 h, whereas under the same growth conditions but in a medium containing the cell-free supernatant fluid of strain EM3, *V. shiloi* hardly grew, reaching an A_{600} of only 0.28 after 8 h. Based on the 16S rRNA gene sequence (700 bp length), strain EM3 is a new species and possibly a new genus, since it had only 94.3% identity with the closest known bacterium, *V. hepatarius* (GenBank accession no. AJ345063). By definition, bacteria in the same genus have at least 95% 16S rRNA gene sequence identity (Bosshard et al. 2003).

DISCUSSION

Even though the coral *Oculina patagonica* has developed resistance to infection by the coral pathogen *Vibrio shiloi*, it continues to demonstrate seasonal bleaching. These findings raised 2 important questions: (1) What is the current mechanism of bleaching of *O. patagonica* every summer in the eastern Mediterranean Sea? (2) How did the coral become resistant to *V. shiloi* infection and bleaching? Results from this study provide evidence to explain the role of bacteria in the bleaching process of *O. patagonica.*

Heat-induced bleaching of corals in the sea has been reported frequently and linked to global warming (e.g. Brown 1997, Berkelmans 2002, Rosenberg & Ben-Haim 2002, Hoegh-Guldberg 2004, Jokiel & Brown 2004, Carilli et al. 2012). However, a fundamental question remains: What is the etiology of heat-induced coral bleaching? For this question, there are at least 2 different viewpoints. Many coral biologists take the position that high temperature and light act directly on the symbiotic algae to inhibit photosynthesis and produce reactive oxygen species, leading to the bleaching disease (Jones et al. 1998). According to this hypothesis, microorganisms play no role in the bleaching process, and changes in the microbial community of bleached corals are a result, not a cause, of the process. The second viewpoint, held by certain coral microbiologists, is that high temperature acts on the coral microorganisms as well as on the coral host, causing a change in the microbial community and expression of virulence genes that in some cases contribute directly or indirectly to bleaching, that is, the microbial hypothesis of coral bleaching (Rosenberg et al. 2009). It has been pointed out that the 2 hypotheses are not mutually exclusive (Takahashi et al. 2004). It is possible that both mechanisms act synergistically. It is also possible that other mechanisms are involved, such as temperature-induced virulence of certain viruses. Clearly, further multidisciplinary research, including a combination of coral microbiology together with coral host physiology, is required to clarify the coral bleaching disease process.

One of the arguments that have been raised against the microbial hypothesis of coral bleaching is that several coral species will bleach in aquaria when the temperature is raised to 32°C (Warner et al. 1999), even when bacteria are not added. However, corals contain hundreds of different indigenous bacterial species, and when the temperature is raised, some of them may produce materials that cause or contribute to the bleaching disease. In the present study, antibiotics were shown to inhibit temperature-induced bleaching of *Oculina patagonica* fragments, providing support for the microbial hypothesis of coral bleaching. The bleaching of *O. patagonica* was previously shown to begin at the perimeter of the colony, and as water temperatures increased, bleaching progressed toward the colony center (Shenkar et al. 2005). The spreading nature of the bleaching is highly symptomatic of an infectious disease (Bailey 1975, Rosenberg 2004). It will be interesting to determine whether antibiotics inhibit the bleaching of other corals too.

Although inhibition of heat-induced bleaching demonstrates that bacteria are involved in the bleaching process, it does not indicate which specific bacterium is involved. One candidate for a bleaching pathogen of *Oculina patagonica* is *Vibrio coralliilyticus* strain EM1, because it is present on bleached corals, causes bleaching of antibiotic-treated coral fragments and can be recovered from the experimentally bleached corals. These data are suggestive but insufficient to demonstrate that *V. coralliilyticus* is the causative agent of the seasonal bleaching disease of *O. patagonica*. It would be interesting to examine if strain EM1 produces similar virulence factors as has been described for *V. shiloi* (Rosenberg & Falkowitz 2004).

Interestingly, in the 2 cases for which the causative agent for coral bleaching has been demonstrated by applying Koch's postulates, the pathogens were *Vibrio* species (Kushmaro et al. 1997, Ben-Haim et al. 2003). Further, Bourne et al. (2008) showed that coral-associated *Vibrio* species increased just prior to a mass bleaching event on the Great Barrier Reef.

The fact that the antibiotic treatment made the corals sensitive to *Vibrio shiloi* infection and bleaching supports the coral probiotic hypothesis. Presumably, the antibiotic treatment killed one or more of the beneficial bacteria which protected the coral

against *V. shiloi* infection. Protection against pathogens is one of the most general and important contributions of resident microbiota to the health of holobionts. Several studies demonstrated that germ-free animals, born and grown under sterile conditions, are considerably more sensitive to infection and death following oral administration of a pathogen than animals grown under natural conditions (Butterton et al. 1996, Round & Mazmanian 2009). In humans, the normal microbiota has been shown to protect against infection by pathogens in the oral cavity (Burton et al. 2011), the intestine (Stecher & Hardt 2008), the skin (Naik et al. 2012) and the vaginal epithelium (Barbes & Boris 1999). The fact that there is an increased frequency of infection by the yeast pathogen *Candida albicans* (Huppert et al. 1955) and by the diarrhea- and colitis-causing bacterium *Clostridium difficile* (Bartlett 2006) following antibiotic therapy is consistent with this concept.

Production of antibacterials by coral-associated bacteria has been reported previously (Koh 1997, Ritchie 2006, Nissimov et al. 2009, Shnit-Orland & Kushmaro 2009). Bacterial strain EM3, isolated in this study from *Oculina patagonica*, produced diffusible material which specifically inhibited the coral pathogen *Vibrio shiloi*. The chemical structure and mode of action of this material is not yet known.

The data presented here indicate that bacteria are responsible for bleaching *Oculina patagonica* at elevated temperatures and that other bacteria can inhibit bleaching. We have not excluded the possibility that *O. patagonica* may bleach via other means and that a primary pathogen may not always be the cause. Whether or not bacteria play a role in the bleaching of other corals remains to be determined.

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