RESEARCH ARTICLE

Vibrio sp. as a potentially important member of the Black Band Disease (BBD) consortium in *Favia* sp. corals

Luba Arotsker[1], Nachshon Siboni[1], Eitan Ben-Dov[1,2], Esti Kramarsky-Winter[3], Yossi Loya[3] & Ariel Kushmaro[1,4]

[1]Department of Biotechnology Engineering, Ben-Gurion University of the Negev, Be'er-Sheva, Israel; [2]Achva Academic College, M.P. Shikmim, Israel; [3]Department of Zoology and the Porter Super Center for Ecological and Environmental Studies, Tel Aviv University, Ramat Aviv, Israel; and [4]National Institute for Biotechnology in the Negev, Ben-Gurion University of the Negev, Be'er-Sheva, Israel

Correspondence: Ariel Kushmaro, Department of Biotechnology Engineering, Ben-Gurion University of the Negev, PO Box 653, Be'er-Sheva 84105, Israel. Tel.: +972 8 647 9024; fax: +972 8 647 2983; e-mail: arielkus@bgu.ac.il

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Abstract

Black Band Disease (BBD) is a well-described disease plaguing corals worldwide. It has been established that ecological and environmental stress factors contribute to the appearance and progression of the disease, believed to be caused by a diverse microbial consortium. We have identified and characterized *Vibrio* sp. associated with BBD in Eilat reef corals using both culture-dependent and -independent methods. Direct sampling using 16S rRNA gene clone libraries showed seasonal dynamics in the diversity of BBD-associated *Vibrios*. In the two sampling periods, BBD-associated *Vibrio* clones showed similarities to different groups: October samples were similar to known pathogens, while December samples were similar to general aquatic *Vibrio* sp. Cultured bacterial isolates of *Vibrio* sp. were highly homologous ($\geq$99%) to previously documented BBD-associated bacteria from the Caribbean, Bahamas and Red Seas, and were similar to several known coral pathogens, such as *Vibrio coralliilyticus*. The proteolytic activity of *Vibrio* sp., as measured using casein- and azocasein-based assays, directly correlated with temperature elevation and peaked at 26–28 °C, with the microorganisms producing more proteases per bacterial cell or increasing the rate of proteolytic activity of the same proteases (potentially metalloproteases). This activity may promote coral tissue necrosis and aid in ensuing progression of the coral BBD.

Introduction

Black Band Disease (BBD) is a widespread and well-described disease plaguing corals worldwide (Antonius, 1985; Bruckner & Bruckner, 1997; Dinsdale, 2002). Several ecological and environmental factors can contribute to the appearance and progression of BBD (Kuta & Richardson, 2002). When active, BBD affects $<$ 1% of the total coral reef (Kuta & Richardson, 1996; Dinsdale, 2002); nevertheless, the disease is considered to be a major factor for coral reef decline, due to the lack of recovery and regeneration of the affected corals (Kuta & Richardson, 1997; Edmunds, 2000). Indeed, Bruckner & Bruckner (1997) reported that once BBD penetrates a reef, it will persist and spread throughout the population, slowly causing the demise of susceptible coral.

Even though BBD has been documented in coral reefs for over 30 years, there is still very little unequivocal information regarding its etiology, and many aspects of this disease still need clarification. BBD symptoms include a dark-red or black band mat that moves across a coral colony, destroying healthy tissues and exposing the calcium carbonate skeleton. Environmental parameters, such as water depth and clarity, as well as temperature, are known to be correlated with disease prevalence and dispersal (Rützler *et al.*, 1983; Antonius, 1985; Kuta & Richardson, 2002). BBD is most active during the summer when water temperatures are elevated, reaching temperatures above 28 °C, and yet disappears almost completely during winter months, when the water temperature declines below 20 °C (Rützler *et al.*, 1983; Kuta & Richardson, 1996).

The BBD mat is made up of a diverse community that includes cyanobacteria, sulfur reducers and oxidizers, vibrios and numerous other microbial groups (Garrett & Ducklow, 1975; Rützler & Santavy, 1983; Bythell *et al.*, 2002;

Cooney *et al.*, 2002; Frias-Lopez *et al.*, 2002, 2004; Viehman & Richardson, 2002; Richardson, 2004; Sekar *et al.*, 2006; Sussman *et al.*, 2006; Barneah *et al.*, 2007). This community is distinct from that of healthy corals or of healthy tissues from diseased corals (Pantos *et al.*, 2003; Barneah *et al.*, 2007). The morphology and chemical characteristic of the black band were found to be similar to typical microbial mats (Carlton & Richardson, 1995; Richardson, 1997). The fact that BBD communities all contained populations of the same (physiologically) functional groups of microorganisms led Carlton & Richardson (1995) to propose that BBD is caused by a pathogenic microbial consortium and that there is no primary pathogen.

BBD in the Red Sea corals exhibits an aggregated distribution, which provides evidence for local transmission of the disease, which does not necessarily require direct contact (Zvuloni *et al.*, 2009). These authors also suggested that the mechanism of BBD transmission is likely to be a waterborne infection. In addition, although the BBD phenotype disappears during the winter, the infected corals contribute to the reintroduction of the reef in the following summer. In Red Sea corals affected by BBD, the mat is made up mainly of cyanobacteria, sulfate-reducing bacteria (SRB), and several strains of *Vibrios* (Barneah *et al.*, 2007). Because several well-characterized coral diseases are known to be caused by pathogenic strains of *Vibrio* (Kushmaro *et al.*, 1996, 1997; Ben-Haim & Rosenberg, 2002; Ben-Haim *et al.*, 2003a, b; Cervino *et al.*, 2004), it is likely that pathogenic strains of these bacteria may also play a role in BBD. This study, therefore, focused on the characterization of the *Vibrio* sp. in the BBD consortium and evaluation of their possible role in the disease, using microbiological and molecular tools.

Materials and methods

Sample collection

Samples from BBD-affected corals were collected from the Eilat coral reef [Inter-University Institute (IUI) for Marine Science, Northern Red Sea, 29°51′N, 34°94′E] during October and December 2007. The seven sampled *Favia* sp. corals were numbered and marked to enable repeated samplings. October samples were collected while the disease was very active and the black band was morphologically evident. The ensuing December samples were collected from the interface area (i.e. between the healthy tissue and the exposed skeleton) of the same marked corals, even when the band was not evident. Mucus and tissue samples were collected using 10-mL syringes and immediately transported to the laboratory. Water temperatures were measured *in situ* adjacent to the colonies in October, 26 °C, and in December, 24 °C (IUI monitoring).

Bacterial isolation and selection media

In the laboratory, the collected volume was spun down by centrifuge and the resuspended pellet was used for cultures and DNA extraction. For isolation, several different selective isolation media were tested: thiosulfate citrate bile sucrose agar (TCBS) with a pH indicator for *Vibrio* selection (Merck), Marine Broth and Marine Agar 2216 (HiMedia Laboratories, Mumbai, India) plates (10% of the recommended final concentration) were used for isolation of bacteria of marine origin (DSMZ GmbH) and *Desulfovibrio* medium (M63) (http://www.dsmz.de/index.htm) for isolation and enrichment of anaerobic SRB. Marine agar (MA) and M63 media were modified using sea salt water [NaCl 4% (w/v)] and used as liquid and for 1.5% agar or 0.8% gellan gum (Fluka, Sigma-Aldrich, St. Louis, MO) plates. M63 cultures were incubated in an anaerobic chamber (BD Gas Pak EZ). All media were incubated at room temperature (22–24 °C).

DNA extraction

Total genomic DNA was extracted from BBD environmental samples using a MoBio Power Soil DNA isolation kit (MoBio Laboratories Inc., Solana Beach, CA) or using the physical bead-shearing technique and precipitation. Silica beads (0.5 mm diameter) in 300 μL double distilled water were used for the shearing, achieved by a 10-min vortex step at maximum speed. The precipitation lasted 10 min at maximum centrifugation speed (13 200 ***g***).

Primer design and PCR amplification

Total DNA was amplified using a Biometra TGradient thermocycler (Biometra, Göttingen, Germany) by PCR, using specific modified 16S rRNA gene primers for bacteria: 8F (GGATCCAGACTTTGATYMTGGCTCAG, modified by shortening from the 5′-end; Falske *et al.*, 1997) and 907R (CCGTCAATTCCTTTRAGTTT; Lane *et al.*, 1985). The reaction mixture included 12.5 μL ReddyMix (ABgene, Surrey, UK), 1 μL of 10 mM of each primer (forward and reverse), 1 μL of 25 mM bovine serum albumin, 1–2 μL of the sample genomic DNA (environmental, 5–90 ng μL^{-1}; culture, 20–100 ng μL^{-1}) and water to bring the total volume to 25 μL. An initial denaturation hot start of 4 min at 95 °C was followed by 30 cycles of the following incubation pattern: 94 °C for 30 s, 54 °C for 30 s and 72 °C for 1 min. In the case of clone library building, a final extension at 72 °C for 30 min concluded the reaction.

Clone library construction and sequencing

The heterologous 16S rRNA gene PCR products were purified from the gel using the Wizard PCR Prep kit (Promega, Madison, WI), cloned into the pCRII-TOPO-TA

cloning vector as specified by Invitrogen (Carlsbad, CA) and transformed into BioSuper $CaCl_2$-competent HD5α *Escherichia coli* cells (Bio-Lab, Israel), according to the manufacturer's instructions. Clones were checked for inserts by PCR amplification using M13-Forward and Reverse primers (Invitrogen) and sent to McLab (San Francisco, CA) for sequencing.

Sequence analysis

The sequences were aligned using CLUSTALW found in the MOLECULAR EVOLUTIONARY GENETICS ANALYSIS package (MEGA, version 4.0; Tamura *et al.*, 2007). Positions not sequenced in all isolates or with alignment uncertainties were removed. Phylogenetic trees were constructed using the neighbor-joining method by the MEGA package. The BLAST database and Ribosomal Database Project II (RDPII) (Cole *et al.*, 2005) were used to classify all 16S rRNA gene sequences.

Metabolic tests

Culture strains were characterized biochemically using the API20 NE and API ZYM systems, according to the manufacturer's instructions (bioMerieux SA, Marcy l'Etoile, France). Oxidase activities of the strains were examined using an oxidase test kit (Fluka, Sigma-Aldrich), according to the manufacturers' instructions. Catalase activity was tested using 3% analytical H_2O_2.

Sensitivity to common commercial antibiotics was tested according to the disk diffusion assay (Awerbuch *et al.*, 1989), using Oxoid antimicrobial susceptibility test disks (Oxoid, Hampshire, UK) containing the following antibiotics: tetracycline, sulfamethoxazole trimethoprim, nalidixic acid, erythromycin, chloramphenicol, methicillin and ampicillin.

Proteinase assay

Casein test

Modified calcium caseinate agar (Sigma-Aldrich) was used for a protease test. Calcium caseinate agar (2 × with sea water) was autoclaved and added to 1% skim milk (2 ×), and heated at 70 °C for 1 h to prepare casein plates. Bacterial isolates were spread evenly and incubated overnight at different temperatures to observe lysed areas produced by extracellular proteinases (Chantawannakul *et al.*, 2002).

Azocasein test and EDTA inhibition

The main principle of the azocasein test is the separation of the azo-molecule from the casein protein during azocasein degradation by bacterial extracellular proteases. The azo-molecules released in this reaction have a unique absorption of ~420 nm. Chosen isolates were grown overnight in marine broth medium at different temperatures (i.e. 20, 22, 24, 26, 28 and 30 °C). The cultures (1.5 mL each) were centrifuged to precipitate the bacteria and 1 mL of each supernatant was added to 1% azocasein in 0.1 M Tris buffer (pH 8) for 1 h of incubation at different temperatures. The reaction was stopped using 2 mL of 5% trichloroacetic acid and left for 20 min at 4 °C. The solution was centrifuged and an identical volume of 1 M NaOH was added to the supernatant. Absorption of the azo-molecules was measured at 420 nm (Vazquez *et al.*, 1995). Relative protease activity units (RAU) were calculated using the following formula (Denkin & Nelson, 1999):

$$\mathrm{RAU} = \left(\frac{\mathrm{OD}_{420\,\mathrm{nm}} \times 10^3}{\mathrm{CFU}}\right) \times 10^9$$

Bacterial isolate supernatants were tested for inhibition of protease activities at different concentrations of EDTA (from 0.1 to 50 mM). Samples were incubated for 1 h at 30 °C and then tested for proteolytic activity by the azocasein assay described above. A control sample contained isolate supernatant with no EDTA.

Nucleotide sequence accession number

The 16S rRNA gene sequences from this study have been deposited in the NCBI GenBank database under accession numbers GQ215061–GQ215096 for the isolate sequences and GQ215097–GQ215227 for the clone library sequences.

Results

BBD-infected *Favia* sp. corals were found on the reef near the IUI shore of the Gulf of Eilat. Diseased *Favia* sp. corals were sampled in October and December 2007 from the black band. Subsequently, 148 sequences were generated from appropriate 16S rRNA gene libraries. Diversity assessment on the class level in black band samples collected in October and December revealed an abundance of *Cyanobacteria* (32% at both time points), 31% and 22% *Alphaproteobacteria*, respectively, followed by 11% and 8% *Gammaproteobacteria*, a class that includes the *Vibrio* sp., respectively (Fig. 1). Only four classes were common to both time periods, namely *Flavobacteria*, *Cyanobacteria*, *Alpha-* and *Gammaproteobacteria*. *Bacteroidetes* and *Clostridia* classes that appeared in October libraries disappeared in December, when several new classes appeared (e.g. *Sphingobacteria*, *Lentisphaerae* and *Deltaproteobacteria*). The proportion of unclassified bacteria had grown from 5% to 17% in the December samples.

Five ribotypes from October and one ribotype from December of the *Gammaproteobacteria* class were found to be highly similar (≥ 99%) to *Vibrio* species (Fig. 2). October

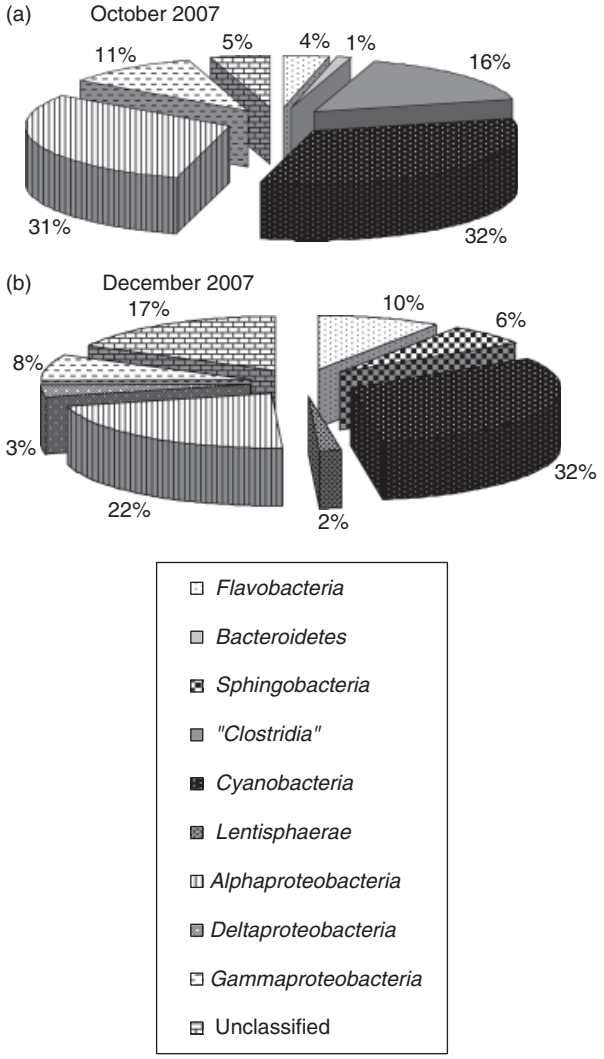

Fig. 1. Bacterial diversity (by class) obtained from clone libraries constructed with universal 16S rRNA gene primers from diseased corals of samples collected in (a) October ($n = 85$) and (b) December ($n = 64$) 2007. Classification was performed using RDPII and BLAST.

clones Oct07-4BB-60, Oct07-5BB-67, 70 and 72, and Oct07-6BB-81 were highly homologous to several well-known pathogenic *Vibrios* (e.g. EU372929 – white syndrome pathogen *Vibrio* sp. PM6A and AJ440004 – *Vibrio coralliilyticus*), and clones that were previously documented in BBD (EF123487 – BBD-associated *Gammaproteobacteria* clone). December clone Dec07-1BB-21 was highly homologous (≥ 99) to previously documented BBD clone BB2INT16SI-5 (EF089440; Barneah *et al.*, 2007) found in the interface between the healthy apparent tissue adjacent to the black band. Other December clones were highly similar (100%) to different aquatic *Vibrio* sp. (AM159567, EF587962, AJ630202, etc.; data not shown).

A total of 110 bacterial isolates from the October 2007 samples and 45 isolates from the December 2007 samples were obtained by culturable methods using TCBS, MA and *Desulfovibrio* media. All isolated bacteria were obtained from the seven marked coral colonies taken from diseased tissue. No *Vibrio* isolates were obtained using TCBS plates from the apparently healthy area of the coral. Alignment analysis of the 16S rRNA genes identified 88% and 93% of isolates as *Gammaproteobacteria* from the October and December samples, respectively. At the genus level, most *Gammaproteobacteria* belonged to the genus *Vibrio* (65% and 87%) and *Beneckea* (15% and 5%) in the October and December samples, respectively.

Phylogenetic tree analysis clustered the sequences from isolates retrieved from different time periods and different corals into five separate *Vibrio* subclusters (Fig. 3). All sequences showed high homology ($\geq 99\%$) to diverse *Vibrio* sp., most of which were previously associated with different coral diseases. The sequences of cluster 1 (isolated from marked corals 1, 3, 6 and 7 at different time periods) displayed high similarity to *V. coralliilyticus* (AJ440004), a well-known coral pathogen that causes bleaching and tissue lysis (Ben-Haim *et al.*, 2003a), and to a BBD clone (EF123487) from the Caribbean. The sequences of clusters 2 and 4 (Fig. 3) showed high resemblance to apparent *Vibrio* clones (EF089442 and EF089451) previously retrieved from BBD by Barneah *et al.* (2007) showing high homology to *Vibrio* sp. (AY876051) associated with the brown band syndrome from the Great Barrier Reef. Cluster 3 showed isolates highly similar to *Vibrio shiloi* (AF007115), a known pathogen of Mediterranean coral *Oculina patagonica* that causes coral bleaching (Kushmaro *et al.*, 1996, 1997). Cluster 5 included an isolate similar to a pathogen *Photobacterium eurosenbergii* (AJ842345) associated with coral bleaching (Thompson *et al.*, 2005). The last *Alphaproteobacteria* cluster includes isolates from different corals and time periods that were similar to previously studied BBD clones (i.e. DQ446157 and EF123485; Sekar *et al.*, 2006).

Out of the 110 isolates obtained in the October 2007 sampling, 20 isolates were chosen for further research. The choice was made according to isolate abundance and similarity to a known coral pathogen (metabolic and antibiotic resistance test results are summarized in Supporting Information, Tables S1 and S2).

Bacterial proteolytic activity is known to be a key virulent characteristic of pathogenic *Vibrio* sp. (Filkelstein & Hanne, 1982). In this study, we used the qualitative casein test and the semi-quantitative azocasein test to assess the proteolytic activities of our strains. Following incubation at 20 °C, no lysed area was observed on the casein agar plates around the bacterial colonies (Fig. 4), although several isolates showed slight activity beneath the colony itself. As the temperature increased, so did the activity area around all tested isolates (Fig. 4). Isolates MA-3BB-3 and M63-7BB-2 display relatively high activity levels, with the latter being the most

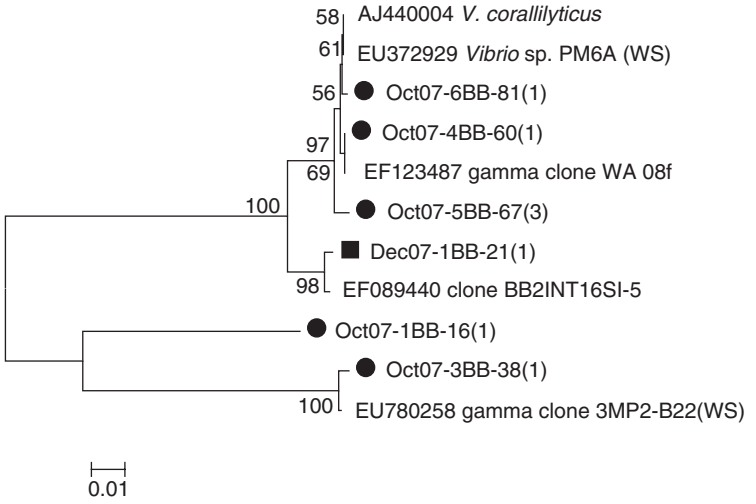

Fig. 2. Phylogenetic trees of the *Gamma proteobacteria* class representatives of the BBD clone library constructed using the 16S rRNA gene and environmental genomic DNA of samples obtained from the marked corals in October and December 2007. The numbers in parentheses indicate the total number of similar clones from the same coral colony on the basis of ≥99% identity for each representative sequence. ● – October 2007, coral BB area; ■ – December 2007, coral BB area; unmarked – clones with high homology to GenBank entries. WS, white syndrome.

active, taking over the entire Petri dish upon overnight incubation at 30 °C (Fig. 4).

The azocasein test allows us to quantify protease activity as a function of CFU. Seventy-five percent of isolates showed relatively moderate or high proteolytic activity. Several examples of this activity are exemplified in Fig. 5. Isolate M63-7BB-2 showed a relatively high activity at all temperatures and peaked around 26–28 °C. Isolate MA-3BB-3 was linearly dependent on temperature between 20 and 24 °C, showing equivalent maximal magnitudes at 25 °C (Fig. 5). Similar activity was observed with isolate MA-2BB-3, with the activity peaking at 28 °C. Isolate MA-5BB-3 was almost inactive at all the temperatures tested. Isolates TCBS-6BB-11, TCBS-6BB-12 and TCBS-7BB-3 showed moderate protease activity at all temperatures, peaking around 26 °C (data not shown). Isolate TCBS-6BB-7 had the most distinct profile of all, showing no activity at low temperatures, linear dependence with temperature between 20 and 24 °C, a clear peak of activity at 24 °C and a sharp decrease above this value (Fig. 5).

In addition, the most active isolate M63-7BB-2 was grown overnight at three different temperatures (22, 26 and 30 °C) and extracellular proteases secreted by the strain were tested as a function of temperature (measured between 20 and 30 °C at intervals of 2 °C). Extracellular proteases secreted at 22°C showed a high shift in the relative activity, in comparison with the proteases secreted during high-temperature growth (26 and 30 °C) (data not shown) that display low temperature dependence.

Extracellular proteolytic activity was inhibited using EDTA and bacterial isolate supernatants, as revealed by the azocasein assay. Eighty-seven percent of active (75% moderate and highly active) isolates were totally inhibited by 50 mM EDTA. Others showed partial, but significant inhibition. For example, isolate M63-7BB-2 showed 34% inhibition with 0.1 mM EDTA, 51% inhibition with 5 mM EDTA, up to 68% inhibition with 10 mM and 81% inhibition with 50 mM EDTA.

Protease activity can be characterized as a function of time and temperature. Three isolates were grown overnight at two different temperatures, namely 20 and 30 °C. The growth media, including extracellular proteases, were incubated with azocasein at the same temperatures. The reaction was interrupted every 10 min and the sample absorption was measured. The slope in the figure of the relative activity as a function of time represents the relative activity rate of proteases in 1 mL culture as a function of temperature, CFU and the amount of protein. Isolate M63-7BB-2 showed high activity at all the temperatures tested. Even at 20 °C, it was more active (1.0471 RAU min^{-1}) than isolate MA-3BB-3 at 30 °C (0.9608 RAU min^{-1}). At 30 °C, the former multiplied its activity by 1.53-fold ($a = 1.6128$ RAU min^{-1}). Isolates MA-2BB-2 and MA-3BB-3 showed somehow low rates of 0.3654 and 0.3016 RAU min^{-1} at 20 °C, and 1.2324 and 0.9608 RAU min^{-1} at 30 °C, respectively.

Discussion

In this study, we focused on the specific microbial group of *Vibrio* sp. appearing in BBD-affected corals in the Red Sea. Our BBD samples were collected from the same corals in October 2007 (when the disease was very active and the black band was evident, at a water temperature of 26 °C) and again during December 2007 (when the water temperature declined to 24 °C and the black band disappeared). Our findings on the general microbial diversity from the 16S rRNA gene libraries obtained from direct sampling during October and December 2007 were also compared with the findings of Barneah *et al.* (2007). These earlier results showed that diseased corals contained mainly *Cyanobacteria* (25%) and *Alphaproteobacteria* (30%) sequences, which were comparable to values of 32% and 27%, respectively, in

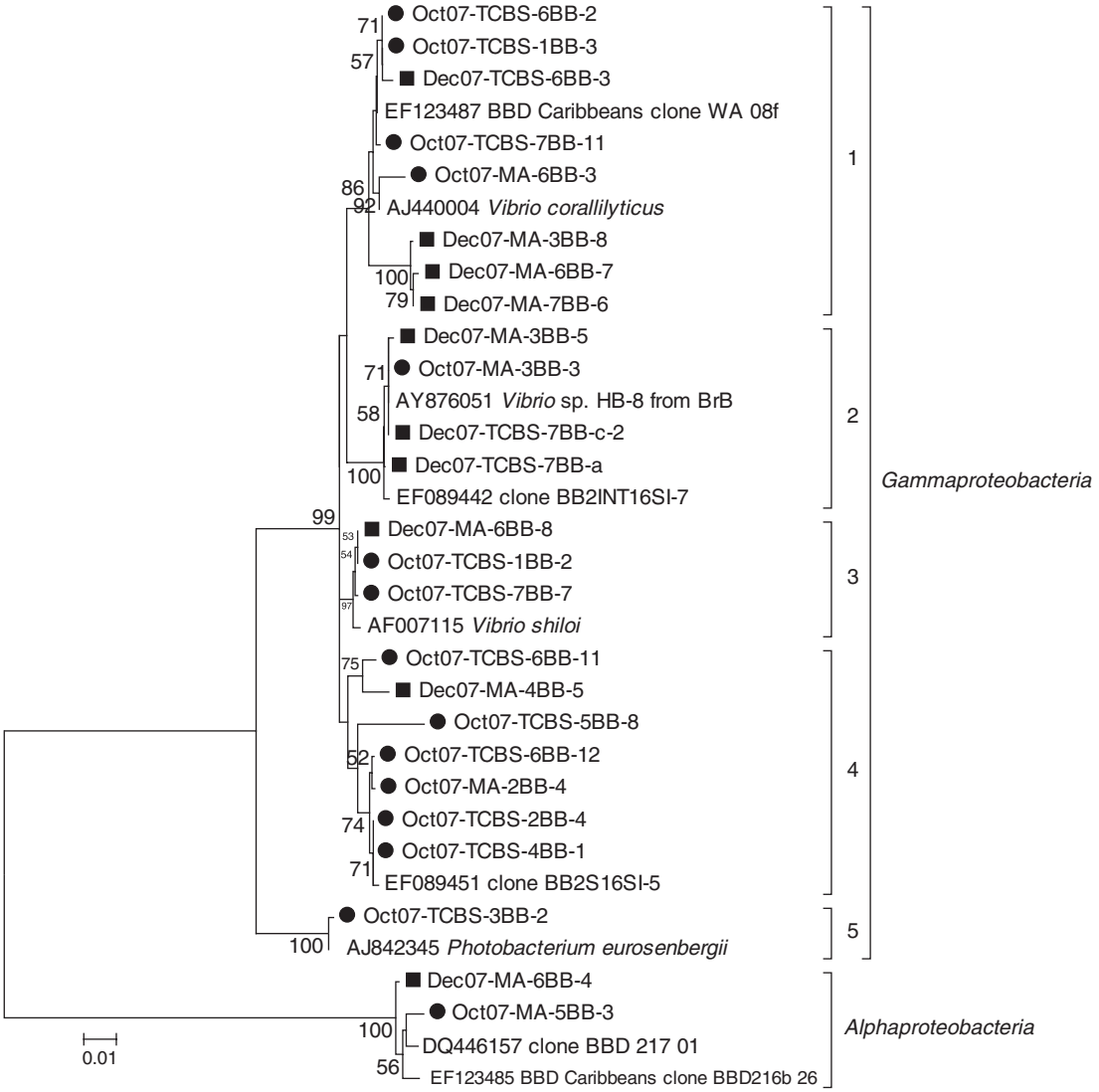

Fig. 3. Phylogenetic tree of bacterial isolates representatives from *Favia* sp. corals infected with the BBD, together with best-matched sequences from GenBank. Subtrees are marked by genus or class (according to RDPII, BLAST). ● – October 2007, coral BB area; ■ – December 2007, coral BB area; unmarked – clones with high homology to GenBank entries.

the present study. In addition, 10% of the sequences from the clone libraries (Figs 1 and 2) demonstrated high similarity (≥99%) to *Gammaproteobacteria*, in general, and to *Vibrio* genus sequences reported in Barneah *et al.* (2007). These findings strengthen our hypothesis that *Vibrio* sp. plays a role in BBD appearance and progression. October and December 2007 *Vibrio* sequences showed similarities to different *Vibrio* sp. groups. While the October samples included clones similar to known coral pathogens (Fig. 2), the December clones were similar to general aquatic *Vibrio* sp. sequences.

In addition to clone library sequences, *Vibrio* sp. originating from the BBD coral mucus and tissue samples from October and December 2007 were collected and cultured (Fig. 3). No *Vibrio* sp. were isolated from the apparently healthy area of the coral, probably due to their low concentration in the coral tissue and mucus. In addition, *Vibrio* sp. found in the BB area are distinct from those found in the apparently healthy area (Barneah *et al.*, 2007). The possibility exists, therefore, that the TCBS selective medium was not suitable for isolation of these strains. Some of the *Vibrio* strains isolated in this study were highly similar (≥99%) to BBD *Vibrio* clones identified previously by Barneah *et al.* (2007) using molecular techniques. Moreover, on examining the sequences, we noticed high similarity between isolates from different coral colonies sampled in the same time

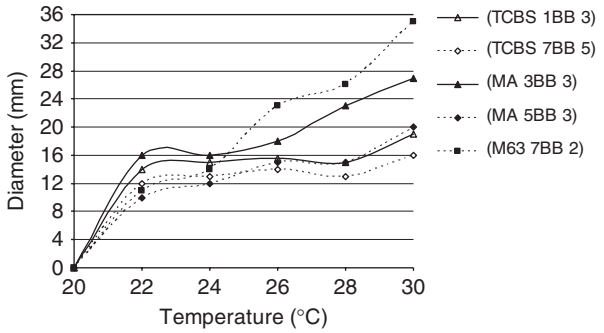

Fig. 4. Protease activity of *Vibrio* sp. isolates as detected on casein agar plates. The figure summarizes the diameter of the lysed area under and around the bacterial colony, as a function of temperature. Fifty microliters of bacterial culture, grown overnight in marine broth ($\sim 10^9$ CFU) at 22 °C, was placed on casein agar plates and incubated at different temperatures overnight.

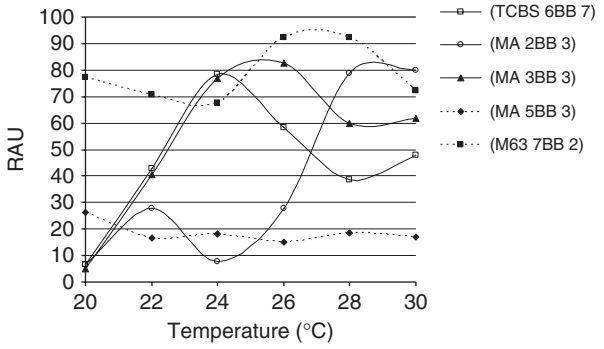

Fig. 5. Proteinase (azocasein) assay of representative *Vibrio* sp. isolates. RAU vs. temperature are plotted. The values were calculated using the azocasein formula and mean CFU at a specific temperature.

period. Similarities were also found between isolates from samples collected from the same coral, but at different times (see Fig. 3). These findings suggested that some *Vibrio* sp. may be ubiquitously associated with the diseased coral, both in the symptomatic and in the asymptomatic state. Furthermore, we isolated several strains (9%) that were highly homologous (> 99%) to *V. coralliilyticus*, a coral pathogen known to cause bleaching and tissue lysis at elevated temperatures in the branching coral *Pocillopora damicornis* (Ben-Haim *et al.*, 2003a). We also succeeded in isolating several bacterial strains that were similar to other *Vibrio* sp. associated with known coral diseases, such as the brown band syndrome (Great Barrier Reef, AY876051) and coral bleaching (Mediterranean Sea, AF007115; Kushmaro *et al.*, 1996) (Fig. 3). Additional *Gammaproteobacteria* ribotypes were obtained in this study. These *P. eurosenbergii*-like isolates were found to belong to a group known to be associated with coral bleaching (Thompson *et al.*, 2005). Interestingly, we also isolated a group of *Alphaproteobacteria*, a class of bacteria that appear in all BBD studies to date (Cooney *et al.*, 2002; Frias-Lopez *et al.*, 2003; Richardson, 2004; Barneah *et al.*, 2007). This class also includes several known pathogens and particularly a specific coral pathogen, *Aurantimonas coralicida*, which is known to cause white plague type II disease in several coral hosts (Denner *et al.*, 2003) and that is often a natural inhabitant of corals (Rohwer *et al.*, 2001, 2002). This pathogen is a constant feature of BBD mats in several locations in the world and, therefore, should be taken into consideration in assessing BBD etiology.

*Vibrio*s are known pathogens with characteristic pathogenic and virulence properties (Thompson *et al.*, 2004). One characteristic of pathogenic *Vibrios* is the activity of their extracellular proteases. This activity could be a mechanism by which the coral tissue is penetrated and degraded (Ben-Haim & Rosenberg, 2002; Ben-Haim *et al.*, 2003b; Halpern *et al.*, 2006; Sussman *et al.*, 2008). Two protease activity tests were conducted in this study, namely the casein and azocasein tests. All isolates tested by the casein test displayed elevated temperature-dependent activity (Fig. 4). We found significant differences (Kruskal–Wallis nonparametric test, followed by multiple comparisons analysis, $P < 0.05$) between activity at 20 °C, and at the other tested temperatures (i.e. 26, 28 and 30 °C), as well as between 30 °C and the other tested temperatures (i.e. 22 and 24 °C). Interestingly, isolate M63-7BB-2 showed the highest levels of proteolytic activity on casein agar plates (Fig. 4). This enhanced activity was reinforced by the additional azocasein proteolytic test (Fig. 5). In the azocasein test, most of the isolates' proteolytic activity peaked at around 26–28 °C. This finding is consistent with BBD appearance, which is associated with elevated water temperatures (Rützler *et al.*, 1983; Kuta & Richardson, 1996). Accordingly, we can speculate that when water temperatures increase, the general proteolytic activity of the black band microbial mat also increases, probably due, at least in part, to the increased activity of *Vibrio* sp. (in addition to the increase in the biomass). This phenomenon could explain the increased progression of the disease during periods of high temperatures, and may shed light on the mechanism of the disease. It is also possible to attribute the shift in bacterial virulence properties as a function of temperature to the different natures/types of the extracellular proteases secreted at different incubation times.

EDTA inhibition indicates that the extracellular proteases are a family of metalloproteases. This protein family was previously identified with several *Vibrio* sp. pathogens, such as the human pathogen *Vibrio cholerae*, *V. coralliilyticus* that causes bleaching in *P. damicornis* coral and *Vibrio* sp. associated with Indo-Pacific coral white syndrome (Ben-Haim *et al.*, 2003b; Halpern *et al.*, 2006; Sussman *et al.*, 2009).

The proteolytic activity of the M63-7BB-2 isolate, which may be an important component of BBD, was quantified as a function of time and temperature using 'relative activity units.' Our results demonstrated that this strain was highly active, showing 1.0471 RAU min^{-1} at 20 °C and 1.6128 RAU min^{-1} at 30 °C. The strain thus either produces more extracellular protease or enhances protease activity at the higher temperature. This isolate may be a major contributor to the tissue necrosis observed in BBD; however, its activity on coral tissue *in situ* still needs to be assessed. Additional isolates, for example MA-2BB-2 and MA-3BB-3, also increased their protease activity between 20 and 30 °C (by more than threefold) and may also play a role in tissue necrosis. Our results indicate a shift in bacterial virulence as a function of temperature.

We showed that proteolytic activity increases with temperature between 20 and 30 °C (Fig. 4), either due to the production of more proteases per bacterial cell (Fig. 5) or by an increase in the rate of proteolytic activity of the same proteases. The shift in the proteolytic activity usually occurred between 20 and 24 °C, peaking around 24–28 °C. These features correlate highly with the appearance and progression of the BBD in the Red Sea corals (Zvuloni *et al.*, 2009).

Carlton & Richardson (1995) suggested that the microbial consortium in the BBD mat operates synergistically to exploit a source of nutrients, as the BBD migrates across the coral. They suggested that tissue degradation releases a rich variety of organic nutrients for use by the mat population. Our findings regarding the possible increase in the proteolytic activity of BBD-associated *Vibrio* sp. as a function of elevated temperatures support this hypothesis. Such proteolytic activity could, therefore, play a major role in the observed black band progression as a function of elevated seawater temperature, as described previously (Rützler *et al.*, 1983; Kuta & Richardson, 1996).

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Supporting Information

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

Table S1. Antibiotic resistance test results and colony color on TCBS.

Table S2. API 20 NE test of bacterial isolates.

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