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The Possible Role of Cyanobacterial Filaments in Coral Black Band Disease Pathology

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Abstract Black band disease (BBD), characterized by a black mat or line that migrates across a coral colony leaving behind it a bare skeleton, is a persistent disease affecting massive corals worldwide. Previous microscopic and molecular examination of this disease in faviid corals from the Gulf of Eilat revealed a number of possible pathogens with the most prominent being a cyanobacterium identified as *Pseudoscillatoria coralii*. We examined diseased coral colonies using histopathological and molecular methods in order to further assess the possible role of this cyanobacterium, its mode of entry, and pathological effects on the coral host tissues. Affected areas of colonies with BBD were sampled for examination using both light and transmission electron microscopies. Results showed that this dominant cyanobacterium was found on the coral surface, at the coral–skeletal interface, and invading the polyp tissues and gastrovascular cavity. Although tissues surrounding the invasive cyanobacterial filaments did not show gross morphological alterations, microscopic examination revealed that the coral cells surrounding the lesion were dissociated, necrotic, and highly vacuolated. No amoebocytes were evident in the mesoglea of affected tissues suggesting a possible repression of the coral immune response. Morphological and molecular similarity of the previously isolated BBD-associated cyanobacterium *P. coralii* to the current samples strengthens the premise that this species is involved in the disease in this coral. These results indicate that the cyanobacteria may play a pivotal role in this disease and that the mode of entry may be via ingestion, penetrating the coral via the gastrodermis, as well as through the skeletal–tissue interface.

Esti Kramarsky-Winter and Luba Arotsker provided equal contribution to this work.

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Introduction

Recent studies cite increases in coral tissue loss due to diseases as one of the major causes of coral mortality and ensuing reef decline [26, 49, 63]. Black band disease (BBD), one of the first coral diseases to be recorded, is characterized by a black band or line ranging from 0.2 mm to 7 cm in width that migrates across a colony leaving behind it a bare skeleton. It affects 19 of 66 Caribbean species and 45 of approximately 400 species in the Indo-Pacific [60], including some of the major reef framework builders [65], as well as some acroporids. The disease was first described by Antonius in 1973 on the reefs of the Florida Keys and in the Caribbean, and in the following decades was found to occur virtually worldwide. Though this disease usually affects less than 1 % of the total number of surveyed corals, its persistence and spread through coral populations makes it an important player in reef health [3, 4, 16, 24, 40, 67].

The black band (or mat) that is characteristic of this disease migrates across a colony at rates as fast as 1 cm/day, causing

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demise of small colonies within a few weeks and large colonies over months [2, 18, 67]. The disappearance of the black band signs usually occurs with the decrease in water temperatures [10]. On the other hand, the disease signs may persist year-round depending on the species and environmental conditions (personal observation).

Previous microscopic and molecular searches for the primary pathogens involved in BBD in different coral species from widely dispersed geographic locations have revealed a number of possible candidates, including heterotrophic and gliding bacteria, as well as a number of cyanobacteria species [2, 10, 16, 20, 24, 32, 45, 48, 50, 55, 67]. The fact that BBD communities from different corals and different geographic locations all contained abundant populations of the same physiologically functional groups of microorganisms led Carlton and Richardson [10] to propose that BBD is caused by a microbial consortium with a dominant cyanobacterial component.

While the search for the pathogen or pathogens continues, there is little attention being given to the micro-morphological effects of the disease on the coral tissues. In a pioneering study, Rützler et al. [51] reported morphological observations of BBD infection revealing that cyanobacterial filaments were found along the boundary of living coral tissue and skeleton, occasionally penetrating the tissue. They reported a subectodermal tunneling by the band, progressing at rates as fast as 20 mm in 12 h. Repeating the work by Antonius [2], Rützler et al. [51] showed that infection could be artificially induced by inserting the cyanobacterial mat (including all accompanying microorganisms) between the coral tissue and skeleton or by putting injured colonies in direct contact with a BBD-infected coral. Additionally, in a recent study, Richardson et al. [48] succeeded in infecting colony fragments of *Montastraea annularis* with the disease using freshly collected BBD mat that was attached to a healthy coral.

There are still many unresolved questions concerning BBD etiology. Of particular interest are the role of the cyanobacterium play in this disease and its mode of action [13, 20, 47]. To attempt to clarify this, we recently isolated (as monoalgaic culture) and molecularly and morphologically identified a cyanobacterium from BBD-affected tissues of the coral *Favia favus* from the Gulf of Eilat, Israel [44]. This cyanobacterium showed high molecular and morphological similarity to BBD-associated cyanobacteria from other geographical areas [6, 44]. In the present study, we use morphological and molecular identification to verify that the cyanobacterial filaments found in and adjacent to the diseased coral tissue in situ are indeed those previously isolated and cultured [44] from BBD-affected colonies. Furthermore, we describe the morphological and histopathological changes occurring in the BBD-affected *Favia* tissues surrounding the cyanobacteria, in an attempt to understand possible points of entry and pathological effects on coral tissues.

Materials and Methods

BBD-affected *F. favus* colonies (Fig. 1) were tagged, photographed, and observed monthly for gross morphological changes over a period of 1 year. During peak disease periods in late summer, affected corals were photographically recorded, and disease mean progression was assessed. Samples of the BBD microbial communities were collected from the surface of a number of BBD-affected colonies ($n=7$). These were used for culturing and molecular identification. A number of colonies ($n=3$) with black band disease infections were sampled using sterile corers (two cores/colony). Samples were taken in the area of the BBD and healthy portions of the corals and were used for histology and transmission electron microscopy (TEM) studies. Cultured samples were also identified molecularly and morphologically (TEM).

Morphological Identification of the Affected Tissue and Cyanobacterial Community Associated with It

Cored samples of the band and samples of BBD microbial communities were collected using sterile corers and fixed for microscopic examination. Microscopic evaluation of the affected tissues and cultures included gross morphological examination as well as histological (using light microscopy) and TEM examination of the affected coral tissues surrounding the BBD lesion. To this end, samples of affected tissues were

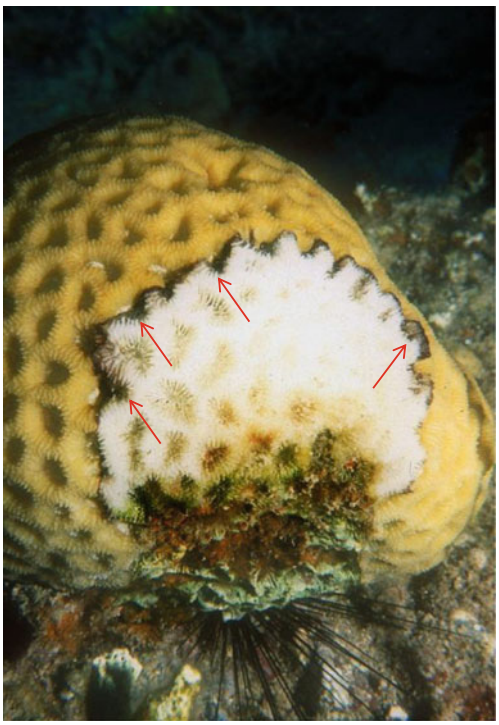

Fig. 1 Black band disease lesion on *F. favus* in shallow water in Eilat, Red Sea. Note the healthy yellowish tissue bordered by a black band followed by the bare skeleton. *Arrows* indicate the black band microbial mat

fixed in 4 % paraformaldehyde in sterile sea water (SW) for histopathology or in 2.5 % glutaraldehyde in filtered sea water for TEM. The fixed samples were enrobed in 1.5 % agarose and decalcified using 10 % EDTA. For histological studies, the enrobed tissues were processed in a Citadel tissue processor, embedded in paraffin, and sectioned (5 μm). The sections were stained with hematoxylin/eosin and observed and photographed using a Nikon microscope and digital camera, respectively. For TEM, the decalcified tissues were washed in buffer and post-fixed in 4 % buffered osmium tetroxide, washed again in SW buffer, and dehydrated in graded ethanol (30, 50, 70, and 100 %) and in propylene oxide, followed by a gradual embedding in Araldite epoxy resin 502 (Electron Microscopy Sciences, Fort Washington, PA, USA). One-micrometer semi-thin sections were stained with toluidine blue to target areas for examination by TEM. The samples were then sectioned (60–90 nm) using an ultramicrotome, mounted on 300-mesh copper grid, and coated with lead citrate. Sections were examined using a JEM-1230 at 80 kV. In order to compare morphologies, these micrographs were compared to those of cultured BBD-derived cyanobacterial filaments.

Molecular Identification of the Cyanobacteria

Molecular identification of cyanobacteria was carried out in order to identify the cyanobacteria found within the diseased tissues. Samples from the affected and healthy areas from BBD-affected colonies were analyzed by PCR and cloning. To probe the BBD community in the tissues, universal primers for 16S rRNA genes were used as phylogenetic markers. Genomic DNA was extracted from the BBD tissue and mucus samples (MoBio PowerSoil kit, MoBio Laboratories, Solana Beach, CA, USA). Total DNA was amplified by PCR using universal 16S rRNA gene primers for bacteria (8F–GGATCCAGACTTTGATYMTGGCTCAG, modified by shortening from the 5′- end [19], and 907R–CCGTCAATTCCTTTRAGTTT [29]) and specific 16S rRNA gene primers for cyanobacteria (106F–CGGACGGGTGAGTAACGCGTGA and 781R–GACTACTGGGTATCTAATCCCATT [39, 59]) by Arotsker et al. [5]. Clone libraries were generated from the PCR products using pCRII-TOPO-TA cloning vector as specified by Invitrogen (Carlsbad, CA, USA) and transformed into BioSuper $CaCl_2$-competent *HD5a Escherichia coli* cells (Bio-Lab, Israel) according to the manufacturer's instructions, and selected clones were sequenced. Sequences were analyzed using MOTHUR [54] and MEGA5 (Molecular Evolutionary Genetics Analysis) [61] packages and compared with those in the GeneBank database using the BLAST network service to find closely related sequences to be used in subsequent analyses.

Results

Description of the Disease Process

BBD in the Red Sea was found to be prevalent in a number of massive faviid species [67]. The disease lesion was usually focal in nature. The BBD lesion began either in the central or peripheral region of the colony and was characterized by defined black or dark brown edges. The lesion morphology included tissues with smooth margins or irregular shapes (see [66]) and revealed intact bare skeleton distinctly delineated from healthy tissue by a thin dark band. Tissue loss revealed the intact underlying skeleton, which in some cases was then overgrown by filamentous algae. Movement of the microbial mat into the tissue ceased during the winter and continued during the following warm season. Thus, BBD-affected corals that were not completely decimated by the disease and where the disease signs persisted showed a continuation of tissue loss during the following summer months. Moreover, colonies in which the black band was not evident during the winter, showed a recurrence during the following summer.

Morphology of BBD Lesion

The BBD lesion is characterized by a mat of cyanobacteria and accompanying microorganisms, interspersed by degraded tissue (Fig. 2). Most of the cyanobacterial filaments were found at the tissue–skeleton boundary in association with necrotic tissues. Filaments also penetrated or burrowed into the calicoblastic epithelia (Fig. 2b, c). Coral tissues in proximity to the band lost cell-to-cell adhesion and were characterized by numerous intracellular vesicles characterizing necrosis (Fig. 2).

Cyanobacterial cells (mean and SD, 2.83±0.36 μm in diameter and 2.20±0.23 μm in length) were identified in the lesions, both in juxtaposition to and penetrating the polyps (Figs. 2 and 3). They were found in the gastrovascular cavity as well as penetrating the gastrodermal tissue (Fig. 2). Filaments were also evident underlying the calicodermis (Fig. 2) as well as penetrating the mesoglea (Fig. 4) and in juxtaposition to the coral's skeleton. Filaments were rarely evident in the surface body wall epidermis in areas of the lesion. Careful examination of the histological and TEM sections (Figs. 2 and 4) revealed that BBD cyanobacteria penetrate the coral tissue between cells as well as in areas rich in collagen, such as the mesoglea.

The coral tissues associated with numerous cyanobacteria were characterized by loss of tissue confluence and by cell to cell adhesion (Figs. 2 and 3). In these areas, mesoglea denuded of surrounding epithelial cells was evident. Most of the coral host cells associated with the cyanobacteria showed cytoplasmic disintegration, which precluded nuclear breakdown. TEM revealed that coral

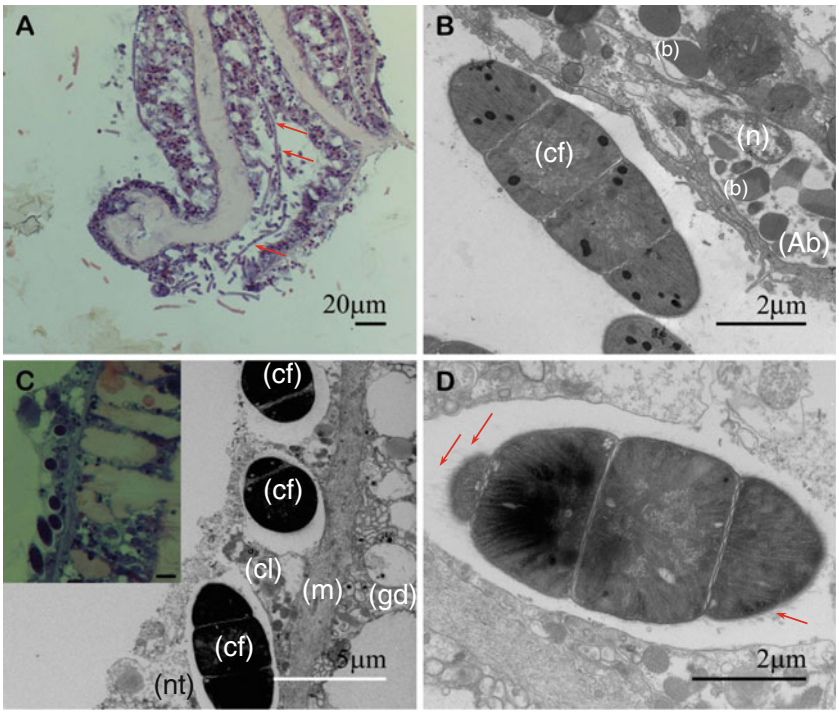

Fig. 2 Gastrodermis and calicodermis of a BBD-affected colony. **a** Histology: note the cyanobacterial filaments and trichomes in the gastrodermis (*arrows*). **b** TEM micrograph showing the cross section of cyanobacterial filament (*cf*) found in and adjacent to the gastrodermal cells, nucleus (*n*), and bacteria (*b*) inside an autophagous body (*Ab*). **c** Filaments in calicodermis (*cl*) and intracellular vesicles (necrotic tissue (*nt*)); *m* denotes mesoglea, while *gd* denotes gastrodermis. **d** Release of filamentous material by proximal trichome of burrowing filament

cells associated with the lesions were characterized by numerous autophagous bodies, pyknotic nuclei, and few apoptotic bodies (Figs. 2 and 3). Autophagous bodies are identified as vacuole-containing sub-cellular organelles (phagophore, phagosome, [27]), while pyknotic nuclei are characterized by nuclear condensation [22]. Cyanobacterial trichomes were found in spaces in the mesoglea of affected tissues (Fig. 4), which were bordered by electron dense areas (Fig. 4b), indicating localized alterations in the mesoglea. Filamentous material was evident at the apical cells of the trichome (Fig. 3c, d). No amoebocytes were evident in the coral tissues.

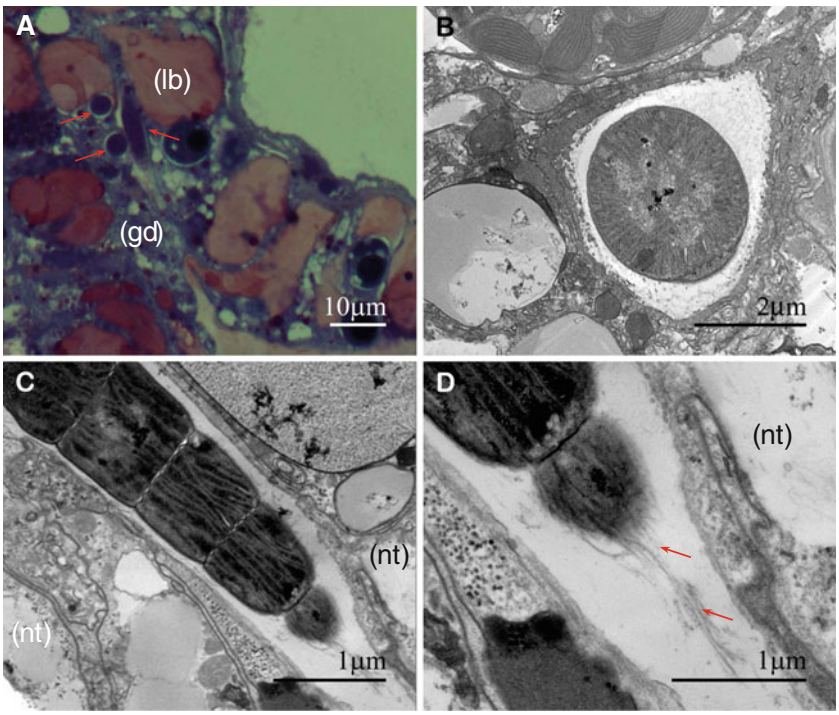

Fig. 3 Gastrodermis of BBD-affected colony. **a** Histology: affected gastroderm (*gd*) with numerous trichomes (*arrows*); lipid bodies (*lb*). **b** Cross section of a trichome borrowing in the tissue. **c** Trichomes burrowing into the tissue (note the necrotic tissue (*nt*) surrounding them). **d** Closeup of trichome releasing filamentous material

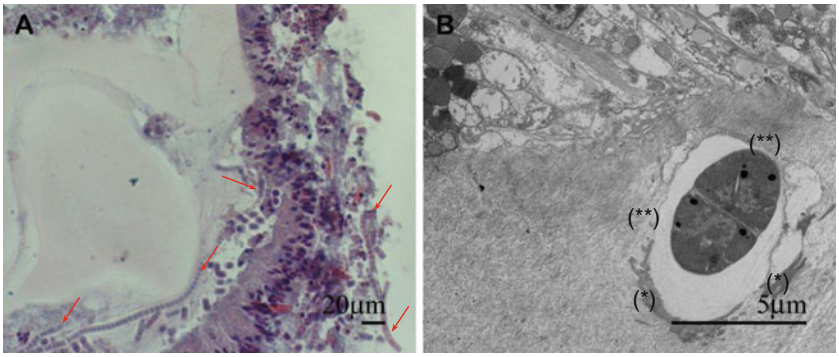

Fig. 4 Actinopharynx mesoglea with embedded cyanobacterial trichomes. **a** Histological section revealing numerous filaments and trichomes in the mesoglea. **b** TEM micrograph of trichome burrowing in the mesoglea showing changes to surrounding mesoglea, including higher density on one side (*asterisk*) versus the other (*double asterisk*)

Cyanobacterial Filament Morphology and Molecular Identification

Non-sheathed filaments of the cyanobacteria associated with the lesion appeared as two or more trichomes with a pointed terminal cell (Figs. 2, 3, and 5). Each non-terminal cell in the trichome was approximately 2.20±0.23 µm (mean and SD) in length and averaged 2.83±0.36 µm in width, and contained numerous granules. The resulting non-terminal trichome cell length to width ratio was 0.78+0.043 µm. Thylakoids surrounding the central nucleoplasm appeared parallel in longitudinal section and were radially arranged in cross section (Figs. 2b–d and 3b–d). The trichomes were surrounded by a multilayered cell wall and were partitioned from each other by characteristic undulating partitions (membrane/wall) (Fig. 5). The cell wall of the filaments had the typical layered structure.

Direct molecular identification of cyanobacteria from BBD-affected tissues of the *Favia* sp. from the reef of Eilat using 16S rRNA gene primers displayed a number of cyanobacterial sequences. These sequences showed over 97 % similarity to cyanobacteria found in BBD from corals from the Caribbean and GBR Australia [41] (Fig. 6). *Pseudoscillatoria coralli* sequence (FJ210722, 1,416 bp) had more than 97 % similarity to all sequences in the *P. coralli* clade (Fig. 6). In addition, *P. coralli* had 93 % similarity to cyano clone 37-CL28-OTU12-28 (GQ204792, 663 bp) and

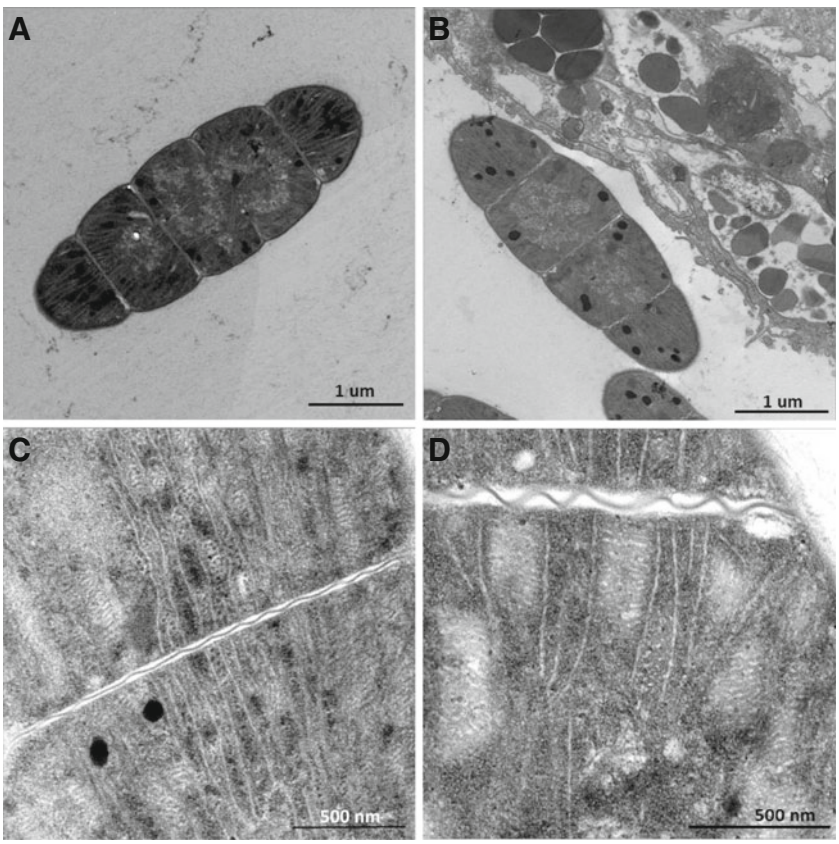

Fig. 5 Comparison of TEM micrographs of cyanobacterial filaments: trichome from a culture (**a**) and in the tissue of BBD-affected coral (**b**) and undulating septa between cells from the culture (**c**) and in the BBD tissue (**d**)

Fig. 6 Phylogenic tree of all cyanobacterial sequence (cultured and uncultured) representatives from different studies on the black band disease. *Black diamond*, *P. coralli*; *white circles*, sequences from our group's previously published studies; *black circles*, sequences from this study. The tree was built using MEGA 5.10 software, neighbor-joining statistical method, and bootstrap phylogeny test (500 replications). The names of the sequences were built as follows: accession number, sequence name - BBD-affected coral host - geographic location, *parentheses* denote reference

88–90 % similarity to all *Geitlerinema* sp. (DQ151461, DQ680351, EF110974, EF725580; 1,363 bp), *Leptolyngbya* sp. (EU743966, 422 bp), clone CL35-CP-OTU1 (GQ204788, 663 bp), and cyano clone PNG-50 (AY123040, 615 bp).

Discussion

In the Northern Gulf of Eilat, BBD is a persistent disease affecting mainly faviid corals [67]. Depending on their size, the corals affected by the disease can be completely decimated within a few weeks of the appearance of the microbial mat. Indeed, in the Gulf of Eilat, the disease advanced as quickly as 1.0±0.72 mm/day during the warm summer months (August/September), resulting in the decimation of colonies of 5-cm diameter within weeks. Spatio-temporal patterns of the disease signs provide evidence that this is a persistent disease and that surviving infected corals may lack the black band during the cool winter months and redevelop it as the water warms. Thus, the affected corals may also act as reservoirs of the disease during the winter months, infecting nearby corals when the environmental conditions permit [33, 67].

During the active phase of the disease, a polymicrobial mat is evident adjacent to, penetrating, and covering both intact and necrotic coral tissues, as is characteristic of the BBD lesion. This mat includes filamentous cyanobacteria, together with numerous heterotrophic bacteria, interlaced with disrupted and necrotic coral tissue fragments and cells. Both molecular and morphological identification of the cyanobacterial component from the affected coral tissue showed close similarity and even identity to those previously isolated and cultured from BBD in these and other coral species [11, 33, 44]. This indicates that these cyanobacteria are an important component of this disease. The BBD lesion is characterized by some loss of coral tissue integrity, resulting in a mass of loose disaggregated single cells and cell clumps similar to what was previously reported [9], providing evidence for their possible role in causing coral tissue necrosis. In the present study, cyanobacterial filaments were not only found in the disintegrating tissues but were also found adjacent to and penetrating what appeared to be contiguous coral tissues. Moreover, similar to what was reported by Miller et al. [33], filaments were also found underlying and in close proximity to the coral's calicodermis. This strengthens the hypothesis of Rutzler et al. [51] that the cyanobacterial filaments could penetrate the coral via the calicodermis. In the present study, filament penetration was not only evident in the calicodermis and in the mesoglea of the calicodermis but also in the gastrovascular cavity, gastrodermis, and the mesoglea of the surface body wall. Thus, penetration may not only be via

the calicodermis–skeleton interface but may also occur via the gatrovascular cavity, indicating a possible additional venue for penetration presumably by ingestion.

The ability of cyanobacteria to penetrate and survive in live animal tissues is not novel and is evident in a number of aquatic organisms [62]. For example, in the sponge *Tethys orphe*, endosymbiotic cyanobacteria found in the cortex provide the host with photosynthates. In some corals, endosymbiotic cyanobacteria have been reported to aid in nitrogen fixation [30]. Additional studies have shown that endolithic cyanobacteria burrowing in coral skeletons may be beneficial to their host corals, providing them with nutrients [31, 43]. On the other hand, in organisms ranging from arthropods to humans, there are reports of cyanobacteria as causative agents or as secondary pathogens in disease processes [7, 15]. Indeed, Ainsworth et al. [1] revealed the presence of a cyanobacteria-dominated microbial mat deep within the coral polyp structure in white plague-affected faviids. They posited that pathogenic cyanobacteria that are present in the interface between the tissues and skeleton in seemingly healthy corals can only penetrate into tissues of immuno-compromised or damaged tissues, thus producing visible and recognizable signs of BBD. In addition, the filamentous material released at the trichome end (Figs. 2d and 3c–d) may play a role in penetration of the tissue.

Results from a number of studies of BBD show that mats from corals from disparate geographic regions, different seasons (highly active during summer time vs. non-active during the winter), or from different species (summarized in Fig. 6) all contain closely related or identical cyanobacteria. Indeed, the molecular similarities indicate that they may be the same genus (or species). Moreover, the similarity of filament morphology of the cyanobacteria from Eilat *Favia* BBD reported in the present study to that of cyanobacteria found in BBD from the Caribbean *M. annularis* [33] may strengthen this premise. Cyanobacteria described in this work were morphologically (Fig. 5) similar to those previously isolated from diseased colonies of *Favia* [44] and to those of other coral species found throughout the Caribbean and Indo-Pacific [33]. Cell length to width ratio of the cyanobacteria in this study was 0.78+0.043 μm, similar to other species of Oscillatoriales [11]. Slight morphological differences in these cyanobacteria may be attributed not only to strain differences but also to different physiological states of the cyanobacteria in the different hosts. Additionally, in some cases, more than one cyanobacteria genus may be found within the same BBD mat [13, 20, 33, 48, 59, 64]. These different species or strains may occur in the same infection and contribute to BBD pathology by producing different toxins at different stages in the disease process [58]. Even though the cyanobacterial strain or species may differ, they are necessary for the development of BBD by providing the necessary functional group for the development of the mat.

Observation of the tissues adjacent to but not bordering the BBD lesions does not reveal changes in gross morphology. Microscopic examination of that area though reveals cellular necrosis and no characteristics of tissues undergoing repair or immune reactions. In general, during tissue repair following damage, there are increases in amoebocyte numbers in the mesoglea of tissues adjacent to the lesions [26, 36, 41, 63]. This localized increase in amoebocytic cells was also reported in *Aspergillosis* infections in gorgonians [36]. In BBD-affected corals, there is no evidence for amoebocyte mobilization, and the cells in affected tissues appear necrotic with few autophagic bodies. This lack of amoebocyte mobilization may be a sign that there is a failure of the coral's immune system, ultimately resulting in the massive infiltration of these filaments into the tissue. Indeed, cyanobacteria may actively inhibit recruitment of amoebocytes as evident by lack of tissue repair [28].

The mechanism of cyanobacterial penetration into the coral tissue is as yet unclear. Damage to the tissues by tunneling may, on the one hand, depend on environmental factors that promote the production of certain toxic enzymes by the cyanobacterium and, on the other hand, may induce stress to the coral that results in complete loss of resistance [35]. This is supported by recent studies that have shown that increased temperature and light intensities enhance progression and spread of BBD [8, 46, 52]. The cellular necrosis characteristic of the BBD lesion may have been the result of the production of toxins or enzymes by the cyanobacteria in or adjacent to the affected tissues. Cyanobacterial microcystins were found to affect cells of vertebrates and invertebrates via inhibition of protein phosphatase, enhancing reactive oxygen species (ROS) production and promoting apoptosis [7, 12, 14, 25, 34, 42].

Richardson et al. [47] showed that different strains of cyanobacteria associated with BBD produce different toxins. Furthermore, Richardson et al. [48] showed that exposure to even low concentration of BBD cyanobaterial microcystin (1 μg L^{-1}) may promote bacterial growth in the coral tissue enhancing tissue breakdown. Thus, toxins such as microcystin may be both directly toxic to the coral hosts and/or may indirectly affect the host by stimulating growth of some species of pathogenic bacteria. The necrotic/autophagous processes evident in the tissues surrounding the cyanobacterial lesions are the result of a direct action of the toxins on the adjacent tissues. In addition to cyanobacteria in the BBD mat, there are toxin-associated heterotrophic bacteria, including sulfide-reducing bacteria [5, 48, 55, 56] that may also cause necrosis of coral tissues due to cellular intolerance to sulfides [17, 32, 34].

Although cyanobacteria are normally intolerant to high levels of sulfide, there are reports of some isolates of BBD cyanobacteria tolerant to sulfide and conduct sulfide-resistant oxygenic photosynthesis [37]. Thus, pathogenesis of BBD

could involve a combination of mycrocistin-associated tissue necrosis, leading to high sulfide and promotion of invasiveness by sulfide-tolerant cyanobacteria into the tissues. The present study provides a snapshot of the cellular effects of the cyanobacterial filaments on the coral tissue and may aid in the assessment of the role this organism plays in the BBD pathology.

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