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ORIGINAL ARTICLE

Successional changes in bacterial communities during the development of black band disease on the reef coral, *Montipora hispida*

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Black band disease (BBD) consists of a mat-forming microbial consortium that migrates across coral colonies causing rapid tissue loss. Although BBD-associated microbial communities have been well characterized, little is known regarding how these complex bacterial consortia develop. This study analyzed successional changes in microbial communities leading to the development of BBD. Long-term monitoring of tagged corals throughout outbreaks of BBD in the central Great Barrier Reef documented cyanobacterium-infected lesions, herein termed cyanobacterial patch(es) (CP), which were macroscopically distinct from BBD and preceded the onset of BBD in 19% of the cases. Dominant cyanobacteria within CP lesions were morphologically distinct from ones dominating BBD lesions. Clone libraries and terminal restriction fragment length polymorphism analysis confirmed shifts within cyanobacterial assemblages, from *Blennothrix* sp.-affiliated sequences dominating CP lesions, to *Oscillatoria* sp.-affiliated sequences, similar to those retrieved from other BBD samples worldwide, dominating BBD lesions. Bacterial 16S ribosomal RNA clone libraries also showed shifts in bacterial ribotypes during transitions from CP to BBD, with *Alphaproteobacteria*-affiliated sequences dominant in CP libraries, whereas gammaproteobacterial and cyanobacterial ribotypes were more abundant in BBD clone libraries. Sequences affiliated with organisms identified in sulfur cycling were commonly retrieved from lesions showing characteristic field signs of BBD. As high sulfide concentrations have been implicated in BBD-mediated coral tissue degradation, proliferation of a microbial community actively involved in sulfur cycling potentially contributes to the higher progression rates found for BBD compared with CP lesions. Results show how microbial colonization of indistinct lesions may facilitate a common coral disease with proven ecological effects on coral populations.

Introduction

Black band disease (BBD), which manifests as a darkly pigmented microbial mat that migrates across live coral colonies causing necrosis of coral tissues (reviewed in Richardson, 2004), is a major contributor to ongoing loss of reef building corals worldwide, particularly in the Caribbean (Bruckner and

Bruckner, 1997; Goreau *et al.*, 1998; Green and Bruckner, 2000; Sutherland *et al.*, 2004; Kaczmarsky, 2006; Page and Willis, 2006; Sato *et al.*, 2009). BBD has been termed a poly-microbial disease because a primary causative agent(s) has not been identified and a tightly organized, complex microbial consortium appears to act in concert to cause coral tissue necrosis (Richardson *et al.*, 1997, 2007; Richardson, 2004). Currently, however, little is known regarding how bacterial pathogens form the black band on host corals or how changes in microbial communities lead to onset of the disease. Given the potential for BBD to cause substantial tissue loss in coral populations (reviewed in Green and Bruckner, 2000; Sato *et al.*, 2009) and the likelihood that effects of the disease will be exacerbated as seawater

temperatures warm with predicted climate change (Bruckner *et al.*, 1997; Kuta and Richardson, 2002; Borger and Steiner, 2005; Voss and Richardson, 2006; Rodriguez and Croquer, 2008; Sato *et al.*, 2009), there is need for greater understanding of early stages in the onset of the disease if BBD is to be managed effectively.

It is well-established that the microbial consortium making up the black band is dominated by cyanobacteria, but also includes sulfate-reducing bacteria, sulfide-oxidizing bacteria, marine fungi and other heterotrophic microbes (reviewed in Richardson, 2004). Molecular analyses of BBD-dominating cyanobacteria have retrieved bacterial 16S ribosomal RNA (rRNA) gene sequences affiliated with many cyanobacterial species/strains, such as a *Geitlerinema* sp. (Cooney *et al.*, 2002; Myers *et al.*, 2007) (formerly known as *Phormidium corallyticum*), *Trichodesmium* spp. (Frias-Lopez *et al.*, 2002, 2003), *Leptolyngbya* sp. (Myers *et al.*, 2007) and an *Oscillatoria* sp., which has been most commonly detected in BBD samples collected from the wider Caribbean, Indo-Pacific and the Red Sea (reviewed in Myers *et al.*, 2007; Myers and Richardson, 2009). To what extent each of these cyanobacterial strains is involved in BBD pathogenesis is unclear, however, there is emerging evidence that BBD-associated cyanobacteria vary between geographical and regional locations and host coral species (Voss *et al.*, 2007), yet each may occupy the same ecological niche in BBD microbial mats (Myers and Richardson, 2009).

Pathogenesis of BBD is currently thought to involve light-associated, vertical microgradients in oxygen and sulfide (Carlton and Richardson, 1995), accompanied by down-migration of the filamentous cyanobacteria when light levels are high (Richardson, 1996; Viehman and Richardson, 2002). Subsequent cyanobacterial penetration of coral tissue (Barneah *et al.*, 2007) suggests that mechanical and/or chemical degeneration of coral tissue is also involved in pathogenicity. In addition, genes for the biosynthesis of microcystin have been detected from *Geitlerinema* sp. and *Leptolyngbya* sp. derived from BBD samples, and production of the toxin has been confirmed with high-performance liquid chromatography/mass spectrometry and a protein phosphatase inhibition assay (Richardson *et al.*, 2007). These findings indicate that the cyanotoxin may contribute to coral tissue necrosis caused by BBD. *Desulfovibrio* spp. are also thought to have a role in BBD pathogenesis through production and accumulation of sulfide, which is present at high concentrations under the BBD mat and can be lethal to coral tissue when combined with the anoxic microenvironment (Richardson *et al.*, 1997). Sequences affiliated with other potential pathogenic bacteria, including *Cytophaga* sp., *Clostridium* sp., *Campylobacter* sp., *Arcobacter* sp. and an alphaproteobacterium responsible for juvenile oyster disease, have been retrieved from Caribbean BBD samples (Cooney *et al.*, 2002;

Frias-Lopez *et al.*, 2002), although it is unknown whether these organisms have any role in the pathogenesis of BBD.

Little is known regarding how bacterial pathogens form the characteristic black band on host corals or regarding microbial communities during the early onset of BBD in nature. In one published observation, a new BBD infection on *Diploria strigosa* was documented as a small pigmented ring a few centimeter in diameter, which apparently had a BBD microbial mat already formed (Antonius, 1981). Given that BBD-associated bacteria may be present on or within healthy coral tissues at background levels (Frias-Lopez *et al.*, 2002), the disease band may be formed when environmental factors change and/or host coral health is biologically or physically compromised (Rohwer *et al.*, 2002). For example, the BBD-associated cyanobacterium designated as CD1C11 is commonly detected in healthy tissues of *Montastraea annularis* with culture-independent methods (Klaus *et al.*, 2007). It has also been shown that predation of octocorals by corallivorous animals (for example, the gastropod *Cyphoma gibbosum* and polychaete *Hermodice carunculata*) facilitates BBD infection where BBD is present (Antonius, 1985). Lack of knowledge regarding transmission modes and early developmental stages of the BBD microbial consortium hinders development of reef management strategies to mitigate the impacts of this disease.

Recently, recurring summer outbreaks of BBD were documented in a long-term monitoring study of tagged corals in an assemblage of *Montipora* species on an inshore reef in the central Great Barrier Reef (GBR) (Sato *et al.*, 2009). In some cases, actively expanding cyanobacterium-infected lesions, whose green or brown colorations were distinct from characteristic BBD lesions, were identified before the onset of BBD (Figure 1). We herein refer to these lesions as 'cyanobacterial patch(es)' or 'CP'. Preliminary microscopic observations of CP indicate that they contain cyanobacteria that are morphologically distinct from ones observed in subsequent BBD lesions, suggesting that CP and BBD have different overall microbial communities. In this study, we analyze successional changes in microbial communities from early CP lesions to fully developed BBD lesions to document development of the complex bacterial consortia that characterize BBD. Seasonal patterns in abundance of CP and BBD were documented to determine how often and when CP microbial communities develop into BBD microbial consortia. We compare the virulence of CP and BBD by recording progression rates of the lesions on host corals and by histological observations. In addition, we determine phylogenetic diversity of bacteria associated with CP and BBD lesions to examine changes in these microbial communities during the development of BBD from CP.

Materials and methods

Study site and field surveys

Montipora assemblages on an inshore reef at Pelorus Island in the central GRB were monitored between September 2006 and January 2009 to examine seasonal patterns in prevalences of CP and BBD. The study site is exposed to minimal levels of terrestrial run-off or human impact (see Sato *et al.*, 2009 for a detailed description of the study site). In addition to the three previously described quadrats at the southeast corner of the island (18°33.6'S, 146°30.1'E), three replicate 10 m × 10 m permanent quadrats were established at the northeast corner of the island (18°32.5'S, 146°30.0'E) in the same manner in May 2007. CP-infected colonies were individually tagged and monitored approximately monthly to determine whether CP lesions developed into BBD. Progression rates of CP and BBD lesions were measured throughout the study period as described previously (Sato *et al.*, 2009), and annual means and means for summer (December 2007 ~ February 2008) and winter (June 2007 ~ August 2007) were statistically compared between disease signs and seasons.

Sample collection

Samples of CP and BBD lesions were collected from five tagged colonies of *Montipora hispida* adjacent to the monitoring plots at depths of 2.5–3.0 m between August 2007 and February 2008. For bacterial examination, samples were collected at approximately 2-week intervals to monitor changes in microbial communities as CP lesions progressed into characteristic BBD lesions. To minimize sampling effects on disease progression, a small (approximately 5 mm in diameter and 2 mm in depth) portion of the cyanobacterial mat and skeleton underneath the mat was collected from the front of each lesion, using a separate sterilized stainless steel chisel for each sample to avoid cross contamination. The samples were placed in individual 1.5 ml screw cap tubes underwater and kept on ice upon returning to the surface. After examining the cyanobacterial morphology under a phase contrast microscope (BX41TF, Olympus Corporation, Tokyo, Japan) at a magnification of 400 times, excess seawater was removed and lesion samples were preserved in 100% ethanol at −20 °C. A small portion of coral tissue with attached skeleton was also sampled from two healthy colonies and preserved in the same manner. For histological observations, small (3 cm × 3 cm) diseased coral fragments were sampled from each of five CP- and BBD-infected colonies and immediately fixed in 10% seawater formalin. In addition, an ambient seawater sample (1 l) was collected from approximately 30 cm above the sampled colonies and immediately filtered through a 0.22 µm Millipore Sterivex filter unit (Sigma-Aldrich, St Louis, MO, USA). The filter unit
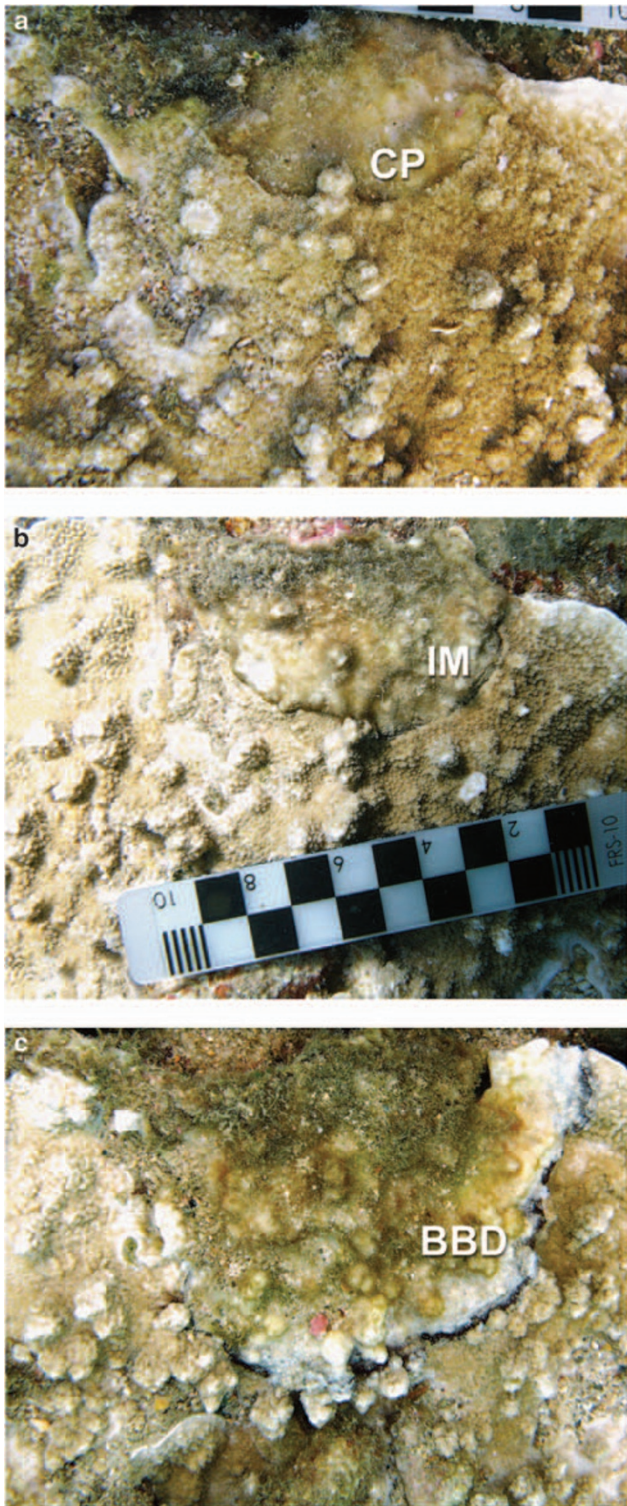
Figure 1 Transition in macroscopic signs of a disease lesion at the identical site on a colony of *Montipora hispida* (colony 3) from: (**a**) a typical CP (7 October 2007), through (**b**) an intermediate stage between CP and BBD (25 October 2007), to (**c**) a well-developed BBD lesion (10 November 2007). Abbreviations: BBD, black band disease; CP, cyanobacterial patch; IM, intermediate stage between CP and BBD.

was kept on ice while transported to the laboratory before being stored at $-20\,^\circ$C.

Histological preparation

Formalin fixed samples were placed between sponge pads in histology cassettes to avoid detachment of the disease mat from the underlying coral tissue during decalcification in a 1 to 3% series of formic acid washes. After paraffin wax embedding of decalcified samples, transverse sections of coral tissue (5 µm thickness) from the edge of each lesion were prepared. Sections were stained with hematoxylin and eosin to examine the extent of tissue structure degradation.

DNA extraction

DNA extraction from ethanol preserved lesions and coral tissue samples was performed with the Power-Plant DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) as described by the manufacturer with the following modifications. After the initial preparation, 2×30-s bead-beating cycles with a 30-s interval were performed with a Mini-Bead-beater (Biospec Products, Bartleville, OK, USA). DNA from the seawater filter unit was isolated according to the methods outlined in Schauer et al. (2000). The quality of extracted DNA was verified on 1% agarose gel stained with ethidium bromide and quantified with a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

PCR amplification, cloning and sequencing

Cyanobacterial clone libraries were constructed from microbial mat samples derived from two tagged corals, each with characteristic CP lesions (colonies 1 and 3, sampled on 24 August and 7 October, respectively). Lesions on these two colonies were repeatedly sampled as they visually transitioned into characteristic BBD lesions (fully developed BBD samples were collected on 22 September and 10 November, respectively). Cyanobacteria-specific PCR primers CYA106F (CGGACGGGTGAGTAACGC GTGA) and CYA781R (an equimolar mixture of CYA 781R(a) (GACTACTGGGGTATCTAATCCCATT) and CYA781R(b) (GACTACAGGGGTATCTAATCCCTTT)) (Nubel et al., 1997) were used to amplify an approximately 700-bp region of the 16S rRNA gene. Each reaction mixture of 50 µl contained 0.2 µM of each primer, 0.2 mM of each dNTP, $1 \times$ PCR Reaction Buffer (TAQ DNA Polymerase Kit, Scientifix, Clayton, Australia), 0.08% (w/v) bovine serum albumin, 1.25 U of TAQ DNA Polymerase (Scientifix) and 10–20 ng of template DNA. Amplification was performed with initial melting at 95 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR products were purified with gel extraction using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned with the T&A Cloning Kit (Real Biotech Corporation, Taipei, Taiwan) and TOP10F' Competent Cells (Invitrogen, Carlsbad, CA, USA). A total of 72 clones were randomly selected from each library and the inserted product was PCR re-amplified with the M13 primers supplied with the T&A Cloning Kit. Operational taxonomic units (OTUs) were identified with restriction fragment length polymorphism using enzymes MspI and RsaI (New England BioLabs, Beverly, MA, USA). Clones representative of each OTU group were sequenced with the M13 forward primer (Macrogen Inc., Seoul, Korea). A cyanobacterial 16S rRNA gene clone library was also constructed from seawater sampled above the coral colonies using the method outlined above. Cloning and sequencing of a total of 96 randomly selected clones of this sample were performed at Australian Genome Research Facility Ltd (Brisbane, Australia).

Bacterial 16S rRNA gene clone libraries were constructed from microbial samples derived from three coral colonies (colonies 1, 2 and 3), each with a characteristic CP lesion that was repeatedly sampled throughout its transition into BBD (lesions were sampled from 24 August to 22 September, 7 October to 10 December and 24 August to 23 November, respectively). PCR amplification of an approximately 1300-bp region was performed with bacterial-specific forward primer 63F (CAGGCCTAACA CATGCAAGTC) and reverse primer 1387R (GGGCG GWGTGTACAAGGC) (Marchesi et al., 1998). Each reaction mixture of 50 µl contained 0.2 µM of each primer, 0.2 mM of each dNTP, $1 \times$ Buffer (HotStarTaq DNA Polymerase Kit, Qiagen), 1 U of HotStarTaq DNA Polymerase (Qiagen) and 2–10 ng of template DNA. Amplification was performed with initial heating at 95 °C for 15 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, followed by a final extension step at 72 °C for 10 min. PCR products were purified with the QIAquick Gel Extraction Kit (Qiagen) and ligated into the TOPO TA cloning vector (Invitrogen). Cloning and sequencing of a total of 96 random clones per library using the M13 forward primer were performed by the Australian Genome Research Facility Ltd.

Terminal restriction fragment length polymorphism (T-RFLP) analysis

Cyanobacterial community profiles of five lesions that visually changed from CP to BBD (lesions were sampled from 24 August to 22 September, 7 October to 10 December, 24 August to 23 November, 25 October to 10 December and 27 January to 15 February, respectively) were examined by T-RFLP. Cyanobacterial 16S rRNA genes were PCR amplified using the same reaction mixture and conditions as outlined for the cyanobacterial clone libraries, except that 0.1 µM CYA106F with the WellRED D4 fluorescent label at the 5' end (Sigma-Aldrich) and

0.1 μM CYA781R were used as the primers. Labeled PCR products were quantified with a NanoDrop Spectrophotometer (Thermo Fisher Scientific) and 75 ng of each product was digested with the restriction enzyme RsaI (New England Biolabs). Digested fragments were purified with the DyeEx 2.0 Spin Kit (Qiagen). Lengths of terminal restriction fragments (T-RFs) were determined and visualized using the CEQ8800 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA) with a size standard (600bp, Beckman Coulter) following the manufacturer's instructions. Resulting T-RFLP profiles were compared with ones obtained from cyanobacterial clones representing each of the OTUs in the same manner.

Phylogenic analysis of clone sequences

Retrieved cyanobacterial and bacterial 16S rRNA gene clone sequences were visualized and vector sequences were removed with the sequence analysis package Sequencher (Gene Codes Corporation, Ann Arbor, MI, USA). Sequences were imported into the ARB software package (http://www.arb-home.de) (Ludwig et al., 2004), and aligned against the Greengenes database (http://greengenes.lbl.gov) (DeSantis et al., 2006), followed by a manual correction of the alignment when necessary. Additional reference sequences not available in the Greengenes database at the time of analysis were identified from BLAST searches (Altschul et al., 1997) of sequences retrieved in this study, imported and aligned using ARB. Phylogenetic trees were constructed using the neighbor-joining (Jukes–Cantor correction) (Saitou and Nei, 1987) algorithms implemented in ARB. The robustness of the inferred tree topologies was evaluated after 1 000 bootstrap replicates of the neighbor-joining data. Partial sequences (∼700 bp) were inserted into the tree without changing the tree topology by using the ARB parsimony interactive method.

Nucleotide sequence accession numbers

All unique sequences retrieved from this study have been deposited in the GenBank database under accession numbers GQ204788–GQ204978.

Results

Field observations of CP and BBD

Cyanobacterial patch lesions on laminar and encrusting colonies of *Montipora* characteristically appeared either as small (1–5 cm in diameter), pale green/brown, semicircular lesions at the edge of colonies or as round patches surrounded by apparently healthy coral tissue in the middle of colonies (Figure 1a: CP). CP lesions typically expanded radially across coral tissues and subsequently darkened at their edges (intermediate stage; Figure 1b: IM), leaving exposed skeleton that gradually became covered with algal turf behind the lesion front. Darkened edges of CP lesions visually changed into the dark microbial mat characteristic of BBD, which also migrated across coral colonies, killing tissue and leaving freshly exposed white skeleton (Figure 1c: BBD). From a total of 262 CP lesions recorded on *Montipora* colonies monitored between September 2006 and January 2009, 18.7% developed into the visually characteristic BBD lesions. The remaining CP lesions disappeared leaving exposed coral skeleton covered with turf algae without an actively progressing microbial mat. These CP-derived BBD lesions accounted for 18.6% of a total of 263 BBD cases that were observed within this *Montipora* assemblage during the same period. Sources of infection for the remaining BBD lesions were unknown, except for 1.5% of infections that were apparently caused by direct contact with a BBD lesion on a neighboring colony.

The 2.3-year monitoring program documented that maximum prevalence of CP occurred between November and December each year, followed by a peak in BBD prevalence 40–50 days later (Figure 2). Specifically, characteristic BBD lesions developed from tagged CP lesions 62 ± 5 days (mean $\pm$ s.e., $n = 49$ lesions) after the corresponding CP was first recorded.

Annual mean ($\pm$ s.e.) rates of linear progression for lesion fronts were 0.47 ± 0.03 mm per day for CP ($n = 55$ lesions) and 2.38 ± 0.12 mm per day for BBD ($n = 65$ lesions). Linear progression rates of BBD were significantly higher than those of CP lesions, regardless of the season (two-way ANOVA, $F = 160.5$, df $= 1$, $P < 0.001$: Figure 3). CP and BBD lesions both progressed more rapidly in summer than in winter.
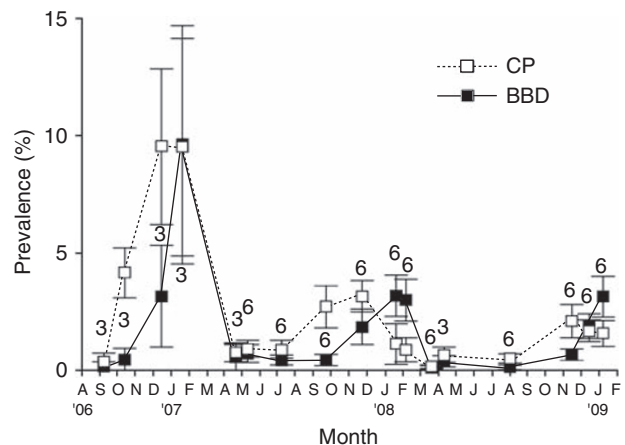
Figure 2 Temporal patterns in the prevalence of CP and BBD in a *Montipora* species assemblage on the east coast of Pelorus Island, central Great Barrier Reef. Plots and error bars represent mean prevalence ± s.e. Numbers above or below plots indicate sample size (quadrats; note that three quadrats were established in May 2007 and were not accessible in April 2008). Abbreviations: BBD, black band disease; CP, cyanobacterial patch.
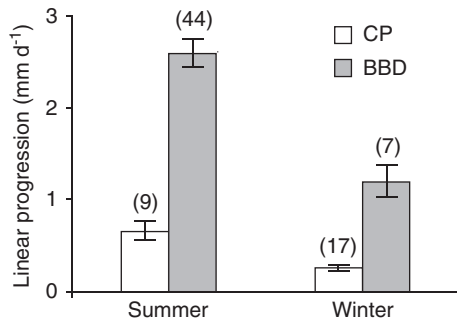
Figure 3 Linear progression rates of CP and BBD on *Montipora* species during summer and winter. Columns and bars represent means ± s.e. Numbers in brackets indicate sample size (lesions). Abbreviations: BBD, black band disease; CP, cyanobacterial patch.

Microscopic observations of CP and BBD
Histological examination revealed that microbial mats of both CP and BBD were dominated by cyanobacterial filaments, although the dominant cyanobacteria within the respective microbial mats were morphologically distinct (Figures 4a and b). Cyanobacterial filaments within CP lesions were typically twofold greater in diameter (9.0–9.2 µm) than filaments in the BBD mat (4.0–4.2 µm). Both CP- and BBD-derived cyanobacteria showed gliding motility. At the interface between the CP lesion and apparently healthy tissue, free body wall coral tissues (for example, tentacles, oral disks and coenosarc) were degraded beneath the CP bacterial mat, however, aboral structures (calicoblastic layer and lower mesentries) appeared intact (Figure 4c). In contrast, tissue structure beneath the BBD mat at the interface area was severely disorganized from the oral to the aboral surface (Figure 4d). Trichomes penetrating into CP-infected coral tissue were observed at the base of apparently intact epidermis (Figure 4e), whereas BBD cyanobacteria were observed throughout the necrotic tissue mass including the gastrodermis (Figure 4f).

Molecular comparisons of cyanobacteria between CP and BBD
Cyanobacterial-specific 16S rRNA gene clone libraries derived from microbial mats were distinct for CP and BBD lesions (Table 1). CP clone libraries ($n = 2$ lesions) were dominated by sequences (designated as CL35–CP–OTU1) closely affiliated with a *Blennothrix* sp. In contrast, BBD libraries ($n = 2$ lesions) were dominated by sequences (designated as CL36–BBD–OTU1) closely related to ones that have been retrieved from BBD lesions on Red Sea corals (BB1S16SI-18). This BBD dominant clone type, CL36–BBD–OTU1, also appeared in the CP libraries, although it represented only 1.5% of each library. Similarly, the CP dominant clone type, CL35–CP–OTU1, was retrieved from the BBD lesion of colony 1, representing 11.6% of the clone library. Ambient seawater collected above the diseased corals showed

a higher diversity of cyanobacterial ribotypes, although they were distinct from ribotypes retrieved from CP and BBD lesions, with only one sequence (CL37–SW–OTU1), related to an uncultured *Synechococcus* species MC21, shared with one CP library (colony 1).

Terminal restriction fragment length polymorphism (T-RFLP) profiles of amplified cyanobacterial 16S rRNA genes illustrated successional changes in dominating T-RFs from 343/344 to 149 nucleotide length fragments as CP changed into BBD (Figure 5). Five replicate coral colonies showed this identical T-RF pattern (including colonies 1–3, data not shown). T-RFLP analyses of representative cyanobacterial 16S rRNA gene clones confirmed that the 343/344 and 149 peaks corresponded to CL35–CP–OTU1 and CL36–BBD–OTU1, respectively, which also matched with theoretical lengths of T-RFs of CL35–CP–OTU1 and CL36–BBD–OTU1 digested with the *Rsa*I restriction enzyme.

Phylogenetic analysis of 16S rRNA gene partial sequences indicated that the dominant cyanobacterial sequences retrieved from the CP and BBD lesions were positioned in two distant groups in comparison with other reference cyanobacteria (Figure 6). CL35–CP–OTU1 formed a cluster related to *Trichodesmium* species, although it was more closely affiliated with a group including a *Blennothrix* species, an *Oscillatoria* sp. PAB-21 and PNG-50 retrieved from a BBD lesion on corals in Papua New Guinea. CL36–BBD–OTU1 and closely related BBD cyanobacterial sequences retrieved from corals in the Red Sea (BB1S16SI-18), Palau (BMS1) and the Caribbean (128–56 and CD1C11) formed a distinct group.

Bacterial shifts during the transition from CP to BBD
The composition of bacterial 16S rRNA gene clone libraries derived from CP and BBD lesions sampled from the same colony showed distinctive phylogenetic shifts in higher bacterial taxonomic groups as the lesions transitioned from CP to BBD ($n = 3$ colonies, Supplementary Tables S1). In general, *Alphaproteobacteria*-affiliated sequences were dominant in CP clone libraries (56.8–63.6% of libraries), followed by *Gammaproteobacteria*-related sequences (26.1–33.0% of libraries). In contrast, BBD libraries were dominated by sequences affiliated to *Gammaproteobacteria* (23.0–57.0% of libraries). *Alphaproteobacteria* represented 30.1% of the BBD library of colony 1 but were only a minor constituent (5.5–11.5%) of the other two replicate libraries. Cyanobacteria were also more commonly retrieved from BBD libraries than CP libraries, representing 57.7% of clones in the BBD library of colony 3. *Deltaproteobacteria*-affiliated sequences, including *Desulfobacter* sp. and *Desulfovibrio* sp., were retrieved from two BBD libraries, although one *Desulfovibrio* sp.-affiliated sequence was also retrieved from one CP library (colony 2).
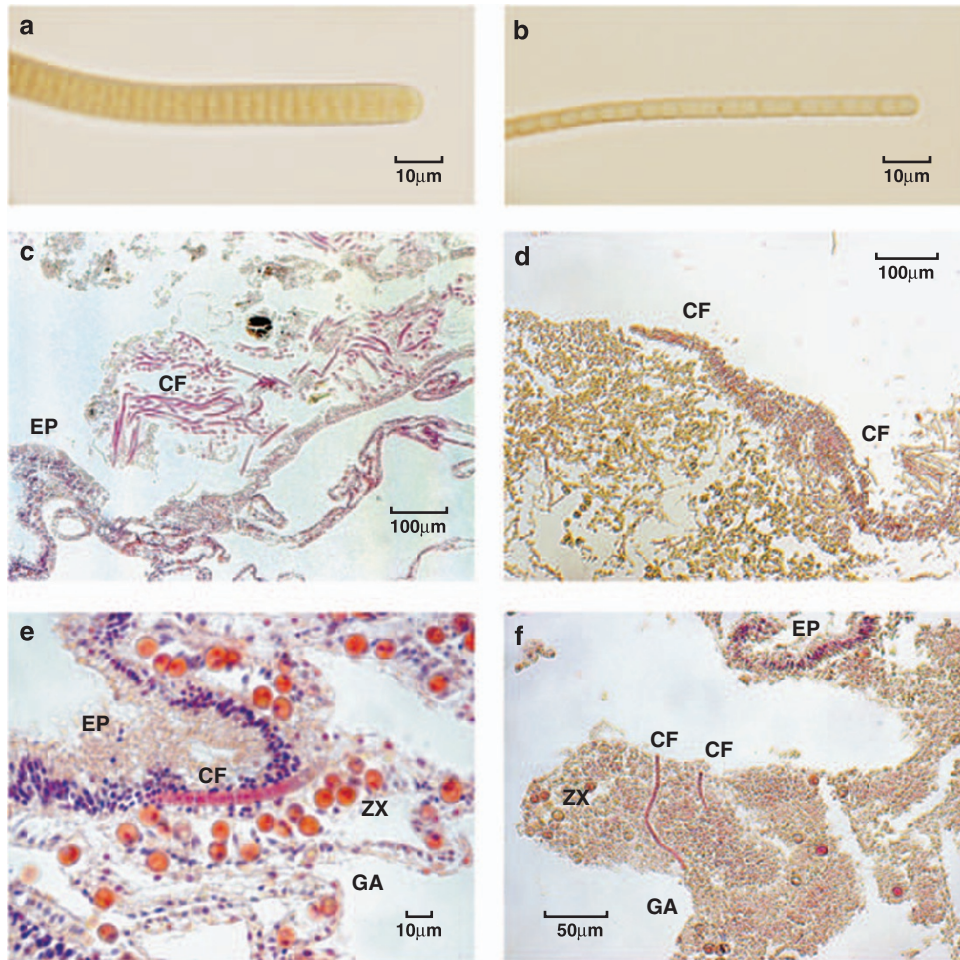
Figure 4 Microscopic images showing typical appearance of cyanobacterial filaments derived from: (**a**) CP and (**b**) BBD. Histological appearance of the interface between a microbial mat and tissues of the coral, *Montipora hispida,* for: (**c**, **e**) CP; and (**d**, **f**) BBD. Abbreviations: BBD, black band disease; CF, cyanobacterial filament; CP, cyanobacterial patch; EP, epidermis; GA, gastrodermis; ZX, zooxanthella.

Epsilonproteobacteria- and *Deferribacteres*-related sequences were retrieved only from BBD clone libraries.

The bacterial communities of disease lesions showed successional changes during the transition from CP to BBD, as illustrated by clone libraries constructed for a lesion on colony 3 that was repeatedly sampled (Figure 7 and Supplementary Table S2). First, the libraries from the early stages of the CP lesion (CP1 and CP2) contained a limited number of cyanobacterial-affiliated sequences. Even at the intermediate stage (IM), the relative number of cyanobacterial sequences represented only 7.3% of the clone library, although interestingly, these cyanobacterial sequences shifted from those dominating the early CP libraries (CP1 and CP2) to sequences closely related to previously retrieved BBD cyanobacterial sequences (Supplementary Table S2). When the characteristic BBD signs were first visually observed (BBD1), the relative number of cyanobacterial sequences increased to 42.6% and increased further as the lesion developed into a rapidly expanding BBD lesion (57.7% of BBD2 library). Secondly, coinciding with this increase in relative number of cyanobacterial-affiliated sequences, *Epsilonproteobacteria*-affiliated sequences were retrieved in the BBD1 and BBD2 libraries, although these represented only a small relative percentage of clones within the libraries (1.1–2.6%), whereas the relative abundance of alphaproteobacterial sequences declined. Thirdly, *Desulfovibrio* spp.-affiliated sequences first appeared in the IM library and subsequently formed small percentages (1.3–4.6%) of the BBD1 and BBD2 libraries. The identity of sequences retrieved from all clone libraries and their closest affiliations are compared in Supplementary Table S3. Supplementary Table S3 also illustrates bacterial sequences retrieved from a healthy coral tissue sample (colony 4—HC) and highlights the distinctiveness of this library, which shares only a few individual sequences with CP- and BBD-derived clone libraries.

Table 1 Compositions and identity of partial 16S rRNA gene sequences amplified with cyanobacterial-specific primers (CYA106F/CYA781R) in clone libraries derived from microbial mats of cyanobacterial patch and black band disease on two *Montipora hispida* colonies and ambient seawater above the sampled colonies

Sequence ID	Accession number	Closest relative[a] (Accession no.)	Identity[a]	colony 1[b]		colony 3		SW (24 Aug.'07)
				CP (24 Aug.'07)[c]	BBD (22 Sep.'07)	CP (7 Oct.'07)	BBD (10 Nov.'07)	
CL35–CP–OTU1	GQ204788	*Blennothrix* sp. PNG05–4 (EU253968)	98%	61	8	60		
CL36–BBD–OTU1	GQ204789	Uncultured BBD cyanobacterium BB1S16SI-18 (EF433097)	99%	1	55	1	68	
CL37–SW–OTU1	GQ204790	Uncultured *Synechococcus* sp. MC21 (DQ903987)	99–100%	1		1		21
CL35–CP–OTU3	GQ204791	Uncultured cyanobacterium 1DP1-B21 (EU780374)	99%					
CL28–BBD–OTU3	GQ204792	*Trichodesmium erythraeum* IMS101 (CP000393)	94%		1			
CL37–SW–OTU2	GQ204793	*Prochlorococcus marinus* MIT 9312 (CP000111)	99%					49
CL37–SW–OTU3	GQ204794	Uncultured *Plectonema* sp. C8 (DQ072876)	96%					2
CL37–SW–OTU4	GQ204795	*Synechococcus* sp. RCC307 (CT978603)	99%					2
CL37–SW–OTU5	GQ204796	Uncultured cyanobacterium A822 (EU283559)	98%					1
Non cyanobacterial sequences			—	5	5	5		8
Sum				68	69	67	68	83

Abbreviations: BBD, black band disease; CP, cyanobacterial patches; SW, seawater sample.
[a]Sequences were aligned to the closest relative over 700bp using BLAST (Altschul *et al.*, 1997). The similarity was calculated with gaps not taken into account.
[b]Labeling of colonies corresponds with Materials and methods.
[c]Dates represent when each sample was collected.
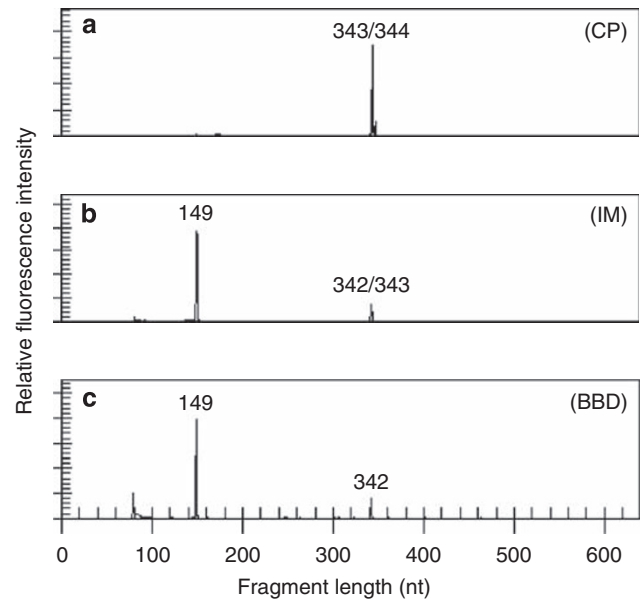
Figure 5 Results of terminal restriction fragment length polymorphism (T-RFLP) analyses of microbial communities associated with lesions on the coral, *Montipora hispida* (colony 3), showing cyanobacterial communities from: (**a**) CP (7 October 2007), (**b**) an intermediate stage between CP and BBD (25 October 2007) and (**c**) BBD (10 November 2007). Approximately 700-bp partial sequences of 16S ribosomal RNA (rRNA) genes were PCR amplified with cyanobacterium-targeting primers and digested with the *Rsa*I restriction enzyme. Numbers above peaks indicate exact sizes of T-RFs. Abbreviations: BBD, black band disease; CP, cyanobacterial patch; IM, intermediate stage between CP and BBD.

Discussion

Field monitoring throughout seasonal outbreaks of BBD within an assemblage of *Montipora* species in the central GBR confirmed that, in some cases, characteristic BBD develops from visually distinctive, cyanobacterium-infected lesions, herein named 'cyanobacterial patch(es) (CP)'. The 40- to 50-day earlier peak in the prevalence of CP compared with the peak in BBD prevalence, which roughly coincides with the average length of time for individually monitored CP lesions to develop into BBD, provides corroborative evidence that CP represents an early successional stage in the development of some BBD cases.

Histological and microbiological investigations indicated that CP and BBD lesions comprised morphologically and phylogenetically distinct cyanobacterial assemblages and that this shift occurred through successional changes in microbial assemblages, which correlated with field observations of disease signs. Cyanobacterial 16S rRNA gene clone libraries derived from CP lesions were dominated by *Blennothrix* sp.-affiliated sequences (CL35–CP–OTU1), whereas libraries from BBD lesions were dominated by a phylogenetically distinct sequence (CL36–BBD–OTU1), which formed a tight cluster with sequences retrieved from BBD mats in the Red Sea (Barneah *et al.*, 2007), Palau (Sussman
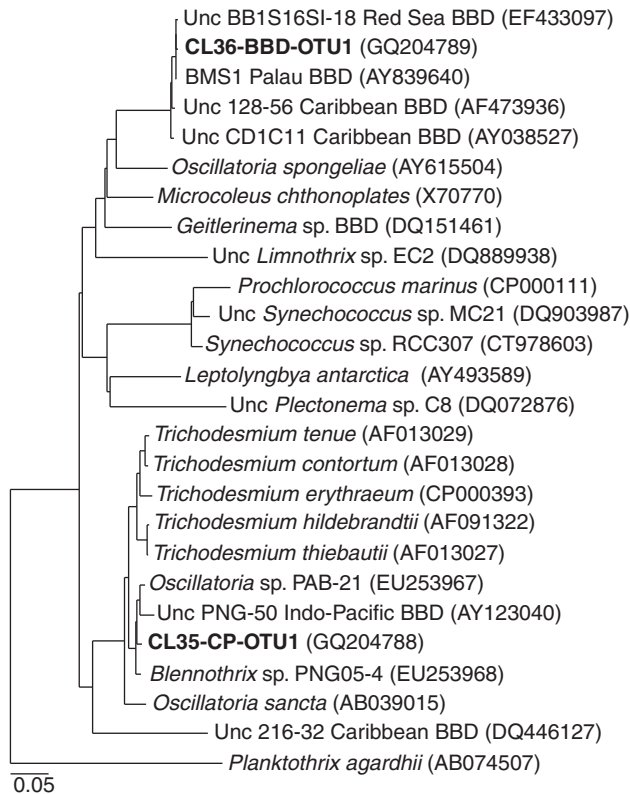
Figure 6 Phylogenetic neighbor-joining tree of approximately 700-bp partial 16S ribosomal RNA (rRNA) gene sequences of cyanobacteria derived from cyanobacterial patches (CP) (CL35–CP–OTU1) and black band disease (BBD) (CL36–BBD–OTU1) with other reference sequences including cyanobacteria previously retrieved from BBD samples (labeled with 'BBD'). Bar indicates 0.05 change per base position.
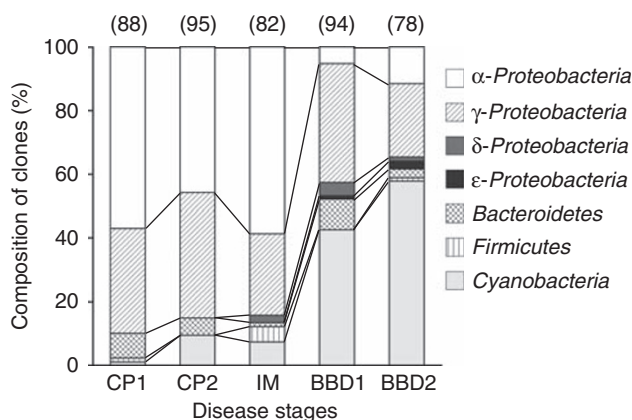
Figure 7 Comparison of clone library compositions of bacterial 16S ribosomal RNA (rRNA) genes amplified with bacterial-specific primers (63F and 1378R) throughout the transition from cyanobacterial patch (CP1, 25 August 2007; CP2, 07 October 2007), intermediate stage (IM, 25 October 2007) and BBD (BBD1, 10 November 2007; BBD2, 23 November 2007) from the identical lesion on a *Montipora hispida* colony (colony 3). Numbers in brackets above columns indicate sizes of clone libraries (sequences).

et al., 2006) and the Caribbean (Cooney *et al.*, 2002; Frias-Lopez *et al.*, 2003). T-RFLP analyses of replicate samples ($n = 5$ colonies) all supported this

successional change in dominant T-RFs, with peaks corresponding to CL35–CP–OTU1 in CP and CL36–BBD–OTU1 in BBD samples. Cyanobacterium-affiliated sequences in 16S rRNA gene clone libraries constructed with the bacterial-specific 63F/1387R primers also support distinct CP and BBD cyanobacterial assemblages: Red Sea BBD cyanobacterium (BB1S16SI-18)-affiliated sequences dominated cyanobacterial portions of BBD libraries, whereas sequences affiliated with an *Oscillatoria* sp. PAB-21 appeared in CP libraries, which are phylogenetically close to *Blennothrix* sp. PNG05–4 (98% sequence identity).

Phylogenetically, the dominant cyanobacterial sequence derived from CP lesions (CL35–CP–OTU1) clusters with *Trichodesmium* spp. sequences and is closely related to a *Blennothrix* (=*Hydrocoleum*) sp.-affiliated sequence. *Hydrocoleum* spp. are the most common mat-forming benthic cyanobacteria in tropical oceans (Abed *et al.*, 2003), and they contribute the major component of nitrogen fixation in tropic lagoons (Charpy *et al.*, 2007). If this cyanobacterium within the CP mat functions similarly, it may have an important role in bacterial succession from CP to BBD by increasing local concentrations of fixed nitrogen and creating new niches for other bacteria utilizing these products. *Hydrocoleum* spp. are also morphologically and phylogenetically close to planktonic species of *Trichodesmium*, suggesting a common evolutionary origin (Abed *et al.*, 2006). Interestingly, *Trichodesmium* spp.-related 16S rRNA gene sequences have been retrieved previously from BBD bacterial communities as dominant cyanobacterial sequences, such as a *Trichodesmium tenue*-affiliated (93% sequence identity) sequence from the Caribbean (Frias-Lopez *et al.*, 2002) and a sequence designated as PNG-50 from the Indo-Pacific (Frias-Lopez *et al.*, 2003). Together, these results suggest that the '*Trichodesmium/Hydrocoleum* cluster' (Abed *et al.*, 2006) includes benthic cyanobacterial species that dominate pathogenic bacterial mats on live coral colonies such as BBD and CP. Sequences affiliated with planktonic species recovered from a seawater clone library, such as *Trichodesmium erythraeum* IMS101 and *Synechococcus* sp. MC21, also occurred in CP and BBD clone libraries. However, as these sequences constitute only minor components in the CP and BBD clone libraries, it is unclear whether they are contaminants from the ambient water column or cyanobacteria playing specific roles in CP and BBD microbial communities.

Linear progression rates of BBD lesions were fivefold higher than those of CP lesions, suggesting that the BBD bacterial community is more virulent than the CP community. Although trichomes of both CP- and BBD-dominating cyanobacterial morphotypes showed gliding motility and penetration into coral tissues, BBD cyanobacteria were observed at deeper histological layers within coral polyps than

CP cyanobacteria, suggesting their greater potential for mechanical and/or chemical destruction of coral tissues. However, it is unclear whether this is because of a greater ability of the BBD cyanobacteria to penetrate into coral tissue or because the coral tissue was already degraded by other virulence factors associated with the BBD bacterial community. Higher biomass of cyanobacteria in the BBD mat, suggested by the higher representation of cyanobacterial-affiliated sequences within BBD clone libraries, is also possibly an important factor that promotes faster progression of BBD lesions through formation of a dense microbial community that allows development of anoxic zones and consequently substantial diurnal changes between oxic and anoxic conditions (Carlton and Richardson, 1995). Darkening in the color of lesions at the onset of characteristic BBD signs may be attributed to an increase in cyanobacterial densities. Furthermore, cyanobacterial toxins have been suggested to have a role in the pathogenicity of BBD (Richardson et al., 2007). The toxicity of cyanobacterial blooms of *T. erythraeum* and *Trichodesmium thiebautii*, phylogenetically close to CL35–CP–OTU1, has been reported from the GBR and Caribbean waters, respectively (Hawser and Codd, 1991; Endean et al., 1993). However, the presence of toxins produced by cyanobacterial strains dominating CP and BBD lesions cultured from the study site was not detected (unpublished results), although further assessment of the role of toxins in the etiology of CP and BBD is required.

Sequences involved in sulfur cycling appeared during successional changes in bacterial communities from CP to BBD, as illustrated by bacterial 16S rRNA gene clone libraries. In addition to anoxic conditions, the presence of highly concentrated sulfide at the base of the BBD mat has been shown to cause coral tissue necrosis (Richardson et al., 1997) and there is clear evidence that *Desulfovibrio* spp. and other sulfate-reducing bacteria occupy microenvironmental niches within the BBD mat (Cooney et al., 2002; Frias-Lopez et al., 2002; Sekar et al., 2006, 2008; Viehman et al., 2006; Barneah et al., 2007). This study retrieved sequences affiliated with *Deltaproteobacteria*, including *Desulfobacter* sp. and *Desulfovibrio* sp. from BBD clone libraries. One *Desulfovibrio* sp.-affiliated sequence was detected in one CP library, suggesting that these organisms may already be present in the CP bacterial mat. Interestingly, *Epsilonproteobacteria*- and *Deferribacteres*-affiliated sequences were retrieved only from BBD clone libraries. Epsilonproteobacterial sequences retrieved from BBD libraries were affiliated with *Candidatus* Arcobacter sulfidicus (94–97% sequence identity), a marine sulfide-oxidizing autotrophic bacterium that produces filamentous sulfur in sulfide-oxygen gradients (Wirsen et al., 2002). The *Deferribacteres* bacteria include iron-, manganese- and nitrogen-reducers, found in anaerobic microenvironments (Greene et al., 1997;

Myhr and Torsvik, 2000). The sulfur cycle is often tied to other elemental cycles such as nitrogen, iron, phosphorus and carbon within a microenvironment of highly diverse bacterial communities (Sievert et al., 2007). Thus, the presence of these *Epsilonproteobacteria* and *Deferribacteres* species, as well as *Gammaproteobacteria and Deltaproteobacteria*, within BBD libraries likely indicates that BBD lesions possess a more dynamic sulfur cycle with higher concentrations of toxic sulfide than CP lesions. Combined with higher cyanobacterial biomass, these bacterial element cycles may promote further anoxic sulfide rich microenvironments and hence faster lesion progression of BBD compared with CP. As the BBD band forms, diurnal fluctuations of sulfide and oxygen concentrations along the vertically stratified microenvironment are expected to occur (Carlton and Richardson, 1995). Under these rapidly changing microenvironmental conditions, bacteria that can withstand such extreme fluctuations possibly become dominant in the microbial community (Sekar et al., 2008). To verify microenvironmental regimes within the changing microbial mat from CP to BBD, isolation and physiological characterization of CP- and BBD-dominating cyanobacterial cultures along with microsensor studies are currently underway.

Sequences affiliated with bacteria involved in sulfur metabolism were, however, potentially underrepresented in the clone library analyses. The bacterial-specific primers used in this study (63F/ 1387R) match only 28% of *Desulfovibrio* spp.-, and <1% of *Epsilonproteobacteria*-affiliated sequences that are available in public databases (assessed by primer comparison to the Ribosomal Database Project II). Sulfide-oxidizing *Beggiatoa* spp. have been reported as constituents of the BBD microbial mat (Richardson, 1996; Viehman and Richardson, 2002; Sekar et al., 2008), although ribotypes affiliated with *Beggiatoa* spp. were not retrieved from CP or BBD clone libraries in this study. This may be due to primer bias or limitations, bias in DNA preparation (Sekar et al., 2009), and/or because sequences of *Beggiatoa* strains specifically associated with BBD have not been deposited in public databases (Frias-Lopez et al., 2002). Nonetheless, all samples in this study were treated similarly and therefore major shifts in bacterial ribotypes are deemed to reflect the actual transition in bacterial compositions from CP to BBD. Further investigation of bacterial communities using probes and primers targeting specific bacterial groups and their functional genes (for example, Barneah et al., 2007) will provide further insight into the development of the BBD bacterial consortium and roles of key members in the transition from CP to BBD.

Approximately, 19% of CP lesions developed into BBD, however, it is unclear what factors govern whether a CP lesion develops into BBD or loses the active microbial front. Overall, bacterial ribotype

composition derived from a CP lesion that did not develop into BBD was similar to that of CP lesions which did develop into BBD (Y Sato, 2008, unpublished data). Further investigations of intra-colony variation in bacterial members within CP lesions and of host immune responses are required to identify factors driving the development of BBD from CP lesions. Although BBD has been reported from throughout the GBR on multiple host species (Page and Willis, 2006), CP lesions have been observed only on *Montipora* species in the central, inshore GBR. Therefore species- and location-specific disease mechanisms leading to the onset of BBD may exist. Earlier reports have shown that BBD can be triggered from white band disease (Antonius, 1985) on scleractinian corals despite the absence of a visible microbial mat in this case. This study indicates that microbial lesions with a distinct bacterial community such as CP may develop into the characteristic BBD through successional changes in bacterial communities, but this may not be the only or even the major mode of onset of BBD.

In summary, BBD lesions showed faster progression and hence higher virulence than CP lesions, potentially caused by distinct bacterial assemblages in the BBD microbial mat, such as the dominant cyanobacterial and sulfur-cycle-associated species. Formation of the characteristic BBD band may be facilitated by the bacterial community in CP lesions through alteration of microenvironmental conditions and creation of microecological niches. Differences in bacterial composition between CP and BBD also suggest that bacterial pathogen(s) that trigger the BBD infection may become less abundant during subsequent successional changes. This may be one reason why identifying the primary causative agent(s) of BBD has been difficult. Further investigations of key functional genes and their expressions at different disease stages in the CP–BBD system will provide valuable information to elucidate what role each bacterial member performs within the microbial communities and thus, which bacteria are key players in the development of BBD pathogenesis.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

References

Abed RMM, Golubic S, Garcia-Pichel F, Camoin GF, Sprachta S. (2003). Characterization of microbialite-forming cyanobacteria in a tropical lagoon: Tikehau Atoll, Tuamotu, French Polynesia. *J Phycol* **39**: 862–873.

Abed RMM, Palinska KA, Camoin G, Golubic S. (2006). Common evolutionary origin of planktonic and benthic nitrogen-fixing oscillatoriacean cyanobacteria from tropical oceans. *FEMS Microbiol Lett* **260**: 171–177.

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W *et al.* (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**: 3389–3402.

Antonius A. (1981). The 'band' diseases in coral reefs. *Proc Fourth Int Coral Reef Symp* **2**: 7–14.

Antonius A. (1985). Black band disease infection experiments on hexacorals and octocorals. *Proc Fifth Int Coral Reef Cong* **6**: 155–160.

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.

Borger JL, Steiner SCC. (2005). The spatial and temporal dynamics of coral diseases in Dominica, West Indies. *Bull Mar Sci* **77**: 137–154.

Bruckner AW, Bruckner RJ. (1997). The persistence of black-band disease in Jamaica: impact on community structure. *Proc Eighth Int Coral Reef Symp* **1**: 601–606.

Bruckner AW, Bruckner RJ, Williams EH. (1997). Spread of a black-band disease epizootic through the coral reef system in St Ann's Bay, Jamaica. *Bull Mar Sci* **61**: 919–928.

Carlton RG, Richardson LL. (1995). Oxygen and sulfide dynamics in a horizontally migrating cyanobacterial mat — Black band disease of corals. *FEMS Microbiol Ecol* **18**: 155–162.

Charpy L, Alliod R, Rodier M, Golubic S. (2007). Benthic nitrogen fixation in the SW New Caledonia lagoon. *Aquat Microb Ecol* **47**: 73–81.

Cooney RP, Pantos O, Tissier MDAL, 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.

DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K *et al.* (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol* **72**: 5069–5072.

Endean R, Monks SA, Griffith JK, Llewellyn LE. (1993). Apparent relationships between toxins elaborated by the cyanobacterium *Trichodesmium erythraeum* and those present in the flesh of the narrow-barred spanish mackerel *Scomberomorus commersoni*. *Toxicon* **31**: 1155–1165.

Frias-Lopez J, Bonheyo GT, Jin QS, Fouke BW. (2003). Cyanobacteria associated with coral black band disease in Caribbean and Indo-Pacific Reefs. *Appl Environ Microbiol* **69**: 2409–2413.

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.

Goreau TJ, Cervino J, Goreau M, Hayes R, Hayes M, Richardson L *et al.* (1998). Rapid spread of diseases in Caribbean coral reefs. *Rev Biol Trop* **46**: 157–171.

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Green EP, Bruckner AW. (2000). The significance of coral disease epizootiology for coral reef conservation. *Biol Conserv* **96**: 347–361.

Greene AC, Patel BKC, Sheehy AJ. (1997). *Deferribacter thermophilus* gen nov, sp nov, a novel thermophilic manganese- and iron-reducing bacterium isolated from a petroleum reservoir. *Int J Syst Bacteriol* **47**: 505–509.

Hawser SP, Codd GA. (1991). The toxicity of *Trichodesmium* blooms from Caribbean waters. In: Carpenter EJ, Capone DG, Rueter JG (eds). *Marine Pelagic Cyanobacteria: Trichodesmium and Other Diazotrophs*. Kluwer Academic Publishers: Dordrecht. pp 319–327.

Kaczmarsky LT. (2006). Coral disease dynamics in the central Philippines. *Dis Aquat Org* **69**: 9–21.

Klaus JS, Janse I, Heikoop JM, Sanford RA, Fouke BW. (2007). Coral microbial communities, zooxanthellae and mucus along gradients of seawater depth and coastal pollution. *Environ Microbiol* **9**: 1291–1305.

Kuta KG, Richardson LL. (2002). Ecological aspects of black band disease of corals: relationships between disease incidence and environmental factors. *Coral Reefs* **21**: 393–398.

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

Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ *et al.* (1998). Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Appl Environ Microbiol* **64**: 795–799.

Myers JL, Richardson LL. (2009). Adaptation of cyanobacteria to the sulfide-rich microenvironment of black band disease of coral. *FEMS Microbiol Ecol* **67**: 242–251.

Myers JL, Sekar R, Richardson LL. (2007). Molecular detection and ecological significance of the cyanobacterial genera *Geitlerinema* and *Leptolyngbya* in black band disease of corals. *Appl Environ Microbiol* **73**: 5173–5182.

Myhr S, Torsvik T. (2000). *Denitrovibrio acetiphilus*, a novel genus and species of dissimilatory nitrate-reducing bacterium isolated from an oil reservoir model column. *Int J Syst Evol Microbiol* **50**: 1611–1619.

Nubel U, Garcia-Pichel F, Muyzer G. (1997). PCR primers to amplify 16S rRNA genes from cyanobacteria. *Appl Environ Microbiol* **63**: 3327–3332.

Page C, Willis B. (2006). Distribution, host range and large-scale spatial variability in black band disease prevalence on the Great Barrier Reef, Australia. *Dis Aquat Org* **69**: 41–51.

Richardson LL. (1996). Horizontal and vertical migration patterns of *Phormidium corallyticum* and *Beggiatoa* spp. associated with black-band disease of corals. *Microb Ecol* **32**: 323–335.

Richardson LL. (2004). Black band disease. In: Rosenberg E, Loya Y (eds). *Coral Health and Disease*. Springer-Verlag: Heidelberg. pp 325–336.

Richardson LL, Kuta KG, Schnell S, Carlton RG. (1997). Ecology of the black band disease microbial consortium. *Proc Eighth Int Coral Reef Symp* **1**: 597–600.

Richardson LL, Sekar R, Myers JL, Gantar M, Voss JD, Kaczmarsky L *et al.* (2007). The presence of the cyanobacterial toxin microcystin in black band disease of corals. *FEMS Microbiol Lett* **272**: 182–187.

Rodriguez S, Croquer A. (2008). Dynamics of black band disease in a *Diploria strigosa* population subjected to annual upwelling on the northeastern coast of Venezuela. *Coral Reefs* **27**: 381–388.

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

Saitou N, Nei M. (1987). The Neighbor-Joining Method—a New Method for Reconstructing Phylogenetic Trees. *Mol Biol Evol* **4**: 406–425.

Sato Y, Bourne DG, Willis BL. (2009). Dynamics of seasonal outbreaks of black band disease in an assemblage of *Montipora* species at Pelorus Island (Great Barrier Reef, Australia). *Proc R Soc B Biol Sci* **276**: 2795–2803.

Schauer M, Massana R, Pedros-Alio C. (2000). Spatial differences in bacterioplankton composition along the Catalan coast (NW Mediterranean) assessed by molecular fingerprinting. *FEMS Microbiol Ecol* **33**: 51–59.

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

Sekar R, Kaczmarsky LT, 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**: 85–98.

Sekar R, Kaczmarsky LT, Richardson LL. (2009). Effect of freezing on PCR amplification of 16S rRNA genes from microbes associated with black band disease of corals. *Appl Environ Microbiol* **75**: 2581–2584.

Sievert SM, Kiene RP, Schulz-Vogt HN. (2007). The sulfur cycle. *Oceanography* **20**: 117–123.

Sussman M, Bourne DG, Willis BL. (2006). A single cyanobacterial ribotype is associated with both red and black bands on diseased corals from Palau. *Dis Aquat Org* **69**: 111–118.

Sutherland KP, Porter JW, Torres C. (2004). Disease and immunity in Caribbean and Indo-Pacific zooxanthellate corals. *Mar Ecol Prog Ser* **266**: 273–302.

Viehman S, Mills DK, Meichel GW, Richardson LL. (2006). Culture and identification of *Desulfovibrio* spp. from corals infected by black band disease on Dominican and Florida Keys reefs. *Dis Aquat Org* **69**: 119–127.

Viehman TS, Richardson LL. (2002). Motility patterns of *Beggiatoa* and *Phormidium corallyticum* in black band disease. *Proc Ninth Int Coral Reef Symp* **2**: 1251–1255.

Voss JD, Mills DK, Myers JL, Remily ER, Richardson LL. (2007). Black band disease microbial community variation on corals in three regions of the wider Caribbean. *Microb Ecol* **54**: 730–739.

Voss JD, Richardson LL. (2006). Coral diseases near Lee Stocking Island, Bahamas: patterns and potential drivers. *Dis Aquat Org* **69**: 33–40.

Wirsen CO, Sievert SM, Cavanaugh CM, Molyneaux SJ, Ahmad A, Taylor LT *et al.* (2002). Characterization of an autotrophic sulfide-oxidizing marine *Arcobacter* sp that produces filamentous sulfur. *Appl Environ Microbiol* **68**: 316–325.

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