RESEARCH ARTICLE

Cyanotoxins are not implicated in the etiology of coral black band disease outbreaks on Pelorus Island, Great Barrier Reef

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Abstract

Cyanobacterial toxins (i.e. microcystins) produced within the microbial mat of coral black band disease (BBD) have been implicated in disease pathogenicity. This study investigated the presence of toxins within BBD lesions and other cyanobacterial patch (CP) lesions, which, in some instances (∼19%), facilitated the onset of BBD, from an outbreak site at Pelorus Island on the inshore, central Great Barrier Reef (GBR). Cyanobacterial species that dominated the biomass of CP and BBD lesions were cultivated and identified, based on morphology and 16S rRNA gene sequences, as *Blennothrix*- and *Oscillatoria*-affiliated species, respectively, and identical to cyanobacterial sequences retrieved from previous molecular studies from this site. The presence of the cyanotoxins microcystin, cylindrospermopsin, saxitoxin, nodularin and anatoxin and their respective gene operons in field samples of CP and BBD lesions and their respective culture isolations was tested using genetic (PCR-based screenings), chemical (HPLC-UV, FTICR-MS and LC/MS^n) and biochemical (PP2A) methods. Cyanotoxins and cyanotoxin synthetase genes were not detected in any of the samples. Cyanobacterial species dominant within CP and BBD lesions were phylogenetically distinct from species previously shown to produce cyanotoxins and isolated from BBD lesions. The results from this study demonstrate that cyanobacterial toxins appear to play no role in the pathogenicity of CP and BBD at this site on the GBR.

Introduction

Black band disease (BBD) lesions consist of a complex microbial mat dominated by phototrophic cyanobacteria that appear on corals as bands up to 10 cm wide, separating healthy coral tissue from exposed coral skeleton (Antonius, 1981; Ruetzler & Santavy, 1983). The band migrates at a rate of up to 2 cm day^{-1}, resulting in the death of the underlying coral tissue (Richardson, 1997). BBD is widespread (Sutherland *et al*., 2004) and occurs in the families of *Pocilloporidae*, *Acroporidae*, *Faviidae*, *Poritidae*, *Pectiniidae*, *Mussidae*, *Dendrophylliidae* and *Siderastreidae* and the orders of *Alcyonacea* and *Hydrocorallina* in the GBR (Page & Willis, 2006).

Early investigations of BBD revealed three distinct potential pathogens based mainly on microscopic observations: (1) gliding, filamentous cyanobacteria that were later termed *Oscillatoria submembranaceae*; (2) gliding bacteria with sulfide inclusions; and (3) sulfate-reducing bacteria (Antonius, 1981). Later, a marine fungus was detected in a number of diseased corals (*Montastrea annularis*) in the Caribbean and proposed as a pathogen (Ramosflores, 1983). An additional cyanobacterial species was also detected that was morphologically different from *O. submembranaceae* and named *Phormidium corallyticum* (Ruetzler & Santavy, 1983). This species was believed to be the primary pathogen of BBD following the success of infection experiments with this cyanobacterial strain (Ruetzler & Santavy, 1983). However, these experiments were performed with nonaxenic clumps of BBD, and so designation of this species as pathogenic was not justified according to Koch's postulates. Further studies proposed that there is no 'primary pathogen' and that BBD lesions and transmissions are caused by a

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consortium of microorganisms (Carlton & Richardson, 1995). This conclusion was supported by data that indicated that BBD always contained dominant mixed populations of the same microorganisms. These populations included sulfide-oxidizing and sulfate-reducing bacteria as well as the phototrophic filamentous cyanobacteria.

Although BBD has been extensively studied, there are many questions still to be answered about this coral disease, not least its etiology. Microsensor studies on the BBD microbial mat provided the first insight into the oxygen and sulfide dynamics within the mat. Measurements indicated that the base of the mat in contact with the coral tissue is always anoxic, that sulfide is present and that the oxygen and sulfide interface migrates upon varying light intensities (Carlton & Richardson, 1995; Richardson *et al.*, 1997). Further, experiments demonstrated that the combination of anoxia and sulfide at the base of the microbial mat is lethal to coral tissue and therefore sulfide production is suspected to be a substantial component of BBD pathogenicity (Richardson *et al.*, 1997). Enrichment and isolation of bacteria from BBD lesions at different locations and from different coral species in the Caribbean identified two *Desulfovibrio* spp., which were proposed as essential for BBD pathogenicity (Viehman *et al.*, 2006). Other earlier studies succeeded in cultivating (nonaxenic) the sulfide-oxidizing bacteria of the BBD lesion and classified them morphologically as *Beggiatoa* spp., although their role in pathogenicity is unknown (Nelson, 2000).

A recent study suggested that the production of cyanobacterial toxins within the microbial mat of BBD may contribute to the pathogenicity of the disease (Richardson *et al.*, 2007). In this study, 22 different field samples from five coral species of the wider Caribbean were tested for the presence of cyanobacterial toxins. Furthermore, two cyanobacterial species grown in culture were tested, which had previously been isolated from a BBD lesion in the Caribbean and genetically identified as belonging to the genus of *Geitlerinema* (isolated 1991) and *Leptolyngbya* (isolated 2004). Analysis of field samples by liquid chromatography (LC)/MS identified the presence of six different microcystin (MC) variants (MC-LY, -LR, -LF, -LA, -RR and -LW) within BBD. Microcystins are cyclic peptides with a range of toxic mechanisms including inhibition of protein phosphatase and can affect a variety of organisms from terrestrial, marine and freshwater environments (Ibelings & Havens, 2005). The identification of microcystins in association with BBD mats therefore raised the hypothesis that microcystin production may function as an important factor in disease progression. Further recent studies also detected toxins in cyanobacterial cultures derived from BBD lesions as well as other marine-derived cyanobacterial cultures (Gantar *et al.*, 2009) and highlighted the potential role both microcystin and sulfide play in BBD pathobiology (Richardson *et al.*, 2009).

A long-term monitoring project of a BBD-infected *Montipora* assemblage on an inshore reef around Pelorus Island (GBR) documented recurring summer outbreaks of BBD between 2006 and 2008 (Sato *et al.*, 2009). Actively expanding green or brown cyanobacterial-infected lesions termed 'cyanobacterial patch(es)' (CP), distinct from the characteristic BBD signs, preceded the onset of BBD lesions in some cases (~19%; Sato *et al.*, 2010). These lesions appear to constitute a predisposition for BBD development through successional changes in the microbial community, demonstrating a shift in dominant cyanobacterial species as the lesion transitions from CP into BBD (Sato *et al.*, 2010). The current study tested for the presence of cyanobacterial toxins within the CP and BBD lesions to characterize the contribution of toxin production to the pathogenicity of these lesions at this site on the GBR. Dominant cyanobacterial species within both CP and BBD microbial mats, as assessed by molecular and morphological techniques, were isolated and cultivated to attain sufficient biomass for genetic and chemical analyses. Along with microbial biomass collected directly from CP and BBD lesions in the field, the presence and the production of cyanobacterial toxins were tested by both molecular and chemical methods for all samples (Table 1). Molecular probes were chosen from the literature to target cyanotoxin gene clusters of microcystin, cylindrospermopsin, saxitoxin and nodularin (Table 2). Chemical analyses for the presence of these respective toxins (including anatoxin) were performed using a stepwise approach of HPLC coupled with UV detection (HPLC-UV), MS and a protein phosphatase assay (PP2A) (Table 1).

Materials and methods

Sampling

An assemblage of *Montipora* spp. on an inshore reef at Pelorus Island (18°33′S, 146°30′E; central region of the GBR Marine Park) was monitored between September 2006 and January 2009 with recurring outbreaks of BBD observed (Sato *et al.*, 2010). Lesions of CP and BBD were sampled from freshly collected fragments of *Montipora* spp., which represents the dominant genus (33% coral cover) at this site, mainly belonging to *Montipora hispida* and *Montipora aequituberculа*, with *Montipora mollis* constituting a minor component. Coral fragments infected with either CP or BBD were immediately transported separately in natural seawater to aquarium facilities at the Australian Institute of Marine Science in Townsville, where they were maintained in the aquarium system at 25–30 °C (diurnal range) and 70% daylight shading. Microbial mats from CP and BBD lesions were sampled using sterile forceps.

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Table 1. Methods used to identify cyanotoxins and summary of results for the presence of cyanotoxins in field samples of BBD and CP mats, and cultured isolates

Method	Method summary	Specificity*	NS	Sample distribution	Collection date of field samples	Field CP	Field BBD	Cultured isolates	+ve controls
HPLC-UV	HPLC screening with UV photodiodearray detection at 238 nm. Identification of toxins confirmed by comparison of retention times, absorption spectra and spiking with standards. Detection limit = 10 $\mu g\,g^{-1}$(dried cell biomass)	MC	10	4ci (2BBD, 2CP), 6fs (4BBD, 2CP)	BBD (20/01/09, 21/01/09), CP (08/01/09)	− (2)	− (4)	− (4)	+ (1)
FTICR-MS	Fourier transform ion cyclotron resonance ESI MS. Identification of toxins by comparison of accurate molecular masses (within 0.001 AMU)	MC, CYN, ND, STX	2	2fs (2BBD)	BBD(17/01/07, 31/10/08)	NT	− (2)	NT	+ (1)
LC/MS^n ion trap	HPLC-UV detection and ESI ion trap MS. MS and MS/MS in parent ion scan to selectively monitor characteristic toxin ions and fragments. Detection limit = 10 $\mu g\,g^{-1}$(dried cell biomass)	MC	52	45ci (28BBD + 17CP), 7fs (5BBD, 2CP)	BBD (17/01/07, 25/10/07, 31/10/08, 13/11/08, 17/12/08), CP (25/10/07, 13/11/08)	− (2)	− (5)	− (45)	+ (1)
LC/MS^n quadrupole	HPLC-UV detection and triple quadrupole MS (TIS source). MS/MS fragmentation in precursor ion mode to selectively monitor the presence of the Adda side chain (*m/z* 135). Detection limit = 10 $\mu g\,g^{-1}$(dried cell biomass)	MC, CYN, ND, STX, ANA	2	2fs (BBD, CP)	BBD (21/04/07), CP (11/07/07)	− (1)	− (1)	NT	+ (1)
PP2A	Colorimetric phosphatase inhibition assay as per Heresztyn & Nicholson (2001). Detection limit = 0.02 $\mu g\,g^{-1}$(dried cell biomass)	MC	10	4ci (2BBD, 2CP), 6fs (4BBD, 2CP)	BBD (17/01/07, 25/10/07, 13/11/08, 17/12/08), CP (25/10/07, 13/11/08)	− (2)	− (4)	− (4)	+ (1)
PCR	Genetic screening for cyanotoxin gene operons	MC, CYN, ND, STX	52	45ci (28BBD + 17CP), 7fs (5BBD, 2CP)	BBD (17/01/07, 25/10/07), CP(25/10/07, 13/11/08)	− (2)	− (5)	− (45)	+ (6)

Parentheses indicate the number of respective samples.
NS, number of samples processed; fs, field sample; ci, culture isolates; CYN, cylindrospermopsin; STX, saxitoxin; ND, nodularin; ANA, anatoxin; −, not detected; +, detected; NT, not tested.

Cultivation and isolation

BBD and CP lesions were cultivated in triplicate ($n = 3$) and from two separate sampling dates (26/10/08, 16/12/08) on four different solid and liquid media [BG11, ASN III, GRUND and enriched seawater media (ESWM)]. BG11 medium was prepared as described by Rippka *et al.* (1979) with double-distilled water and used as a freshwater medium to test for obligate halophiles or halotolerance of associated cyanobacteria. ASN III was prepared as described by Rippka *et al.* (1979) and supplied with a 1000 × vitamin stock solution S-3 as described by Provasoli (1963). GRUND medium was used according to Sussman *et al.* (2006) and ESWM according to Bell *et al.* (2005). Agar plates were scored with a sterilized glass slide (Vaara *et al.*, 1979) to test and facilitate the growth of motile filamentous cyanobacteria. Cultures were incubated at 26 °C under unidirectional cool white light (12 : 12 h dark : light), and the growth response

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Table 2. Oligonucleotide primers used in this study for the amplification of cyanobacterial 16S rRNA genes and cyanotoxin synthetase genes

Primers	Sequence [5′–3′]	Target region	Annealing temperature (°C)	References
CYA106F CYA781Ra CYA781Rb	CGGACGGGTGAGTAACGCGTGA GACTACTGGGGTATCTAATCCCATT GACTACAGGGGTATCTAATCCCTTT	16S rRNA gene	60 °C	Nuebel *et al.* (1997)
HEPF HEPR	TTTGGGGTTAACTTTTTTGGGCATAGTC AATTCTTGAGGCTGTAAATCGGGTTT	Aminotransferase (AMT) domain in *mcyE* and *ndaF* modules of MC and ND operons	52 °C	Jungblut & Neilan (2006)
mcyA-Cd 1R mcyA-Cd 1F	AAAAGTGTTTTATTAGCGGCTCAT AAAATTAAAAGCCGTATCAAA	Condensation domain of *mcyA* module of the MC operon	53 °C	Hisbergues *et al.* (2003)
ndaF8452F ndaF8640R	GTGATTGAATTTCTTGGTCG GGAAATTTCTATGTCTGACTCAG	*ndaF* module of the ND operon	61 °C	Koskenniemi *et al.* (2007)
cynsulF cylnamR	ACTTCTCTCCTTTCCCTATC GAGTGAAAATGCGTAGAACTTG	Sulfotransferase domain of the *cyrJ* module of the CYN operon	60 °C	Mihali *et al.* (2008)
Sxt1-F Sxt1-R	GCTTACTACCACGATAGTGCTGCCG GGTTCGCCGCGGACATTAAA	*sxt1* module of the STX operon	65 °C	Kellmann *et al.* (2008)

was monitored under three different light regimes: 50, 35 and 25 μmol photons m^{-2} s^{-1} for the solid media and 80, 22 and 10 μmol photons m^{-2} s^{-1} for the liquid media. Nystatin was used in media (10 μg mL^{-1}) to destroy the integrity of fungal cell membranes. Germanium dioxide (GeO_2) was used at concentrations of 0.5 μg mL^{-1} to destroy the siliceous shells of diatoms, which preferentially incorporate the compound (Shea & Chopin, 2007).

Cultures were monitored at regular intervals for a period of up to 3 months and purity was assessed by microscopic observations and documented using a digital camera before sampling. Biomass (> 50 mg) was sampled from plates with sterile forceps. Liquid cultures were sampled into sterile centrifuge tubes using sterile Pasteur-pipettes or forceps, centrifuged and the pellet (> 50 mg) was stored at − 20 °C for genetic and chemical analyses.

DNA extraction, PCR amplification and phylogenetic analysis of cyanobacterial cultures

Total genomic DNA was extracted from cyanobacterial enrichment cultures and field samples using the PowerPlant DNA Isolation Kit (MO Bio Laboratories Inc.), checked by agarose (1% w/v) gel electrophoresis (1 × TAE buffer, 0.5 μg mL^{-1} EtBr) and stored at − 20 °C. Cyanobacterial 16S rRNA genes were amplified with cyanobacterial-specific primers CYA106F and an equimolar mixture of CYA781Ra and CYA781Rb, amplifying a PCR product of ~650 bp (Nuebel *et al.*, 1997). PCRs were performed as described in Sato *et al.* (2010). Sequences were manually edited (using CHROMAS LITE 2.01, Technelysium Pty Ltd) and aligned using CLUSTALW 2.04 (Thompson *et al.*, 1994) with existing closely related sequences from GenBank (NCBI) identified by BLAST analysis (NCBI). Sequences with > 97% sequence identities were grouped and treated as the same operational taxonomic unit (OTU) (Schloss & Handelsman, 2005). Phylogenetic trees were established in MEGA 4.0.2 (Tamura *et al.*, 2007) using the neighbor-joining method and the Jukes–Cantor algorithm (Jukes & Cantor, 1969). Phylogeny testing was performed by bootstrapping with 1000 iterations (Felsenstein, 1978).

Sample preparation for chemical analyses

Samples from microbial mats and cultures (> 10 mg estimated dry weight) were resuspended in 1 mL of Milli-Q water (Millipore) and cells were disrupted using a probe sonicator (60–80 W, 5 s pulsations for 1–3 min, Cole Parmer). Samples were then centrifuged at 13 000 ***g*** for 10 min at 4 °C, the supernatant was filtered through 0.45-μm membranes (Millipore) and stored at − 20 °C until later analysis. Authentic MC-LR was purchased from Merck (Germany).

Screening of BBD and CP lesions and cultures for cyanotoxins

A series of spectrometric techniques were used to chemically screen samples for microcystins, cylindrospermopsins, anatoxins, nodularins and paralytic shellfish toxins in BBD and CP microbial mats and in cultivated isolates (the detection limits for each method are listed in Table 1).

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HPLC-UV was used to identify microcystins in field samples of BBD and CP, tank cultivations and cultured isolates (Table 1). Positive controls included standard MC-LR (1 $\mu g\,mL^{-1}$ Merck) and extracts of *Microcystis aeruginosa* PCC7806. HPLC-UV was performed using a Shimadzu (Kyoto, Japan) HPLC system including an FCV-10AL gradient mixer, an LC-10AT pump and an SPD-M10A UV diode array detector. Microcystins were separated on a C18 Alltima column (250 × 4.6 mm, 5 μm, 100 Å). The mobile phases consisted of 8 mM ammonium acetate (solvent A) and 100% acetonitrile (solvent B). A 25-min gradient run was performed from 20% B to 35% B and holding for 10 min at 1 $mL\,min^{-1}$ and 25 °C. Chromatograms were monitored at 238 nm and microcystins and interfering hydroperoxyeicosatetraenoic acids (HETEs) were identified by comparing the peak retention times, absorbance maxima and spiking with the authentic MC-LR standard.

Fourier transform ion cyclotron resonance MS (FTICR-MS, Bruker BioAPEX 47e, Bruker Daltonics, Germany) was used to identify microcystins in the authentic MC-LR standard, extracts of *M. aeruginosa* PCC7806 as well as test for its presence in two BBD field sample extracts (Table 1). This method is able to determine the accurate mass of microcystins to within 0.001 AMU, targeting $[M\text{-}H]^{-}$ and $[M\text{-}2H]^{2-}$ in direct injection experiments.

The authentic MC-LR standard, extracts of *M. aeruginosa* PCC7806 and the 52 samples (from BBD- and CP-derived culture isolations as well as field samples) were subsequently analyzed by ion trap LC/MS for the presence of the expected six microcystins (MC-LY, -LR, -LF, -LA, -RR and -LW) as reported by Richardson *et al.* (2007) (Table 1). Samples were chromatographed on a C18 Alltima column (Alltech, 250 × 4.6 mm, 5 μm, 100 Å), using an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara) equipped with a quaternary solvent delivery pump, column oven (25 °C), a Gilson 215 Liquid Handler autosampler/collector, an Agilent photo diode array detector and coupled to a Bruker Esquire3000 ion trap mass spectrometer with an Apollo ESI ion source operating in the positive mode. The mobile phase consisted of 53 mM formic acid and 5 mM ammonium formate in water–acetonitrile (9 : 1 v/v) (solvent A) and 53 mM formic acid and 5 mM ammonium formate in water–acetonitrile (1 : 9 v/v) (solvent B). Each sample (200 μL) was eluted at a flow rate of 1 $mL\,min^{-1}$ with the following gradient: 80% A : 20% B to 50% A : 50% B over 25 min and isocratic 50% A : 50% B for 10 min. The molecular masses of the expected microcystins (singly and doubly charged, protonated and sodiated parent ions: $[M+H]^{+}$, $[M+2H]^{2+}$, $[M+Na]^{+}$ and $[M+H+Na]^{2+}$) were screened by analyzing the total ion chromatograms (TICs) and the individual extracted ion chromatograms (EICs). Chromatograms were also monitored at 238 nm. Microcystins were identified by comparing the peak retention times, absorbance maxima and spiking with authentic standards.

LC/MS precursor ion analysis was also performed using an API hybrid triple quadrupole/linear ion trap mass spectrometer system as per Hiller *et al.* (2007) (Table 1). Extracts were analyzed for microcystins, cylindrospermopsins, nodularins, saxitoxins and anatoxins in the precursor ion mode, allowing for the simultaneous monitoring of characteristic diagnostic fragments of different classes of cyanotoxins.

A colorimetric phosphatase inhibition assay using PP2A (Promega, Australia) and *p*-nitrophenyl phosphate as a substrate was performed according to the method of Heresztyn & Nicholson (2001) to detect trace concentrations of microcystins and nodularins in 10 representative BBD and CP lesion samples and cultured cyanobacterial samples derived from the lesions (Table 1).

Genetic screening for cyanotoxin gene operons

A total of 52 samples from BBD- and CP-derived culture isolations as well as field samples were screened by PCR for the presence of the respective cyanotoxin gene operons microcystin, nodularin, cylindrospermopsin and saxitoxin (Table 1). PCRs consisted of 0.3 μM of each primer, 1 × reaction buffer (10 × reaction buffer containing 20 mM $MgCl_2$, Scientifix), 0.2 mM of a deoxynucleotide triphosphate mix (2.5 mM dNTP Mix, Scientifix), 0.625 U of Taq-polymerase (5 $U\,\mu L^{-1}$, Scientifix) and between 10 and 100 ng of template DNA to a final reaction volume of 25 μL. Cycling was performed in an ABI 2720 thermocycler (Applied Biosystems) with an initial denaturing step of 94 °C for 3 min, followed by 35 cycles of 92 °C for 20 s, individual annealing temperature (Table 2) for 30 s and 72 °C for 1 min. The final extension at 72 °C was for 7 min. The PCR products were verified by agarose (1% w/v) gel electrophoresis (1 × TAE buffer, 0.5 $\mu g\,mL^{-1}$ EtBr).

Microcystis aeruginosa PCC7806 was used as a positive PCR control for microcystin (Tillett *et al.*, 2000), *Nodularia spumigena* NSOR10 was used for nodularin (Moffitt & Neilan, 2004; Jungblut & Neilan, 2006), *Cylindrospermopsin raciborskii* AWT205 was used for cylindrospermopsin (Mihali *et al.*, 2008) and *C. raciborskii* T3, *Anabaena circinalis* AWQC131C and *A. circinalis* AWQC118C were used for saxitoxin (Kellmann *et al.*, 2008). All 52 sample PCR reactions were tested for contaminant inhibition by seeding with 10–100 ng of the respective positive control DNA.

Results

Isolation and identification of cyanobacterial strains dominant within lesions

All cyanobacterial cultures exhibited best growth under cool-white light, 12 : 12 h light : dark cycling and showed an

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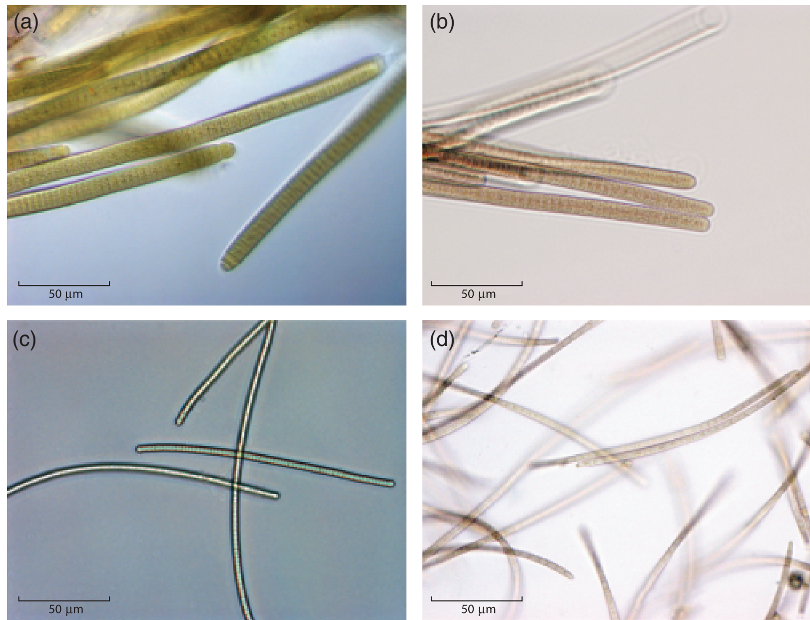

Fig. 1. Microscopic images of cyanobacterial species isolated from CP lesion (a) and the respective CP field sample (b), isolated from BBD lesion (c) and the respective BBD field sample (d).

initial phase of growth increase within the first 3–5 days. Phototactic directional movement of filaments was not observed in any of the cultures (both agar plates and liquid cultures) as reported in other studies (Sussman *et al.*, 2006; Richardson *et al.*, 2007; Myers & Richardson, 2009), although freshly collected cyanobacteria from both CP and BBD lesions demonstrated gliding motilities and cyanobacterial filaments grew in and along the groves of scored plates. Because of the lack of directional movement of cyanobacteria, culture growth on plates was limited and in general less than in liquid media. The medium ASN III resulted in the highest growth for both plate and liquid cultures, closely followed by GRUND medium and ESWM. Cultures on BG11 ceased growing after the initial growth phase and died about a week later, indicating that associated cyanobacteria are potentially obligately halophilic. Cyanobacteria isolated from CP lesions showed the greatest growth under the strongest light regimes in both solid and liquid media. For BBD-associated cyanobacteria, no significant growth trends with varying light intensities were observed (data not shown). Higher incubation temperatures, up to 35 °C, promoted the growth of BBD cultures.

Microscopic observations of cultures derived from BBD and CP lesions indicated that the cultures were monoalgal and identified distinct cyanobacterial morphologies from each lesion source (Fig. 1). Isolates from CP lesion enrichments were filamentous nonheterocystous cyanobacteria, 9.0–9.2 µm in diameter, several 100 µm in length and round at the apical ends. Trichomes were motile, straight, ensheathed and not separated by deep constrictions. Cells comprising the trichomes were highly stacked, disc-shaped and reproduction occurred by transcellular trichome breakage and the formation of hormogonia. Importantly, strains growing in culture were morphologically similar to strains observed directly from the lesions (Fig. 1a and b). Filamentous nonheterocystous cyanobacterial isolates from BBD lesion enrichments were smaller, having a diameter of 4.0–4.2 µm, length of a few 100 µm and round apical ends. Trichomes were motile, also straight, ensheathed and not separated by deep constrictions. Reproduction also occurred by transcellular trichome breakage and the formation of hormogonia; however, cells comprising the trichomes were more elongated and less packed. BBD cyanobacterial isolates derived from BBD lesions displayed morphological characteristics similar to the dominant cyanobacteria within the lesion (Fig. 1c and d).

Primers specifically targeting the cyanobacterial 16S rRNA gene were tested against a range of cyanobacterial cultures. A total of 27 isolates derived from BBD lesions displayed high sequence identities (≥98%) and were therefore identified as the same OTU. All of these obtained sequences were closely related (≥97%; Fig. 2) to other BBD cyanobacterial species (*Oscillatoria* spp. related sequences) retrieved from Indo-Pacific (AY839639), Caribbean (AF473936, AY038527, EF123645) and Red Sea (EF433097) diseased lesions. Five retrieved 16S rRNA gene sequences derived from CP isolations were closely affiliated with a *Blennothrix* spp. sequence retrieved from the reef water column in Papua New Guinea (≥98%, EU253968, Fig. 2). Importantly, the 16S rRNA gene sequences retrieved from BBD and CP isolations were identical to the dominant cyanobacterial sequences (GQ204788, GQ204789) retrieved

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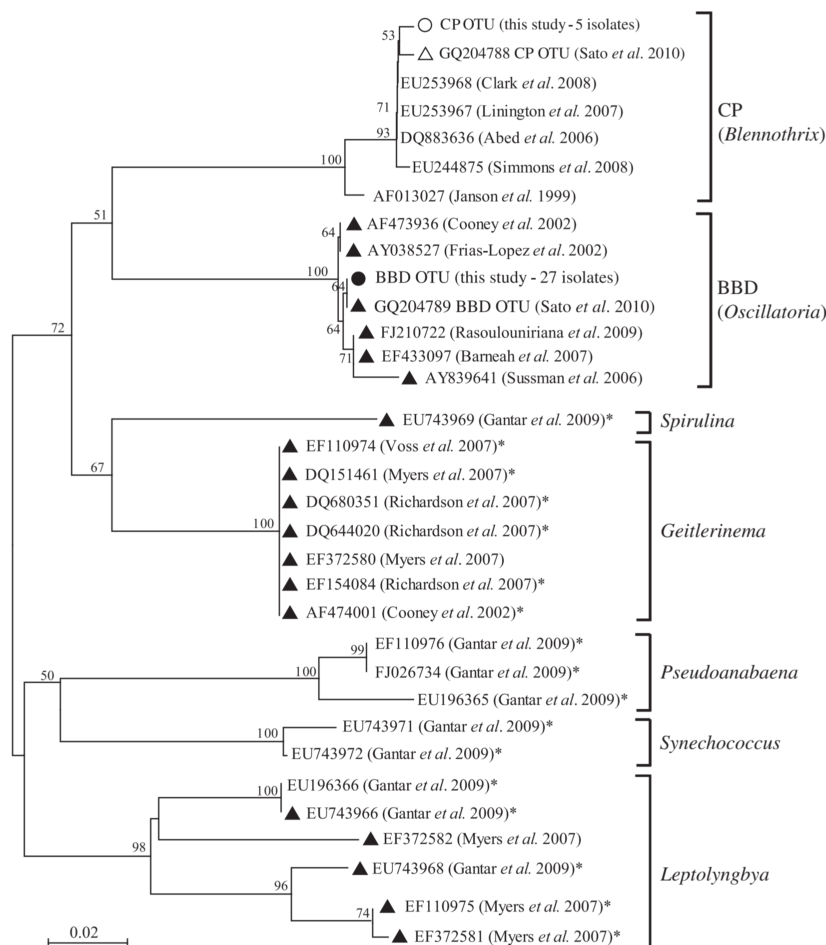

Fig. 2. Neighbor-joining phylogenetic tree of cyanobacterial 16S rRNA gene sequences from this study (●/○), their closest relatives, as well as cyanotoxin-producing strains (*). Sequences attained by other studies from BBD lesions are marked with ▲, and from CP with △. All sequences are indicated by their GenBank accession number. The tree was constructed from partial sequences (450 bp) using the neighbor-joining method and the Jukes–Cantor algorithm (Jukes & Cantor, 1969). The bar represents two substitutions per 100 nucleotide positions (2% sequence divergence). Bootstrap values > 50% (Felsenstein, 1978) are indicated at the branch nodes.

from *in situ* lesion samples from this site (Sato *et al.*, 2010), indicating that the dominant cyanobacterial species associated with BBD and CP were isolated successfully (Fig. 2).

Screening for cyanotoxin production

MC-LR was detected in extracts of the positive control cyanobacterium *M. aeruginosa* PCC7806 at approximately $100\,\mu g\,g^{-1}$ dry weight using HPLC-UV. This method also identified several peaks in BBD mat samples exhibiting UV spectra corresponding to microcystins; however, these peaks were confirmed as interfering HETEs, which absorb UV at identical wavelengths to microcystins, based on their retention times and spiking experiments (Table 1). None of the other samples showed any characteristic peaks for microcystins.

Negative mode ESI FTICR-MS analysis of *M. aeruginosa* PCC7806 generated two diagnostic signals, *m/z* 993.5493 being the singly charged $[M\text{-}H]^-$ ion and *m/z* 496.2706 the doubly charged $[M\text{-}2H]^{2-}$ ion. Comparison of exact mass measurements with the literature data and the calculated mass confirmed the molecular formula as $C_{49}H_{74}N_{10}O_{12}$ (calculated m/z 993.5415, $\Delta = 8$ ppm, calculated m/z 496.2671, $\Delta = 7$ ppm) and the compound as MC-LR. Analysis of two BBD lesion samples did not detect any ions diagnostic of any known microcystins (Fig. 3 and Table 1), confirming that the peaks observed in the HPLC-UV chromatographs of the BBD isolates were HETEs.

Positive mode LC/MS/MS parent ion analysis of *M. aeruginosa* PCC7806 extract resulted in a peak at the same retention time and with the same parent and fragment ions as the MC-LR standard, again confirming that MC-LR was present in the positive control culture. For each field and culture sample, the TIC, 24 EICs and the UV chromatogram were examined for the presence of the six expected microcystins (MC-LY, -LR, -LF, -LA, -RR and -LW). None of the six microcystin variants could be detected in any of the samples. The masses of some peaks revealed by the EICs matched those of expected doubly charged ($[M+H+Na]^{2+}$) microcystin derivatives; however, they did not match the retention times of the respective variants. Also, closer inspection of the isotope distribution of these peaks revealed that, without exception, they were singly charged molecules and thereby not related to any known microcystin derivatives.

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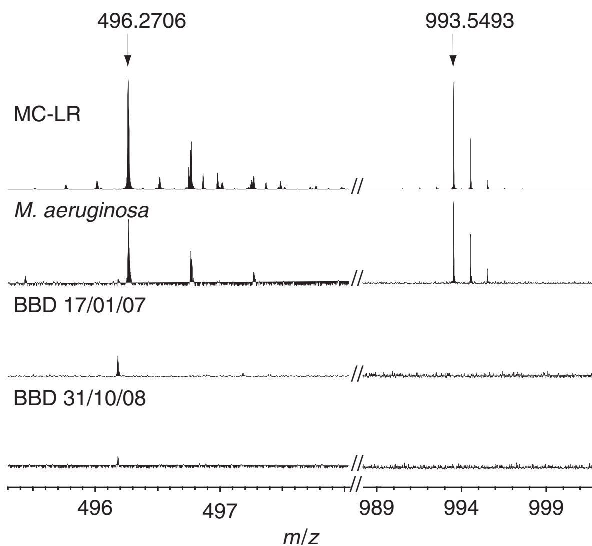

Fig. 3. Negative mode FTICR-MS analysis of the MC-LR standard, the aqueous extract of *Microcystis aeruginosa* PCC7806 and two BBD field samples (sampled 17/01/07 and 31/10/08, Pelorus Island, GBR). The $[M-H]^-$ and $[M-2H]^{2-}$ ions diagnostic for MC-LR (*m/z* 993.5493 and 496.2706, respectively) were present only in the spectra of the MC-LR standard and *M. aeruginosa*. Ions corresponding to known microcystin, cylindrospermopsin, nodularin, saxitoxin or anatoxin cyanotoxins were not observed in any of the experimental samples.

A subset of BBD and CP extracts were further analyzed using a quadrupole MS in precursor ion mode. Characteristic Adda fragmentation peaks (*m/z* 135) were detected in the *M. aeruginosa* PCC7806 extracts; however, none of the potential cyanotoxins microcystins, cylindrospermopsins, nodularins, anatoxins or paralytic shellfish poisoning toxins were detected in the blank or the two tested lesion samples (data not shown) (Table 1).

Testing for microcystin variants by PP2A assay

A total of 10 representative samples derived from cyanobacterial isolations and field samples of CP and BBD lesions tested negative for the presence of microcystins within a standard PP2A assay. Comparison of PP2A activities indicated that six samples demonstrated an increase in PP2A activity (Table 3), although this increase in absorbance is likely due to residual cellular matter such as cell debris and photopigments, as extracts derived from these samples were highly colored. Slight inhibition of PP2A was observed for samples derived from cyanobacterial strains isolated from CP lesions (ci1 and ci2) and a BBD lesion (fd1). However, the decreases in activity were not significant in those samples (8%, 5% and 5%, respectively) and below the detection limits of the assay (1 $\mu g\,g^{-1}$ dry weight). Such low levels of inhibition could not be correlated with PP2A inhibition and therefore all of the tested samples were negative for the presence of microcystins within the detection limits of the assay.

Screening for cyanotoxin gene operons

PCR using primers specifically targeting currently known cyanotoxin gene operons of microcystin, cylindrospermopsin, nodularin and saxitoxin showed specific gene amplification with positive control samples. No specific amplified PCR products were detected from the 52 samples prepared from cyanobacterial cultures and microbial mat biomass derived directly from sampled BBD and CP lesions (Table 1). Seeding of positive control DNA extracts into the 52 samples resulted in positive amplification, indicating that contaminant inhibition of the PCR did not occur. The results of this extensive PCR screening therefore demonstrated that synthetase genes of known cyanotoxins were not present in BBD and CP biomass sampled directly from the lesions or in the respective cyanobacterial culture isolations.

Discussion

The combined results of sensitive genetic and chemical screening suggest that no known microcystins or other cyanotoxins were present within the microbial mats of both BBD and CP coral disease lesions collected from the study site. The dominant filamentous cyanobacterial species within the BBD and CP lesions were successfully isolated, and similarly, these strains were shown not to produce microcystins or other cyanotoxins. Extensive chemical screening of samples including three MS approaches failed to detect parent ions or potential fragment ions characteristic of known toxins. An adapted protein phosphatase bioassay routinely used for the assessment of cyanotoxins (Heresztyn & Nicholson, 2001) produced only negative results. Finally, all samples, including field-collected lesion biomass, aquarium-maintained microbial mats and isolated cyanobacterial cultures derived from the lesions, were screened for four characterized cyanotoxin gene operons including microcystin, cylindrospermopsin, nodularin and saxitoxin using molecular approaches, and yet none of these gene operons were detected. These synthetase operons presently represent all known cyanotoxin gene operons, with the exception of anatoxin. Primer sets targeting these operons have been verified extensively in previous studies (Hisbergues *et al.*, 2003; Jungblut & Neilan, 2006; Koskenniemi *et al.*, 2007; Kellmann *et al.*, 2008; Mihali *et al.*, 2008) and therefore provide a robust assessment of the ability of cyanobacteria to produce toxins and a comprehensive approach for genetic cyanotoxin screening, further strengthening evidence that cyanobacteria isolated from both CP and BBD lesions at Pelorus Island do not produce cyanotoxins. While the presence of toxin-producing cyanobacteria may depend on seasonal variations or different stages of the lesion's

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Table 3. PP2A activity of culture isolates (ci) and microbial mats sampled from CP and BBD coral lesions both maintained in aquaria (aq) and sampled directly from the field (fd) at Pelorus Island (GBR)

			BBD isolates		CP isolates		BBD lesion*		BBD lesion†		CP lesion‡		
Sample	Control	– ve control	ci-1	ci-2	ci-1	ci-2	aq-1	aq-2	fd-1	fd-2	fd-1	fd-2	MC-LR-Std
PP2A activity (%)	100	0	104	106	92	95	159§	173§	95	100	104	143§	62

BBD and CP isolates are from two different culture enrichments.
*BBD aq-1 sampled 13/11/08 and BBD aq-2 sampled 17/12/08.
†BBD fd1 sampled 25/10/07 and BBD fd2 sampled 17/01/07.
‡CP fd-1 sampled 25/10/07 and CP fd-2 sampled 13/11/08.
§Possible color interference from the sample.

progression, such influences seem highly unlikely, given that samples from this study were collected in different seasons over 3 consecutive years (Table 1).

Sato *et al*. (2010) demonstrated that dominant cyanobacteria within CP lesions were morphologically different from species associated with BBD lesions *in situ* and confirmed, using molecular techniques, that *Blennothrix* spp. affiliated 16S rRNA gene sequences dominated CP lesions, while BBD was dominated by *Oscillatoria* spp. related sequences. The cyanobacterial species isolated from CP and BBD in this study possessed 16S rRNA gene sequences highly similar (> 98%) to those retrieved through direct DNA extraction and cloning of environmental samples (Sato *et al*., 2010). In addition, microscopic morphological characterization of isolated cyanobacterial species was consistent with the observations of field-derived samples (Fig. 1), further confirming successful isolation of the dominant species from each lesion type. The cyanobacterial species isolated from BBD lesions at Pelorus Island were closely affiliated, based on 16S rRNA gene sequence comparisons, with *Oscillatoria* spp. and other cyanobacterial ribotypes previously retrieved from BBD lesions analyzed by molecular studies around the world (Cooney *et al*., 2002; Frias-Lopez *et al*., 2003; Sekar *et al*., 2006; Sussman *et al*., 2006; Barneah *et al*., 2007; Myers *et al*., 2007; Myers & Richardson, 2009; Rasoulouniriana *et al*., 2009). The dominant cyanobacterial species isolated from CP showed a close phylogenetic affiliation to sequences retrieved from both water column samples and microbial mats from widely dispersed geographic reef environments including the Caribbean [EU244875 (Simmons *et al*., 2008), EU253967 (Linington *et al*., 2007), AF013027 (Janson *et al*., 1999)] and Indo-Pacific [EU253968 (Clark *et al*., 2008), DQ883636 (Abed *et al*., 2006)] (Fig. 2).

Optimal growth of cyanobacterial cultures derived from BBD lesions occurred under low light intensities of approximately 30 μmol photons $m^{-2} s^{-1}$. However, different light intensities (ranging from 10 up to 80 μmol photons $m^{-2} s^{-1}$) had little effect on this culture performance. High light tolerance might be a special characteristic of cyanobacterial species within BBD lesions, as many cyanobacteria are sensitive to photosystem damage at high irradiances (Castenholz, 1988). The highest cell biomass was achieved with the medium ASN III, followed by GRUND and ESWM, which is also in accordance with previous studies (Sussman *et al*., 2006; Richardson *et al*., 2007). Higher temperatures facilitated increased cell biomass in the BBD-derived cyanobacterial cultures, which was consistent with the growth dynamics of coral lesions within aquarium tanks, also observed by Boyett *et al*. (2007), and correlates with the measured seasonal BBD dynamics observed in the field (Sato *et al*., 2009). For example, at Pelorus Island (GBR), the highest prevalence of BBD lesions occurred when water temperatures reached a maximum of 32 °C in the summer months. However, cyanobacterial cultures derived from CP lesions showed greater growth under higher light levels while elevated temperatures (35 °C) slowed their growth. Interestingly, these trends in optimal culture conditions of BBD- and CP-derived cyanobacteria coincide with seasonal peaks in the prevalence of CP, which are 40–50 days earlier than the maximum prevalence of BBD and annual fluctuations in seawater temperature and light level at the study site (Sato *et al*., 2009, 2010). Together with temperature-driven transitions from CP into BBD observed under tank conditions, these results suggest that both temperature and light are environmental drivers for the development of BBD derived from CP lesions.

Cyanobacterial strains isolated in this study were phylogenetically distinct (see Fig. 2) from the two toxin-producing cyanobacterial strains *Geitlerinema* and *Leptolyngbya* previously isolated from BBD mats (Richardson *et al*., 2007; Myers & Richardson, 2009). The *Geitlerinema* and *Leptolyngbya* strains were not detected within the CP and BBD microbial mats from Pelorus Island, using molecular profiling methods (Sato *et al*., 2010), and were not isolated from the lesions in this study using targeted isolation techniques, indicating that they are unlikely to be members of the complex microbial community of coral lesions at Pelorus Island. *Geitlerinema* and *Leptolyngbya* strains were associated with BBD lesions affecting corals from a study in Papua New Guinea (Myers & Richardson, 2009), although

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other studies characterizing BBD microbial communities from the Indo-Pacific region have not reported the presence of closely affiliated strains (Frias-Lopez *et al.*, 2003; Sussman *et al.*, 2006; Richardson *et al.*, 2007). These results further underscore the complexity of microbial communities associated with BBD lesions from different reef environments being dominated by different cyanobacterial species.

Richardson *et al.* (2007) previously reported that microcystins were not universal in BBD lesion samples, indicating the potential for the regional specificity of BBD microcystins. Toxin presence within lesions is therefore likely to be dependent on the presence of toxic strains, which possess the relevant biosynthetic pathways and that are likely limited to specific cyanobacterial phylogenetic groups such as *Geitlerinema* and *Leptolyngbya* within the BBD microbial communities. Recent studies have expanded the genera of marine cyanobacteria that are able to produce cyanotoxins to include *Phormidium*, *Pseudanabaena* and *Spirulina*, although not all are associated with lesions on corals (Gantar *et al.*, 2009). All currently known marine cyanobacterial strains that produce toxins are therefore phylogenetically distinct (Fig. 2) from strains associated with BBD and CP lesions at our study site. The results from this study suggest that cyanobacterial toxins are not an essential etiological agent in CP or BBD progression or required for disease pathogenicity at this study site. Nevertheless, toxins could function to facilitate lesion progression and increase disease virulence if present. Factors other then cyanotoxins are therefore likely to be primarily responsible for BBD etiology, including anoxic conditions, high sulfide levels and proteolytic activity at the base of the microbial mat (Carlton & Richardson, 1995; Richardson *et al.*, 1997; Arotsker *et al.*, 2009; Richardson *et al.*, 2009).

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