The presence of the cyanobacterial toxin microcystin in black band disease of corals

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Abstract

Black band disease (BBD) is a migrating, cyanobacterial dominated, sulfide-rich microbial mat that moves across coral colonies lysing coral tissue. While it is known that BBD sulfate-reducing bacteria contribute to BBD pathogenicity by production of sulfide, additional mechanisms of toxicity may be involved. Using HPLC/MS, the cyanotoxin microcystin was detected in 22 field samples of BBD collected from five coral species on nine reefs of the wider Caribbean (Florida Keys and Bahamas). Two cyanobacterial cultures isolated from BBD, *Geitlerinema* and *Leptolyngbya* sp. contained microcystin based on HPLC/MS, with toxic activity confirmed using the protein phosphatase inhibition assay. The gene *mcyA* from the microcystin synthesis complex was detected in two field samples and from both BBD cyanobacterial cultures. Microcystin was not detected in six BBD samples from a different area of the Caribbean (St Croix, USVI) and the Philippines, suggesting regional specificity for BBD microcystin. This is the first report of the presence of microcystin in a coral disease.

Introduction

Coral diseases are significant factors contributing to the observed decline in living coral cover worldwide (Harvell *et al.*, 1999; Rosenberg & Loya, 2004). Sixteen of the 18 characterized diseases of corals cause lesions that actively degrade coral tissue, yet little is known about the mechanisms by which this occurs (Sutherland *et al.*, 2004). While toxins have been hypothesized to facilitate this tissue loss, little is known about coral disease-associated toxins or coral disease-associated bacteria that produce toxins (Rosenberg & Loya, 2004). For example, the bacterium *Vibrio coralliilyticus* produces an extracellular protease that causes bleaching and tissue lysis of *Pocillopora damicornis* (Ben-Haim *et al.*, 2003).

Black band disease (BBD) is a complex microbial consortium with an active sulfuretum and a diverse microbial community of phototrophs, heterotrophs, sulfate reducers and sulfur oxidizers (Richardson, 2004). It exists as a cyanobacterial mat, several millimeters to centimeters wide, which migrates across living coral colonies lysing coral tissue. Oxygen and sulfide microelectrodes revealed that the 1-mm-thick BBD mat is oxygenated at and near the surface, is anoxic and sulfide-rich in the middle to lower zones and contains an oxygen/sulfide interface that vertically migrates within the band based on light intensity (Carlton & Richardson, 1995). Thus BBD is chemically analogous to the well-studied cyanobacterial mats found in many sulfide-rich, illuminated environments (Jørgensen *et al.*, 1979).

In a previous study, experimental exposure of living coral fragments to conditions of anoxia, and to sulfide together with anoxia, caused coral tissue lysis (Richardson *et al.*, 1997). Only the combination of sulfide and anoxia resulted in tissue death over the time period in which a migrating BBD mat exposes the coral surface to the BBD microenvironment. These results indicated that BBD pathogenicity involves at least two mechanisms: production of anoxic conditions due to oxygen consumption by the BBD community and production of sulfide by BBD-sulfate reducing

bacteria. In this report, further results are presented on toxins associated with BBD microorganisms.

Materials and methods

Microcystin, saxitoxin and anatoxin analyses

Field samples for toxin analysis were collected while SCUBA diving using sterile, needleless 10-mL syringes. Samples were maintained at low light at ambient seawater temperature and, once on shore, decanted into sterile cryovials and placed on ice for transport to the laboratory where they were frozen at −20 °C. Frozen samples were shipped to the USDA-ARS laboratory in Stoneville, MS for toxin analysis. Samples were collected on reefs of the northern Florida Keys (June 2004) and Lee Stocking Island, Bahamas (July and August 2004) (Table 1) as well as on St Croix, USVI (June 2005 and July 2006) and the Philippines (August 2005) (see 'Results'). Laboratory cultures of the BBD cyanobacteria *Geitlerinema* sp. (isolated in 1991) and *Leptolyngbya* sp. (isolated in 2004) were grown in ASNIII media, harvested by filtration onto GF/F filters and frozen at −20 °C. For HPLC/MS analysis, samples (field and culture) were thawed and split into two subsamples for toxin extraction. Anatoxin-a and microcystin were extracted in 70% MeOH for 4 h (4 °C) after sonication and then filtered. HPLC detection of microcystin used a tiered approach in which UV-spectra were used to identify samples containing the 232/239 nm UV-chromophore and then MS was used for confirmation based on characteristic molecular weight and retention time (Harada, 1996). Microcystin variants were identified by comparison with standards and published atomic weight (Harada, 1996; Chorus & Bartram, 1999). Anatoxin-a was assessed using precolumn derivatization (James *et al.*, 1997) with fluorescence detection and by comparing the mass of suspected peaks to native samples for confirmation. Saxitoxins were analyzed following extraction in 5% acetic acid using post column derivatization (Oshima, 1995). A Dionex Summit HPLC equipped with photodiode array and fluorescence detectors was used for cyanotoxin analyses, and a Dionex MSQ mass spectrometer operated in electrospray mode used for mass confirmation.

PCR amplification, cloning and sequencing

Genomic DNA was extracted by the bead beating method using the FastDNA® SPIN Kit for Soil, and verified by electrophoresis in an agarose gel (1% w/v) stained with ethidium bromide. DNA was quantified using a Bio-Rad fluorometer and DNA aliquots (10–20 ng μL^{-1}) stored at −20 °C. *McyA* was PCR amplified with two primer sets: *mcyA*-Cd 1F/R (Hisbergues *et al.*, 2003) and MSF/R

Table 1. Summary of BBD field samples from reefs of Lee Stocking Island (LSI) Bahamas and the northern Florida Keys that were positive for microcystin using HPLC/MS analysis

Field location and reef	Host coral species	Water depth (m)	*T* (°C)	Colony size* (% tissue loss)	N colonies sampled (band width mm)	Microcystin variant detected
LSI, Bahamas						
Big Point	*Siderastrea siderea*	3	29	35 (10)	1 (ND)	-LA†
Horseshoe ‡	*S. siderea*	7–11	28–30	25–110 (< 5–60)	9 (6–22)	-LF†, -LY, -LW, –RR
	Montastraea annularis	10	29–30	25 (70–85)	1§ (22–26)	No id¶
	Colpophyllia natans	11	30	55 (90)	1 (14)	-LY
North Norman's	*Diploria strigosa*	3	29	40 (40)	1 (8)	No id¶
North Perry ‖	*C. natans*	17	29	320 (15)	1 (28)	-LR
Rainbow‡	*S. siderea*	4	31	25 (80)	1 (18)	-LY
Tug and Barge	*S. siderea*	2	30	15 (90)	1 (28)	-LF
White Horse	*S. siderea*	10	29	50 (50–90)	2§ (4–10)	-LY
Florida Keys						
Conch Shallow	*Meandrina meandrites*	7	30	ND (15–20)	2 (3)	-LY
Davis Ledge‖	*M. meandrites*	5	30	ND (5–25)	2 (3)	-LY

The presence of BBD *Geitlerinema* and *Leptolyngbya* (determined by DGGE and sequencing of bands) as well as samples positive for *mcyA* are indicated in footnotes.

*Colony size = maximum diameter (cm); % tissue loss is % of surface area of tissue completely lysed by BBD activity.

†Mass spectrometer signal barely above identification level (3 × baseline) thus variant identification is tentative.

‡Two BBD samples from Horseshoe Reef and one from Watson's Reef in the Florida Keys (not in Table) revealed the BBD *Geitlerinema* by DGGE and sequencing of bands. One BBD sample from Rainbow Reef revealed the BBD *Leptolyngbya* (DGGE and sequencing).

§The same band was sampled on two different dates.

¶Mass spectrometer signal did not match published list of microcystin variants.

‖The *C. natans* BBD sample and one of the *M. meandrites* BBD samples were positive for the microcystin synthesis gene *mcyA*.

See text for details.

(Tillett *et al.*, 2001). The former set is specific to the condensation (C) domain of the *mcyA* gene, and the latter specific to the *N*-methyl transferase (NMT) domain. PCR amplification was carried out in a Peltier Thermal Cycler (PTC-200, MJ Research, Waltham, MA). For *mcyA-Cd 1F/R*, an initial denaturing step at 94 °C for 10 min was followed by 40 cycles of 94 °C for 30 s, 53 °C for 30 s and 72 °C for 30 s and the final extension for 5 min at 72 °C. For MSF/R, an initial denaturing step at 94 °C for 10 min was followed by 40 cycles of 94 °C for 1 min, 47 °C for 30 s and 72 °C for 1 min and the final extension for 7 min at 72 °C. PCR products were verified by agarose (1% w/v) gel electrophoresis.

Amplified *mcyA* gene fragments from both primer sets were purified with the QIAquick® PCR purification Kit (Qiagen Inc., Valencia, CA). The purified PCR product using *mcyA-Cd 1F/R* was directly sequenced using the *mcyA*-Cd 1F primer. PCR products using MSF/R were cloned using the TOPO TA Cloning Kit® version N (Invitrogen, Carlsbad, CA). Clones were screened for inserts, plasmids extracted from positive clones (eight clones in each library) and plasmid inserts sequenced with an ABI Prism™ 3100 genetic analyzer (Applied Biosystems, Foster City, CA) at Florida International University's (FIU) DNA Core Facility using the MSF primer. Sequences were analyzed using the BLAST queuing system. *Microcystis aeruginosa* (UTCC 299) was used for a positive control and a strain of *Anabaena* that does not produce microcystin (Hisbergues *et al.*, 2003) used as a negative control.

Denaturing gradient gel electrophoresis (DGGE) analysis

DNA was extracted as described above. Extracted DNA was amplified via PCR with the cyanobacterial-specific primers GC-CYA359F and CYA781R(b) (Nübel *et al.*, 1997). PCR amplication consisted of an initial denaturing step at 94 °C for 3.5 min followed by 40 cycles of 94 °C for 30 s, 55 °C for 45 s and 72 °C for 30 s with a final extension step at 72 °C for 10 min. PCR products were analyzed by agarose gel electrophoresis before DGGE, in which PCR products were separated on a vertical polyacrylamide gel using a denaturant gradient of 45–80%. Individual bands were excised from the gel and eluted in 0.5 mL nuclease-free water for 48 h. Eluted DNA was reamplified with 0.5 μM of each primer, CYA359F and CYA781R(b). Purified products were sequenced using the CYA359F primer and analyzed using BLAST.

Protein phosphatase inhibition assays and enzyme-linked immunosorbent assay (ELISA)

At FIU BBD *Geitlerinema* and *Leptolyngbya* were grown in BG11 medium (prepared with artificial seawater) under cool-white fluorescent light (50 $\mu E\, m^{-2}\, s^{-1}$) at 24 °C. Cultures were aerated with air and harvested by filtration. Freeze-dried biomass (100 mg) was suspended in 10 mL of 50% (v/v) aqueous methanol, sonicated for 10 s and refrigerated overnight for extraction. After centrifugation the supernatant was filtered through a membrane filter (0.2 μm). The protein phosphatase inhibition assay was performed using the supernatant according to the method of Ward *et al.* (1997). The PP2A enzyme was obtained from Millipore (Temecula, CA) and microcystin-LR from Dr Kathleen Rein (FIU). Assays were run in 96-well plates and read on a Bio-Tek Instruments plate reader. A standard inhibition curve was constructed by diluting microcystin-LR in 50% methanol and by determination of the percentage inhibition of enzyme activity. These assays were conducted in triplicate. Blind samples for protein phosphatase inhibition assays, from BBD *Geitlerinema* and *Leptolyngbya* grown and filtered at the USDA laboratory, were also sent to SUNY-ESF in Syracuse, NY, for analysis using PP1A (New England Biolabs, Ipswich, MA) following the methods of Carmichael & An (1999). Additionally, culture samples were analyzed for microcystin at the USDA laboratory using an ELISA test (Envirologix QuantiPlate, Portland, ME).

Results

Anatoxin, microcystin and saxitoxin analysis

Twenty-two BBD samples were analyzed, collected from five species of corals on nine reefs of the Bahamas and the Florida Keys (Table 1), from five BBD samples on *Siderastrea siderea* and *Diploria strigosa* from two reefs in St Croix, USVI, and one sample from BBD on *Porites lutea* on a reef in the Philippines. All Bahamas and Florida Keys samples were positive for microcystins (Fig. 1) and negative for anatoxin-a and saxitoxins. BBD microcystins included the variants -LY, -LR, -LF, -LW and -RR (Table 1). None of the St Croix or Philippines samples were positive for microcystin. Both the BBD *Geitlerinema* and BBD *Leptolyngbya* cultures produced microcystin-LR as determined by HPLC/MS analysis.

Protein phosphatase inhibition and ELISA

The two BBD cyanobacterial cultures were tested for bioactivity using protein phosphatase inhibition assays in laboratories at FIU and SUNY. The FIU results indicated positive inhibition by both cultures in each of three experiments, with averaged inhibition values, as compared with the control (50% methanol) of 93% for BBD *Leptolyngbya* (0.06 μg MC-LR_{equiv} /mg dry wt) and 42% for BBD *Geitlerinema* (0.03 μg MC-LR_{equiv} /mg dry wt). Results from the SUNY laboratory were positive for BBD *Leptolyngbya* (1.89 ± 0.02 mg MC-LR_{equiv} /L extract) and negative (< 6 μg MC-LR_{equiv}/L) for BBD *Geitlerinema* (duplicate

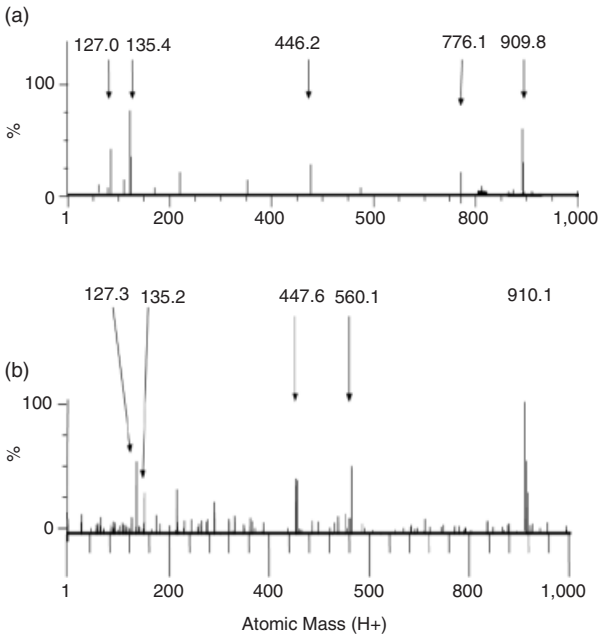

Fig. 1. Electrospray ionization mass spectra for microcystin-LA standard (a) and a field sample (b) acquired using a cone voltage of 80 V. Five diagnostic fragments and the indicative peak (atomic mass 910) are indicated for both samples. The field sample was from BBD on a colony of *Siderastrea siderea*, Horseshoe Reef, Lee Stocking Island, Bahamas.

assays performed for each). One of the two cultures (BBD *Geitlerinema*) also tested positive using the ELISA assay at 0.3 p.p.b. microcystin-LR equivalents (USDA laboratory).

Molecular analysis

Direct sequencing of the *mcyA* gene using the *mcyA*-Cd 1F primer produced partial sequences of 267 and 285 bp for *M. aeruginosa* (positive control) and BBD *Leptolyngbya*, respectively. The former sequence was closely related to *Microcystis* sp. 205 (100% match to partial *mcyA* gene, accession no. AJ515453, 96% query coverage) and *Microcystis* sp. 199 (partial *mcyA* gene, AJ515452). The BBD *Leptolyngbya* sequence (deposited as EF432065 in GenBank) was most closely related (100% to partial sequence) to the *Planktothrix* sp. NIVA-CYA34 partial *mcyA* gene (AJ515474 , 8% query coverage). Using the *mcyA*-Cd 1F primer, a good partial sequence for BBD *Geitlerinema* was not obtained. However, amplification of the *mcyA* gene using MSF/R primers was possible and subsequent cloning produced a useable partial sequence for the BBD *Geiterinema*. The partial sequence (608 bp, deposited as EF432064 in GenBank) was closely (100% to partial sequence, 3% query coverage) related to the *M. aeruginosa mcyA*, *mcyB* and *mcyC* genes (AB019578). The *mcyA* gene from other *Geitlerinema* and *Leptolyngbya* species have not been previously reported and other *mcy* sequences from these organisms are not available in the GenBank database.

McyA was detected in two field samples collected from BBD on *Colpophyllia natans* in the Bahamas and *Meandrina meandrites* in the Florida Keys using the *mcyA*-Cd 1F/R primer set (Table 1). Despite the detection of microcystin by HPLC/MS analysis in 20 additional field samples, the *mcyA* gene was not detected in molecular analysis of these samples. BBD field samples and the BBD cyanobacterial cultures were coanalyzed using DGGE. DGGE analysis of 11 BBD field samples from the Bahamas and Florida Keys regions, all from BBD on *S. siderea*, yielded three bands that matched BBD *Geitlerinema* and one band that matched BBD *Leptolyngbya* (Table 1). Sequencing of the bands confirmed that they corresponded with BBD *Geitlerinema* and *Leptolyngbya*.

Discussion

Many cyanobacteria produce a range of bioactive compounds, including cyanotoxins, that are implicated in the deaths of amphibians, fish, birds, mammals and invertebrates (Dow & Swoboda, 2000). Finding of microcystin in BBD and in cultures of cyanobacteria isolated from BBD suggests that corals may be additional targets of this cyanotoxin. Microcystin was detected in all of the BBD field samples collected from reefs of the Florida Keys and Bahamas, and in cultures of two different genera of BBD cyanobacteria (Florida isolates) using three distinct tests: HPLC/MS, two different protein phosphatase inhibition assays (PP1A and PP2A) and ELISA.

The presence of microcystin in the BBD cyanobacterial cultures and in two of the field samples of this study was confirmed by detection of the microcystin gene *mcyA*. This gene has been targeted in a number of studies aimed at detection of microcystin in diverse aquatic ecosytems (Hisbergues *et al.*, 2003; Ouahid *et al.*, 2005; Ouellette *et al.*, 2006). Although this gene was detected in only two of the 22 positive (HPLC/MS) field samples, previous results have indicated that PCR detection of microcystin genes is often difficult when toxin concentrations are low (Nonneman & Zimba, 2002). BBD field samples of this study were < 0.2 g wet weight, and besides the two positive samples, contained < 10 ng microcystin per sample. This small sample size may have influenced the negative results of this study.

The microcystin-producing BBD cyanobacteria *Geitlerinema* and *Leptolyngbya* were found in BBD on reefs of the Florida Keys and the Bahamas using both cultivative and molecular techniques (Richardson & Kuta, 2003; Voss *et al.*, 2007). The authors have, to date, isolated four BBD *Geitlerinema* strains into culture: the Florida strain used in the present study, isolated from BBD on *Montastraea annularis* in 1991 and originally identified as *Phormidium corallyticum*

but now known as *Geitlerinema* (accession no. AF474001) (Cooney *et al.*, 2002; Richardson & Kuta, 2003); a strain isolated from *S. siderea* on a second Florida reef (accession no. EF154084); and two Bahamas strains, both from *S. siderea* (DQ680351 and EF110974). The BBD *Leptolyngbya* culture (accession no. EF110975) was isolated from *C. natans* on a Florida reef. In the present study DGGE analysis revealed BBD *Geitlerinema* and *Leptolyngbya* in BBD field samples from the Bahamas and the Florida Keys (Table 1). Using a different approach, PCR, cloning and sequencing of the 16S rRNA gene, a sequence (accession no. DQ644020) has been detected that matches (99%) the 16S rRNA gene of the 1991 BBD *Geitlerinema* (AF474001) in a clone library constructed from BBD on Florida *S. siderea*. Other groups have failed to detect this BBD *Geitlerinema* sequence using molecular techniques that target the 16S rRNA gene. Their studies used BBD samples from the southern Caribbean (Curaçao), the eastern Caribbean (St Croix and Barbados) and Indo-Pacific (Papua New Guinea and Palau) (Cooney *et al.*, 2002; Frias-Lopez *et al.*, 2003; Sussman *et al.*, 2006). The fact that microcystin was not detected in the St Croix and Philippines BBD samples of this study may be based on the absence of the BBD *Geitlerinema* or *Leptolyngbya* on reefs in these regions. On the other hand, the absence of BBD *Geitlerinema* or *Leptolyngbya* in seven of 11 samples analyzed using DGGE from BBD on host corals and reefs where microcystin was consistently detected by HPLC/MS suggests the potential presence of other microcystin-producing BBD cyanobacteria. It has been reported (Rützler *et al.*, 1983; Sussman *et al.*, 2006) that there are several different cyanobacterial genera present in BBD.

Two different protein phosphatase inhibition assays run by two different laboratories both indicated that BBD *Leptolyngbya* was positive using this assay and was more toxic than BBD *Geitlerinema*. In contrast, BBD *Geitlerinema* was positive for microcystin using an ELISA against microcystin-LR, but this assay was negative for BBD *Leptolyngbya*. This may be a reflection of the antibody used in this assay and the different microcystin congeners present in these cultures. While there are a number of commercially available ELISA kits, each provides different results depending on the microcystin congeners present. The fact that the MSF/R primer amplified *mcyA* in both BBD cyanobacteria, but *mcyA*-Cd 1F/R amplified this gene only in BBD *Leptolyngbya*, suggests sequence (and thus structural) differences between the two isolates. *McyA* codes for the ala[1] position and the ELISA used in these tests is sensitive to changes at that end of the molecule (i.e. it is three- and 10-fold less sensitive to MC-YR and [Dha[7]] MC-LR than MC-LR; Carmichael & An, 1999). In contrast, toxin congeners with changes in that portion of the ring are easily detected using the PPIA assays. Thus molecular results, ELISA assays and PPIA/PP2A results of this study from three different laboratories are all in agreement. Further chemical studies characterizing the toxin congeners in *Geitlerinema* and *Leptolyngbya* are currently in progress.

Pathogenicity mechanisms in BBD may be as synergistic and complex as the BBD community itself. Oxygen consumption by BBD microorganisms results in anoxia at the base of the band, promoting development of the population of BBD-associated sulfate reducers and generation of sulfide, lethal to eukaryotes due to disruption of electron transport in respiration pathways. The finding of microcystin in BBD samples and cultures of BBD cyanobacteria suggests another mechanism of BBD toxicity. Microcystin is known to have several toxic properties, including inhibition of protein synthesis, inhibition of protein and nuclear protein phosphatases, disruption of both membrane integrity and membrane conduction (Codd *et al.*, 2005), and induction of apoptosis (Chen *et al.*, 2005). The authors have also recently detected (Sekar *et al.*, 2006) the presence of numerous sequences in BBD clone libraries that closely match sequences of bacteria associated with toxin-producing dinoflagellates. It can be concluded that mechanisms of pathogenicity associated with BBD are more complex than those of many pathogenic systems. Additionally, the presence of toxin-producing cyanobacteria in marine systems may be more widespread than previously thought (Cox *et al.*, 2005; Ramos *et al.*, 2005).

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