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Pseudoscillatoria coralii gen. nov., sp. nov., a cyanobacterium associated with coral black band disease (BBD)

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ABSTRACT: Black band disease (BBD) is a widespread coral disease which mainly infects massive framework-building corals. BBD is believed to be caused by a consortium of microorganisms and may not result from the actions of a primary pathogen. The BBD microbial community is dominated, in terms of biomass, by filamentous cyanobacteria. Here we describe a cyanobacterial strain, designated BgP10_4S^T, cultured from a BBD-affected *Favia* sp. coral from the northern Red Sea (Gulf of Eilat, Israel). This dark-green pigmented cyanobacterium showed optimal growth at salinities of 5.0 to 5.5% (w/v), pH of 7 to 8 and cultivation temperatures of 25°C. Morphological examination revealed cylindrical, unbranched trichomes with tapering and blunt cells at the ends which leave a thin mucilaginous trail as they glide. No sheath was evident under these conditions. Inclusion bodies and straight thylakoids were clearly discerned by transmission electron microscopy. Pigment analysis revealed absorption spectra for phycocyanin, carotenoid and chlorophyll *a*. The sequence of the 16S rRNA gene in this cyanobacterium isolate showed high similarity (99%) to cyanobacterial sequences retrieved from BBD-affected corals from different geographical sites (i.e. the Caribbean Sea, Palau and the Red Sea). The BgP10_4S^T strain is observed to be a persistent component of the BBD mat of Faviid corals and may thus be an important agent in the disease etiology. On the basis of its morphological, physiological and phylogenetic distinctiveness, strain BgP10_4S^T represents a novel genus and species of Subsection III (formerly Oscillatoriales), for which the name *Pseudoscillatoria coralii* gen. nov., sp. nov. is proposed.

KEY WORDS: Black band disease · Marine cyanobacteria · Oscillatoriales · *Pseudoscillatoria coralii* · Stony corals

INTRODUCTION

Black band disease (BBD) is a widespread coral malady infecting massive framework-building corals (Frias-Lopez et al. 2004). The disease is globally distributed and has been described on reefs throughout the Caribbean (Weil 2004), in the Indo-Pacific and Red Sea (Antonius 1985, Al-Moghrabi 2001, Barneah et al. 2007, Zvuloni et al. 2009) and in the Great Barrier Reef (Willis et al. 2004). BBD is believed to be caused by a consortium of microorganisms (Antonius 1981, Richardson 1998, Dinsdale 2002) and may not have a primary pathogen (Richardson 2004). It was found that the BBD-associated microbial community is dominated, in terms of biomass, by filamentous gliding cyanobacteria (Rützler & Santavy 1983, Bythell et al.

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2002, Cooney et al. 2002, Frias-Lopez et al. 2003, Richardson 2004). The progression of the disease is characterized by a black band or mat of microorganisms dominated by cyanobacteria that migrate across the coral colony (Rützler & Santavy 1983, Taylor 1983, Barneah et al. 2007). Indeed, most of the cyanobacterial sequences retrieved from BBD-affected corals from different geographic regions showed relationships to the genera *Geitlerinema*, *Leptolyngbya* and *Oscillatoria* of the order Oscillatoriales (Rützler & Santavy 1983, Frias-Lopez et al. 2003, Sekar et al. 2006, Sussman et al. 2006, Barneah et al. 2007, Myers et al. 2007, Richardson et al. 2007, Richardson & Ragoonath 2008).

Oscillatoriales are known in the marine environment, with many being pathogenic to marine invertebrates and vertebrates through the production of toxins (Smith 1996, Mez et al. 1997, Carmichael & Li 2006). Moreover, strains of this order are known to form marine cyanobacterial mats (Antonius 1981). In the present study we describe the taxonomic properties of a cyanobacterium strain BgP10_4S^T that originated from BBD-affected *Favia* sp. from the northern Red Sea (Gulf of Eilat, Israel). Here we describe phenotypic, genotypic and phylogenetic characteristics of this strain.

MATHERIALS AND METHODS

Sample collection and culturing. Using 10 ml syringes, samples of the black band were collected from the surface of the coral *Favia* sp. (at depths ranging from 1 to 5 m) from a site opposite the Interuniversity Institute for Marine Science, Eilat, Israel (IUI, 29° 51′ N, 34° 94′ E). Samples were brought to the surface and immediately processed. For isolation of the BgP10_4S^T cyanobacterial strain, initial liquid samples were plated on BG11 Fluka (Buchs) agar plates and incubated at 22°C (typical late spring–early summer seawater temperatures, a period when the disease is first observed in the shallow water of the Gulf of Eilat) under a 12 h light:12 h dark cycle. The BG11 medium was prepared using artificial seawater (Instant Ocean) in order to maintain the BgP10_4S^T culture at the desired salinity (4% w/v). From this culture, single cyanobacterial filaments were transferred to a liquid BG11 medium, grown and then plated again on BG11 plates, thus producing monocyanobacterial cultures of this strain. To ascertain temperature optima for growth, BgP10_4S^T biomass were plated on BG11 agar and incubated at 22, 25 and 30°C. After 2 wk of incubation, the expansion zones of the colonies were measured with a ruler on the undersurface of the Petri dish. The salinity range for growth of the cyanobacteria was determined using a sea salt mixture (Instant Ocean) for preparing artificial seawater at concentrations of 3.5 to 6.5% (w/v at intervals of 0.5%) and 8 to 12% (w/v at intervals of 1%). The pH of a BG11 liquid culture medium (with 4% w/v sea salt) was adjusted by adding alkaline (NaOH) or acid (Tris-citrate buffer) compounds to produce pH of 4.5, 5, 7, 9, 10 and 11. Optimal pH was assessed by the qualitative appearance of BgP10_4S^T biomass which attached and coated walls of the Erlenmeyer flasks.

DNA extraction and PCR amplification. For molecular identification, genomic DNA was extracted from the cyanobacterial cultures (Wilson 1987) using a PowerSoil DNA Isolation kit (Mo Bio Laboratories) according to the manufacturer's instructions. Genomic DNA was eluted using 40 µl of elution buffer or double distilled water and stored at –20°C. Total DNA was amplified by PCR with a Mastercycler gradient thermocycler (Eppendorf) using the specific 16S rRNA gene cyanobacterial primers, 106F (CGG ACG GGT GAG TAA CGC GTG A) and 781R (GAC TAC TGG GTA TCT AAT CCC ATT) (Nübel et al. 1997, Sussman et al. 2006).

Phylogenetic analysis. Amplified 16S rRNA gene sequences were compared with those in the GenBank database with the basic local alignment search tool (BLAST) network service (www.ncbi.nlm.nih.gov/blast/blast.cgi) and aligned with representative species from the order Oscillatoriales using CLUSTALW, part of the MEGA package (Kumar et al. 2004). A phylogenetic tree was constructed by the neighbor-joining method (Saitou & Nei 1987) using the MEGA package (Kumar et al. 2004). Bootstrap resampling analysis (Felsenstein 1985) for 100 replicates was performed to estimate the confidence levels of tree topologies.

Nucleotide sequence accession number. The 16S rRNA gene sequence was deposited in GenBank and given accession number FJ210722.

Pigment analysis. Chlorophyll extraction was carried out using methanol, followed by heating for 2 min at 70°C. The extract was centrifuged and the clear supernatant solution was used for measurement (Talling 1969). Water-soluble phycobilin pigment extraction was carried out by overnight freezing of the cell suspension. After thawing and vortex-mixing, each sample was centrifuged to obtain an aqueous supernatant (Francis 2001). Absorption spectra of the cyanobacteria samples were examined using a UV/visible spectrometer (Agilent 8453, Agilent Technologies).

Cell structure identification. For ultrastructural characterization, cells of strain BgP10_4S^T cultivated in BG11 liquid media were fixed with 2.5% gluteraldehyde in phosphate-buffered saline (simulating seawater osmolarity) overnight, then washed, post-stained with osmium tetraoxide, serially dehydrated in ethanol

(30, 50, 70 and 100%), embedded in Araldite epoxy resin (Electron Microscopy Sciences) and sectioned in 70 to 80 nm slices (Hoppert & Holzenburg 1998). The resulting sections were stained with uranyl acetate and lead citrate, and examined using a JEM-1230 transmission electron microscope at an excitation voltage of 80 kV (Harel et al. 2008).

Fatty acid content. Fatty acid content was analyzed by GC-MS using a protocol adjusted for cyanobacteria (Caudales et al. 2000). The composition of the fatty acid content of the cells was described by co-chromatography with reference standards (MIST02).

RESULTS

A monoalgaic culture of strain BgP10_4S^T originating from BBD-affected *Favia* sp. coral was obtained. Strain BgP10_4S^T is a gram-negative gliding filamentous cyanobacterium 5 to 6 µm in width (Fig. 1), with phenotypic characterizations listed in Table 1. The strain is a slow grower in liquid culture, forms a dark-green coat on the walls of the Erlenmeyer flasks and a bead-like coat on agar plates. No growth was evident when the cultures were maintained in the dark. On BG11 agar plates, trichomes were detected only 2 wk after plating, therefore making it difficult to determine generation time. No heterocysts were evident in this strain when examined under both light microscopy and transmission electron microscopy. Morphological examination revealed cylindrical unbranched trichomes with tapering and blunt cells at the ends (Fig. 1). Trichomes were found to leave a thin mucilaginous trail as they glide, although no sheath of mucilage was noted (Fig. 2a). Several pigments were identified spectrophotometrically, namely water-soluble phycocyanin, with an absorption maximum at 623 nm, chlorophyll *a*, with 2 absorption peaks at 436 and 666 nm, and carotenoid, with an absorption maximum at 336 nm. The detection of chlorophyll and the fact that when exposed to light this organism grows on BG11, a medium that is almost free of organic nutrients, suggests that the strain comprises photoautotrophic bacteria.

Table 1. Differential characteristics of strain BgP10_4S^T. Lipid content does not include trace amounts and compounds showing similarity to library compounds lower than 90%. Yes: positive; no: negative

Characteristic	Strain BgP10_4S^T
Morphology	Filamentous
Trichome width	5–6 µm
Hetrocyst formation	No
Motility	Yes
Color of cultures	Dark green
Pigments	Chlorophyll *a*, phycocyanin, carotenoid
Photosynthetic activity	Yes
Optimal salinity	5–5.5% (w/v)
Optimal pH	7–8
Optimal temperature	25°C
Aerobic growth in the dark	No
Lipid content	
16:0	58.61%
16:1	6.39%
16:3	14.08%
17:1	6.99%
18:1	7.13%
18:2	6.80%

The optimal growth temperature for BgP10_4S^T was 25°C. At this temperature, the pH range for growth was between 5 and 11, with optimal growth occurring between pH 7 and 8. Optimal growth was observed from 5 to 5.5% (w/v) salinity, while there was no

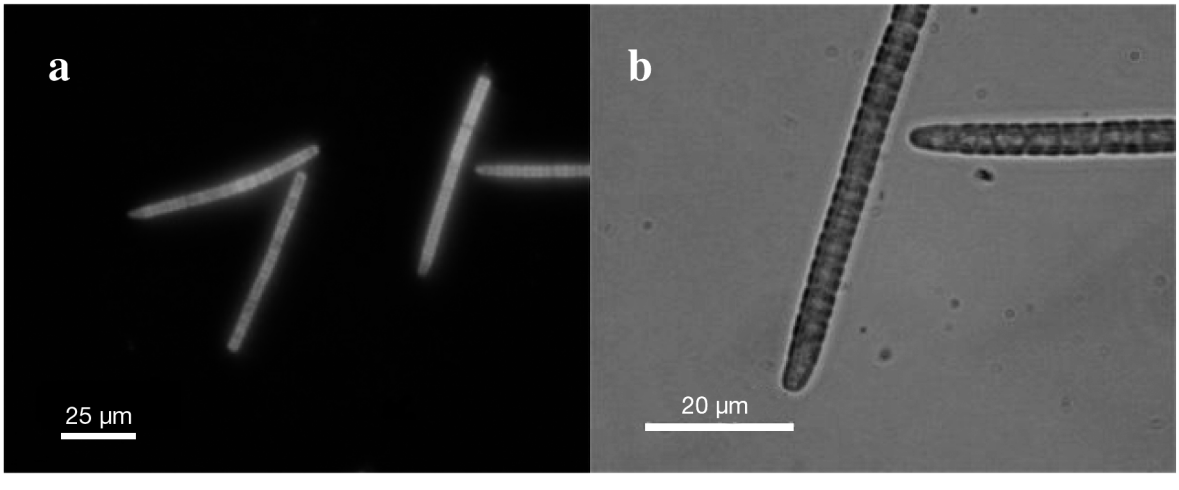

Fig. 1. Photomicrographs of *Pseudoscillatoria coralii* strain BgP10_4S^T under (a) UV and (b) phase contrast conditions. Images were taken at 400× and 1000× magnification, respectively

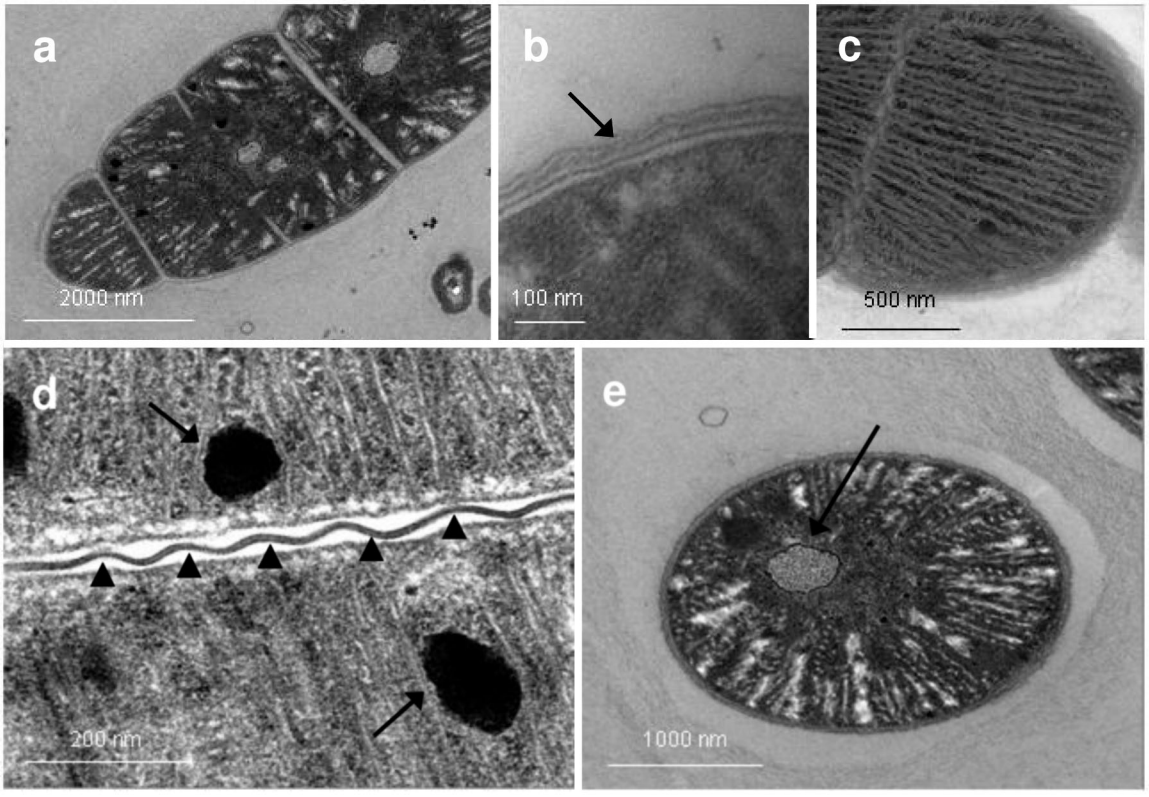

Fig. 2. Transmission electron micrographs of *Pseudoscillatoria coralii* strain BgP10_4S^T. (a) Apical oriented trichome; (b) enlargement of the cell wall, demonstrating the typical gram-negative layered structure (arrow); (c) thylakoids (note the parallel arrangement as seen in long-section); (d) undulating septa between cells (arrowheads) and black inclusion bodies within the cells (arrows); and (e) cross-section of trichome, revealing light inclusion bodies (arrow)

growth observed below 4% (w/v). The major cellular straight, long-chain fatty acid (>50%) was C16:0. In addition, 16:1, 16:3, 17:1, 18:1 and 18:2 fatty acids were present (Table 1), with ratios of mono- to polyunsaturated fatty acids of nearly 1:1.

Microscopic observation showed single trichomes with tapered and blunt ends (Figs. 1 & 2a) that displayed fluorescence when excited under UV light. Several identifying morphological features were detected. These include a multilayered cell wall (Fig. 2b) consisting of a number of membrane structures divided by electron opaque layers containing darkly stained inclusion bodies. In addition, separate parallel thylakoids; these appear parallel in long-section (Fig. 2c), but radial in distribution in cross-section, were evident (Fig. 2e), as were undulating partitions between the trichomes (Fig. 2d). Various inclusions, such as dark (Fig. 2d) and white bodies (Fig. 2e), were also visible. Strain BgP10_4S^T (SAG NA 2008.006; temporary number) is under deposition at the Culture Collection of Algae (SAG) at Georg August University, Göttingen, Germany. The 16S rRNA gene sequence of BgP10_4S^T (accession no. FJ210722) was found to have 91% similarity to a previously isolated cyanobacterium from a marine sponge, *Oscillatoria spongeliae* (AY615503; Fig 3).

DISCUSSION

Cyanobacteria strain BgP10_4S^T isolated and cultured from a BBD-affected colony of the coral *Favia* sp. from the northern Red Sea (Gulf of Eilat) are gram-negative, gliding filamentous non-heterocystous, photoautotrophic cyanobacteria containing parallel thylakoids and various inclusion bodies (Figs. 1 & 2). Filament pigments include phycocyanin, carotenoid and chlorophyll *a*. The cells contain long-chain fatty acids of a straight-chain saturated nature, over 50% of which are C16:0. In our cyanobacterial specimens, multilayered cell walls, consisting of a number of membrane structures divided by electron opaque layers, were evident (Fig. 2b). In addition, the filaments contained darkly stained inclusion bodies, and separate parallel thylakoids were observed (Fig. 2a,c–e). Moreover, the cylindrical, unbranched trichomes with conical and blunt apical cells (Figs. 1 & 2a) morphologically define the cultured cyanobacteria strain

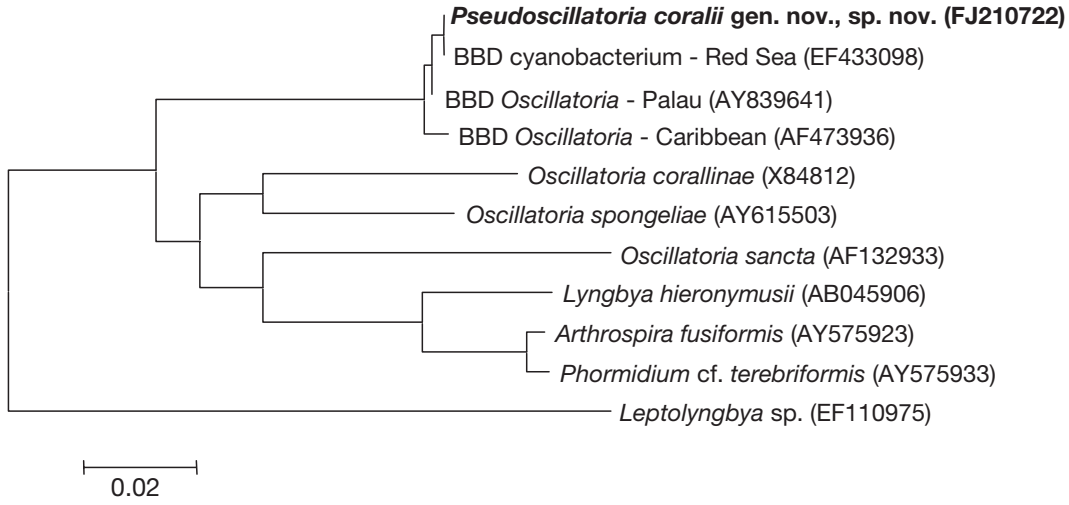

Fig. 3. Neighbor-joining 16S rRNA gene phylogenetic tree showing the relationship between strain BgP10_4S^T (*Pseudoscillatoria coralii* gen. nov., sp. nov.) in bold type and representative species of Subsection III. Sequence accession nos. are given. The scale bar represents 2 substitutions per 100 nucleotide positions

BgP10_4S^T as belonging to Subsection III (Oscillatoriales in the traditional sense), which includes almost all filamentous cyanobacteria with uniplanar binary fission with no heterocyst (Castenholz et al. 2001). According to Castenholz et al. (2001), the genus *Oscillatoria* is widely distributed in fresh, marine and brackish waters. Some species seem almost black, and can synthesize chlorophyll, phycoerythrin and phycocyanin without chromatic adaptation. Some are known as the motile components of microbial mats. *Oscillatoria* species divide exclusively by binary fission in one plane and are further characterized by cylindrical straight trichomes and, in some cases, cap-like thick terminal cells. Sheaths of this genus may be nearly invisible; however, although trichomes of the strain BgP10_4S^T were found to leave a thin mucilaginous trail as they glide, no sheath of mucilage was present. Members of the closely related genus *Oscillatoria* are well known for their motility, with their trichomes gliding by means of undulation of their microfibrils while the cells maintain contact with a solid substrate. Mucilage is secreted through pores in the cell wall and may help to provide better contact with the substrate surface (Van den Hoek et al. 1995).

The optimal growth of BgP10_4S^T (originating from BBD-affected *Favia* sp.) was obtained at pH 7 to 8, 25°C, in 5 to 5.5% (w/v) salinity; low or no growth was evident at lower salinities (<4% w/v). In the northern Gulf of Eilat in the summer months during the active spread of the disease, the water temperature at 1 to 3 m depth where the disease is most prevalent (Barneah et al. 2007) is approximately 26°C, salinity is 4% (w/v) and pH is around 8. Hence, these conditions are sufficient for reproduction of the strain on coral surface. In general, BBD cyanobacteria contend with harsh surroundings existing in BBD mats. Recently, Richardson & Ragoonath (2008) demonstrated the ability of the BBD cyanobacterium *Geitlerinema* (AF474001) to use exogenous organic carbon to enhance survival in the dark under the anaerobic (as well as illuminated aerobic), sulfide-rich conditions existing in BBD mats.

Recent cyanobacterial 16S rRNA gene sequences retrieved from BBD-affected corals distributed globally were identified as belonging to the genera *Geitlerinema*, *Leptolyngbya* and *Oscillatoria* of Subsection III (Sekar et al. 2006, Sussman et al. 2006, Barneah et al. 2007, Myers et al. 2007, Richardson et al. 2007, Richardson & Ragoonath 2008). Molecular identification of the 16S rRNA gene sequence of BgP10_4S^T (1432 bases; FJ210722) displayed a 99% similarity to the cyanobacteria (i.e. AF473936, AY839641 and EF433098) retrieved from BBD species collected at different sites, such as the Caribbean Sea, Palau and the Red Sea, respectively (Cooney et al. 2002, Sussman et al. 2006, Barneah et al. 2007). Moreover, the BgP10_4S^T sequence showed a 91% similarity to the closest cultured cyanobacterial relative, *Oscillatoria spongeliae* (AY615503), previously isolated from a marine sponge (Fig. 3). These findings suggest that strain BgP10_4S^T is a ubiquitous member of the BBD community and may be an important agent in the disease etiology. Hence, based on its morphological properties, growth characteristics, fatty acid composition and phylogenetic distinctiveness, we propose that strain BgP10_4S^T be classified as a novel genus and species in Subsection III, for which the name *Pseudoscillatoria coralii* gen. nov., sp. nov. is proposed.

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