JOURNAL OF PURE AND APPLIED MICROBIOLOGY, September 2013. Vol. 7(3), p. 2397-2403

Predominant Bacterial Candidates Associated with Diseased Corals from Gulf of Mannar, India

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(Received: 20 November 2012; accepted: 28 January 2013)

Black band disease (BBD) and white band diseases (WBD) are well-described diseases in corals worldwide and believed to be caused by a diverse microbial consortium. To expand our understanding of specific dominant bacterial community associated with the black band and white band diseased coral tissue, a culture-dependent approach was applied to diseased coral tissues of *Acropora cytherea* and *Montipora digitata* inhabiting Shingle Island of the Gulf of Mannar. Morphologically different dominant bacteria were isolated from corals affected with BBD and WBD for molecular identification. 16S rRNA gene sequence analysis revealed the dominance of isolates belongs to three genera *Bacillus, Micrococcus,* and *vibrio* in black band diseased *Aropora cytheria*. Moreover, two cyanobacterial species *Phormidium* sp. and *Synechocystis* sp also observed in diseased *Aropora cytheria*. In the case of white band disease in *Montipora digitata*, dominance of isolates belongs to three genera: *Micrococcus*, *Psychrobacter* and *Palnomicrobium* were found. The observed predominant bacterial candidate associated with BBD and WBD can be considered to find out the disease etiology.

Key words: Black band disease, White band disease, Phylogeny, *Acropora cytherea*, *Montipora digitata*.

In the last few decades, coral disease has emerged as a significant threat to coral reef ecosystems with declines in coral cover and diversity[1]. Despite this vast destruction of coral reefs, the causative agents of coral diseases have yet to be conformed in most cases, though many are demonstrated to have microbial origins[1-3]. The black band disease (BBD) and white band disease (WBD) are among the most important coral diseases responsible for vast destruction of coral ecosystems in Gulf of Mannar[4]. BBD is a lethal disease that contributes to coral decline in the wider Caribbean[5], Red Sea[6] and the Great Barrier Reef[7]. The BBD spreads across healthy coral tissue as a distinctive dark-colored band at a rate between 3 mm and 1 cm per day[7]. Cause of BBD has been reported as a consortium of microorganisms rather than a single pathogen[9]. Microbial population associated with BBD has been dominated by a filamentous, non heterocystous, cyanobacterium, *Phormidium corallyticum*[10, 11]. Further optical microscopy studies revealed that there were other organisms accompanying *P. corallyticum* in the BBD mat: the motile sulfide-oxidizing bacterium *Beggiatoa*, the sulfate-reducing bacterium *Desulfovibrio*, numerous heterotrophic bacteria, and marine fungi[12]. The 16S rRNA gene sequence based studies has revealed occurrence of different cyanobacteria in association with BBD[13]. In the Caribbean, the BBD mat is dominated by an unidentified cyanobacterium most closely related

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to the genus *Oscillatoria*[14, 15]. In the Indo-Pacific, the BBD associated cyanobacterium is most closely related to the genus *Trichodesmium*[13].

WBD is also destructive coral disease, first described in 1977[15], and has progressively spread throughout the Caribbean causing massive mortality of Caribbean *Acroporid* coral, *Acropora cervicornis* and *A. palmata*. The WBD causes rapid tissue necrosis at a rate of up to 1-2 cm per day that progress from the base of the branch towards the tip leaving behind a characteristic band of bare white skeleton that is rapidly colonized by algae[15, 16]. Despite the unprecedented levels of mortality caused by WBD, very little is known about its epistemology[15]. It has been suggested that WBD is caused by a bacterial infection and gram negative rod shaped bacterial aggregates have sometimes been found associated with diseased *Acroporoid* corals[17, 18]. An analysis using culture independent techniques, of the bacterial communities in healthy and WBD infected *Acropora cervicornis* found that bacterial communities associated with both were dominated by *α-Proteobacterium*[19].

Early descriptions of BBD and WBD were dominantly made by optical and electron microscopy. Recently, in addition to optical and electron microscopy, 16S rRNA gene sequences has been identified as an effective tool to detect various microbes of the microbial causation responsible for BBD and WBD. The etiology of both WBD and BBD is poorly understood and basic information about the microbial association is remaining unclear. To know the process of infection and to reveal the possible environmental factors favoring causation, it is important to identify those organisms that play a crucial role in the disease. The aim of the present study is to investigate the predominant bacterial candidates associated with BBD and WBD by using 16S rRNA gene through the culture dependent method.

MATERIALS AND METHODS

Collection of diseased coral samples

The BBD mat of *Acropora cytherea* and WBD affected tissue of *Montipora digitata* tissues were sampled using sterile cotton swab in replicates using scuba at a depth of 3m in Shingle Island of the Gulf of Mannar. The samples were placed separately in sterile screw cap tubes to avoid contact with air and were transported to the laboratory within two hours by keeping in ice (4ºC).

Isolation of bacteria associated with diseased coral samples

A set of samples were serially diluted and spread over the Zobell marine agar medium and incubated at room temperature 37ºC for 48 hours. Morphologically distinct, dominant bacterial strains were selected and purified by repeated streaking on Zobell marine agar plates. The other set of samples was observed under the microscope and photomicrographed using Motic Digital Microscope (Model no.DMB1-223) with imaging software. Cyanobacterial strains were identified using the keys described elsewhere[20, 21].

Amplification, sequencing and analysis of 16SrRNA genes

All the dominant bacterial isolates were grown in 25 ml of Zobell marine broth at 37°C for 36 h. The cells were harvested from the broth by centrifugation at 10,000 rpm for 3 min and DNA was extracted following standard phenol-chloroform extraction procedure

The 16S rRNA gene of the coral associated bacterial isolates was amplified in a PCR with the primers: 27F 5'-AGA GTT TGA TCC TGG CTC AG-3' and 1492R 5'-GGT TAC CTT GTT ACG ACT T-3'. The 50 µl PCR reaction mixture contained 1X Reaction Buffer (10 mM Tris [pH 8.3], 50 mM KCl, 1.5 mM MgCl2), 200 µM of dNTPs, 10 pM of each primer, 5 to 25 ng of genomic DNA and 0.05 U of Taq DNA polymerase (Sigma, USA). The PCR condition was reaction cycles 35 times, 94ºC predenaturation 4 min, 94ºC denaturation 1 min, 55°C annealing 30 sec, 72ºC extension 1 min, 72ºC final extension 7 min, 4ºC hold. The PCR product was purified by column DNA gel extraction kit (Hi media) and sequenced by Applied Biosystems 3730XL DNA Analyzer.

Phylogenetic analysis

The 16S rRNA sequences of the strains were compared with 16S rRNA gene sequences of the other strains from the GenBank database using BLAST[22]. The 16S rRNA sequences of the strains were aligned with 16S rRNA gene sequences of selected members of bacterial community. The phylogenetic tree was constructed with the neighbour-joining method in phylogenetic analysis program PHYLIP[23] following Jose et al[24]. The

topology of phylogenetic tree was appraised by using bootstrap value with 1,000 repeats.

RESULTS AND DISCUSSION

Microscopic observation of BBD

On microscopic observation of BBD mat, two cyanobacterial genera: *Synechocystis* and *Phormidium* were identified and are given in the fig. 1. The strain *Synechocystis* sp. was coccoid in shape and measured 2 - 3 µm in diameter, the cells divided by binary division. In the case of *Phormidium* sp, trichomes were simple, cylindrical, individual sheath thin, non-branched filaments without any heterocytes and akinetes, cells 2-3 times longer than wider, not attenuated and apical cell rounded. The result conforms concisely to Richardson[25], and Rutzler and Santavy[26]; they reported the presence of *Phormidium* in the BBD mat along with *Synechocystis*. Research on cyanobacteria revealed that *Phormidium uncinatum* have gliding movement with the help of extracellular glycoprotein, oscillin[27]. *Synechocystis* has gliding or twitching motility while in contact with a surface[28]. In the present study, identification of such motile cyanobacterial strains *Synechocystis* and *Phormidium* in BBD suggests them as possible candidates contribute to spreading of BBD over healthy coral tissue.

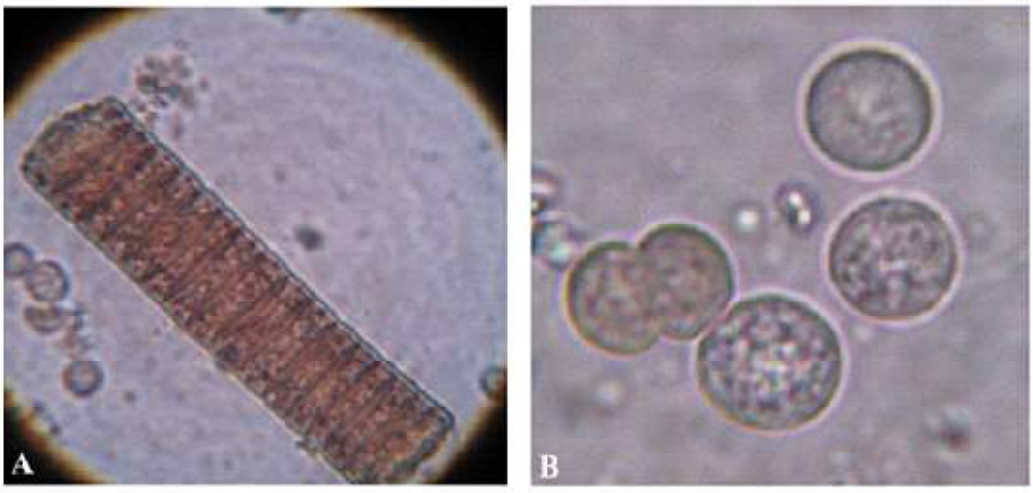

Fig. 1. Morphology of cyanobacteria A) *Phormidium* sp. and B) *Synechocystis* observed in *Acropora cytherea* affected with BBD

Marine cyanobacteria *Synechocystis sp.*[29] and *Phormidium sp.*[30] have already been reported to produce toxins. These toxins have been reported to affect structural integrity of the coral tissue and results in extrusion of zooxanthallae from the normal location in the gastrodermis[31]. Experiments proved that lowest concentration of cyanobacterial toxin (Microcystin) promote bacterial growth in the coral tissue[32] and thus appearing to be directly toxic to corals. In the present study, occurrences of toxin producing stains such as Phormidium and *Synechocystis* within the BBD mat were found. Disease affected corals were often found dead and it might be due to the toxin producing strains and bacterial accumulation on the BBD mat.

Dominant bacteria associated with diseases corals

A total of 6 dominant and unique bacterial colony morphologies were obtained on marine agar from both *Montipora digitata* infected with WBD and *Acropora cytherea* infected with BBD. These organisms were observed at high Colony Forming Unit (CFU ml^{-1}) numbers in more than 50% of replicate plates.

Amplification and sequencing of 16S rRNA gene

Universal eubacterial primers were used to amplify the 16S rRNA gene from the genomic DNA of all the isolates and the electrophoresis pattern showed that the products were about 1,500 bp in size. The nucleotide sequences of 16S rRNA genes have been submitted to GenBank nucleotide

sequence data base under accession numbers JF268250 to JF268255. Since late 1970s, the 16S rRNA gene based bacterial identification method has been used as a standard method for documentation of bacterial population in all habitats[32, 33] and associationships including detection of the bacteria associated with WBD and BBD.

Phylogenetic affiliation of Isolates

The16S rRNA gene sequence of strains SDMRI-W1, SDMRI-W2 and SDMRI-W4 for WBD and SDMRI B, B7 and B9 for BBD were compared with the nucleotide sequences available in the Gen bank database using BLAST search.

Dominant bacterial candidates associated with WBD

Phylogenetic tree was constructed based on the 16S rRNA gene sequence of bacteria associated with WBD (Fig. 2). The bacterial isolates, SDMRI-W1, SDMRI-W2 and SDMRI-W4 were identified as *Micrococcus* sp, *Planomicrobium* sp, and *Psychrobacter* sp, respectively. The strain SDMRI-W1 fall in cluster of genus *Micrococcus* in phylogenetic tree and shared 99% sequence similarity in 16S rRNA gene with *Micrococcus luteus* (FJ999946).The strain SDMRI-W2 was posed with uncultured *Psychrobacter* sp. CI2 (FJ695523) as one branch in phylogenetic tree and shared 99% sequence similarity in 16S rRNA gene. The strain SDMRI-W4 falls in cluster of genus *Planomicrobium* in phylogenetic tree and shared 99% sequence similarity in 16S rRNA gene with *Planomicrobium okeanokoites* (D55729).

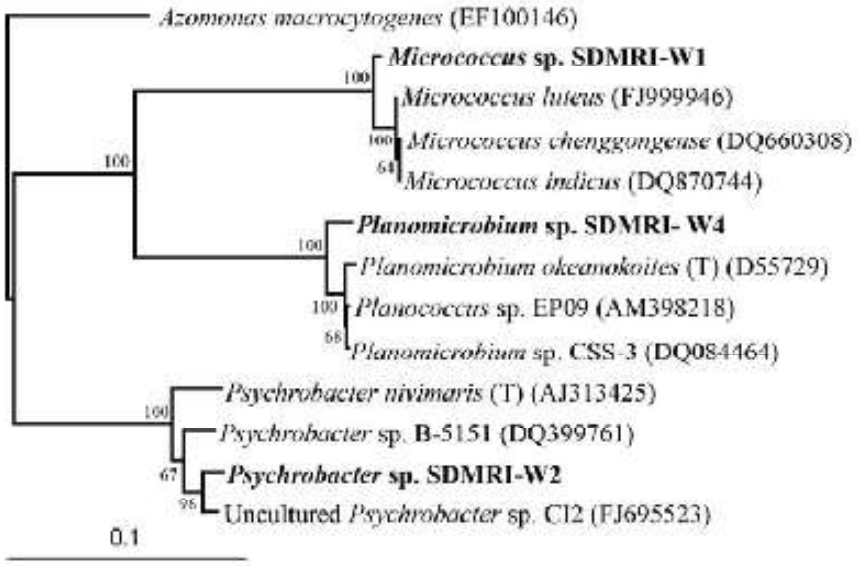

Fig. 2. Neighbour-joining phylogenetic tree inferred by 16S rDNA sequences shows phylogenetic affiliation of bacteria isolated from WBD. Numbers at nodes are bootstrap values. The scale bar represents the number of substitutions per 100 positions per a unit branch length

In the case of WBD, according to Ritchie and Smith[34] *Vibrio carchariae* dominates *Pseudomonas* sp. contrasting to the healthy condition where *Pseudomonas* sp. is dominant. The present study *Vibrio* sp. was not found as the dominant bacteria, while higher number of *Micrococcus* sp. was detected in the WBD affected portions of the corals. This finding aligns with a previous report[35] claimed a higher number of *Micrococcus* sp. associated with the disease. Moreover, two γ *Proteobacterium*: *Planomicrobium* and *Psychrobacter* were also found in the WBD affected portion.

Dominant bacterial candidates associated with BBD

To find out the phylogenetic position of the strains SDMRI-B, SDMRI-B7, SDMRI-B9 and SDMRI-B11 associated with BBD, another phylogenetic (Figure 3) tree was constructed based on their 16S rRNA gene sequence. The isolates

were found to be belonging to *Pseudoalteromonas* sp*, Bacillus* sp*, Vibrio* sp and *Pseudomonas* sp. The isolates SDMRI-B and SDMRI-B9 fall in cluster of genus *Pseudoalteromonas* in phylogenetic tree and shared sequence similarity of 98% and 99% respectively with *Micrococcus luteus* (FJ999946). The strain SDMRI-B7 fall in cluster of genus *Bacillus* in phylogenetic tree and shared 100% sequence similarity in 16S rRNA gene with *Bacillus subtilis subsp. subtilis*; GB4.1 (EU287468). The strain SDMRI-B11 fall in cluster of genus *Vibrio* in phylogenetic tree and shared 99% sequence similarity in 16S rRNA gene with *Vibrio* sp. 5H12 (EU517636).

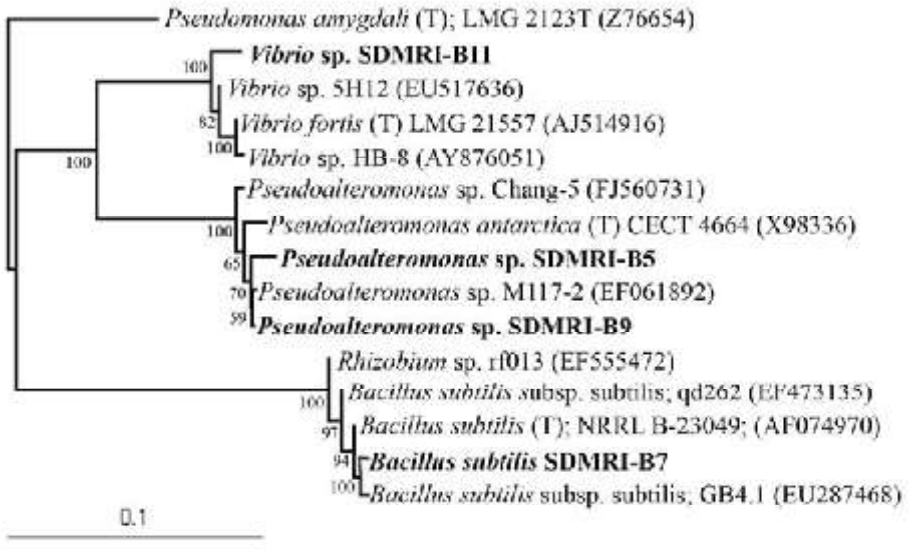

Fig. 3. Neighbour-joining phylogenetic tree inferred by 16S rDNA sequences shows phylogenetic affiliation of bacteria isolated from BBD. Numbers at nodes are bootstrap values. The scale bar represents the number of substitutions per 100 positions per a unit branch length

Numerous heterotrophic bacteria associated with BBD mat has been reported in diseased corals[36]. Likewise, present study revealed the prevalence of bacterial species belonging to taxonomic groups: Gamma proteobacteria and Firmicutes. This indicates dominance of Gamma Proteobacteria and corroborates with the work of Ritchie and Lewis[37], who documented higher Gamma Proteobacteria composition in diseased coral samples. Moreover, earlier studies have revealed the occurrence of a list of bacterial genera: *Pseudomonas, Salmonella, Yersinia, Vibrio*, *Desulfovibrio* and B*eggiatoa* and reported them as the common pathogens causing BBD[38, 39]. The present study expands the list through the identification of *Pseudoaltromonas* and *Bacillus* along with other bacteria constitute the bacterial consortium associated with BBD. Members of *Vibrio* are considered as one of the most important coral pathogens[2, 40] and it is supported by the current identification of *Vibrio* sp. among the predominant bacteria associated with diseased coral tissues.

The complex community of microorganisms present in BBD mats and WBD has made it difficult to understand the onset and progression of the disease. However, a subset of bacterial species likely to play important roles in the etiology of BBD and WBD has been identified in the present study. In order to understand the mechanisms of the disease in corals, further focused research should be conducted to explore the virulent components of all identified bacterial communities. Identification of changes in the bacterial community over the climatic change is also needed to save the corals of Gulf of Mannar from the disease threats post by several factors.

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