Microbial 16S rDNA Sequencing of Cultivable Bacteria Associated with Toxic Dinoflagellate, *Pyrodinium bahamense* var. *compressum*

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ABSTRACT Bacteria associated with toxic dinoflagellate, *Pyrodinium bahamense* var. *compressum* play an important role in paralytic shellfish toxin production during harmful algal bloom occurrences, which frequently spotted in the coastal waters of Sabah. The outbreak has caused a remarkable impact on economic losses to Sabah aquaculture industries, even worse, human illness and fatalities. The expression of PST during a toxic bloom remains elusive, furthermore studies on the bacterial diversity associated with Sabah *P. bahamense* are limited. Thus the study aimed to examine the cultivable bacteria diversity associated with *P. bahamense* through 16S rDNA sequence analysis. A total of 74 isolates were successfully obtained, which were predominated by the phyla proteobacteria. 41 of the isolates were gamma-proteobacteria; *Alteromonas* sp. (37), *Marinobacter* sp. (3), *Methylophaga* sp. (1), and 34 of the isolates were alpha-proteobacteria; *Ruegeria* sp. (29), *Lutimaribacter* sp. (5).The major bacteria strains identified were *Alteromonas* sp. and *Ruegeria* sp. which were previously reported as a possible PST-producing bacterium. The information from this study will provide important insights into the understanding the relationship between dinoflagellate-bacteria association as well as the role of these bacteria in toxin production.

KEYWORDS: 16S rDNA sequencing; bacteria; toxic dinoflagellate; *Pyrodinium bahamense* var. *compressum*; harmful algal bloom.

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TRANSACTIONS ON SCIENCE AND TECHNOLOGY

INTRODUCTION

Harmful algal bloom (HAB) has been regularly occurring in the coastal water of western Sabah (Roy, 1997) and mainly caused by a toxic dinoflagellate, *Pyrodinium bahamense* var. *compressum* (PBVC), which is responsible for paralytic shellfish poisoning (PSP) in human (Usup *et al*., 2002).

Previous studies have suggested the involvement of symbiotic bacteria in the PSP toxin production during HAB event (Gallacher & Smith, 1999; Alavi *et al*., 2001; Alverca *et al*., 2002; Córdova *et al*., 2003; Azanza *et al*., 2006). In addition, the bacteria-algal interaction has been also reported to play an important role in the process of bloom initiation, maintenance, and decline (Doucette, 1995; Ferrier *et al*., 2002).

Prior research using bacterial culture-dependent method identified some potential paralytic shellfish toxin (PST)-producing bacteria such as, *Hyphomonas* sp. *Roseobacter* sp., and *Alteromonas* sp. from Sabah PBVC (Chin *et al*., 2013), while *Moraxella* spp., *Erythrobacter* spp., and *Bacillus* spp. in PBVC culture from the Philippines (Azanza *et al*., 2006). Based on Gallacher & Smith (1999), major cultivable bacteria commonly observed were from Alteromonas and Roseobacter clade. *Alteromonas* sp. observed to be a fundamental part of the bacterial community responsible for growth enhancement for toxic dinoflagellates, such as *Alexandrium* sp. (Doucette & Trick, 1995).

The16S rDNA, a small subunit of the ribosomal RNA, have been widely used in DNA barcoding of microbes. This is because the region is present across all bacterial genomes and its nucleotide

sequence is highly-conserved owing to their functional stability (Pace, 1997). In this study, 16S ribosomal RNA gene was used to investigate the bacterial population associated with the toxic dinoflagellate, *Pyrodinium bahamense* var. *compressum*.

METHODOLOGY

Sample Collection

Seawater samples containing PBVC was collected from Sepanggar Bay (6.08° N, 116.12° E) in December of 2012 that coincided with the period when high concentrations of PSP toxins were detected in the seawater. A pure, monoalgal culture of PBVC (herein referred to as strain CC-UHABS-040(M)) was established in sterile seawater-based enriched f/2 medium as described in Guillard and Ryther (1962). The culture flasks were kept at 28°C in a light/dark cycle of 12:12 h under 150 μE m^{-2} s^{-1} irradiation.

Culture-Dependent Assessment of Bacterial Diversity

Isolation and characterization of the bacteria followed the method delineated in Hold *et al.* (2001) with an additional sonication step to disrupt the bacterial-dinoflagellate cell attachments. A total of 1 ml of cultured PBVC strain CC-UHABS-040(M) in the late exponential phase was sonicated using Ultrasonic Cleaner, 4 L for 15 min at 37°C (Tosteson *et al.*, 1989). The sample was serially diluted 10-fold in sterile seawater, following which 100 μL of each dilution were plated, in triplicate, onto sterile marine agar (Difco). The plates were incubated 37°C for over 72 h. Bacteria from the dilution containing between 50 and 100 colonies were re-plated onto fresh marine agar medium and incubated at 37°C overnight.

DNA Barcoding of the Cultured Bacterial Isolates

Prior to DNA extraction, each isolate was cultured in 5 ml marine broth (Difco) overnight in an incubator-shaker set at 37°C. Genomic DNA was isolated using DNeasy® Blood & Tissue DNA Isolation Kit (Qiagen) following the manufacturer's protocol. The partial 16S region of the isolates was PCR-amplified using S-D-Bact0341-b-S-17 and S-D-Bact-0785-a-A-21 (Klindworth *et al.*, 2013), targeting the V3-V4 region of the 16S rRNA gene. The PCR reaction contains 1x Phusion Flash PCR Master Mix (Thermo Scientific), 0.5 μM of each primer, and 20-fold diluted DNA template to a final volume of 20 μL. The amplification cycling is as follows: 10 s initial denaturation at 98°C, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 5 s, and extension at 72°C for 8 s, and a final extension at 72°C for 1 min. The PCR products (≈ 20 ng/μL) were then sequenced on an ABI 3130 Genetic Analyzer (Applied Biosystems), using the BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems).

Bioinformatics Analyses of DNA Barcode Sequences

Sequencing outputs were pre-analyzed using CLC Genomics Workbench v.7.5.1 (CLC bio) with the default parameters (p=0.05, equivalent to quality score of Q≈13), where raw sequences were trimmed, edited and concatenated to produce multi fasta sequence file. Chromatogram files of the pre-processed sequences exported to BioEdit (Hall, 1999) for manual base-calling of ambiguous sites. Identity of the sequences was determined by similarity search using the Basic Local Alignment Search Tool for nucleotide sequences (BLASTN) against the NCBI 16S Microbial Database. The annotated DNA barcoding sequences were deposited at the NCBI GenBank database (accession numbers: KT895998–KT896071).

RESULT AND DISCUSSION

In overall, the result of the BLAST similarity search for the 74 sequences showed near 100% sequence similarity to the reference sequences currently available in the public NCBI database, in which the isolates were limited to the phylum Proteobacteria specifically gamma (γ)-proteobacteria and alpha (α)-proteobacteria. As illustrated in Figure 1, the diversity of the cultivable bacteria associated with Sabah PBVC were predominated by genera of *Alteromonas* sp. (47 % of the total 74 isolates) and *Ruegeria* sp. (39%), while *Lutimaribacter* sp. (7%), *Marinobacter* sp. (4%), and the single isolate identified as *Methylophaga* sp. (1%) comprised of the minor isolates.

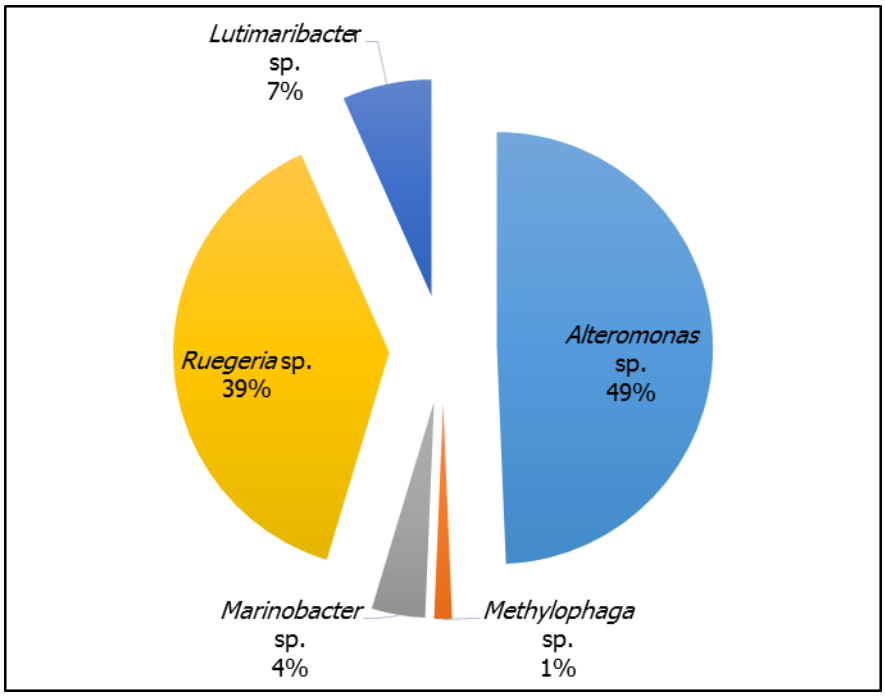

Figure 1. Genus-level distribution of the 16S rDNA gene sequences of cultivable bacteria associated with *Sabah Pyrodinium bahamense* var. *compressum*

The number of bacterial phyla reported here concurred with the phylum-level diversity detected in the culture-dependent assessment by Chin *et al.* (2013) on Sabah PBVC, in which the majority of the isolates were from the phyla Proteobacteria. Azanza *et al.* (2006) reported congruent findings based on screening of cultivable endosymbionts in axenic PBVC occurring in the Philippines, with the addition of bacteria under the phylum Firmicutes. The most likely explanation as to why the other phyla had not been detected previously is maybe because the representative taxa for these phyla could not be grown under standard culture protocols; i.e. the "unculturable' bacteria (Stewart, 2012).

BLASTN analysis (not shown here) showed that 29 isolates showed 100 % similarity of the partial partial 16S rRNA gene sequences with the species *Ruegeria pomeroyi* of the Roseobacter clade. This species also frequently reported to be closely associated with other dinoflagellates such as *Alexandrium tamarense* (Hold *et al.*, 2001), *A. ostenfeldii* (Allgaier *et al.*, 2003), *Scrippsiella trochoidea* (Hold *et al.*, 2001), and *Gymnodinium catenatum* (Green *et al.*, 2004). The second major isolates showed 100 % sequence homology with bacteria from the member of species *A. macleodii* in the *Alteromonas* clade. Similar to bacteria from *Roseobacter* clade, member of the *Alteromonas* clade reported capable of synthesizing multiple PSP derivatives (Gallacher *et al.*, 1997). Bacteria from this two major distinct clade reported to contain STXs toxin as shown from high-performance liquid chromatography (HPLC) assay (Gallacher and Smith, 1999), thus suggested that bacteria belonging to the clade of

Roseobacter and Alteromons capable of producing PSTs, and may subsequently play a role in the production of PST during HABs bloom.

In addition, five isolates in this study also showed 100 % sequence homology to members of *Lutimaribacter pacificus* and believed to have the capability of utilizing cyclohexylacetate (CHAA), and useful for bioremediation in marine ecosystems (Iwaki *et al*., 2013). Meanwhile, three isolates were found to have 100 % similarity with the sequence for *M. salsuginis*. This species was recently reported to have the ability to influence intracellular saxitoxin production in its host dinoflagellate (Albinsson *et al*., 2014) and capable of supporting the survival of dinoflagellates *G. catenatum* (Bolch *et al*., 2011). Finally, one isolate yielded 97.3 % 16S rDNA sequence homology with *Methylophaga* sp., an obligate methylotrophic bacterium that was observed to also be present in PBVC isolate from the Philippines (Onda *et al*., 2015). Therefore, this species was suggested to be involved in the secondary metabolism mechanisms.

Overall, the result showed that the bacterial diversity of Sabah strain PBVC was limited to Proteobacteria compared to the previous studies (Azanza *et al*., 2006; Chin *et al*., 2013). This inconsistency could resulted from the approach used such as culture condition or any unknown factors, as some bacteria could only be cultured under condition that vary from the traditional methods and still unable to grow (Schut *et al*., 1993). Hence, some of the bacterial communities associated with Sabah PBVC CC-UHABS-040(M) strain may unable to grow even when the natural growth conditions are met *in vitro*.

Information from this study will elucidate the thorough description of biodiversity cultivable bacteria through culture-dependent approach and the possible contribution of the associated bacteria in the toxicity PBVC isolated from Sabah.

CONCLUSION

Overall, the phylogenetic diversity was limited to bacteria phyla Proteobacteria (α- and γ-Proteobacteria) with Altermonas and Roseobacter clade reveals as a potential bacteria association with PST production. The finding from this study provides important information on the bacteria-dinoflagellate association and further characterization of the associated bacteria will enable the thorough analysis of potential associated bacteria involved in toxin production and its function.

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