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A metagenomic study of bacterial communities associated with the saxitoxin producing dinoflagellate, *Pyrodinium bahamense* var. *compressum*

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ABSTRACT

Aim: A number of reports have implicated the role of the symbiotic bacterial communities associated with toxic dinoflagellates in the biosynthesis of saxitoxin during harmful algal blooms (HABs). However, the exact mechanisms by which the bacteria facilitate toxin production remain inconclusive. The toxic dinoflagellate, *Pyrodinium bahamense* var. *compressum*, is the causative organism responsible for paralytic shellfish poisoning in the coastal waters of Sabah, and it is caused by the consumption of filter-feeding shellfish contaminated with the neurotoxin, saxitoxin. The present study aimed at characterizing the species diversity of symbiotic bacteria occurring within a monoalgal culture of *P. bahamense* var. *compressum*.
Methodology and results: The total bacterial DNA was amplified using paired-end 16S community sequencing on the Illumina platform, targeting the V3–V4 region of the 16S ribosomal RNA gene. Bacteria were classified into 20 classes, 43 orders, 60 families, and 105 genera. A total of 10 phyla were present, where the major phylum was Proteobacteria (69.5%). The major genera were *Pseudoruegeria* (32%), *Roseibium* (16%), *Hyphomonas* (16%), *Phaeobacter* (7%), *Lutimaribacter* (5%) and *Methylophaga* (5%). This study showed that the previous method of assessing microbial diversity occurring in *P. bahamense* var. *compressum* has underestimated the actual species diversity.
Conclusion, significance and impact of study: The high-throughput sequencing of the 16S metagenomes revealed hitherto unreported bacterial taxa associated with *P. bahamense* var. *compressum*. The findings of the present work will pave the way for further studies aimed at isolating and characterizing symbiotic bacteria that are likely to be associated with the biosynthesis of toxins.

Keywords: Pyrodinium bahamense var. *compressum*, harmful algal bloom, 16S ribosomal RNA gene, 16S metagenomic sequencing, bacteria association

INTRODUCTION

The thecate, chain-forming dinoflagellate, *Pyrodinium bahamense* var. *compressum* (PBVC) is the main causative organism responsible for paralytic shellfish poisoning (PSP) and has been recognised as the most important species responsible in harmful algal blooms (HAB) outbreaks in the Southeast Asian region. PSP is caused by Paralytic Shellfish Toxins (PSTs) and a suite of neurotoxins collectively known as saxitoxins (SXTs). These toxins act by blocking the movement of sodium ions through the nerve cell membranes thus inhibiting the flow of nerve impulses that leads to symptoms of PSP characterised by muscular paralysis (Mosher *et al.*, 1964) and possibly death from respiratory failure in severe cases (Tan and Ransangan, 2015).

In Malaysia, HABs have been frequently recorded in the west coast of Sabah since the first report of PBVC bloom in 1976 (Roy, 1977). These frequent HAB episodes have led to economic losses mainly to the mariculture industry as well as illness and fatalities due to consumption of PST-contaminated seafood (Usup *et al.*, 2012). According to the Sabah Department of Fisheries, the HAB that occurred in December of 2012 was one of the worst episodes recorded during which 40 cases of PSPs were reported including three fatal ones (Tan, 2014). Following reports on the 2012 HAB, seawater samples containing toxic PBVC were collected by the Unit for Harmful Algal Bloom studies of the Borneo Marine

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Research Institute, Universiti Malaysia Sabah. A monoalgal culture of the PBVC was then established, enabling an in-depth study of its symbiotic bacterial community.

The interaction between a dinoflagellate and the heterotrophic bacteria associated with them during HAB events is complex and dynamic in that they may undergo changes throughout the span of the bloom (Cole, 1982). The association is frequently linked with secretion of extracellular products by both organisms in the marine ecosystem (Baines and Pace, 1991; Agustí and Duarte, 2013). These heterotrophic bacteria include, but are not limited to, flavobacteria and roseobacter that are capable of recycling organic matter and fixing carbon secreted by their host into several possible fates in the ocean (Buchan *et al.*, 2014). Subsequently, there has been a growing volume of literature postulating the contributions by bacteria occurring symbiotically with toxic dinoflagellates in the synthesis of neurotoxins during a HAB event (Hold *et al.*, 2001; Alverca *et al.*, 2002; Green *et al.*, 2004; Azanza *et al.*, 2006; Pérez-Guzmán *et al.*, 2008).

The association between a toxic dinoflagellate and the bacteria they host could be implicated either directly (Plumley, 1997) or indirectly (Yoshinaga *et al.*, 1995) in toxin production. More importantly, these bacteria may play a role in modulating STX biosynthesis in dinoflagellates (Ho *et al.*, 2006; Stüken *et al.*, 2011). Besides that, these symbionts are capable of both stimulating (Cole, 1982) and inhibiting (Ferrier *et al.*, 2002; Zheng *et al.*, 2013; Li *et al.*, 2014) phytoplankton growth. Therefore, knowledge of microbial association with toxic dinoflagellates may help answer fundamental questions about their roles in the algal-bacterial symbiosis, particularly in the initiation, maintenance, and decline of HABs.

Taxonomic assessments on bacterial diversity found in environmental samples previously involved cultivating the bacteria in a nutrient-rich media prior to characterization. However, the culture-dependent approach can be selective in the sense that only cultivable bacteria are viable for screening while taxa that are difficult to grow in standard microbial media would be rendered obscure (Stewart, 2012). In addition to this, the pipeline for screening clone libraries of metabarcodes is laborious (Rastogi and Sani, 2011) and has been reported to be biased due to selective amplification (González and Moran, 1997). Consequently, bacterial diversity identified through conventional techniques has been recognised to account for less than one percent of microbial diversity *in vivo* (Rastogi and Sani, 2011). The advent of high-throughput sequencing has surmounted this oversight and has provided opportunities to explore the microbial diversity in complex environments in a time- and cost-effective manner (Wooley *et al.*, 2010).

The 16S rDNA, a small subunit of the ribosomal RNA, has been extensively applied to the DNA barcoding of microbes. This is because the region is present across all bacterial genomes and its nucleotide sequence is highly-conserved owing to its functional stability (Pace, 1997). In DNA metabarcoding of environmental samples using 16S rDNA, the paired-end short reads can be easily aligned while a standard method for subsequent data analysis has been established (Chaudhary *et al.*, 2015). Studies on the diversity of bacterial communities associated with toxic PBVC had hitherto been limited to culture-dependent methods (Azanza *et al.*, 2006; Chin *et al.*, 2013, Law *et al.*, 2017). This study extends the knowledge pertaining to non-culturable bacteria associated with PBVC isolated in Sabah, Malaysia by adopting a metagenomic approach based on next generation sequencing of the V3–V4 region of the 16S rRNA gene. The remainder of the discussion consists of an overview of symbiotic bacteria that are potentially significant in the context of HAB toxicity.

MATERIALS AND METHODS

Study site and sample preparation

Seawater samples containing PBVC were 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 (Tan, 2014). A pure, monoalgal culture of PBVC, CC-UHABS-040(M), was established and has been maintained through continuous laboratory culture 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. The cultures were left to grow under 150 µE/m^2/s irradiation and sub-cultured every two weeks into fresh f/2 medium following the culture conditions in Gedaria *et al.* (2007).

Total bacterial DNA isolation

Extraction of bacterial genomic DNA for 16S rDNA amplification was carried out using PowerLyzer® PowerSoil® DNA Isolation Kit (MoBio Laboratories). Approximately 300 mL of PBVC culture in the late exponential phase was gradually harvested by centrifugation (10,000 × *g*) in 50 mL tubes to finally obtain a total amount of 0.10–0.15 g of starting samples. The cell pellet was resuspended in the lysis buffer and transferred to a 1.5 mL tube, following which subsequent purification steps were undertaken according to manufacturer's instructions. The presence of genomic DNA was verified with 1% (w/v) agarose gel electrophoresis while the purity (260/280 nm and 260/230 nm ratios) was assayed with NanoVue Plus Spectrophotometer (GE Healthcare). The concentration was quantified with Qubit® dsDNA HS Assay Kit (Life Technologies) on a Qubit 2.0 Fluorometer (Life Technologies) and then analysed with Agilent 2100 Bioanalyzer (Agilent Technologies).

Amplification of 16S rDNA and high-throughput sequencing

The 16S rDNA was amplified using the following oligonucleotides: 5'-TCGTCGGCAGCGTCAGATGTGTAT

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AAGAGACAGCCTACGGGNGGCWGCAG-3' and 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3' which target the V3 and V4 hypervariable regions of the 16S. The underlined oligonucleotides are the Illumina adapter overhang sequences, while the non-underlined regions are the locus-specific primer sequences of S-D-Bact0341-b-S-17 and S-D-Bact-0785-a-A-21, respectively, as designed by Klindworth *et al.* (2013). A limited-cycle amplification step was performed to add multiplexing indices and Illumina sequencing adapter using the Nextera® XT Index Kit. A 25 µL reaction mixture containing 12.5 µL 2× KAPA Hifi HotStar ReadyMix (KAPA Biosystems, USA), 5 µM of each primer, and 50 ng template DNA was set up and PCR amplification was performed on a PTC-200 thermal cycler (MJ Research). Thermal cycling conditions were as follows: an initial denaturation at 95 °C for 3 min followed by 25 cycles at 95 °C for 30 sec, 55 °C for 30 sec, and 72°C for 30 sec, with a final extension at 72 °C for 5 min. The PCR products were then purified using Agencourt AMPure XP System (Beckman Coulter). The purified amplicons were subsequently subjected to index PCR in which Nextera XT dual indices (5 µL) and 2× KAPA HiFi HotStar Ready Mix (25 µL) were added to a final total volume of 50 µL. Thermal cycling conditions were as follows: an initial denaturation at 95 °C for 3 min followed by 8 cycles at 95 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 30 sec, with a final extension at 72 °C for 5 min. Second PCR clean-up was again performed using Agencourt AMPure XP System. The amplified libraries were validated by performing library quantification with Qubit® dsDNA HS Assay Kits (Life Technologies) and Agilent High Sensitivity DNA kit (Agilent Technologies). Next, concentration of the PCR amplicons was normalised to 10 nM prior to pooling, where the library concentration was verified using KAPA Library Quantification Kit (KAPA Biosystem) on Eco Real Time PCR system (Illumina). Finally, 600 µL of the pooled library including 10% of PhiX control library was loaded into a load sample reservoir. Paired-end sequencing of the library was then performed using the MiSeq Reagent Kit v2 (500-cycles) with the 2 × 250 bp library using an MiSeq sequencer (Illumina) at the Biotechnology Research Institute, Universiti Malaysia Sabah.

Analysis of DNA metabarcodes

The raw sequences obtained were firstly filtered based on quality using Fastq Quality Filter in Fastx Toolkit. Sequences with less than 80% of the expected length (as indicated by their Q20 scores) were discarded. PEAR (version 0.9.6) (Zhang *et al.*, 2014) was used to merge the paired-end reads by aligning overlapping regions of both reads. Alignments with low Q20 scores including those with short alignment lengths or those with high proportion of mismatches were further eliminated. Analysis of sequence similarities of the merged fragments against the National Center for Biotechnology Information (NCBI) 16S Microbial Database was carried out using BLASTN (E-value ≤ 10^{-6}) in the BLAST+ Package. The NCBI database was selected since it contains a larger amount of data compared to its alternatives and is therefore capable of providing a greater depth of taxonomic information from the bacterial 16S sequences (McDonald *et al.*, 2011). Analysis of taxonomic distribution was performed by analyzing the taxonomic distribution profile of the 16S sequences based on the similarity search results using the lowest common ancestor (LCA) algorithm (parameter: MinScore = 50, Top Percent = 10, and MinSupport = 5) in MEGAN 5.10.5 (Huson *et al.*, 2007). Rarefaction curves of this profile were generated using the same software. The original sequencing output files have been deposited in the Sequence Read Archive (SRA) service of the European Bioinformatics Institute (EBI) database under Accession Number SRR2877576.

RESULTS

The microbial diversity reported in the present work constitutes the most comprehensive assessment on the ecto- and endosymbiotic bacteria associated with *P. bahamense* var. *compressum* to date. A large portion (98%) of the bacterial 16S sequences generated through the high-throughput sequencing pipeline was phylogenetically assigned into operational taxonomic units (OTUs). Symbiotic bacteria associated with the Sabah PBVC were classified into 10 phyla, 20 classes, 43 orders, 60 families, and 105 genera (Figure 1). The remaining 2% could not be attributed to any known taxonomic divisions that could be due to limitations in the taxonomic coverage of representative 16S rDNA sequences in the public database.

Bacteria belonging to the phylum Proteobacteria were the most abundant type (69.5% of the assigned OTUs). In terms of proportion, over half of the Proteobacteria comprised of Alphaproteobacteria (60.5%) while Gammaproteobacteria formed a third (34.6%). Deltaproteobacteria constitutes 3.9% of the Proteobacteria diversity and Beta- and Epsilonproteobacteria accounted for the remaining 1% (Figure 2). Bacteroidetes was the second most abundant bacterial phylum (24% of the OTUs), followed by Planctomycetes (6%). Meanwhile, diversity of OTUs classified under the phyla Actinobacteria, Acidobacteria, Firmicutes, Lentisphaerae, Verrucomicrobia, Gemmatimonadetes, and Cyanobacteria were relatively scarce, making up approximately 0.2% of the total diversity.

Major bacterial genera identified to be associated with *P. bahamense* var. *compressum* from Sabah belonged to *Pseudoruegeria* (14.56% of the entire set of genera), *Roseibium* (7.52%), *Hyphomonas* (7.38%). Genera *Phaeobacter*, *Lutimaribacter*, *Methylophaga*, *Donghicola*, *Tropicibacter*, *Azotobacter*, and *Marinobacter* collectively accounted for 12.43% of the total 16S rDNA diversity, while the remaining genera represented 4.08% of the total population. In total, these genera represented approximately 45.97% of the total bacterial population associated with the Sabah PBVC; the status of the

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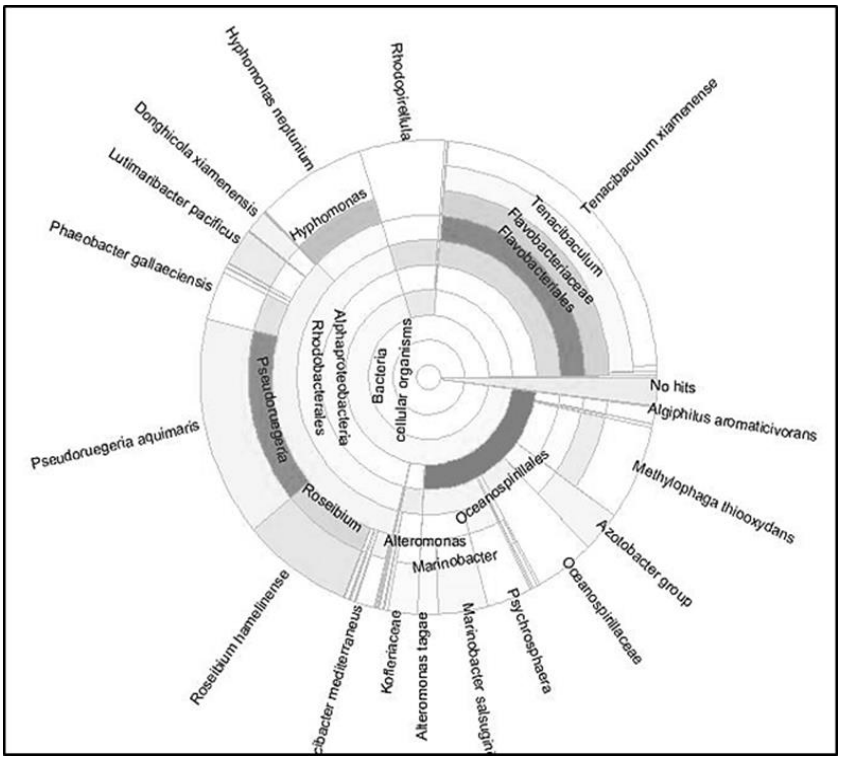

Figure 1: Taxonomic distribution of the 16S rDNA sequences for the overall population.

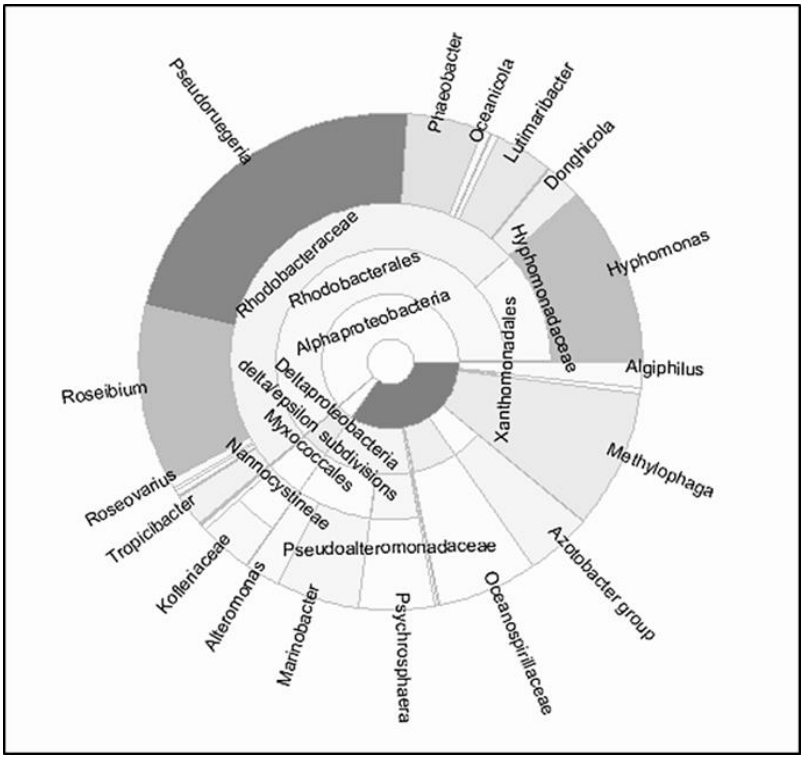

Figure 2: Breakdown of taxa under the Proteobacteria clade based on OTUs assessment of culture-independent study.

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remaining 16S rDNA sequences was only determined up to the family level (Figure 3).

DISCUSSION

A total of 10 phyla were identified via the culture-independent assessment on Sabah PVBC, in which only one percent (less than two phyla) were detected by using traditional 16S rDNA method from the previous studies by Azanza *et al.* (2006) and Chin *et al.* (2013). In addition, it is generally known that less than one percent of the total bacterial flora is capable of isolation through traditional culturing techniques as reported by Kennedy *et al.* (2010). The most likely explanation as to why the other phyla had not been detected previously is probably because the representative taxa for these phyla could not be grown under standard culture protocols; i.e. the "unculturable' bacteria (Stewart, 2012). Therefore, by using the 16S metagenomics approach, the diversities of both culturable and viable cells that are non-culturable (VBNCs) taxa could be isolated and further identified.

Interestingly, OTUs obtained in the culture-independent assessment belonged exclusively to the phylum Proteobacteria; specifically under classes α- and γ-proteobacteria (Figure 2). Thus, the phylum-level diversity reported here falls short compared to similar assessments by Azanza *et al.* (2006), Chin *et al.* (2013) and Law *et al.* (2017). Nonetheless, the predominance of symbiotic α- and γ-proteobacteria in the cells of PBVC is not surprising as it is a common feature that has been found across other algal hosts (Hold *et al.*, 2001; Sapp *et al.*, 2007; Liu *et al.*, 2011; Miranda *et al.*, 2013; Klindworth *et al.*, 2014). However, the proportion of α-proteobacteria to γ-proteobacteria enumerated through DNA metabarcoding with high-throughput sequencing was 2:1.

Based on the bacterial diversity assessment, the clade Roseobacter (family: Rhodobacteraceae; class: α-proteobacteria) were the most dominant in the PBVC culture. Roseobacter genera that were identified as dominant in this study include *Pseudoruegeria*, *Roseibium*, *Phaeobacter*, *Donghicola*, *Roseovarius*, and *Ruegeria*. These genera have been reported to be frequently involved in interactions such as dimethylsulfoniopropionate (DMSP) metabolism and the utilisation of dinoflagellate-derived compounds as their source of organic carbon (Buchan *et al.*, 2014). Species such as *Ruegeria pomeroyi* and *Phaeobacter gallaeciensis*, in particular, have been found to harbour corresponding genes for DMSP catabolism (Onda *et al.*, 2015). Hence, the abundance of these DMSP-decomposing bacteria could facilitate the catabolism of dinoflagellate by-products, which supports the hypothesis of a definitive association between the symbiotic bacteria and their host dinoflagellate cells.

Individuals belonging to the Roseobacter clade have been reported to be capable of synthesizing a range of secondary metabolites including signalling molecules, i.e. quorum sensing molecules (Miller and Bassler, 2001), antimicrobial compounds (Cude *et al.*, 2012), and growth-promoting compounds (Buchan *et al.*, 2014), which could justify the intimate relationship of symbiotic bacteria with the dinoflagellate cells. However, these types of interactions need to be further elaborated to provide better understanding on their contributions to the dinoflagellate cells. To a greater extent, such scrutiny will potentially reveal the roles of symbiotic bacteria in toxin

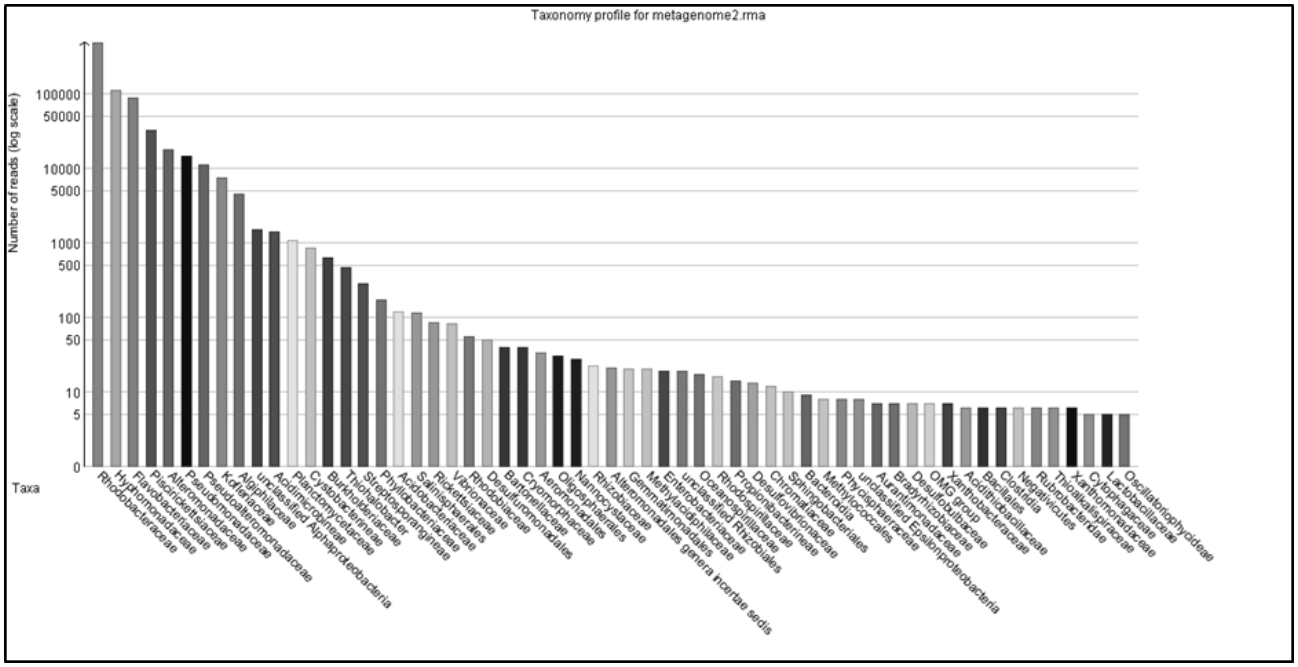

Figure 3: The frequency (in log scale) of the number of reads assigned at the Family level.

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production. Roseobacter clades have been suggested to excrete mostly N-acyl homoserine lactones (AHLs), a small quorum signalling molecule which is most commonly associated with cell-to-cell communications (Miller and Bassler, 2001). The ability of Roseobacters to communicate with one another via the quorum sensing molecule provides them the capacity to regulate gene expression, which is believed to be triggered in response to inconsistencies in cell population density, therefore presumably linked to control of toxin biosynthesis.

Interestingly, a major genus with 7.38% relative abundance possessed high similarity with the genus of *Hyphomonas* (class: α-proteobacteria; family: Hyphomonadaceae), which is likely to be involved in toxin biosynthesis. Similar congeneric bacteria have been reported in previous studies on toxic dinoflagellates namely PBVC (Chin *et al.*, 2013) and *Alexandrium* spp. (Hold *et al.*, 2001). The detection of this major genus could suggest their contribution in the toxicity of PBVC in the waters of Sabah, comparable to that in other toxic dinoflagellates. Thus, the dominance of bacteria from this genus may subsequently influence the bacterial-dinoflagellate interaction, which further enhances toxic production.

Bacteria identified as *Marinobacter* sp. were found to occur abundantly in this study and was reported to greatly influence the toxicity of the dinoflagellate *Gymnodinum catenatum* based on an *ex situ* experiment by Albinsson *et al.* (2014). In addition to being one of the major genus present, *Alteromonas* sp. was discovered to be abundant based on the culture-dependent approach and has been reported to be capable of synthesizing PSTs in *Alexandrium* spp. dinoflagellates (Gallacher *et al.*, 1997). These findings on major bacterial taxa occurring symbiotically in the Sabah PBVC could provide early insights into the identification of putatively toxic bacteria associated with HABs as reported in previous studies.

Moreover, bacteria belonging to Flavobacteria in the phylum Bacteroidetes (the second major phylum) have also been found to be dominant in other toxic dinoflagellates, *Alexandrium* spp. (Jasti *et al.*, 2005), and *Ostreopsis lenticularis* (Pérez-Guzmán *et al.*, 2008). More interestingly, several members of Flavobacteria such as *Flavobacterium* sp. were previously reported to cause diseases in red algae as reported by Goecke *et al.* (2010). Therefore, the abundance of Flavobacteria associated with PBVC is not surprising as it is a shared feature in other toxic dinoflagellates and their presence could potentially be related to toxin production or bloom initiation.

The biodiversity assessment of bacteria associated with PBVC in this study has been useful in surveying putatively toxin-producing bacteria associated with toxic dinoflagellates. Despite the frequently observed associations between marine bacteria and toxic dinoflagellates, the roles of the former in bloom toxicity remain elusive. Thus, the findings from this study may provide preliminary insights into the diversity of bacteria associated with PBVC isolated from the tropical waters of Borneo.

CONCLUSION

We discovered a high diversity of bacteria associated with PBVC culture in Sabah that may potentially be constituted by novel species. The bacteria identified were classified into the 10 phyla with Proteobacteria being the most abundant. The major genus present was *Pseudoruegeria*, which is classified within the Roseobacter clade and frequently proposed to have an indirect involvement in HAB occurrences. We have also demonstrated that culture-independent metagenomic analysis targeting 16S rDNA is the most promising method that is able to furnish a thorough description of microbial community in environmental samples. Based on previous studies, several major bacteria present in the 16S metagenomics study are potentially putatively toxic or are involved toxin biosynthesis. These include members of the Roseobacter clade such as *Hyphomonas* sp., *Marinobacter* sp., *Alteromonas* sp., and Flavobacteria. Thus, the findings presented here furthered our understanding of the biodiversity of bacteria associated with toxic PBVC and may shed light on their involvement in toxin production during HABs. Further analyses on the genome of some of the putative PST-producing bacteria, *Rugeria* sp. and *Marinobacter* sp. are currently being carried out as a follow-up to this study.

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