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Ribosomal DNA Analysis of Marine Microbes Associated with Toxin-producing *Pyrodinium bahamense* var. *compressum* (Böhm), a Harmful Algal Bloom Species

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ABSTRACT

Blooms of the toxic alga, *Pyrodinium bahamense* var. *compressum* (Böhm), have become a problem in Malaysia over the past three decades. The alga is a causative agent of paralytic shellfish poisoning, a potentially fatal neurological disorder. Past research suggest that bacteria-algae association may play a direct or indirect role in toxin production. As such, ribosomal DNA-based restriction enzyme analysis for the identification of bacteria associated with *Pyrodinium* sps. was undertaken. A total of 16 bacterial isolates were successfully obtained from the clonal cultures of *Pyrodinium* sps. The diversity of the extracellular bacteria associated with *Pyrodinium bahamense* var. *compressum* was limited to the Phyla Proteobacteria and Actinobacteria. The major bacterial species identified included *Alcanivorax* spp. and *Hyphomonas* spp., whereas *Kocuria* spp., *Nesterenkonia* spp., *Alteromonas* spp., *Roseobacter* spp., *Xanthomonas* spp., and *Acinetobacter* spp. were identified as minor isolates. The identified bacterium *Hyphomonas* spp. exhibited high sequence identity with an unknown bacterium strain, SCRIPPS_739, in the GenBank database that is known to be associated with toxic and non-toxic dinoflagellates, *Alexandrium* spp. and *Scrippsiella trochoidea*, respectively.

Keywords: Bacteria, harmful algal bloom, paralytic shellfish poisoning, *Pyrodinium bahamense* var. *compressum*, ribosomal DNA

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INTRODUCTION

In Malaysia, toxic harmful algal bloom (HAB) only occurs in the coastal waters of

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west Sabah, where the causative organism is the dinoflagellate, *Pyrodinium bahamense* var. *compressum* (*Pyrodinium)*. *Pyrodinium* blooms are very common in the Southeast Asian region, and where the blooms have often been reported in the waters of Malaysia (Anton *et al*., 2008), Brunei (Seliger, 1989), Indonesia (Wiadnyana, 1996) and the Philippines (Azanza-Corrales *&* Hall, 1993). The photosynthetic alga is one of the causative agents of paralytic shellfish poisoning (PSP), a potentially fatal neurological disorder.

Paralytic shellfish toxins consist of saxitoxin and at least 20 other chemically related derivatives, which block sodium channels in mammalian nerve cells, and thus prevent the flow of signals along the neuron (Gallacher *et al*., 1997; Plumley *et al*., 1999). Initially, marine dinoflagellates were considered to be the sole contributor for toxin production during a toxic algal bloom; however, according to Plumley *et al*. (1999), it is postulated that certain marine bacteria attached to or associated with algae may able to synthesize toxins and/or influence the toxicity of the algae.

Bacteria-algae interactions play an important role in HAB dynamics, where it has been postulated that they are regulators in the processes of algal bloom initiation, maintenance and decline (Ferrier *et al*., 2002). The diversity of bacteria associated with microalgae belongs to two bacterial phyla, namely, the Proteobacteria (α-Proteobacteria and γ-Proteobacteria) and the Cytophaga-Flavobacter-Bacteroides (Alverca *et al*., 2002).

In addition, it has been suggested that bacteria-algae association may play a direct or indirect role in toxin production. These toxin-producing bacteria can autonomously produce toxin and have the ability to metabolize the toxins, converting them from one derivative to another (Córdova *et al*., 2003). Thus, bacteria may be involved in both the production and modification of these toxins. Bacteria that are involved in PSP toxin production have been identified in dinoflagellate *Alexandrium lusitanicum* (Plumley *et al*., 1999) and *Alexandrium* sp. (Gallecher *et al*., 1997).

Due to the close relationships between algae and bacteria, it is necessary to identify the genetic diversity of the bacteria to better understand the occurrence of toxic blooms and to assess the bacteria-algae association in the marine ecosystems. In this study, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of 16S ribosomal DNA (rDNA) gene was used to investigate the bacterial population associated with the toxic dinoflagellate, *Pyrodinium bahamense* var. *compressum*.

MATERIALS AND METHOD

The culture and isolation of *Pyrodinium bahamense* var. *compressum* were carried out based on the methods described by Guillard (1975) and Guillard and Morton (2004). All the cultures and isolation procedures were carried out under aseptic condition to prevent contamination. The isolated *Pyrodinium* cells were transferred through ten drops of sterile f/2 media (≈

20 µL) for serial washing, and this was done to do away with those marine bacteria that were not associated with *Pyrodinium bahamense* var. *compressum*.

Then, the cultures were checked for bacterial growth after two weeks from the initial isolation of *Pyrodinium* cells. Approximately 100 µl of *Pyrodinium* cultures (≈ 200 cells/ml) were spread on the surface of marine agar media (Difco, USA) and kept at 37°C for overnight incubation. All bacterial isolation and culture procedures were performed in a laminar flow cabinet under sterile conditions.

Preparation of pure bacteria culture was done based on the streak plate method (Beveridge & Daview, 1983), where the isolated bacteria were then used for *gram-staining* and 16S rDNA PCR amplification. The *gram-staining* method was used to differentiate bacteria into two major groups, namely, *gram-positive* and *gram-negative* bacteria, based on the method described by Beveridge and Daview (1983).

The bacterial genomic DNA was obtained by lysing the bacterial cells at 100°C. PCR was then carried out in a 20 µl reaction mixture containing 1 U Taq DNA polymerase (Promega, USA), 1 x PCR buffer (Promega, USA), 3.75 mM $MgCl_2$ (Promega, USA), 500 µM dNTPs (Promega, USA), 1.25 µM each primer and 1 µl of the supernatant of the lysed bacterial cells (≈ 50 ng genomic DNA). Universal PCR primers, 27F (5'-AGAGTTTGATCMTGGCTCAG -3') and 1492R (5'- TACGYTACCTTGTTACGACT-3') were used for the 16S rDNA amplification of the marine bacteria. The PCR amplification was performed as follows: 2 min of initial denaturation step at 94°C, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 1 min. A final extension at 72°C for 2 min was also included. The PCR was carried out on a PTC-200 thermal cycler (Bio-Rad, USA). After gel electrophoresis, the resulting PCR bands within the expected size of 1,500 bp were excised and purified by using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's protocol.

The purified PCR products were digested with six restriction enzymes (RE), *Hae*III, *Dpn*II, *Alu*I, *Rsa*I, *Bam*HI and *Xho*I (New England Biolabs, USA). The RE analysis was carried out in a 10 µl reaction mixture containing 1 x restriction enzyme buffer, 10µg/µl BSA, 5U of RE and 5µl of DNA template. A total of 20 µl of mineral oil was added to prevent evaporation of the sample. The RE digestion was carried out in a PTC-200 thermal cycler (Bio-Rad, USA). The reaction conditions of each RE were according to the manufacturer's protocol.

The purified PCR products (≈ 20 ng/µl) were then sequenced using the BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, USA). Sequencing was performed using the ABI 3130 Genetic Analyzer (Applied Biosystems, USA).

Meanwhile, the analysis of the 16S rDNA sequences of the bacteria isolates was conducted using the software Lasergene 6.1 (DNASTAR, Inc., USA). The rDNA sequences of the bacteria isolates were

then aligned with DNA sequences of the marine bacteria obtained from GenBank (www.ncbi.nih.gov). The construction of phylogenetic tree (neighbour-joining) for the assembled sequences was performed using MEGA 4.0 software (Tamura *et al*., 2007), whereby the sequences of the other bacteria were also retrieved and incorporated: *Alteromonas* sp., DQ412075; *Hyphomonas* sp., AY258084; *Alcanivorax venustensis*, DQ768632; *Roseobacter* sp., EF512125; *Acinetobacter* sp., DQ366086; *Xanthomonas* sp., DQ213024; *Luteimonas aestuarii*, EF660758; *Lysobacter gummosus*, AB161361; *Stenotrophomonas* sp., AM400231; *Kocuria rhizophila*, AY030315; *Nesterenkonia* sp., AY914062.

RESULTS AND DISCUSSION

A total of 16 individual bacterial colonies were successfully isolated from the *Pyrodinium* cultures. The isolated bacterial colonies (Pyro-Bac) were labelled as 19A, 22A, 25A, 27A, 27B, 27C, 27D, 27E, 27F, 27G, 28A, 29A, 30B, B5, B9 and B18. The majority of the Pyro-Bac samples were *gram negative*, except for two Pyro-Bac, 27A and B5, which were stained in purple colour (*gram positive*). In addition, the majority of the Pyro-Bac samples were rod-like or bacillus shape, except for three Pyro-Bac (27A, 27E and B5), whereby their morphology was cocoid or spherical shape and sometimes short rod-like shape could also be seen.

The PCR amplification was conducted using the universal ribosomal DNA (rDNA) primers 27F and 1492R, which yielded positive results of a single band at the expected size of approximately 1.5 kb. It is an alternative way for identifying bacteria, which does not require prior knowledge in the bacterial characteristics. Compared to the conventional morphological and metabolic identification techniques, 16S rDNA sequence-based bacterial identification is simpler and accurate because it is based on highly conserved stretches of DNA sequences. Meanwhile, the 16S rDNA gene has always been the choice for bacterial identification by bacteriologists because it has a large and authentic sequence database, where comparisons of the sequences around the world could be done and the BLAST search programme could also be used even by users with limited expertise in the field of bacterial systematic (Mehnaz *et al*., 2006).

Restriction enzymes *Hae*III, *Dpn*II, *Alu*I and *Rsa*I yielded positive results, whereas for RE *Bam*HI and *Xho*I, no clear restriction patterns were observed. The four restriction enzymes had successfully produced eight riboprint (riboprint A – H) according to their restriction patterns (Fig. 1). The major population of bacteria associated with *Pyrodinium bahamense* var. *compressum* belonged to riboprint C, representing seven Pyro-Bac samples. This was followed by riboprints D with three Pyro-Bac samples. The remaining 6 riboprint had one Pyro-Bac sample each.

According to Clark (1997), twelve enzymes with four-based recognition sequence could yield up to 15 % of the interested gene sequence without necessary cloning or DNA sequencing. This is because

rDNA fingerprinting is a cost-effective method meant to evaluate the DNA sequence variation without DNA sequencing (Clark, 1997; Weising *et al*., 2005). The main advantages of rDNA fingerprinting are cost-effectiveness and high reproducibility. However, there are also some drawbacks, such as tedious experimental procedures and the requirement of microgram amounts of relatively pure and intact DNA (Weising *et al*., 2005).

The 16 Pyro-Bac samples were then subjected to direct sequencing to confirm their identity. The restriction enzyme analysis data should be combined with the sequencing data to produce a better data comparison and interpretation (Dowling *et al*., 1996). The sequences of the Pyro-Bac samples were obtained (GenBank accession nos: EF688604 to EF688619) after the PCR amplification using the universal rDNA primers, 27F and 1492R.

The sequencing results showed that the diversity of the extracellular bacteria associated with *Pyrodinium* was limited to the Phyla Proteobacteria (α- proteobacteria and γ-proteobacteria) and Actinobacteria, which are similar to the findings by Alverca *et al*. (2002) and Azanza *et al*. (2006). Alverca and colleagues reported that γ-proteobacteria was found outside the dinoflagellate, *Gyrodinium instriatum*, whereas β-proteobacteria and *Cytophaga-Flavobacterium-Bacteroides* were observed in the cytoplasm and nuclear; however, no α-proteobacteria was detected either free-living or intracellular (Alverca *et al*., 2002).

An investigation of the bacteria associated with *Pyrodinium bahamense* var. *compressum* was also conducted by Azanza and co-workers (2006). They investigated bacterial endosymbionts of *Pyrodinium bahamense* var. *compressum,* where bacteria from the phyla Proteobacteria, Actinobacteria and Firmicutes were identified. The identified microorganisms included *Moraxella* spp., *Bacillus* spp., *Erythrobacter* spp., *Micrococcus* spp., *Pseudomonas putida* and *Dietzia maris*. Meanwhile, a comparison with the findings

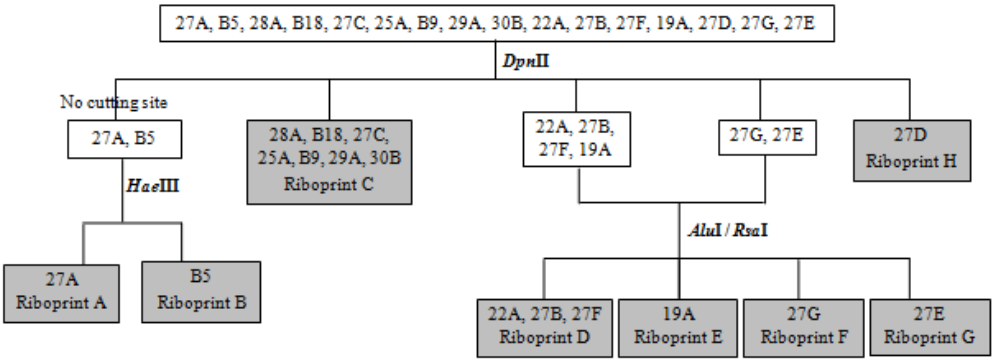

Fig.1: Division of the 16 Pyro-Bac samples based on the restriction patterns produced by the restriction enzymes *Hae*III, *Dpn*II, *Rsa*I and *Alu*I

of Azanza *et al*. (2006) showed that both proteobacteria and actinobacteria were also identified in this study; however, the identity of the microorganisms was different. This can be attributed to the presence of two different groups of microorganisms predominating intracellularlly and extracellularly, as mentioned in the findings of Alverca *et al*. (2002).

The major bacteria associated with *Pyrodinium* was from the genus *Alcanivorax* (97 to 100% identity), which belongs to the γ-proteobacteria class. This common marine bacterium has been previously described by Fernandez-Martinez *et al*. (2003) and Liu and Shao (2005), where γ-proteobacteria was isolated from the Mediterranean Sea and Bohai Sea, respectively. Another major bacterium associated with *Pyrodinium* was from the genus *Hyphomonas* (98 to 99% identity), belonging to α-proteobacteria class. In fact, the three isolates (22A, 27B, 27F) exhibited high sequence identity with an unknown bacterium strain, SCRIPPS_739, that is associated with toxic and non-toxic dinoflagellates, *Alexandrium spp*. and *Scrippsiella trochoidea* (GenBank accession no.: AF359546), as reported by Hold *et al.* (2001).

The minor bacteria isolates identified in this study were *Alteromonas* spp., *Xanthomonas* spp., *Acinetobacter* spp., *Roseobacter* spp., *Kocuria* spp., and *Nesterenkonia* spp. (one isolate each). *Alteromonas* spp., *Xanthomonas* spp., *Acinetobacter* spp., and *Roseobacter* spp. belonging to the Proteobacteria class, whereas *Kocuria* spp. and *Nesterenkonia* spp. belonging to the Actinobacteria class. Bacteria from the genus *Alteromonas* (Pyro-Bac 19A) and *Roseobacter* (Pyro-Bac 27D) have been reported to be associated with the harmful algal bloom species of the genera *Alexandrium*. Jasti *et al*. (2005) investigated that the genera *Roseobacter* showed a higher degree of association with the PSP toxin-producing dinoflagellate, *Alexandrium* spp. than with other bacterial groups, whereby *Alteromonas* spp. was one of the identified bacteria associated with *Alexandrium*. However, no toxicity test was carried out by in their studies (Jasti *et al*., 2005).

The same findings were also observed by Wichels *et al*. (2004), whereby both *Roseobacter* and *Alteromonas* bacteria were isolated from the toxic *Alexandrium tamarense* blooms off the Orkney Isles and the First of Forth of Scotland. Gallacher *et al*. (1997) provided strong evidence that a range of bacterial species isolated from the *Alexandrium* spp. cultures were capable of autonomous production of paralytic shellfish toxin. However, the identity of each bacteria species remains unknown.

The *Roseobacter* clade of marine bacteria was also found to be associated with the harmful alga *Pfiesteria*, one of the major producers of dimethylsulfoniopropionate (DMSP). It has been suggested that *Roseobacter* bacteria benefit from the association with DMSP-producing dinoflagellates because of the high metabolic rate at which *Roseobacter* can degrade them (Miller & Belas 2004).

The *gram-negative* bacteria strain, Pyro-Bac 27G, isolated in the study

exhibited a high identity (>95%) with a few different bacteria such as *Xanthomonas*, *Luteimonas*, *Lysobacter*, *Stenotrophomonas*, and several unknown gammaproteobacteria. Therefore, the identity of the Pyro-Bac 27G isolate remains uncertain. Pyro-Bac 27E (γ-proteobacteria) and 27A (Actinobacteria) were considered as contaminated, whereby the species were distributed in widespread, diverse habitats (Bull *et al*., 2005). The remaining bacteria isolate, Pyro-Bac B5, was identified as *Nesterenkonia* (95 to 98% identity), whereby the bacterium is a common marine actinobacterium and has previously been isolated from Lake Abjata in Ethiopia (Delgado *et al*. 2006).

According to Fox *et al*. (1992), 16S rDNA sequence identity may not necessarily be sufficient to guarantee species identity. Fox and colleagues had compared the sequences of three different psychrophilic *Bacillus* strains. The strains exhibited more than 99.5% sequence identity and the results could be regarded as identical. In contrast, previously published DNA-DNA hybridization results have convincingly established that the three strains did not belong to the same species. These results emphasize that the identity of the 16S rDNA sequence is not a good criterion to guarantee species identity. Although 16S rDNA sequences could be routinely used to distinguish and establish relationships between the genera and well-resolved species, very diverged species might not be recognizable (Fox *et al*., 1992). For this reason, all the sequences of the sixteen Pyro-Bac samples were identified only to its genus level.

The molecular phylogenetic tree of the partial 16S rDNA of bacteria associated with *Pyrodinium bahamense* var. *compressum*, together with sequences of other marine microbes obtained from the GenBank, was constructed and is shown in Fig.2. The tree was constructed using the Neighbour-Joining (NJ) algorithm.

Eight clusters of the bacterial groups (clusters A to H) were identified according to the phylogenetic NJ tree. The phlylogenetic results were corroborated by the ribotyping restriction fragment patterns analysis data as well as the sequencing data. The phylogenetic tree also distinctively separated the three bacterial phyla, the Actinobacteria, the α-Proteobacteria and the γ-Proteobacteria. Similar results were also reported by Wichels *et al*. (2004), who investigated the bacterial diversity in toxic *Alexamdrium tamarense* blooms in the Scotland waters and the phylogenetic tree they constructed also separated the tree into two phyla Proteobacteria (α and γ subdivisions) and Bacteroids.

According to Erko Stackebrandt (2002), the available phylogenetic branching pattern reflects the actual situation in nature quite incompletely. Phylogenetic reconstructions are based on the similarities from only a few nucleotides and thus can be considered as an approximation. The gradually emerging 16S rRNA tree is probably best considered as presenting a hypothesis about the relationships which should be tested on the basis of supporting data. The phylogenetic branching pattern serves as an aid to recognize the clusters of phylogenetically related strains but the delineation of

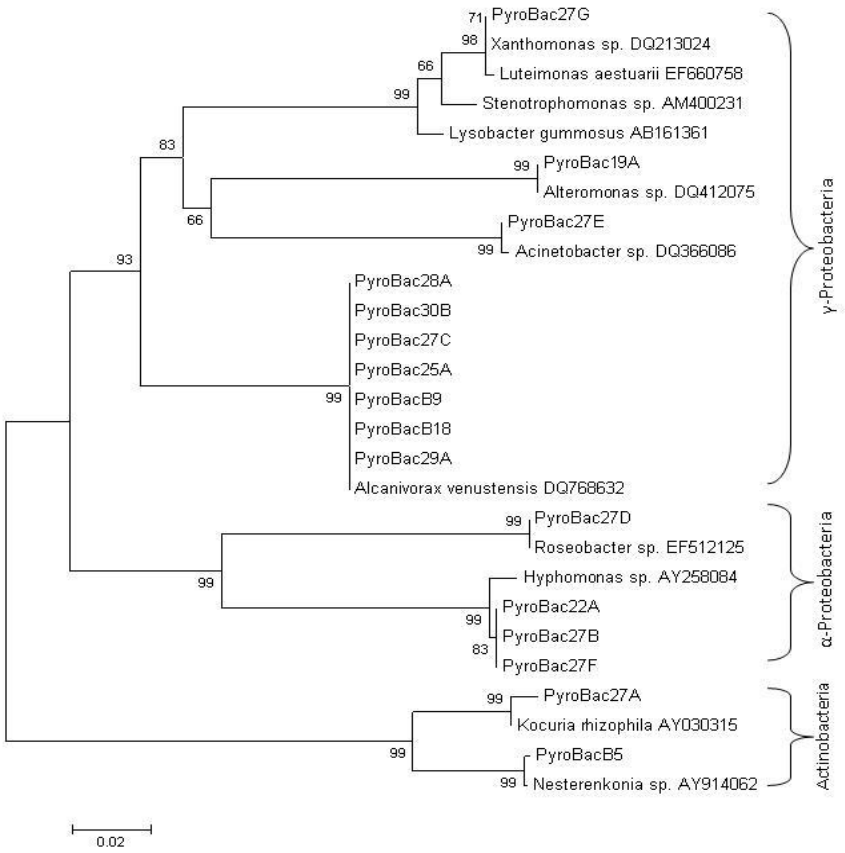

Fig.2: Molecular phylogenetic tree (neighbour-joining) of the partial 16S rDNA for bacteria associated with *Pyrodinium bahamense* var. *compressum*

phylogenetically neighbouring clusters is predominantly made on the basis of morphology, biochemical properties and episematic molecules (Stackebrandt, 2002).

CONCLUSION

The diversity of cultivable extracellular marine microbes associated with Malaysian *Pyrodinium bahamense* var. *compressum* strain has been shown to be limited to the Phyla Proteobacteria (α-proteobacteria and γ-proteobacteria) and Actinobacteria. Majority of the bacterial isolates are *gram-negative* rods which are common in the marine environment. Some of the isolates (*Hyphomonas* spp., *Roseobacter* spp. and *Alteromonas* spp.) were shown to be associated with other toxin-producing HAB species, such as *Alexandrium spp*. and *Scrippsiella trochoidea*. Although toxicity assessments were not part of this study, the species have previously been described as producing toxin. Therefore, an approach combining the information in this study with toxin detection methods could add much pertinent information regarding the participation of the bacteria in the event of harmful algal bloom. Further studies using

metagenomic techniques can be used in the future to characterize a complete diversity of the marine microbes that are associated with *Pyrodinium bahamense* var. *compressum* as to represent both culturable and non-culturable bacteria groups, and thereby enlarging the limited information available about the natural bacterial environment associated with dinoflagellates. In addition, studies on the interactions between algae and bacteria are beneficial to better understand the way both organisms interact during algal bloom initiation, maintenance and decline.

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