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# Research Article
# Diversity of Bacterial Communities Associated with Toxic and Non-toxic Dinoflagellates from Malacca, Malaysia

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## Abstract
**Background:** Association between bacteria and marine dinoflagellates is one of the important factors in harmful algae bloom formation and toxin production by dinoflagellates. The diversity of the bacteria associated with clonal cultures of both toxic and non-toxic dinoflagellates, *Alexandrium tamiyavanichii* and *Alexandrium leei* were assessed by using culture dependent and culture independent approaches. **Materials and Methods:** In culture-dependent approach, isolated bacteria were identified based on 16S rDNA sequences. In culture-independent approach bulk nucleic acid was extracted directly from the cultures of dinoflagellates. The extracted nucleic acid was then used for 16S rDNA amplification to construct the clone libraries. Nucleotide sequences obtained were then compared with sequences from the GenBank Database. **Results:** A total of 17 bacteria were isolated from culture of *Alexandrium tamiyavanichii* and 21 were from *Alexandrium leei*. The 16S rDNA sequences analysis revealed that bacteria associated with two species of dinoflagellates were a diverse group of α-proteobacteria (40%) followed by γ-proteobacteria and cytophaga flavobacter bacteroides (CFB) (21%) and firmicutes (14.7%). In 16S PCR cloning and sequencing analysis, a total of 50 phylotypes were obtained from the directly amplified DNA of both cultures, of which 22 phylotypes were obtained from *Alexandrium tamiyavanichii* and 28 from *Alexandrium leei*. Sequence analysis of the clones also revealed that associated bacteria belonged to α-proteobacteria (48.4%), CFB (21.2%), unknown bacteria group (18.9%) and γ-proteobacteria (10.5%). In addition, one phylotype belonged to Planctomycetes was discovered from culture of *Alexandrium leei*. The results suggest that a number of different bacterial species are associated with dinoflagellates, some of which are common to each of the dinoflagellate cultures examined, whereas others appear to be unique to a particular dinoflagellate. **Conclusion:** Present study showed that culture-independent method is necessary to capture better diversity of these associated bacteria since a large percentage of uncultured bacteria sequences were obtained.

Key words: Bacterial community, 16S rDNA analysis, dinoflagellates

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Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Phytoplankton and bacterioplankton act as base components of marine ecosystems. Microbes use broken up natural matter processed by phytoplankton. In the meantime, phytoplankton uses the supplements remineralized by microscopic organisms and microorganisms and phytoplankton might rival one another under supplement constrained conditions for both living beings[1]. Hagstrom *et al*.[2] and Glockner *et al*.[3] in their respective study had discovered that $\alpha$ and $\gamma$-proteobacteria, as well as Cytophaga Flavobacter Bacteroidetes (CFB) and Planctomycetes have been distinguished as paramount parts of seaside bacterioplankton.

Dinoflagellates are a very large and diverse group of eukaryotic organisms that play a major role in aquatic food webs of both, fresh water and marine habitats. Nonetheless, many dinoflagellates are known as Harmful Algal Blooms (HABs) species. An algal bloom is a rapid increase of algal population in a water system and those algal bloom that cause adverse effects are called HABs[4]. Some dinoflagellates species can produce toxins known as Paralytic Shellfish Toxins (PST) which causes paralysis and writhing in people who consume the contaminated shellfish[5], thereby, causing damage to the fisheries industry and public health. Almost all PSP outbreaks have been caused by 10 toxic *Alexandrium* species including *A. tamiyavanichii* and *A. minutum*[6].

Cultures of dinoflagellates contain a considerable amount of bacteria which probably accompanied the dinoflagellates in the original sample. Interactions between algae and bacteria are commonly observed in both freshwater and marine ecosystems with bacteria increasingly cited as potentially important regulators in the process of algal bloom initiation, maintenance and decline[7]. This community of bacteria is believed to be associated with each dinoflagellate, which also believed can contribute to dinoflagellate physiology and toxigenesis[7].

Interactions between bacteria and phytoplankton such as dinoflagellates may play an important role in regulating dinoflagellate toxin production[8]. Previous studies on the interactions between bacteria and dinoflagellates have been shown to be highly variable and are sometimes specific. Effects of bacteria on toxic dinoflagellates include negative effects such as cell lysis and death[9] and positive effects such as growth enhancement with an addition of bacteria to cultures[10]. In the context of Harmful Algal Blooms (HABs), the specificity of these associations has also been researched[11]. For a few dinoflagellates, it has been speculated that microscopic organisms, either intracellular or appended to the dinoflagellates could be included in the generation of toxins, for example crippled shellfish poisons[12,13]. In fact, paralytic shellfish toxin has been detected in bacteria isolated from *Alexandrium* sp., cultures[13,14].

In Malaysia, many Harmful Algal Blooms (HABs) occurrences had been reported at several coastal areas such as in Sabah, Straits of Malacca, Kelantan and Johor[15,16]. Such phenomenon had given many parties especially aquaculturists problems because it can cause seafood poisoning and a huge loss in profit for the aquaculture industry. In Malaysia, the most significant PSP-toxin producing species is *Pyrodinium bahamense* var., *compressum*[17]. In the Straits of Malacca, however, there is evidence that paralytic shellfish poisoning events are due primarily to *A. tamiyavanichii*. previous report by Kodama *et al*.[18] showed the presence of *A. tamiyavanichii* in Thai waters.

Bacterial assemblage found in the phycosphere of dinoflagellates may play an important role in regulating dinoflagellate toxin production. While several studies have suggested that bacteria-phytoplankton interactions have the potential to dramatically influence harmful algal bloom dynamics, little is known about how bacteria and phytoplankton communities interact at the species composition level. At present, the precise association of bacteria with cultured dinoflagellates is still not well understood. Thus, diversity of bacterial community associated with dinoflagellate should be investigated as the first step to better understanding of the bacteria-dinoflagellate relationships.

Generally, two approaches were used to assess bacterial diversity, i.e., culture dependent and culture independent. A culture dependent method relies on the cultivation of bacteria in the laboratory and are classified and identified by phenotypic or genotypic characteristics. Although, it is convenient to understand the physiological potential of the isolated bacteria, it does not provide complete makeup of mcirobial composition[19,20]. It has been discovered that majority of the microorganisms present in the environments are not readily culturable and therefore not accessible for biotechnology or basic research[20]. Therefore, culture independent approach is essential to evaluate microbial diversity as it circumvents the need of culturing microbes in the laboratory. Moreover, technology to access the genomic DNA or RNA of microorganisms, directly from environmental samples without prior cultivation has opened new ways of understanding microbial diversity and functions[21-23]. Thus, the objective of present study is to investigate the composition of bacteria associated with dinoflagellates by using culture dependent technique complimented with culture independent approach.

## MATERIALS AND METHODS

**Dinoflagellates culture collection:** Clonal cultures of non-toxic and toxic dinoflagellates, *Alexandrium leei* (AlMS02) and *Alexandrium tamiyavanichii* (AcMS01) were obtained from University Kebangsaan Malaysia (UKM) Microalgae Culture Collections. *Alexandrium leei* and *Alexandrium tamiyavanichii* were initially isolated from Sebatu, Malacca during a PSP episode. *Alexandrium leei* confirmed as non-toxic whereas *A. tamiyavanichii* as PSP toxin producer[24]. Cultures were routinely grown in ES-DK medium[25] containing vitamin f/2 stock. The cultures were grown at 26°C under a 14:10 h light:dark cycle.

**Isolation and identification of bacteria associated with dinoflagellates:** Cultures of dinoflagellates with exponential-phase growth had been used in this bacteria isolation. Approximately 1 mL of both toxic and non-toxic dinoflagellates cultures were added to 9 mL sterilized seawater and were serially diluted up to 6 fold. Then, 100 µL of each dilution were spread onto the surface of marine agar (Difco, USA) and incubated at 28°C up to 14 days. Pure bacterial cultures were obtained after successive transfer of individual colony in marine agar plates. Resultant bacterial isolates were categorized by colony morphology and examined microscopically. For the amplification of 16S rDNA by Polymerase Chain Reaction (PCR), a single colony was picked from the overnight culture and resuspended in 500 µL TE buffer (pH 7.8). Then, 200 µL of bacterial suspension was transferred to a PCR tube and subjected to boiling at 100°C for 10 min. About 4 µL of the bacteria suspension were used as the DNA templates for 16S rDNA amplification. The PCR was carried out using the primer pair 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GTTTACCT TGTTACGACTT-3')[26]. The PCR was carried out on PTC-0200G thermo cycler (Bio-Rad Laboratories, Inc., USA) in a 50 µL reaction containing DNA template, 1x reaction buffer, 2.5 mM $MgCl_2$, 200 mM of each dATP, dCTP, dGTP and dTTP (Vivantis Technologies, Malaysia), 0.5 mM of each primer and 2 U of Taq polymerase (Vivantis Technologies, Malaysia). The PCR cycle was as follows: Preheating at 95°C for 5 min, 26 cycles of 95°C for 30 sec, 55°C for 1 min and 72°C for 2 min, followed by 72°C for 10 min. Fragment sizes of the PCR products were determined by electrophoresis in 1% agarose gel stained with 1x SYBR safe DNA gel stain (Life Technologies, USA) in 1x TAE buffer. The gel was then visualized under ultra violet light and images were taken using Alpha Imager 2200 (Alpha Innotech, USA).

**16S rDNA sequencing and sequence analysis:** The PCR products were directly sequenced by using an ABI 3730XL automated DNA sequencer (PE ABI, USA). Bidirectional sequencing was sent and sequenced by FirstBase Sdn. Bhd. The raw and unaligned 16S rDNA full sequences were edited by using sequence assembly software DNA Baser Sequence Assembler v3.x (2012) (Heracle BioSoft SRL Romania). The sequences were then compared with sequences from GenBank using BLASTn[27] (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to obtain the closest phylogenetic neighbours. Phylogenetic analysis[28] were conducted using MEGA version 5.

**Analysis of bacteria community by 16S rDNA cloning and sequencing:** About 10 mL of each dinoflagellates culture were used to extract the bulk DNA. The bulk DNA was extracted using GeneJET™ Genomic DNA Purification Kit (Fermentas, USA) following the manufacturer's protocol. Approximately 200 ng of genomic DNA of bacteria were amplified using 16S rRNA gene-specific primer set 63F (5'-CAGGCCTAACACATGCA AGTC-3') and 1389R (5'-ACGGGCGGTGTGTACAAG-3')[29]. This set of primers were used because it can recover more diverse bacteria taxa. The PCR reagent mixture and protocol[30] were performed under the following conditions: 2 min initial denaturation at 95°C followed by 24 cycles of denaturation (30 sec at 95°C), annealing (1 min at 53°C) and extension (2 min at 72°C) and a final extension at 72°C for 10 min. Fragment sizes of the PCR products were determined by electrophoresis in 1% agarose gel in 1x TAE buffer. The gel was then visualized under ultraviolet light and images were taken using AlphaImager 2200 (Alpha Innotech, USA). Purified PCR products were ligated into a pJET1.2 (Fermentas, USA) vector and transformed into competent XL1-blue *Escherichia coli*.

**Plasmid extraction and sequencing:** A total of 110 clones were selected from each library and were purified by using Vivantis Plasmid DNA Extraction Kit (Vivantis Technologies, Malaysia). Briefly, the procedure of plasmid extraction start with growing the 5-10 mL plasmid-containing bacteria cells in medium with appropriate antibiotic(s) overnight (12-16 h) at 37°C with agitation. Noted that fresh culture must always be used for extraction. Then followed by centrifugation of bacteria cell pellets, pellets resuspension, pellets lysization and neutralization and elution of bacteria plasmid DNA (Vivantis Technologies, Malaysia). The purified plasmids were then sequenced using ABI 3730XL automated DNA sequencer (PE ABI, USA) with primers T7 (5'-AATACGACTCACTATAG-3') and SP6 (5'-ATTTAGGTGACACTATAG-3'). Sequencing was performed by FirstBase Sdn. Bhd.

**Bioinformatic analysis:** The sequences were assembled in the Staden Package[31] and consensus sequences were compared with other 16S rRNA genes in GenBank using NCBI BLAST[32]. Consensus sequences were also analyzed using the Check-Chimera program on the Ribosomal Database Project (RDP) website[33]. For phylogenetic analysis, each full sequence was aligned with closely related sequences identified from the BLAST search. Phylogenetic analysis was conducted[28] using MEGA version 5 with branching support for the inferred tree was established. The phylogenetic tree was inferred from neighbour-joining algorithm. Bootstrapping analysis was carried out with 1000 replications.

**Nucleotide sequence accession numbers:** The 16S rDNA sequences of the bacterial isolates obtained in this study have been deposited at GenBank under accession No. KJ721938 to KJ721975.

## RESULTS

**Phylogenetic diversity based on 16S sequences analysis:** In this study, isolated bacteria of both toxic and non-toxic dinoflagellates had been identified based on 16S rDNA sequences. A total of 38 isolates were sequenced from both toxic dinoflagellates, *Alexandrium tamiyavanichii*, AcMS01 and non-toxic dinoflagellates, *Alexandrium leei*, AlMS02. Results of the most similar sequence obtained from BLAST analysis for both culture of dinoflagellates are shown in Table 1 and 2. Diversified bacterial phylotypes spanned 4 phyla group, Cytophaga Flavobacterium Bacteriodetes (CFB), Firmicutes, α-proteobacteria and γ-proteobacteria. The α-proteobacteria dominated with 39.5% (15/38) followed by both Firmicutes and γ-proteobacteria with 21.0% (8/38) and CFB with 18.5% (7/38).

Bacteria isolates from clonal cultures of toxic *Alexandrium tamiyavanichii* was dominated by both α-proteobacteria and γ-proteobacteria with 35.3%, followed by CFB with 17.7% and Firmicutes with only 11.7%. Meanwhile, bacteria isolates from non-toxic *Alexandrium leei* were dominated by phyla α-proteobacteria with 42.9%, followed by Firmicutes with 28.6% and CFB with 19% while the other 9.5% was γ-proteobacteria group.

Overall, sequences of the bacteria isolates were ranging between 1312-1510 bp. All bacteria isolates showed sequence identity more than 97% in GenBank database. Five bacteria isolates, UMTAT04, UMTAT06, UMTAT13, UMTAT17 and UMTAL06 were identical (100%) to their closest relatives in GenBank. There were 6 distinct bacteria were shared between AcMS01 and AlMS02 culture, *Exiguobacterium profundum*, *Marinobacter salsuginis*, *Ponticoccus litorali*, *Thalassospira tepidiphila*, *Algoriphagus*

Table 1: Phylogenetic affiliation of bacteria isolated from *Alexandrium leei*, AlMS02 based on BLAST analysis

| Bacteria isolate | Closest relative in GenBank database | Accession No. | Identity (%) | Phylogenetic affiliation |
|---|---|---|---|---|
| UMTAL01 | *Exiguobacterium profundum* ( AY818050) | KJ721938 | 99 | Firmicutes |
| UMTAL02 | *Bacillus* sp. (JN119354) | KJ721939 | 99 | Firmicutes |
| UMTAL03 | *Thalassospira* sp. (AB548215) | KJ721940 | 99 | α-proteobacteria |
| UMTAL04 | *Roseobacter* sp. (EF512125) | KJ721941 | 99 | α-proteobacteria |
| UMTAL05 | *Thalassospira* sp. (AB548215) | KJ721942 | 98 | α-proteobacteria |
| UMTAL06 | *Thalassospira* sp. (AB548215) | KJ721943 | 100 | α-proteobacteria |
| UMTAL07 | *Bacillus* sp. (JN119354) | KJ721944 | 99 | Firmicutes |
| UMTAL08 | *Bacillus megaterium* (EU918562) | KJ721945 | 98 | Firmicutes |
| UMTAL09 | *Thalassospira* sp. (AB548215) | KJ721946 | 99 | α-proteobacteria |
| UMTAL10 | *Bacillus* sp. (JN119354) | KJ721947 | 99 | Firmicutes |
| UMTAL11 | *Bacillus* sp. (JN119354) | KJ721948 | 99 | Firmicutes |
| UMTAL12 | *Marinobacter salsuginis* (JQ799060) | KJ721949 | 99 | γ-proteobacteria |
| UMTAL13 | *Flavobacterium* sp. (AJ391201) | KJ721950 | 99 | CFB |
| UMTAL14 | *Muricauda aquimarina* (NR042909) | KJ721951 | 97 | CFB |
| UMTAL15 | *Thalassospira profundimaris* (NR042766) | KJ721952 | 98 | α-proteobacteria |
| UMTAL16 | Uncultured Bacteriodetes (HM79884) | KJ721953 | 99 | CFB |
| UMTAL17 | *Thalassospira profundimaris* (NR042766) | KJ721954 | 98 | α-proteobacteria |
| UMTAL18 | *Ponticoccus litoralis* (NR044174) | KJ721955 | 97 | α-proteobacteria |
| UMTAL19 | *Thalassospira* sp. (EU440820) | KJ721956 | 98 | α-proteobacteria |
| UMTAL20 | *Marinobacter salsuginis* (JQ799060) | KJ721957 | 99 | γ-proteobacteria |
| UMTAL21 | *Algoriphagus zhangzhouensis* (NR109472) | KJ721958 | 99 | CFB |

Table 2: Phylogenetic affiliation of bacteria isolated from *Alexandrium tamiyavanichii*, AcMS01 culture based on BLAST analysis

| Bacteria isolate | Closest relative in GenBank database | Accession No. | Identity (%) | Phylogenetic affiliation |
|---|---|---|---|---|
| UMTAT01 | *Exiguobacterium profundum* ( AY818050) | KJ721959 | 99 | Firmicutes |
| UMTAT02 | *Marinobacter salsuginis* (JQ799060) | KJ721960 | 97 | γ-proteobacteria |
| UMTAT03 | *Marinobacter salsuginis* strain SD (NR044044) | KJ721961 | 99 | γ-proteobacteria |
| UMTAT04 | *Roseobacter* sp., CSQ-2 (EF512125) | KJ721962 | 100 | α-proteobacteria |
| UMTAT05 | *Maricaulis* sp. (AJ227809) | KJ721963 | 99 | α-proteobacteria |
| UMTAT06 | *Roseobacter* sp., CSQ-2 (EF512125) | KJ721964 | 100 | α-proteobacteria |
| UMTAT07 | *Marinobacter salsuginis* (JQ799060) | KJ721965 | 97 | γ-proteobacteria |
| UMTAT08 | *Roseobacter* sp., CSQ-2 (EF512125) | KJ721966 | 99 | α-proteobacteria |
| UMTAT09 | Uncultured bacterium clone (JQ03258) | KJ721967 | 96 | α-proteobacteria |
| UMTAT10 | *Roseobacter* sp., CSQ-2 (EF512125) | KJ721968 | 99 | α-proteobacteria |
| UMTAT11 | *Cytophagaceae* sp., BG16 (JN791284) | KJ721969 | 99 | CFB |
| UMTAT12 | *Flavobacterium* sp. (AJ391201) | KJ721970 | 99 | CFB |
| UMTAT13 | *Bacillus* sp. (JN942138) | KJ721971 | 100 | Firmicutes |
| UMTAT14 | *Marinobacter salsuginis* (JQ799060) | KJ721972 | 99 | γ-proteobacteria |
| UMTAT15 | *Marinobacter salsuginis* (JQ799060) | KJ721973 | 99 | γ-proteobacteria |
| UMTAT16 | *Marinobacter salsuginis* (JQ799060) | KJ721974 | 99 | γ-proteobacteria |
| UMTAT17 | *Algoriphagus zhangzhouensis* (NR109472) | KJ721975 | 100 | CFB |

Table 3: Classifications of 16S rDNA sequences obtained from AlMS02 cultures

| Sequence code | Length of sequence (bp) | Closest match in GenBank | Phylogenetic affiliation | Identity (%) | Abundance among sequence (%) |
|---|---|---|---|---|---|
| UMTPAL01 | 1358 | Uncultured bacterium (JF514249) | Unknown | 97 | 1.01 |
| UMTPAL02 | 1420 | *Flavobacterium* sp. (AJ391201) | CFB | 99 | 8.08 |
| UMTPAL03 | 1386 | *Muricauda* sp. (AY576776) | CFB | 99 | 7.07 |
| UMTPAL04 | 1344 | *Nautella* sp. (JN594623) | α-proteobacteria | 100 | 23.23 |
| UMTPAL05 | 1412 | Uncultured *Methylophaga* sp. (HQ012276) | γ-proteobacteria | 99 | 8.58 |
| UMTPAL06 | 1334 | Uncultured bacterium (JQ347377) | Unknown | 99 | 2.02 |
| UMTPAL07 | 1226 | Uncultured gamma proteobacterium (FM242259) | γ-proteobacteria | 99 | 2.27 |
| UMTPAL08 | 1362 | Uncultured bacterium (JQ032581) | α-proteobacteria | 99 | 3.03 |
| UMTPAL09 | 1384 | Uncultured bacterium (FJ644611) | Unknown | 98 | 2.02 |
| UMTPAL10 | 1396 | Bacterium (AY258118) | Unknown | 99 | 2.02 |
| UMTPAL11 | 1323 | *Roseobacter* sp. (EF512125) | α-proteobacteria | 100 | 3.03 |
| UMTPAL12 | 1311 | Uncultured bacterium (FJ644608) | Unknown | 99 | 2.02 |
| UMTPAL13 | 1379 | Uncultured bacterium (HQ224979) | Unknown | 98 | 1.01 |
| UMTPAL14 | 1328 | *Sphingomonas aquatilis* (AB681116) | α-proteobacteria | 99 | 1.01 |
| UMTPAL15 | 1344 | Uncultured Planctomycete (FR714340) | Planctomycetes | 99 | 1.01 |
| UMTPAL16 | 1373 | Uncultured bacterium (JQ801068) | Unknown | 95 | 1.01 |
| UMTPAL17 | 1336 | Uncultured alpha proteobacterium (EF471669) | α-proteobacteria | 99 | 18.18 |
| UMTPAL18 | 1341 | Uncultured alpha proteobacterium (JN825384) | α-proteobacteria | 97 | 1.01 |
| UMTPAL19 | 1197 | *Marinobacter salsuginis* (JQ799108) | γ-proteobacteria | 99 | 1.01 |
| UMTPAL20 | 1370 | Uncultured bacterium (HM127741) | Unknown | 96 | 1.01 |
| UMTPAL21 | 1375 | *Marivirga* sp. (KC890797) | CFB | 99 | 2.02 |
| UMTPAL22 | 1315 | Uncultured bacterium (GU061952) | Unknown | 96 | 1.01 |
| UMTPAL23 | 1265 | Uncultured gamma proteobacterium (FM242259) | γ-proteobacteria | 97 | 2.27 |
| UMTPAL24 | 1327 | Uncultured bacterium (EU804348) | Unknown | 99 | 2.02 |
| UMTPAL25 | 1252 | *Nautella italica (*KC593286) | α-proteobacteria | 97 | 1.01 |
| UMTPAL26 | 1093 | Bacterium (JF411456) | Unknown | 96 | 0.51 |
| UMTPAL27 | 1389 | Uncultured bacterium (JX391431) | Unknown | 99 | 1.01 |
| UMTPAL28 | 1267 | Uncultured Bacteroidetes bacterium (HM798840) | CFB | 98 | 0.51 |

*zhangzhouensis* and *Muricauda aquimarina*. Phylogenetic analysis of all bacteria isolates was shown in Fig. 1.

**Analysis of bacterial community by 16S rDNA cloning and sequencing:** A total of 197 clone sequences were obtained, 105 sequences from toxic dinoflagellates AcMS01 cultures, *Alexandrium tamiyavanichii* and 91 sequences non-toxic dinoflagellates AlMS02 cultures, *Alexandrium leei* were obtained ranging from 1093-1554 bp in length. Results of the BLASTn analysis are shown in Table 3 and 4. There were 22 distinct phylotypes from AcMS01 culture and 28 distinct phylotypes from AlMS02 culture. About 31 out of these 50 phylotypes matched with sequences of uncultured bacteria and 18 of them have no phylogenetic group assigned. Only 6 of the sequences produced matches to species level, namely *Marinobacter salsuginis* which were present in both

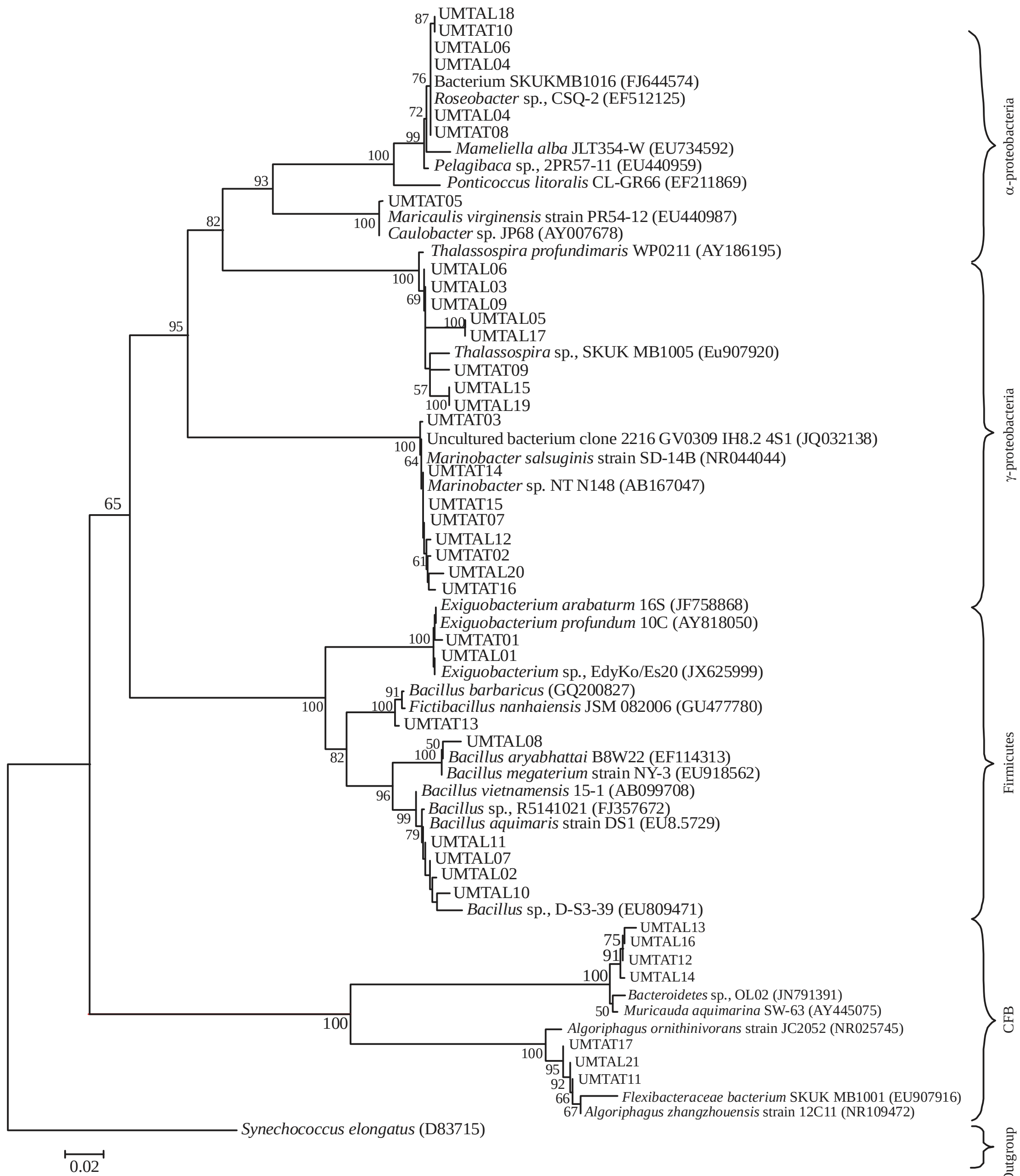


Fig. 1: Phylogenetic tree inferred from neighbour-joining analysis phylogram based on 16S rRNA sequences obtained in this study and related sequences. Sequences of the bacteria isolates from AcMS01 culture are indicated by UMTAT, sequences from the culturable isolates from AlMS02 are indicated by UMTAL bootstrap values based on 1000 replicates are shown at branch nodes, bar: 0.02 substitution per nucleotide position

cultures, *Algoriphagus zhangzhouensis* and *Maritimibacter alkaliphilus* from the AcMS01 culture, *Sphingomonas aquatilis* and *Nautella italica* from the AlMS02 culture.

In general, the bacteria flora can be grouped into three main group, α-proteobacteria, γ-proteobacteria and Cytophaga Flavobacteria Bacteroides (CFB) group. Figure 2 shows the sequences from the AcMS01 culture were dominated by α-proteobacteria (47%) followed by CFB (25%), unknown group bacteria (21%) and γ-proteobacteria (7%). For the AlMS02 culture, the sequences also were dominated by α-proteobacteria (50%), followed by CFB (18%), unknown group of bacteria (17%), γ-proteobacteria (14%) and Planctomyces (1%) are shown in Fig. 3. Phylogenetic relationships of each Operational Taxonomic

Table 4: Classifications of 16S rDNA sequences obtained from AcMS01 cultures

| Sequence code | Length of sequence (bp) | Closest match in Genbank | Phylogenetic affiliation | Identity (%) | Abundance among sequence (%) |
|---|---|---|---|---|---|
| UMTPAT01 | 1349 | Uncultured Rhodospirillales bacterium (HM798908) | α-proteobacteria | 94 | 2.48 |
| UMTPAT02 | 1348 | Alpha proteobacterium (AB821371) | α-proteobacteria | 93 | 1.24 |
| UMTPAT03 | 1334 | Uncultured bacterium (EU805317) | Unknown | 99 | 1.24 |
| UMTPAT04 | 1388 | Uncultured Bacteroidetes bacterium (EF061974) | CFB | 96 | 3.72 |
| UMTPAT05 | 1337 | *Erythrobacter* sp. (DQ104409) | α-proteobacteria | 99 | 2.48 |
| UMTPAT06 | 1405 | Uncultured *Methylophaga* sp. (HQ012276) | γ-proteobacteria | 99 | 4.35 |
| UMTPAT07 | 1341 | Uncultured alpha proteobacterium (EF471669) | α-proteobacteria | 99 | 21.12 |
| UMTPAT08 | 1179 | Uncultured bacterium clone (KF271103) | Unknown | 99 | 0.62 |
| UMTPAT09 | 1185 | Uncultured bacterium clone (KF799423) | Unknown | 93 | 0.62 |
| UMTPAT10 | 1554 | *Flavobacterium* sp. (AJ391201) | CFB | 99 | 12.42 |
| UMTPAT11 | 1324 | Uncultured bacterium (GU061952) | Unknown | 96 | 16.15 |
| UMTPAT12 | 1386 | Uncultured Flammeovirgaceae bacterium (JQ516340) | CFB | 95 | 2.48 |
| UMTPAT13 | 1378 | *Algoriphagus zhangzhouensis* (NR109472) | CFB | 99 | 1.24 |
| UMTPAT14 | 1308 | *Maritimibacter alkaliphilus* (AB681686) | α-proteobacteria | 99 | 1.24 |
| UMTPAT15 | 1310 | *Roseobacter* sp. (EF512125) | α-proteobacteria | 100 | 16.50 |
| UMTPAT16 | 1322 | *Marivita* sp. (HQ871858) | α-proteobacteria | 99 | 1.24 |
| UMTPAT17 | 1375 | Uncultured bacterium (AB694300) | Unknown | 93 | 1.24 |
| UMTPAT18 | 1387 | Uncultured gamma proteobacterium (JF421194) | γ-proteobacteria | 91 | 1.24 |
| UMTPAT19 | 1404 | *Muricauda* sp. (AY576776) | CFB | 98 | 7.45 |
| UMTPAT20 | 1144 | *Marinobacter salsuginis* (JQ799108) | γ-proteobacteria | 98 | 1.24 |
| UMTPAT21 | 1263 | Uncultured bacterium (JQ407269) | Unknown | 97 | 0.62 |
| UMTPAT22 | 1218 | Uncultured bacterium (JX391431) | Unknown | 99 | 0.62 |

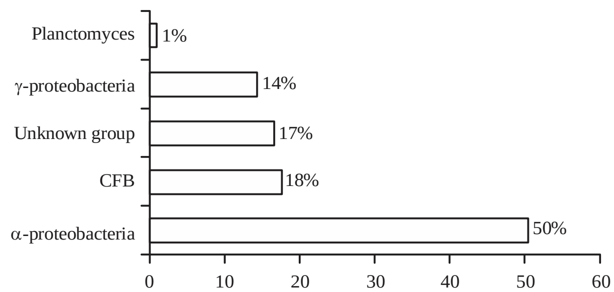


Fig. 2: Abundance of total bacteria with direct extraction from clonal culture *Alexandrium tamiyavanichii*, AcMS01

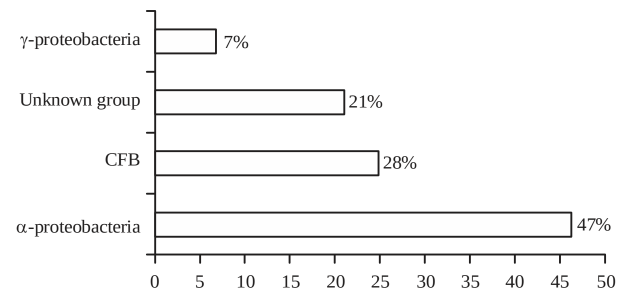


Fig. 3: Abundance of total bacteria with direct extraction from clonal culture *Alexandrium leei*, AlMS02

Unit (OTU) from both cultures are shown in Fig. 4. The OTUs with no phylogenetic group assigned were clustered into specific clades by phylogenetic analysis.

## DISCUSSION

While bacteria are known to be closely associated with phytoplankton and are thought to influence dinoflagellates population dynamics and toxicity[11], the phylogenetic identity and specificity of bacteria-phytoplankton interactions are only beginning to be explored. Cytophaga Flavobacterium Bacteroidetes (CFB) and proteobacteria, usually dominate heterotrophic bacterial communities in the ocean[3]. This group was retrieved from cultured bacteria and represented by *Bacteroidetes* and *Cytophaga* sp. This latter was the most abundant associated for each clone. Bacteria that belong to the phylum CFB are one of the dominant groups of the many microbial populations that inhabit different marine environments[3,34,35] and freshwater ecosystems[36].

All bacterial isolates showed sequence identities more than 97% in GenBank database. The 16S rRNA gene sequence has played an important role in delineating novel taxa and in the identification of isolates. Stackebrandt and Goebel[37] suggested that a 16S rRNA gene sequence identity of 97% should become the boundary for delineation of prokaryotic species, which has been well accepted among microbiologists.

The results showed that bacterial isolates from the non-toxin producing dinoflagellates *Alexandrium leei* harbour the most diverse bacterial populations with many of the strains unique to these particular cultures.

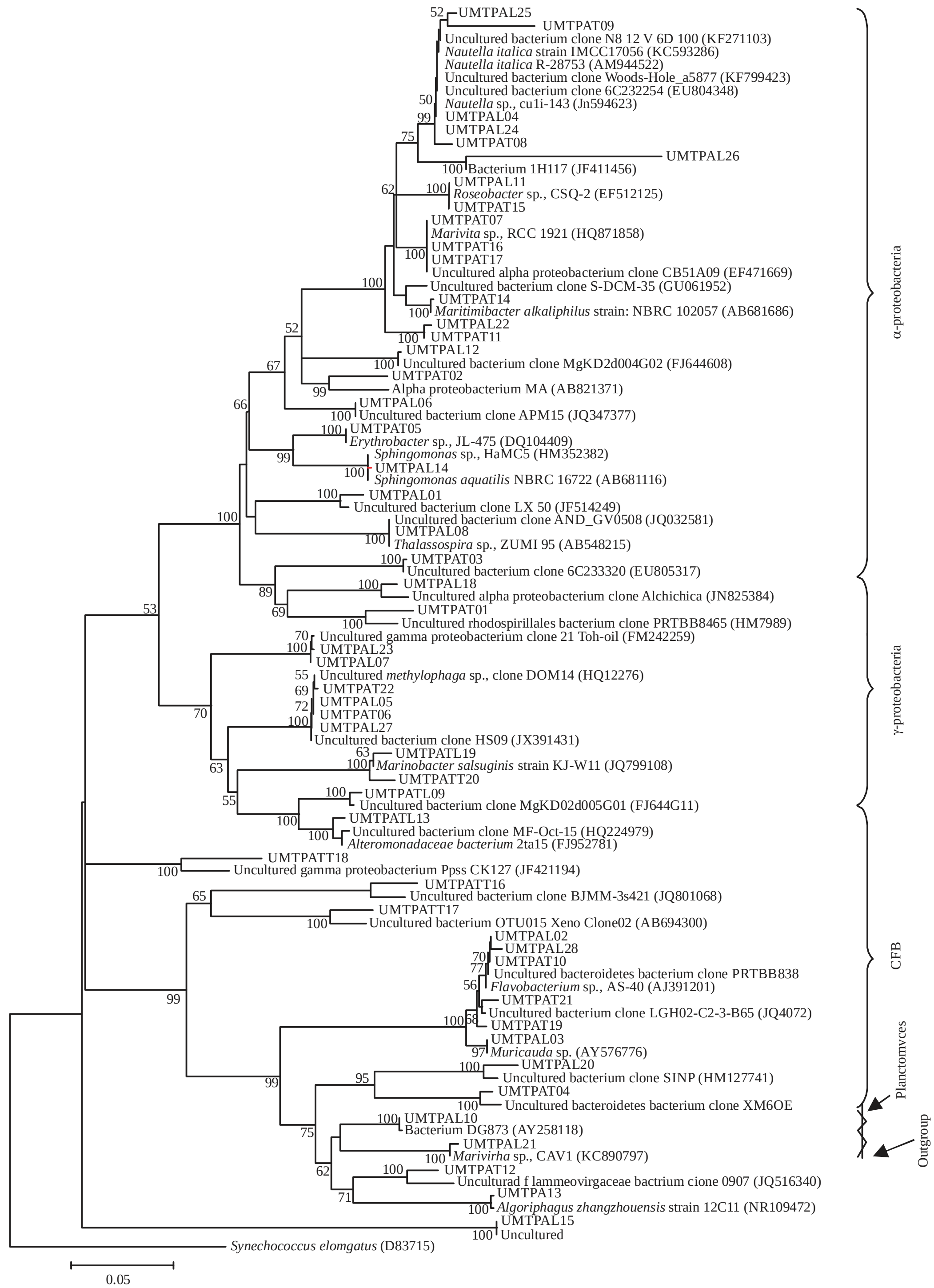

Fig. 4: Neighbour-joining analysis based on 16S rRNA sequences obtained in this study and related sequences. Sequences associated with direct extraction from AcMS01 culture are indicated by UMTPAT and sequences that were associated with direct extraction from AlMS02 culture are indicated by UMTPAL. Bootstrap values based on 1000 replicates are shown at branch nodes, Bar, 0.05 substitution per nucleotide position

This suggests that a species-specific association may exist between some bacteria and certain algal species and that differences exist in the microflora between toxic and non-toxic dinoflagellates. However, further study using more dinoflagellate cultures is required to determine if this is the case.

Previous studies showed only 0.1% of microorganisms from marine dinoflagellates were amenable to culture using traditional techniques[30]. Therefore, the vast majority of microorganisms associated with marine dinoflagellates could not be identified using a culture-based approach. The usage of culture-independent molecular techniques allow the importance of the numerous non-cultivable bacteria present in various ecosystems to be evaluated[30]. For the 16S rDNA clone library, sequencing of the 16S rDNA clones revealed 22 distinct phylotypes from the AcMS01 culture of *Alexandrium tamiyavanichii* and 28 distinct phylotypes from the AlMS02 culture of *Alexandrium leei*, making a total of 50 phylotypes. However, it was not clear whether those numbers equate to actual phenotypic diversity. Recent study revealed a total of 36 distinct phylotypes of bacteria were evaluated from two benthic marine dinoflagellates, *Coolia monotis* and *Ostreopsis ovate*[30]. As comparison, Green *et al.*[38] had obtained 61 distinct cultured bacteria from seven strains of *Gymnodinium catenatum* while Ashton[5] had obtained only seven DGGE bands from an *Ostreopsis lenticularis* strain. To a certain extent the number of bacteria species in these cultures will also depend on the techniques used during the initial isolation of the dinoflagellate cells into culture. Since sterile and aseptic techniques are typically employed in subsequent subculturing, the diversity of the associated bacteria will also be influenced by the diversity of bacteria in the ambient seawater from where the dinoflagellate originated.

BLASTn analysis[27] showed that most of the sequences from this study had no match at species level. In addition, there were a number of sequences with less than 97% SSU rDNA identity to published sequences. Thirteen Operational Taxonomic Units (OTUs) from AcMS01 culture and 18 OTUs from AlMS02 culture matched with sequences of uncultured bacteria. These results showed that there were novel bacteria species in these cultures and some may be unculturable.

Co-occurrence of similar bacteria sequences in cultures of these two dinoflagellate strains suggested that dinoflagellate associated bacteria could be less specific since the two dinoflagellates originated from same geographical regions, Sebatu, Melacca. These groups of bacteria may be of specific importance to the growth and physiology of dinoflagellate cells[6]. Sequences highly similar to that of *Marinobacter salsuginis* were found in both cultures. *Marinobacter salsuginis* belongs to the $\gamma$-proteobacteria group, a member of the genus *Marinobacter* which was originally isolated from brine-seawater interface of the Shaban Deep, Red Sea[39].

Sequences similar to *Roseobacter* sp., occurred in both AcMS01 and AloMS02 cultures. *Roseobacter* clade is often found associated with various dinoflagellates, for example *Alexandrium* spp.[14], *Pfiesteria* spp.[40] and *Prorocentrum* spp.[41]. This bacterium is predominantly responsible for the degradation of dimethylsulfoniopropionate (DMSP). The DMSP is the major source of organic sulphur in the world's oceans and is degraded by marine bacteria to dimethylsulphide (DMS). These bacteria thus play very important roles in the global sulphur cycle[42]. Other than that, the *Roseobacter* clade-affiliated group bacteria were shown to cause algal bloom decline and believed that the widespread of this group of bacteria may exert significant control over phytoplankton biomass and community structure in the oceans[43].

One unexpected phylotypes were found from the culture of AlMS02, sequence UMTPAL15 matched with the sequence of uncultured Planctomycete with 99% identity. So far, we have found only one report of association of this bacterium with phytoplankton by Ruh *et al.*[30]. The molecular taxonomic analysis of dinoflagelllates associated bacteria prove that there is a diverse assemblage of bacteria residing within both toxic and non-toxic dinoflagellates; however, many of these microorganisms were uncultured bacterium. The sequence results indicate that a high diversity of bacterial phylotypes was present within both dinoflagellates. There were about 62% of the 50 phylotypes were assigned to uncultured bacterium, where 38% of the total 50 phylotypes cannot be assigned to any group of bacteria. This provides further evidence to suggest that additional molecular analysis would be required to fully documentation of the microbial diversity associated with the dinoflagellates.

There is increasing evidence that there are specific bacteria taxa associated with phytoplankton[44]. Such specificity would imply some sort of bacteria-dinoflagellate recognition mechanism and also probable mutual benefit for both. Whole genome sequencing of the bacteria community followed by analysis of gene presence and absence could indicate the manner of bacteria contribution to and dependence on the dinoflagellate. This approach would be particularly interesting in the case of toxic dinoflagellates because the role of bacteria in toxigenesis continues to be a subject of speculation.

## CONCLUSION

The use of culture-dependent method and culture-independent method had successfully been applied in order to determine the diversity of bacteria community associated with toxic and non-toxic dinoflagellates. Further work should be carried out which involves direct DNA extraction *in situ* from the environment, to compare the natural and culture collections in order to give us a more comprehensive overview.

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