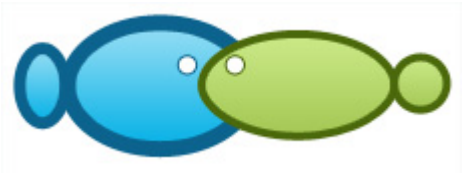

# Microbial communities associated with some harmful dinoflagellates from Malaysian waters revealed by culture-independent and culture-dependent approaches

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**Abstract**. Diversity of bacteria associated with toxic dinoflagellates *Alexandrium minutum*, *Alexandrium tamiyavanichii* and *Pyrodinium bahamense* var *compressum* were assessed using culture-dependent and -independent approaches. Total DNA from the dinoflagellate cultures as well as those of culturable bacteria was extracted and used as templates for PCR amplification of the 16S rDNA. The PCR products from directly extracted genomics were analysed by denaturing gradient gel electrophoresis (DGGE) and direct sequencing. A total of 15 culturable isolates were obtained, of which 4 were from *A. minutum* culture, 7 were from *A. tamiyavanichii* culture and 4 from *P. bahamense* culture. DGGE analysis produced a total of 25 bands of which 6 were from *A. minutum* culture, 9 were from *A. tamiyavanichii* culture and 10 were from *P. bahamense* culture. Fourteen of the bands were unique. Sequence analysis of the unique bands and culturable isolates showed that 15 phylotypes belonged to the α-Proteobacteria, 1 belonged to β-Proteobacteria, 4 belonged to the γ-Proteobacteria, 9 belonged to Cytophaga-Flavobacterium-Bacteroides (CFB) and 1 belonged to Firmicutes. Only 3 of the 14 sequences obtained from the DGGE analysis had matches to known genera in Genbank. In this study, both approaches are important as not all bacteria could be solely detected.

**Key Words**: toxic dinoflagellates, bacterial communties, 16S rDNA sequence analysis, DGGE analysis.

**Introduction**. Dinoflagellate is a member of phytoplanktons which plays an important role in the world's primary productivity. Nonetheless, this microalgae is more well known as harmful algae. Many countries are affected by harmful algal blooms (HABs) and related shellfish toxicity and fish mortality events. The most significant HAB-related intoxication in Malaysia is paralytic shellfish poisoning (PSP) (Usup & Azanza 1998). This poisoning is due to a suite of heterocyclic guanidines, collectively referred to as saxitoxin. The toxins are transferred to humans through consumption of bivalve mollusks such as mussels, clams, oysters or scallops that feed on the toxic dinoflagellates and accumulate the toxin in their tissues. Over the years, PSPs in Malaysia have resulted in many human intoxications including several fatalities. Economic losses due to these events, while difficult to ascertain, could also be substantial. In Malaysia, the known PSP toxin producers are the dinoflagellates *Pyrodinium bahamense* var *compressum*, *Prorocentrum minimum*, *Alexandrium tamiyavanichii*, *A. minutum*, *A. tamarense*, *A. taylori* and *A. peruvianum* (Lim et al 2005; Usup et al 2002a). However, only *P. bahamense*, *A. minutum* and *A. tamiyavanichii* have been reported to cause PSP poisonings (Roy 1977; Usup et al 2002b; Usup et al 2002c).

A specific community of bacteria is believed to be associated with each dinoflagellate. The bacteria are believed to contribute to dinoflagellate physiology and toxigenesis. They may, for example, enhance metabolism, or be involved in co-

metabolism processes. They may even lyse their dinoflagellate hosts. It is also possible that they produce the toxin, or a precursor to the toxin, or perhaps they are involved in degrading the toxin. These bacteria have a potentially more profound role in the development of HAB events. They are able to exert considerable influence on bloom population dynamics through bacterial-algal interactions, such as positive stimulation of growth, promotion of sexuality, antagonism mediated via the production of algicidal factors and inhibition of cyst formation, and protective effects associated with an existing microflora insulating the algal host from antagonistic bacterial activity (Green et al 2004).

Cultures of dinoflagellates contain a considerable amount of bacteria which probably accompanied the dinoflagellate in the original sample. Bacteria in dinoflagellate cultures might produce saxitoxin autonomously or complement the dinoflagellates in either toxin production or growth (Uribe & Espejo 2003). Other than conventional method of bacteria identification such as cultivation prior to biochemical characterization, several molecular techniques have been developed to determine the genetic diversity of microbial communities. Among them includes 16S gene cloning and sequencing and denature gradient gel electrophoresis (DGGE). Technique of DGGE was originally developed to detect single-base mutation in medical research (Myers et al 1985). However, it has been proved to be a fast, sensitive and direct approach to describe bacterial communities of microalgae, bacterial mats and biofilms, hot springs in the marine environment, rhizospheres and soil (Seibold et al 2001).

The present study shows an attempt to investigate the diversity of bacteria in the toxic marine dinoflagellates found in Malaysian waters with cultured and non-cultured approaches. Bacteria isolation and 16S rDNA sequencing were applied in cultured approach whereas direct 16S rRNA gene amplification by PCR followed by DGGE analysis were used in uncultured approach.

**Material and Method**. Clonal cultures of the marine dinoflagellates *Alexandrium minutum* AmKB01, *Alexandrium tamiyavanichii* AcMS01 and *Pyrodinium bahamense* var *compressum* PbSP08 were obtained from the UKM Microalgae Culture Collection and maintained in ES-DK medium (Kokinos & Anderson 1995) throughout the study period from May 2007 to April 2009. Cultures were grown at 26°C under a 14:10 hour light-dark cycle.

***Isolation of bacteria from dinoflagellate cultures***. One mL of dinoflagellate cultures from late exponential growth phase were diluted 7-fold in sterile seawater. Aliquots of 100 uL from each dilution were spread onto Marine Agar (Difco) and subsequently incubated for 10 days at 26°C. Distinct colonies were isolated into pure cultures. Isolates were stored in marine broth plus 20% (v/v) glycerol at -80°C.

***Bacterial nucleic acid extraction, PCR and sequencing***. Isolates were grown overnight in 10 mL of marine broth prior to DNA extraction. Cell pellets were harvested by centrigufation at 8000 rpm (4°C) for 10 min and resuspended in buffer (100 mM EDTA, 10 mM Tris-HCl [pH 8.0]) and treated with proteinase K (0.5 mg $mL^{-1}$)-1% sodium dodecyl sulfate (SDS) for 1 h at 37°C. Lysates were further treated by CTAB extraction (0.5 M NaCl, 1% CTAB) for 10 min at 65°C. DNA was extracted once with equal volumes of chloroform-isoamyl alcohol (24:1) and phenol-chloroform-isoamyl alcohol (25:4:1) and centrifuged at 15 000 rpm for 5 min at 4°C. After that, DNA was precipitated with 0.6 volume of isopropanol followed by centrifugation at 15 000 rpm for 15 min. The DNA pellet was then washed with one volume of 70% ethanol and centrifuged at 15 000 rpm for 10 min. Finally, DNA was resuspended in 50 µL of ddH2O and stored at -20°C. The polymerase chain reaction (PCR) was carried out to amplify the 16S rRNA gene from chromosomal DNA using the primer pair 16SF, 5'-AGAGTTTGATCCTGGCTCAG-3' and 16SR, 5'-GTTTACCTTGTTACGACTT-3' (Wellinghausen et al 2004). PCR was carried out on PTC-100 thermocycler (MJ Research Inc, USA) in a 50 µL reaction containing 10-100 ng DNA template, 1X reaction buffer, 2.5 mM $MgCl_2$, 200 µM of each dATP, dCTP, dGTP and dTTP (Promega, USA), 0.5 µM of each primer and 2 U of Taq polymerase (Promega, USA). The PCR cycle was as follows: preheating at 95°C for 5 min; 26 cycles of 95°C for

30 s, 55°C for 1 min and 72°C for 2 min; followed by 72°C for 10 min. The PCR products were purified using QIAquick column purification kit (Qiagen Inc., USA) according to the manufacturer's instructions. Direct sequencing of the PCR products was performed using dideoxy chain termination (ABI Prism BigDye terminator cycle sequencing ready reaction kit, PE-ABI, USA) and 16SF and 16SR primers. Sequencing was carried out on an ABI 3730 automated DNA sequencer (PE ABI, USA).

***Direct DNA extraction, PCR and DGGE analysis of bacteria community***. Bulk genomic DNA were extracted from a 2.0 L of mid exponential growth phase culture medium. Firstly, culture medium were filtered through 0.2 µm nitrate cellulose membrane (Whatmann, England). Cell pellets were then concentrated and resuspended in buffer (100 mM EDTA, 10 mM Tris-HCl [pH 8.0]) and treated with proteinase K (0.5 mg/mL)-1% sodium dodecyl sulfate (SDS) for 1 h at 37°C. Lysates were further treated by CTAB extraction (0.5 M NaCl, 1% CTAB) for 10 min at 65°C. Then, DNA was extracted as described above. DGGE-PCR was carried out using the primer set 341F-GC and 907R (Muyzer et al 1993). Primer 341F-GC carried the GC clamp. The PCR cycle was as follows: heating at 95°C for 5 min followed by 35 cycles of denaturing at 94°C for 45 s, annealing at 65°C for 45 s, and extension at 72°C for 45 s. The cycle was ended with a final extension at 72°C for 7 min. dNTPs and Taq polymerase were from Promega. PCR was carried out on a PTC-100 thermalcycler (MJ Research Inc.). Size and purity of the PCR products were assessed by electrophoresis in 1% agarose gel, staining with ethidium bromide and visualization on an AlphaImager 2200 (Alpha Innotech, USA). DGGE of the PCR products was carried out using the methods of Muyzer et al (1993). DGGE was carried out in 6% acrylamide gel containing a linear gradient of 30-60% denaturant (100% denaturant = 7M urea, 40% (v/v) formamide). Electrophoresis was carried out at a constant 60°C, 150 V for 7 hours using a Biorad Dcode apparatus (Biorad). The gel was stained with ethidium bromide and visualization on an AlphaImager 2200. All DGGE bands were excised from the gel and suspended in 50 uL of sterile ddH2O. The DNA was then recovered by three cycles of freezing (-20°C) and thawing and subjected to reamplication using the primer pair 341F (without GC clamp) and 907R. The PCR cycle conditions were the same as already described except the annealing temperature was at 60°C. The PCR products were purified using QIAquick column purification kit (Qiagen Inc., USA) according to the manufacturer's instructions. Direct sequencing of the PCR products was performed as already described.

***Sequence determination and phylogenetic analysis***. The 16S rDNA sequences were compared with sequences from GenBank using BLASTn (Altschul et al 1997). The sequences were then aligned using Clustal X (Thompson et al 1997) and adjusted by eye. All unambiguously aligned base positions were used in the phylogenetic analyses. Phylogenetic inference was performed using PAUP* version 4b10 (Swofford 2000) with maximum parsimony (MP) and neighbor-joining (NJ) algorithms. Bootstrapping (Felsenstein 1985) was then conducted for both MP and NJ analyses with 1000 replications ($p < 0.001$) to determine the confidence limits of tree topology using TBR branch-swapping algorithm.

**Results and Discussion**. In culture-dependent analysis, a total of 15 bacteria strains were isolated based on distinct colony morphology. Of these, 4 were isolated from AmKB01 (SKUKMB1001 to SKUKMB1004), 7 from AcMS01 (SKUKMB1005 to SKUKMB1011) and 4 from PbSP08 (SKUKMB1012 to SKUKMB1015), respectively. Table 1 lists the 16S rDNA sequences most closely related to the GenBank. All strains examined were clearly placed in the alpha- , beta- , and gamma-Proteobacteria subphyla, with the exception of strain SKUKMB1004, which was Gram-positive and most closely related to *Micrococcus luteus* and strain SKUKMB1001, which belonged to the Cytophaga-Flavobacterium-Bacteroides (CFB) group. Interestingly, some of the 16S sequences show less than 98% similarity to that from the database and these isolates might be novel species.

Table 1
16S rDNA sequence comparison of bacteria isolated from dinoflagellate cultures with their closest relative from GenBank database

| *Bacterial isolate* | *Closest relative in GenBank database* | *GenBank accession No.* | *Percent identity (%)* | *Phylogenetic affiliation* |
|---|---|---|---|---|
| SKUKMB1001 | Marine CFB-group bacterium MBIC01599 | AB086624 | 96.0 | CFB |
| SKUKMB1002 | *Marinobacter salsuginis* strain SD-14B | EF028328 | 95.0 | Gamma-proteobacteria |
| SKUKMB1003 | *Roseobacter* sp. AzwLept-1c | DQ223021 | 99.0 | Alpha-proteobacteria |
| SKUKMB1004 | *Micrococcus luteus* CV44 | AJ717369 | 96.0 | Gram positive |
| SKUKMB1005 | *Thalassospira profundimaris* WPO211 | AY186195 | 98.0 | Alpha-proteobacteria |
| SKUKMB1006 | *Marinobacter salsuginis* | EF028328 | 99.0 | Gamma-proteobacteria |
| SKUKMB1007 | Uncultured Alteromonas | AB262378 | 98.0 | Gamma-proteobacteria |
| SKUKMB1008 | *Porphyrobacter tepidarius* | AY048657 | 98.0 | Alpha-proteobacteria |
| SKUKMB1009 | *Roseobacter* sp. CSQ-2 | EF512125 | 99.0 | Alpha-proteobacteria |
| SKUKMB1010 | *Ruegeria mobilis* | AB255401 | 94.0 | Alpha-proteobacteria |
| SKUKMB1011 | Uncultured alpha-proteobacterium | DQ446133 | 98.0 | Alpha-proteobacteria |
| SKUKMB1012 | *Thalassospira* sp. MACL12B | EF198251 | 93.0 | Alpha-proteobacteria |
| SKUKMB1013 | *Limnobacter* sp. 9-1V | EF540452 | 99.0 | Beta-proteobacteria |
| SKUKMB1014 | *Erythrobacter flavus* | AF500005 | 97.0 | Alpha-proteobacteria |
| SKUKMB1015 | *Stappia* sp. M8 | AY307927 | 99.0 | Alpha-proteobacteria |

The most likely tree recovered using the NJ and MP analyses are shown in Figure 1. The same pattern of tree topology was observed in both NJ and MP analyses. Phylogenetic analysis revealed the dominance of two major groups of bacteria, the alpha-proteobacteria and the gamma-proteobacteria. Together, these two groups accounted for 80% of all the bacteria isolates. Based on tree topology, only the sequences of SKUKMB1005 and SKUKMB1010 highly matched the sequences of validly described bacteria species. Sequences of SKUKMB1002, SKUKMB1003, SKUKMB1004, SKUKMB1006, SKUKMB1007, SKUKMB1009, SKUKMB1012, SKUKMB1013 and SKUKMB1015 were only phylogenetically closely related to the bacteria identifiable to genus level. The rest of the strains isolated in this study were closely related to unknown bacteria taxa. The phylogenetic characterization of the cultivable bacteria isolated from the three species of marine toxic dinoflagellate cultures demonstrated that there were a number of emergent trends across the cultures examined. In summary, the Alphaproteobacteria dominated the strains isolated. This is similar to the findings of Green et al (2004) and Hold et al (2001). Interestingly, comparative 16S rDNA sequence analyses revealed that many of our isolates showed close identity to clades of predominantly marine bacterial isolates that do not contain validly described representative taxa. This result is common in phylogenetic studies of naturally occurring marine bacterial communities and indicate that novel microbial species can be relatively easily cultivated from marine dinoflagellate cultures using standard marine agar media (Hold et al 2001).

In culture-independent analysis, the diversity of the microbial communities from the three dinoflagellate clonal cultures, AmKB01, AcMS01 and PbSP08 were analyzed by DGGE of partial 16S rDNA fragments obtained by PCR amplification. DGGE profiles (Figure 2) obtained from the analysis shows that the diversity of bacteria associated with each dinoflagellate were different, i.e. between 6 to 10 operational taxonomy unit (OTU). Based on the DGGE band patterns, there are 6 OTU from culture of *A. minutum* AmKB01, 9 OTU from culture of *A. tamiyavanichii* AcMS01 and 10 OTU from culture of *Pyrodinium bahamense* var *compressum* PbSP08.

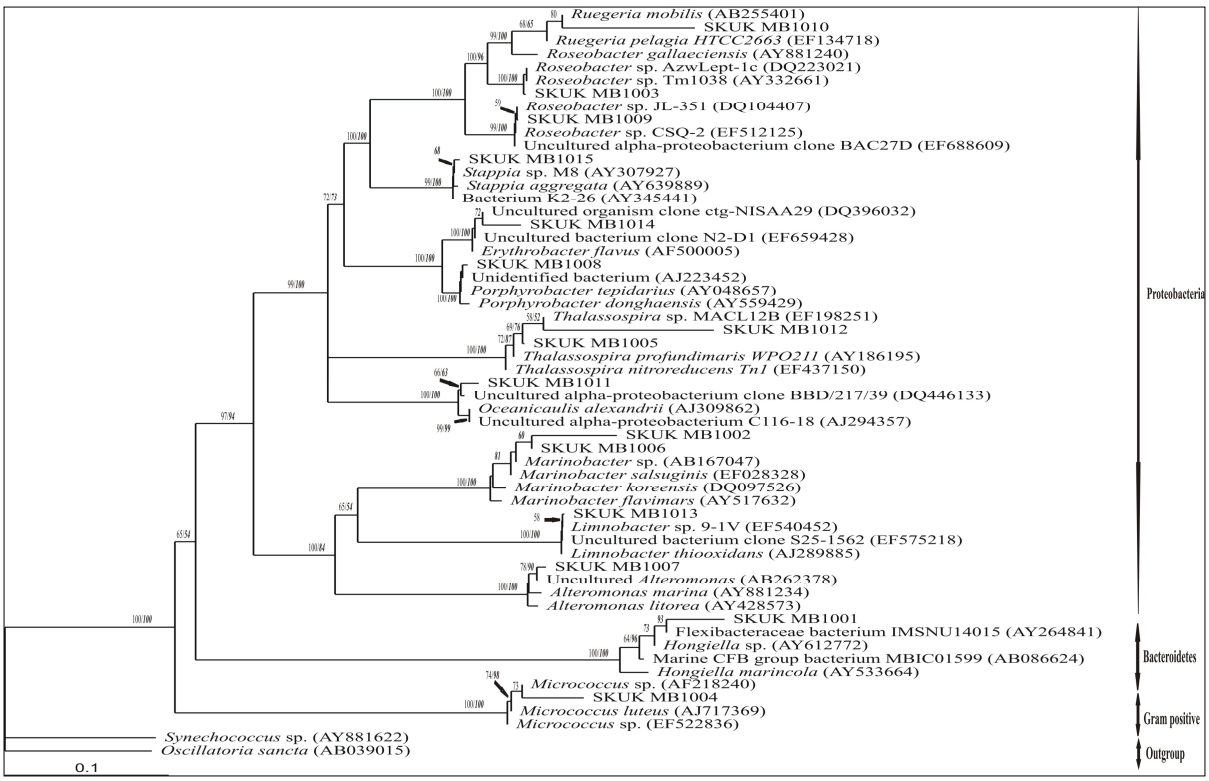


Figure 1. Phylogenetic analysis of bacteria isolated from dinoflagellates culture using the neighbor-joining and maximum parsimony methods. Bootstrap values (1000 replications) of NJ analysis are found in plain text above the internal nodes and in italics with bold are from MP analysis. Scale bar = 0.1 substitution per site.

All the DGGE bands were excised from the gel and subsequently sequenced after reamplification by PCR. However, some of the reamplified DNA were fail to sequenced or with poor quality and were not included for further analysis. Table 2 lists the sequence similarity of reamplified DGGE band with sequences retrieved from GenBank database. Phylogenetic analysis successfully grouped all the sequences into alpha-proteobacteria and Bacteroidetes subphyla with the Bacteroidetes group dominant by two sequences. The same pattern of tree topology reconstructed by NJ and MP analysis was observed and the tree was shown in Figure 3. The DGGE analysis identified some gene sequences which were not detected using the culture-based method. Analysis of sequences retrieved from reamplified DGGE bands shows that the dinoflagellate cultures were dominated by Bacteroidetes bacteria and Alphaproteobacteria. This is not similar to the finding using culturable approach in this study. This may due to some different PCR parameters such as cycle conditions and primers applied in both analysis. All the dinoflagellates cultures used in this study harbored bacteria. Since the medium used in culturing was sterile, the bacteria must have been carried over from the initial isolation. The fact that the dinoflagellate cultures remained healthy over several years suggested that the associated bacteria had minimal detrimental effect on the algal species. Since these dinoflagellate species came from different locations and cultures were established in different years, this result suggested a stable and uniform community of bacterioplankton existed in Malaysian waters. Alternatively, this result could also mean that certain bacteria species selectively associate with dinoflagellates, probably due to trophic interactions. For example some bacteria species will scavenge for dimethysulfoniopropionate (DMSP) and exudates produced by dinoflagellates (Miller & Belas 2004; Miller et al 2004).

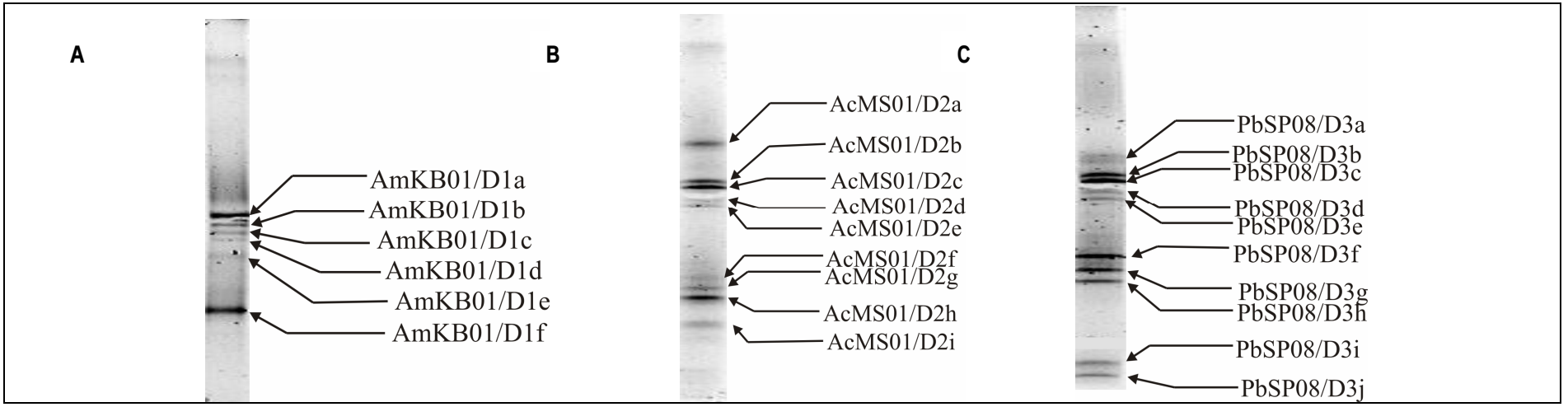


Figure 2. DGGE profiles of bacteria community associated with: A, *Alexandrium minutum*; B, *A. tamiyavanichii* and C, *Pyrodinium bahamense*.

Table 2

Sequence similarity of reamplified DGGE band with sequences retrieved from GenBank database

| *DGGE band* | *Closest relative in the GenBank database* | *GenBank accession No.* | *Percent identity (%)* | *Phylogenetic affiliation* |
|---|---|---|---|---|
| AmKB01/D1c | Flexibacteraceae bacterium | AY264841 | 97.0 | Bacteroidetes |
| AmKB01/D1d | *Erythrobacter* sp. JL-475 | DQ104409 | 94.0 | Alpha-proteobacteria |
| AmKB01/D1e | *Roseobacter* sp. AzwLept-1c | DQ223021 | 99.0 | Alpha-proteobacteria |
| AmKB01/D1f | Uncultured organism clone SIMO-4275 | DQ421640 | 98.0 | Alpha-proteobacteria |
| AcMS01/D2a | Uncultured Bacteroidetes bacterium clone DGGE | AY573533 | 98.0 | Bacteroidetes |
| AcMS01/D2c | Uncultured marine bacterium D030 | AF177564 | 96.0 | Alpha-proteobacteria |
| AcMS01/D2f | Rhodobacteraceae bacterium AZO-C | DQ822569 | 92.0 | Alpha-proteobacteria |
| AcMS01/D2g | *Muricauda* sp. 3IX/A02/243 | AY576776 | 98.0 | Bacteroidetes |
| AcMS01/D2h | Uncultured organism clone SIMO-4275 | DQ421640 | 95.0 | Alpha-proteobacteria |
| PbSP08/D3a | Uncultured CFB group bacterium isolate DGGE band C36 | AF466893 | 91.0 | Bacteroidetes |
| PbSP08/D3b | Bacteroidetes bacterium MO49 | AY553120 | 86.0 | Bacteroidetes |
| PbSP08/D3c | Uncultured Bacteroidetes bacterium clone 302 | DQ482736 | 98.0 | Bacteroidetes |
| PbSP08/D3e | Uncultured bacterium clone WLB16-200 | DQ015862 | 93.0 | Bacteroidetes |
| PbSP08/D3f | Uncultured Cytophagales bacterium clone LA7-B21N | AF513957 | 92.0 | Bacteroidetes |

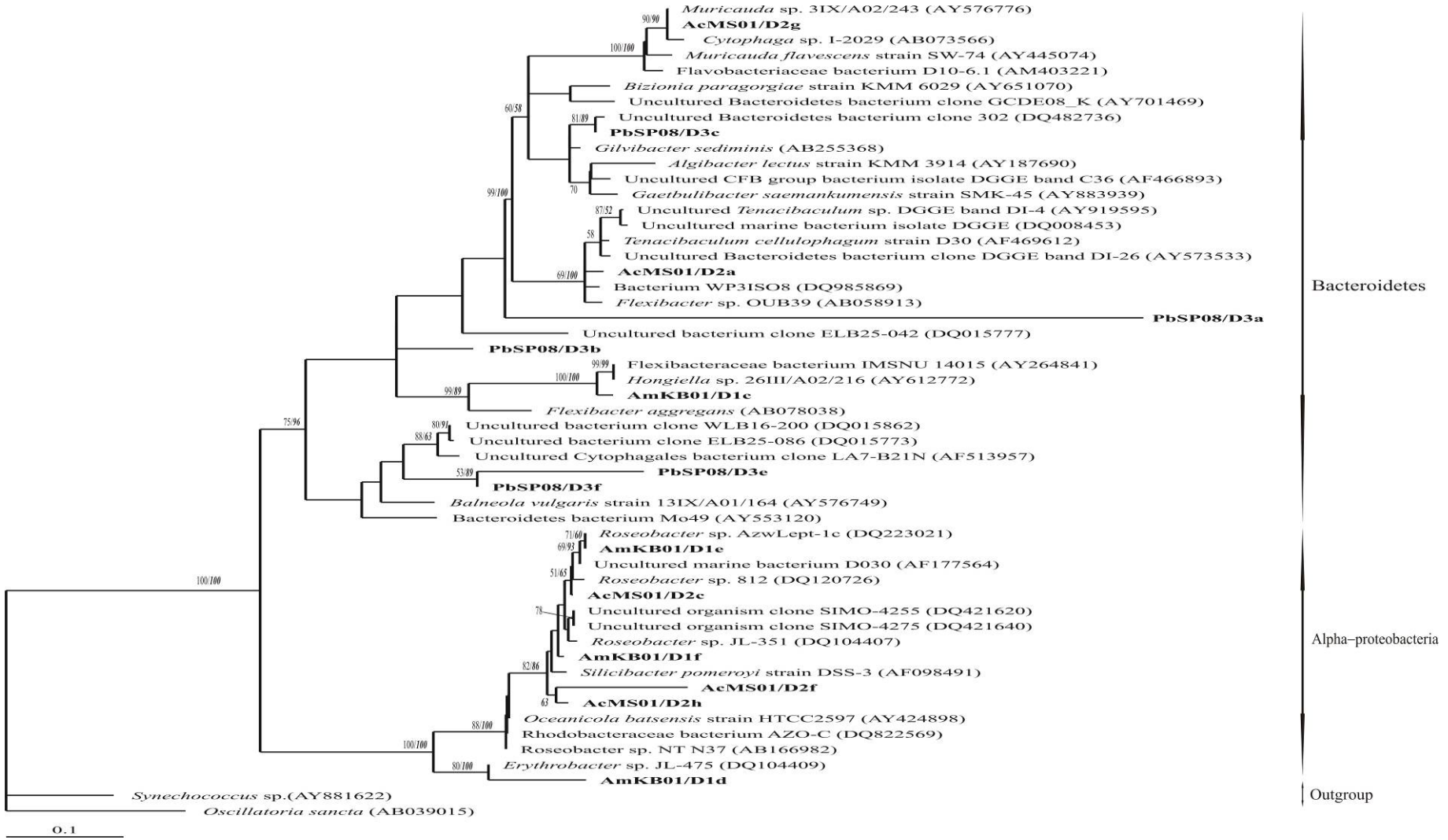


Figure 3. Phylogenetic analysis of sequences obtained from reamplified DGGE bands of bacteria community associated with dinoflagellate cultures using the neighbor-joining and maximum parsimony methods. Bootstrap values (1000 replications) of NJ analysis are found in plain text above the internal nodes and in italics with bold are from MP analysis. Scale bar = 0.1 substitution per site.

**Conclusions**. This study aimed to comprehensively document the bacterial community associated with the toxic marine dinoflagellates found in Malaysian waters as a first step to understanding the influence of bacteria on algal growth, physiology and toxicity and their role in the development of harmful algal blooms. In conclusion, combination of DGGE sequences and 16S rRNA gene sequences of bacterial isolates demonstrates the prevalence of Alphaproteobacteria in the cultures of dinoflagellate, followed by CFB group bacteria and Gammaproteobacteria. Although the presence of bacteria in phytoplankton cultures is a nuisance in certain experiments, analysis of these bacteria could provide useful data. These include discovery of new species, novel metabolic pathways and enzymes, and better insight on interactions between the bacteria and phytoplankton.

**Acknowledgements**. This study was funded by Malaysian government through grants IRPA 09-02-02-008-BTK/ER-36 and IRPA 02-01-02-SF0203.

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Received: 01 August 2016. Accepted: 09 September 2016. Published online: 24 September 2016.
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How to cite this article:
Danish-Daniel M., Ahmad A., Usup G., 2016 Microbial communities associated with some harmful dinoflagellates from Malaysian waters revealed by culture-independent and culture-dependent approaches. AACL Bioflux 9(5):976-984.