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Phylogenetic diversity and specificity of bacteria associated with *Microcystis aeruginosa* and other cyanobacteria

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Abstract

Interactions between bacteria and cyanobacteria have been suggested to have a potential to influence harmful algal bloom dynamics; however, little information on these interactions has been reported. In this study, the bacterial communities associated with five strains of *Microcystis aeruginosa*, three species of other *Microcystis* spp., and four representative species of non-*Microcystis* cyanobacteria were compared. Bacterial 16S rDNA fragments were amplified and separated by denaturing gradient gel electrophoresis (DGGE) followed by DNA sequence analysis. The similarities among bacterial communities associated with these cyanobacteria were compared to the digitized DGGE profiles using the cluster analyses. The bacterial community structure of all cyanobacterial cultures differed. Cluster analysis showed that the similarity values among *M. aeruginosa* cultures were higher than those of other cyanobacterial cultures. Sequence analysis of DGGE fragments indicated the presence of bacteria including, Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Bacteroidetes and Actinobacteria in the cyanobacterial cultures. Members of the Sphingomonadales were the prevalent group among the *Microcystis*-associated bacteria. The results provided further evidence for species-specific associations between cyanoabcteria and heterotrophic bacteria, which are useful for understanding interactions between *Microcystis* and their associated bacteria.

Key words: bacterial diversity; specificity; *Microcystis*; denaturing gradient gel electrophoresis

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Introduction

Interactions between bacteria and algae have been described by many authors ranging from symbiosis to parasitics. As an important region the phycosphere of algae contains algal extracellular organic carbon, and certain bacterial growth is stimulated in it. The associated bacterial flora are considered to benefit from organic substrates released by the algae and be influenced by the nature of algae (Paerl *et al*., 1989). Several studies have shown a specific association of bacteria with different marine algae. In laboratory, diatom cultures harbor distinct bacterial satellite assemblages (Schäfer *et al*., 2002), which also exist between bacteria and dinoflagellates in culture (Hold *et al*., 2001; Jasti *et al*., 2005). The shifts in species composition of the phytoplankton and attached bacterial communities were found to be linked in a coastal marine environment (Rooney-Varga *et al*., 2005). The specific interactions between bacteria and algae would influence the composition of both communities. However, there is little information on specific associations between freshwater cyanobacteria and their associated bacterial communities.

The cyanobacterium *Microcystis* is one of the most widespread and extensively studied harmful algal bloom forming genus in eutrophic lakes. It causes significant issues to drinking water quality, resulting in growing public health concerns all over the world (Falconer *et al*., 1983; Carmichael *et al*., 1985; Galey *et al*., 1987). *Microcystis aeruginosa* is one of the most ecologically damaging species due to its prevalence in eutrophic water bodies (Carmichael, 1992). Interest in harmful algal blooms has revealed that bacteria are capable of stimulating or inhibiting algal growth (Salomon *et al*., 2003), or killing algae (Manage *et al*., 2000). *Microcystis* spp. has numerous bacteria associated with its extracellular mucus zone (Hoppe, 1981). Microcystin-degrading bacteria were detected in mucilage of *Microcystis* (Maruyama *et al*., 2003). The exudates of *M. aeruginosa* are more likely to attract those bacteria co-occurring with *M. aeruginosa* but have antibiotic effects on non-co-occurring bacteria (Casamatta and Wickstrom, 2004). Changes in composition of the bacterial community are moderately correlated

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with *Microcystis* spp. bloom (Xing *et al*., 2007).

Given the complex natural situation, the study of bacteria associated with algal cultures may offer complementary information on bacterial-algal interactions. The extracellular substances from algal cells as bacterial growth substrates may select certain well adapted bacteria. Bacteria, which were initially present in the phycosphere and capable of growing with the algae, can persist in the unialgal cultures without axenic treatment under successive transfer.

In present study, denaturing gradient gel electrophoresis (DGGE) method was employed for understanding bacterial-cyanobacterial associations. The sequenced DGGE fragments were compared with databases to reveal their taxonomic affiliations. The aim of our study was to reveal whether there is specific association of bacteria with *M. aeruginosa*, and to investigate the diversity of bacterial communities associated with the freshwater cyanobacterial cultures.

1 Materials and methods

1.1 Cyanobacterial cultures

To study the specificity of bacteria associated with *M. aeruginosa*, three other *Microcystis* strains and four non-*Microcystis* cyaobacterial strains were used as reference strains (Table 1). All cyanobacterial cultures were obtained from the Freshwater Algae Culture Collection of the Institute of Hydrobiology, Chinese Academy of Sciences. Cultures were grown at (23 ± 1)°C in BG11 medium (Rippka, 1988), at a light intensity of 32 μE/(m^2·s) with 12 h light-darkness cycle. All cyanobacterial cultures were maintained as unialgal, xenic cultures from the time of original isolation and were handled aseptically to prevent cross-contamination among cultures.

1.2 Sampling of bacterial assemblages and DNA extraction

Cyanobacterial cultures samples (1 mL) collected from late exponential growth phases were centrifuged at 10000 ×*g* for 10 min. The cell pellets were stored at –20°C until DNA extraction. Community genomic DNA extraction was conducted using the potassium xanthogenate sodium dodecyl sulfate (XS procedure) method (Tillett and Neilan, 2000).

Table 1 Cyanobacterial cultures used in this study

Species	Source	Source
Microcystis aeruginosa	America	FACHB434
Microcystis aeruginosa	Japan	FACHB918
Microcystis aeruginosa	Australia	FACHB924
Microcystis aeruginosa	Lake Dianchi of China	FACHB905
Microcystis aeruginosa	Lake Taihu of China	FACHB912
Microcystis viridis	Japan	FACHB969
Microcystis wesenbergii	Japan	FACHB929
Microcystis elabens	Japan	FACHB916
Aphanizomenon sp.	Lake Dianchi of China	FACHB1039
Anabaena flos-aquae	Lake Dianchi of China	FACHB1092
Oscillatoria sp.	Lake Dianchi of China	FACHB1097
Cylindrospermopsis sp.	Lake Dianchi of China	FACHB1096

1.3 PCR amplification of 16S rDNA fragments and DGGE

PCR amplification of 16S rDNA fragments was performed by using primers 341f (5'-CCTACGGGAGGCAGCAG-3') with a 40 bp GC-clamp (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3') attached to its 5' end and 907r (5'-CCGTCAATTCMTTTRAGTTT-3') (Teske *et al*., 1996). The PCR reaction mixture with volume of 50 μL contained 1× PCR buffer (without Mg^{2+}), 2.25 mmol/L of $MgCl_2$, 0.8 mmol/L of deoxynucleotide triphosphates, 0.5 μmol/L of each primer, 2 U of Taq DNA polymerase (Takara, Japan), and 10–100 ng of community genomic DNA template. A touchdown PCR (Don *et al*., 1991) was performed with an initial denaturation step at 94°C for 5 min, followed by 10 cycles of 1 min at 94°C, 1 min at 65°C to 56°C (decreasing 1°C every cycle), 1 min at 72°C, 20 additional cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, the tubes were then incubated for 5 min at 72°C (Muyzer *et al*., 1993). PCR reactions were performed in Bio-Rad thermal cycler (USA), and PCR products were detected on 1.2% (*W/V*) agarose gels.

For each sample, about 700 ng of PCR product was loaded on 6% polyacrylamide gels (acrylamide:*N*,*N*'-methylenebisacrylamide, 37.5:1, *V/V*) containing a denaturant gradient from 40% to 70% (100% denaturant contains 7 mol/L urea and 40% deionized formamide). Electrophoresis was performed with the DGGE-2001 system (CBS Scientific Company, USA) using 1× TAE running buffer (40 mmol/L Tris, pH 7.4, 20 mmol/L sodium acetate, 1 mmol/L EDTA) at 60°C for 16 h at 100 V. DGGE gels were stained in SYBR Green I (1:10000 dilution; Molecular Probes) for 30 min. Gel image was recorded with an Omega^{TM} 10 Ultracam Gel Explorer (Omega, USA).

1.4 DGGE analysis

DGGE profiles were analyzed using the Gel-Pro software (Media Cybernetics Inc., USA) to determine the position of individual bands. A numeral one was assigned when there was a band at a certain position in a lane, while zero was allocated when no band was found at the same position in other lanes. The DGGE profiles were compared to each other by using the pairwise similarity values S_D (Dice coefficient), which was determined as the following equation:

$$S_D = \frac{2N_{AB}}{N_A + N_B}$$

where, N_{AB} is the number of bands present in both lane A and lane B, N_A and N_B is the total number of bands in lane A and B, respectively. For cluster analysis, the similarity values among DGGE profiles were analyzed by UPGMA (unweighted pair group with mathematical averages) using the program MVSP (version 3.1, Kovach Computing Services).

1.5 DNA sequence analyses

Prominent DGGE bands were excised from the gel using a sterile scalpel and eluted in 50 μL of sterile Milli-Q water overnight at 4°C. An aliquot of 5 μL was used as template in PCR reamplification as described above. Following amplification, a portion of the PCR products were rerun on DGGE gels to confirm their positions relative to the bands from which they were excised. The PCR products were then purified using a QIAquick PCR purification kit (Sangon Valencia, USA) and sequenced using the ABI Bigdye 3.1 Terminator cycle sequencing kit (Perkin Elmer Corporation) prior to ABI 3730xl DNA Analyzer at Shanghai Sangon Biological Engineering Technology Limited Company, China. Direct sequencing of some of the DGGE bands was unsuccessful, and these bands were reamplified and cloned prior to sequencing.

The obtained sequences were compared with 16S rDNA sequences available in the National Center for Biotechnology Information (NCBI) Database using BLAST search. Phylogenetic tree of 16S rDNA sequences was constructed using neighbor-joining method of Mega 4.0, with Jukes-Cantor corrections for distance values and 1000 bootstrap replicates.

1.6 Nucleotide sequence accession number

The partial sequences of 16S rRNA genes obtained in this study were deposited in GenBank with the following accession numbers: FJ377372-FJ377415.

2 Results

2.1 Comparison of DGGE profiles

Similarity values for DGGE profiles of bacteria associated with different cyanobacteria were compared. All cultures differed in their DGGE fingerprint patterns (Fig. 1), reflecting differences in their bacterial community compositions. Based on the position of bands using unweighted pair groupings of a similarity coefficient matrix, the cluster analysis generated an UPGMA dendrogram (Fig. 2). Regarding the cluster analysis, samples were grouped into 3 clusters. Cluster 1 contained three non-*Microcystis* filamentous cyanobacterial cultures, including *Aphanizomenon* sp., *Cylindrospermopsis* sp., and *Oscillatoria* sp. Cluster 2 was composed of three *Microcystis* cultures: *M. viridis*, *M. elabens* and *M. wesenbergii*. Cluster 3 included all cultures of *M. aeruginosa* and *A. flos-aquae*. Cluster analyses revealed higher similarity values among *M. aeruginosa* cultures than similarity values among *M. aeruginosa* cultures and non-*M. aeruginosa* cyanobacterial cultures, (*t*-test, $P < 0.05$). Also, the similarity values among cultures of *Microcystis* spp. were higher than the similarity values among cultures of *Microcystis* spp. and non-*Microcystis* cyanobacteria (*t*-test, $P < 0.05$).

2.2 Phylogenetic analysis of bacterial communities

Prominent phylotypes in different cyanobacterial cultures were identified by sequence analyses from excised DGGE bands. Sequence data generally revealed the

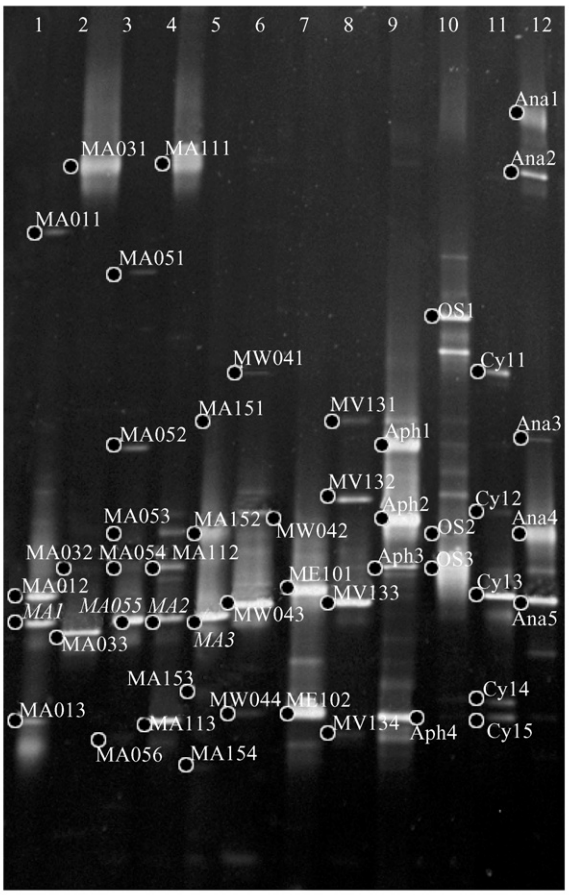


Fig. 1 DGGE profiles of 16S rDNA fragments of bacteria in cultures of the twelve freshwater cyanobacteria used in this study. All labeled bands were excised from the gel, reamplified and sequenced. Sequences (*MA055*, *MA1*, *MA2* and *MA3*) related to cyanobacterial 16S rRNA genes are indicated by italics. Lane 1: *M. aeruginosa* FACHB924; lane 2: *M. aeruginosa* FACHB918; lane 3: *M. aeruginosa* FACHB912; lane 4: *M. aeruginosa* FACHB905; lane 5: *M. aeruginosa* FACHB434; lane 6: *M. wesenbergii*; lane 7: *M. elabens*; lane 8: *M. viridis*; lane 9: *Aphanizomenon* sp.; lane 10: *Oscillatoria* sp.; lane 11: *Cylindrospermopsis* sp.; lane 12: *A. flos-aquae*.

dominance of three major phyla of bacteria: Alphaproteobacteria, Betaproteobacteria and Bacteroidetes. Closest relatives of the sequenced phylotypes retrieved from blast analysis are listed in Table 2. Most (76%) of the analyzed sequences showed more than 99% similarity to their closest relatives in the data base. However, about 14% of the sequences exhibited less than 97% match. The similarity values lower than 97% indicated the two 16S rDNA sequences were originated from different species (Stackebrandt and Goebel, 1994). Therefore, the cyanobacterial cultures may harbor novel bacteria.

Together, 55% of the phylotypes were assigned to the Alphaproteobacteria, and 19% of the phylotypes were related to the Betaproteobacteria or Bacteroidetes. Additionally, we found two phylotypes (MA033 and OS2) were related to Gammaproteobacteria. One sequence (MV4) in the culture of *M. viridis* was related to Actinobacteria.

A neighbor-joining tree of the Alphaproteobacteria revealed that the majority of sequences belonged to the Sphingomonadales clade (43%, Fig. 3a). Only 17% were related to the Rhizobiales and 13% were related to the

Table 2 Similarity values for the closest relatives of 16S rDNA sequences retrieved from cyanobacterial cultures

Source culture	DGGE band	Closest relative (GenBank accession number)	Similarity (%)	Accession number	Phylogenetic group	Taxon
M. aeruginosa FACHB924	MA011	*Sphingomonas alaskensis* (AM403496)	100	FJ377372	Sphingomonadales	Alphaproteobacteria
	MA012	Uncultured *Alphaproteobacterium* (AJ871080)	99	FJ377373	Rhodobacterales	Alphaproteobacteria
	MA013	*Candidatus Chryseobacterium massiliensis* (EF394923)	99	FJ377374	Flavobacteria	Bacteroidetes
M. aeruginosa FACHB918	MA031	*Chryseobacterium* sp. Atl-24 (EF028127)	98	FJ377375	Flavobacteria	Bacteroidetes
	MA032	*Porphyrobacter* sp. A2 (EU770257)	99	FJ377376	Sphingomonadales	Alphaproteobacteria
	MA033	*Xanthomonas* sp. 3C_3 (AY689031)	99	FJ377377	Xanthomonadaceae	Gammaproteobacteria
M. aeruginosa	MA051	Uncultured *Bacteroidetes* bacterium (AJ871049)	99	FJ377378	Bacteroidetes	Bacteroidetes
FACHB912	MA052	Uncultured CFB group bacterium isolate DGGE band W2 (AF423377)	96	FJ377379	Bacteroidetes	Bacteroidetes
	MA053	Uncultured bacterium clone Chlplus CL-040515 OTU-24 (EU808101)	99	FJ377380	Sphingomonadales	Alphaproteobacteria
	MA054	*Porphyrobacter* sp. A2 (EU770257)	99	FJ377381	Sphingomonadales	Alphaproteobacteria
	MA056	*Bosea thiooxidans* strain 187 (EU730912)	100	FJ377382	Rhizobiales	Alphaproteobacteria
M. aeruginosa	MA111	Uncultured bacterium clone R1-10 (EF363041)	99	FJ377383	Sphingomonadales	Alphaproteobacteria
FACHB905	MA112	*Porphyrobacter* sp. A2 (EU770257)	99	FJ377384	Sphingomonadales	Alphaproteobacteria
	MA113	Uncultured *Alphaproteobacterium* clone SM2A11 (AY293404)	99	FJ377385	Alphaproteobacteria	Alphaproteobacteria
M. aeruginosa	MA151	*Sphingopyxis alaskensis* (AM403496)	100	FJ377386	Sphingomonadales	Alphaproteobacteria
FACHB434	MA152	Drinking water bacterium MB11 (AY328842)	96	FJ377387	Rhizobiales	Alphaproteobacteria
	MA153	*Rhodocista pekingensis* (FM177580)	96	FJ377388	Rhodospirillaceae	Alphaproteobacteria
	MA154	Uncultured bacterium clone UWL_CL-080514_OTU-34 (EU809244)	99	FJ377389	Alphaproteobacteria	Alphaproteobacteria
M. wesenbergii	MW041	*Candidatus Chryseobacterium massiliensis* strain URChM1 (EF394923)	99	FJ377390	Flavobacteriaceae	Bacteroidetes
	MW042	*Sphingopyxis* sp. 8_4K (EF540479)	99	FJ377391	Sphingomonadales	Alphaproteobacteria
	MW043	*Devosia insulae* strain A16 (EU794908)	99	FJ377392	Rhizobiales	Alphaproteobacteria
	MW044	Uncultured *Oleomonas* sp. clone I79 (AY692039)	98	FJ377393	Acetobacteraceae	Alphaproteobacteria
M. elabena	ME101	*Sphingomonas* sp. ORS 1497 (AJ968701)	99	FJ377394	Sphingomonadales	Alphaproteobacteria
	ME102	*Paracoccus* sp. DHHS10 (DQ659042)	100	FJ377395	Rhodobacteraceae	Alphaproteobacteria
M. viridis	MV131	*Sphingomonas alaskensis* (AM403496)	99	FJ377396	Sphingomonadales	Alphaproteobacteria
	MV132	Uncultured organism clone B80 (AY707552)	100	FJ377397	Caulobacterales	Alphaproteobacteria
	MV133	*Limnobacter* sp. DG1610 (EU052768)	100	FJ377398	Burkholderiales	Betaproteobacteria
	MV134	*Parkia alkaliphila* (AB376086)	99	FJ377399	Actinomycetales	Actinobacteria
Aphanizomenon sp.	Aph1	Uncultured *Flavobacteriales* bacterium clone MS072A1_F01(EF69740)	100	FJ377400	Flavobacteriales	Bacteroidetes
	Aph2	*Limnobacter* sp. DG1610 (EU052768)	100	FJ377401	Burkholderiales	Betaproteobacteria
	Aph3	*Alphaproteobacterium* BAC247 (EU180521)	99	FJ377402	Alphaproteobacteria	Alphaproteobacteria
	Aph4	*Bosea thiooxidans* strain 187 (EU730912)	99	FJ377403	Rhizobiales	Alphaproteobacteria

Continued

Oscillatoria sp.	OS1	Uncultured bacterium clone ANTLV1_C06 (DQ521478)	100	FJ377404	Alphaproteobacteria	*Alphaproteobacteria*
	OS2	*Pseudomonas* sp. G3DM-33 (EU037280)	99	FJ377405	Pseudomonadaceae	*Gammaproteobacteria*
	OS3	*Acidovorax facilis* strain 228 (EU730927)	99	FJ377406	Burkholderiales	*Betaproteobacteria*
Cylindrospermopsis sp.	Cy1	Uncultured *Flexibacter* sp. (AM989589)	92	FJ377407	Sphingobacteriales	*Bacteroidetes*
	Cy2	Uncultured *Limnobacter* sp. cloneD-15(AF522999)	99	FJ377408	Burkholderiaceae	*Betaproteobacteria*
	Cy4	*Ideonella* sp. IMCC1722 (DQ66424)	98	FJ377409	Burkholderiales	*Betaproteobacteria*
	Cy5	*Hydrogenophaga* sp. TRS-05 (AB166889)	99	FJ377410	Burkholderiales	*Betaproteobacteria*
Anabaena flos-aquae	Ana1	Uncultured bacterium clone Chun-w-43 (EF632773)	99	FJ377411	Bacteroidetes	*Bacteroidetes*
	Ana2	Uncultured *Bacteroidetes* bacterium clone GASP-WC2W3_A12 (EF075333)	93	FJ377412	Bacteroidetes	*Bacteroidetes*
	Ana3	*Chimaereicella* sp. A8-7 (EU313811)	99	FJ377413	Sphingobacteriales	*Bacteroidetes*
	Ana4	Uncultured bacterium clone 6C233273 (EU805273)	99	FJ377414	Betaproteobacteria	*Betaproteobacteria*
	Ana5	*Hydrogenophaga* sp. TRS-05 (AB166889)	99	FJ377415	Burkholderiales	*Betaproteobacteria*

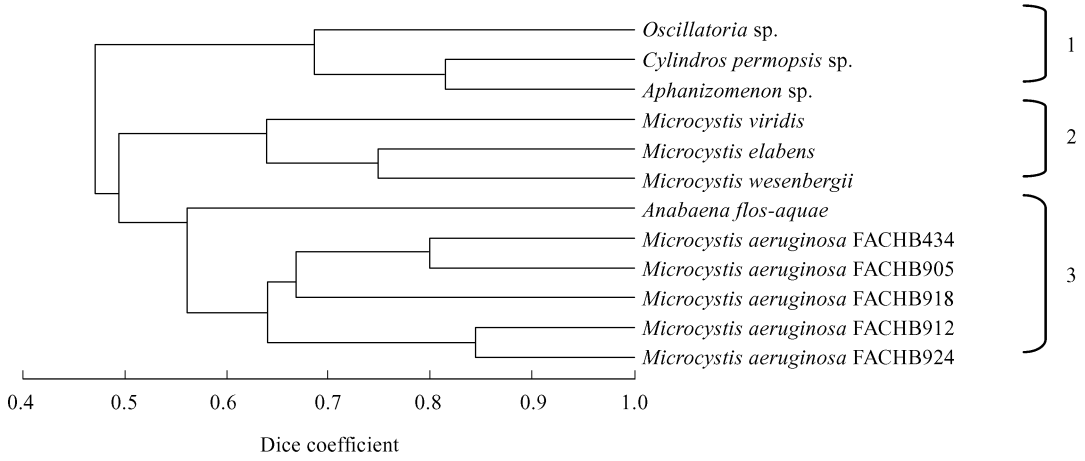


Fig. 2 UPGMA (unweighted pair group with mathematical averages) dendrogram based on similarities of DGGE profiles of bacterial communities associated with the twelve cyanobacterial cultures. Cultures are grouped into three clusters (1, 2 and 3).

Rhodospirillales. In particular, sequences related to Sphingomonadales were retrieved from all *Microcystis* cultures. Most sequences (80%) clustered with *Porphyrobacter* sp., *Sphingomonas* sp. and *Sphingopyxis* sp. Three sequences (MA032, MA054, MA112) retrieved from *M. aeruginosa* (FACHB918, FACHB912, FACHB905) cultures were related to *Porphyrobacter* sp. Three phylotypes (MA011, MV131, ME101) related to *Sphingomonas* were found in cultures of *M. aeruginosa* FACHB924, *M. viridis* and *M. elabens*, while sequences related to *Sphingopyxis* (MA151, MW042) were retrieved from the cultures of *M. aeruginosa* FACHB434 and *M. wesenbergii*. Sequences related to Rhodospirillales were found to appear in cultures of *M. aeruginosa* FACHB924, FACHB434 and *M. elabens*. The sequences related to *Bosea* sp. within the order of Rhizobiales were found in the cultures of *M. aeruginosa* FACHB912 and *Aphanizomenon* sp. (Table 2).

Within the group of Betaproteobacteria, all sequences except Ana4 belonged to the order of Burkholderiales (Fig. 3b). Sequences related to this order were found as associated with *M. viridis* and non-*Microcystis* cyanobacterial species. A cluster of sequences (MV133, Aph2 and Cy2) retrieved from the cultures of *M. viridis*, *Aphanizomenon* sp. and *Cylindrospermopsis* sp. were related to *Limnobacter* sp., whereas one sequence (OS3) from the culture of *Oscillatoria* sp. was related to *Acidovorax facilis*, and two sequences (Cy4, Cy5) from the culture of *Cylindrospermopsis* sp. were related to *Ideonella* sp. and *Hydrogenophaga* sp. Only two phylotypes (OS2 and MA033) related to Xanthomonadaceae and Pseudomonadaceae within Gammaproteobacteria were found only associated with the culture of *M. aeruginosa* FACHB918 and *Oscillatoria* sp., respectively.

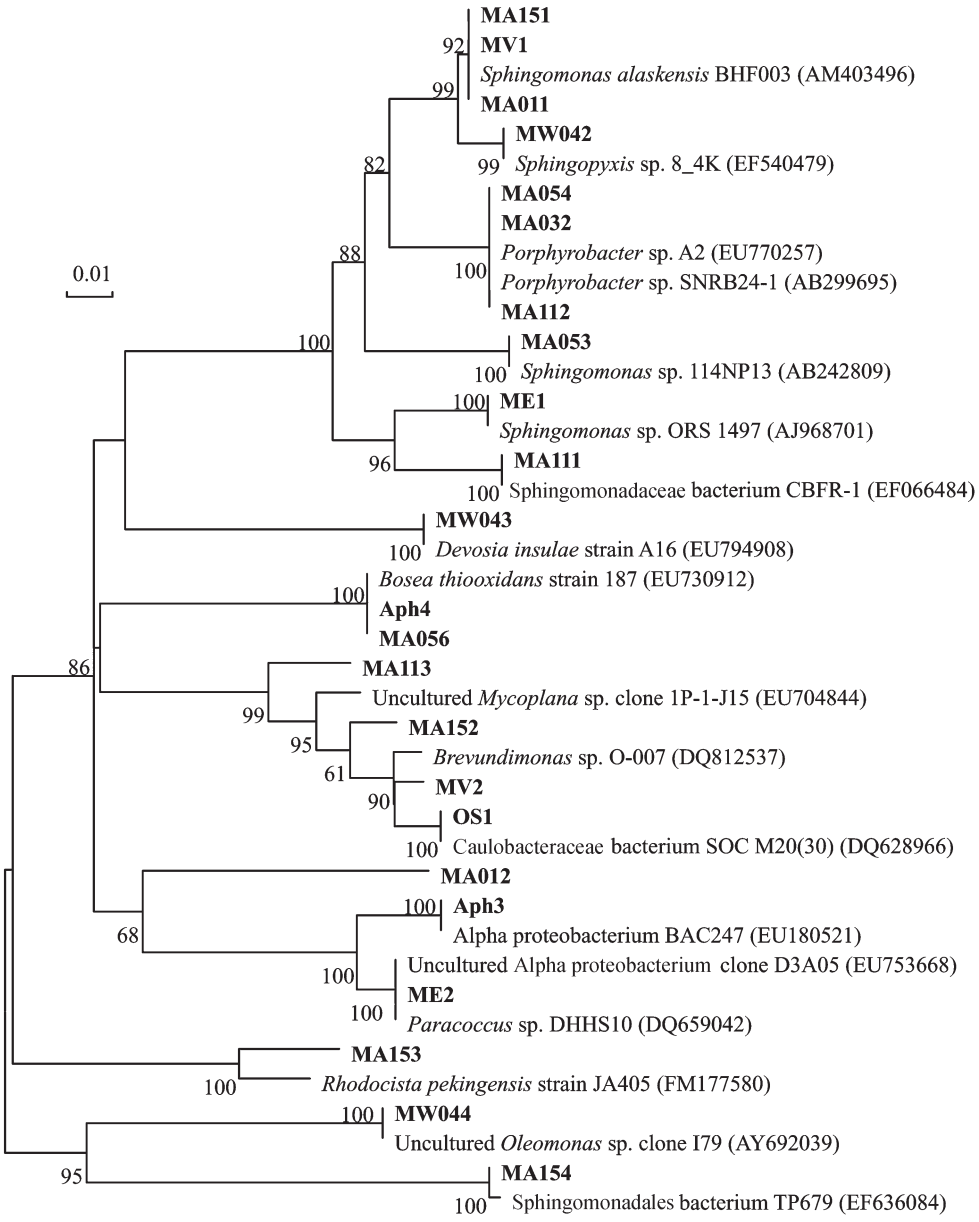
Gene sequences of the Flavobacteriales-Bacteroidetes were assigned to the Flavobacteriales, Bacteroidetes and Sphingobacteriales (Fig. 3c). A cluster of sequences

retrieved from cultures of *M. aeruginosa* FACHB924, FACHB918 and *M. wesenbergii* were related to *Chryseobacterium* sp. One bacterial sequence (Ana3) from the *A. flos-aquae* culture was related to *Chimaereicella* sp., whereas some sequences related to uncultured bacteria were found in the cultures of *M. aeruginosa* FACHB912, *Aphanizomenon* sp. and *Cylindrospermopsis* sp.

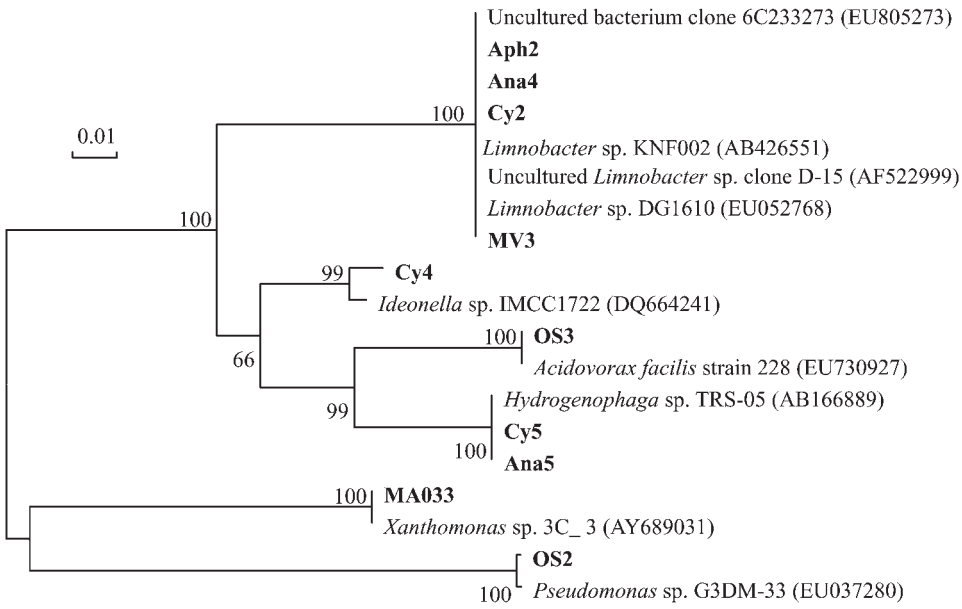
3 Discussion

Since *Microcystis* usually form mucilaginous colonies with some bacteria tightly associated with the colonies (Scott, 1974), and the difficulty in culturing the cyanobacteria without bacteria, the associated bacteria in some way may affect the growth and physiology of *Microcystis*. To study the diversity and specificity of bacteria associated with *M. aeruginosa*, some unrelated cyanobacterial species were selected to minimize potential biases that the process of laboratory culture is partly responsible for the similarities in bacteria seen among different cultures. All of these cyanobacteria were unialgal, without treatment to render the cultures axenic, and grew under similar cultivation conditions.

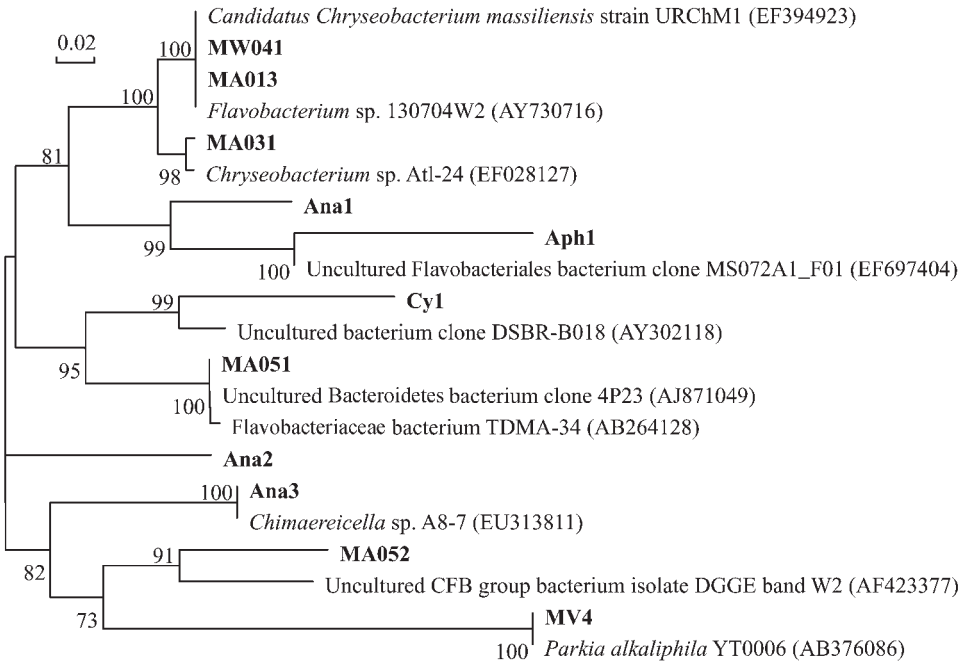
The advantages of using DGGE as a means of studying microbial ecology are well established in that it reduces bias in the detection of unculturable species, and provides a more accurate means of visualizing whole microbial communities (Gafan *et al*., 2005). There are also many difficulties of DGGE, such as primer selectivity, PCR errors and multiple heterogeneous rRNA operons which



a Phylogenetic tree of sequences belonged to Alphaproteobacteria, the scale bar represents 1% sequence divergence.



b Phylogenetic tree of sequences belonged to Betaproteobacteria and Gammaproteobacteria, the scale bar represents 1% sequence divergence.



c Phylogenetic tree of sequences belonged toActinobacteria and Bacteroidetes, the scale bar represents 2% sequence divergence.

Fig. 3 Phylogenetic trees showing the relationships among sequences retrieved from DGGE bands and reference sequences obtained from GenBank (accession numbers of these sequences are given in parentheses). Sequences obtained in this study are in bold. The tree was built using the neighbor-joining method with Jukes-Cantor corrected distances. Bootstrap values > 50% is shown next to its respective branch. The numbers at the branches indicate the percentage of 1000 bootstraps replicates.

may result in more than one DGGE band from one bacterium (Cilia *et al.*, 1996). However, much replicate amplification and multiple DGGE gels show that PCR products and the subsequent DGGE patterns are highly reproducible, therefore, it is reliable to use this method. Our comparisons of DGGE profiles of samples taken during different growth phases of the cyanobacterial batch culture (data not shown), indicated that there was no qualitative difference in the dominant DGGE bands, i.e., the bacterial-cyanobacterial associations were stable. This is consistent

with earlier findings in laboratory dinoflagellates (Hold *et al*., 2001; Jasti *et al*., 2005). As a result, it was not necessary to analyze multiple cyanobacterial growth phases to determine the phylogenetic identities of the dominant bacteria associated with cyanobacterial cultures.

Cluster analysis of DGGE banding profiles, for example, the UPGMA applied in this study, which can be used to identify samples generating similar patterns, is a useful tool to compare and classify different systems (Boon *et al*., 2002; Ibekwe *et al*., 2001). It uses unweighted pair group with mathematical averages analysis and does not take into account the intensities of DGGE bands (Gafan *et al*., 2005). The primers GC-341f and 907r could also amplify cyanobacterial 16S rRNA genes. Some bands (MA055, MA1, MA2 and MA3) related to cyanobacteria in the four *M. aeruginosa* cultures were not considered in further analyses, as many *Microcystis* species showed a similar DNA base composition or DNA relatedness (Kondo *et al*., 2000), and marked similarities in the complete 16S rDNA sequences among different *Microcystis* species were found (Otsuka *et al*., 1998). Cluster analysis of bacterial composition in cyanobacterial cultures showed higher similarity values among *M. aeruginosa* cultures than that among *M. aeruginosa* cultures and other *Microcystis* spp., and also higher than the similarities among *M. aeruginosa* cultures and non-*Microcystis* cyanobacterial cultures. The relationship was true regardless of the geographic location from which *M. aeruginosa* strains were obtained. Also, we found that the similarity values among *Microcystis* spp. were higher than that among *Microcystis* spp. and non-*Microcystis* cyanobacteria. This is consistent with earlier findings that similarity values among *Alexandrium* samples were significantly higher than that among *Alexandrium* samples and non-*Alexandrium* samples (Jasti *et al*., 2005). The organic carbon produced by different algal species may select a variety of bacterial populations. The closely related algae may produce the similar organic carbon, leading to the similar bacterial community associated with the algae. In the cluster analysis, cyanobacterial species including *Aphanizomenon* sp., *Oscillatoria* sp. and *Cylindrospermopsis* sp. were clustered together. High similaity values of some different species were also found in the study of Jasti *et al*. (2005). A selection of more strains of the same species would be needed to make a cluster ananlysis of these algae. However, less similarity values among these non-*Microcystis* reference strains and *M. aeruginosa* strains, and the greatest similarities within the species *M. aeruginosa* were found. This did indicate that specific association between *M. aeruginosa* and associated bacteria may occur.

In this investigation, sequencing of DGGE bands revealed that the majority of the dominant populations associated with twelve cyanobacterial cultures fell into a number of major lineages within the bacteria domain: Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Flavobacteriales-Bacteroidetes and Actinobacteria sp. phylum. Although the cyanobacteria studied in this work were isolated from freshwater, the bacterial affiliations were similar with bacterial sequences associated with Nodularia (cyanobacteria) from the Baltic Sea (Tuomainen *et al*., 2006). However, the majority of bacterial sequences associated with *Microcystis* were different from previously reported bacteria associated with marine diatoms and dinoflagellates. The predominant phylogenetic groups of bacteria found in these marine algal cultures were the Roseobacter clade (Alavi *et al*., 2001; Allgaier *et al*., 2003; Green *et al*., 2004; Hold *et al*., 2001; Schäfer *et al*., 2002.), while the predominant bacterial species associated with *Microcystis* were the Sphingomonadales group, and no phylotype assigned to *Roseobacter* was detected in *Microcystis* cultures. The Sphingomonadales have been found to be associated with other algae (Ferrier *et al*., 2002; Green *et al*., 2004; Hold *et al*., 2001). Compared to the *Microcystis* species associates, there are some differences in the bacterial assemblages found in other cyanobacterial cultures. One bacterium assigned to the order of Burkholderiales was found in only one culture (*M. viridis*) of *Microcystis* spp., while the members of Burkholderiales were found to be prevalent in all of the other cyanobacterial genus. This indicated that *Microcystis* spp. harbored distinct bacterial community compared with other cyanobacteria. Prevalence of the Sphingomonadales order indicated that the group may be well adapted to living in association with freshwater cyanobacteria *Microcystis* spp.

Maruyama *et al*. (2003) have shown that Alphaproteobacteria and Betaproteobacteria tend to dominate in the mucilage of *Microcystis* during the bloom of *Microcystis*. There are some metabolism associations between *Microcystis* and their associated bacteria. Phosphorus exchange between *M. aeruginosa* and attached *Pseudomonas* has been observed in the phycosphere (Jiang *et al*., 2007). *Sphingopyxis* sp. and *Sphingomonas* sp. were found to be capable of degrading microcystin-LR and microcystin-LA, microcystin-RR, respectively (Ho *et al*., 2007; Valeria *et al*., 2006), indicating that toxins produced by *Microcystis* may also play a role in selecting bacteria which could degrade and utilize the microcystin. The identification of the bacteria associated with the *Microcystis* cultures may provide further insight into the bacterial associates of microalgae, also be useful for understanding interactions between *Microcystis* spp. and their associated bacteria.

Bell (1984) pointed out that long term selection should favor a certain bacterial species that could well adapt to the spectrum of organic carbon offered by the algae. The compositions of organic matter released by different algal species are differed greatly (Myklestad, 1995; Biersmith and Benner, 1998). Different organic matter produced by different types of algae causes changes in the composition of bacterial communities utilizing this organic matter (Van Hannen *et al*., 1999). Individual alga would confer a selective advantage to the bacteria capable of utilizing their exudates. Casamatta and Wickstrom (2000) showed that *M. aeruginosa* exudates have three avenues in influencing bacterioplankton communities, that is, chemotaxis, as a source of growth nutrients and antibiotics. Therefore, it is reasonable that specific association between bacterial communities and algae may occur.

4 Conclusions

Our results indicated that the patterns of bacterial community structure associated with different *M. aeruginosa* cultures were more similar than those of other cyanobacterial cultures, and members of the Sphingomonadales were the prevalent group among the *Microcystis* associated bacteria. This provides evidence for specific bacterial-cyanobacterial associations in culture. This study may be useful for understanding the bacterial-cyanobacterial interaction and throw a light for the role of bacteria in cyanobacterial blooms, and will help us to investigate the interactions between *Microcystis* and their associated bacteria *in situ* during water bloom in the future.

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