Effects of the cultivable bacteria attached to *Microcystis* colonies on the colony size and growth of *Microcystis*

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**ABSTRACT**

The interactions between bacteria and algae may play a significant part in the formation and development of algal blooms. The bloom-forming cyanobacterium *Microcystis* occurs mainly as colonial form in natural waters, and thus it is necessary to study the interaction between bacteria and colonial *Microcystis*. This paper aimed to investigate effects of the cultivable bacteria attached to *Microcystis* colonies on the colony size and growth of colonial *Microcystis aeruginosa*. Eleven bacterial strains were isolated from *M. aeruginosa* colonies collected from Lake Taihu. Among these bacteria, seven bacterial isolates significantly influenced the colony size of *M. aeruginosa*, and four bacterial isolates significantly influenced the growth rate of *M. aeruginosa*. Four isolates, related to the *Exiguobacterium*, *Delftia*, *Bacillus* and *Stenotrophomonas*, significantly decreased the colony size of *M. aeruginosa* by 36-51%, and two isolates, assigned to the *Chryseobacterium* and *Pseudomonas chengduensis*, significantly increased the *M. aeruginosa* colony size by 89% and 63%, respectively, while these isolates had no effect on the cyanobacterial growth rate. Two isolates, belonged to the *Rheinheimera* and *Pseudomonas*, significantly decreased the growth rate of *M. aeruginosa* by 47% and 36%, respectively, and one bacterial strain related to *Aeromonas* increased the cyanobacterial growth rate by 22%, while these isolates had no effect on the cyanobacterial colony size. One isolate belonged to *Sphingomonas* was found to significantly increase the colony size of *M. aeruginosa* by 80% and significantly decrease the growth rate of *M. aeruginosa* by 21%. PCR-denaturing gradient gel electrophoresis (PCR-DGGE) analysis showed that all of the bacterial isolates were able to colonize *M. aeruginosa* colonies. Our data suggested that the bacteria attached to *Microcystis* colonies might influence the cyanobacterial colony size and growth, and thus influence the formation and development of *Microcystis* blooms.

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1. Introduction

Cyanobacterial blooms have become a common occurrence in eutrophic freshwater bodies worldwide. Among bloom-forming cyanobacteria, *Microcystis* spp. are the most commonly reported species in lakes and reservoirs. It is well known that *Microcystis* occurs mainly as colonial aggregates constrained by mucilaginous matrix in natural waters (Otsuka et al. 2000). However, *Microcystis* colonies separate into single cells following long-term cultivation under laboratory conditions (Zhang et al. 2007). Colony formation is highly important for the competitive advantage of *Microcystis* over other phytoplankton species in freshwater ecosystems (Yang et al. 2011). Formation of colonies is helpful for *Microcystis* to migrate vertically and defense against predation pressure (Fulton and Paerl 1987; Visser et al. 2005). Colonial *Microcystis* exhibits higher photosynthetic activities and can acclimate to iron deficiencies better than unicellular *Microcystis* (Li et al. 2016; Wu and Song 2008). Large colonies more readily overcome the stirring effects of water flows, waves and perturbations and gain access to the surface layer to form *Microcystis* blooms compared with small colonies (Zhu et al. 2014). Therefore, large *Microcystis* colonies are suggested to favor bloom formation (Li et al. 2013).

The interactions between bacteria and algae may play a significant part in the formation and development of algal blooms (Kodama et al. 2006), and thus have caught increasing attention. Numerous bacteria are embedded in the mucilage of *Microcystis* spp. (Brunberg 1999). The associated bacterial flora depends on both carbon and energy source from *Microcystis*, and supply *Microcystis* with vitamin B12, which is required for growth of *Microcystis* (Xie et al. 2016). Phosphorus exchange occurs between *Microcystis aeruginosa* and its attached bacterium (*Pseudomonas* sp.) (Jiang et al. 2007). Microcystin-degrading bacteria have been found in the mucilage of *Microcystis* colonies (Maruyama et al. 2003). Bacteria attached to *Microcystis* colonies may induce unicellular *Microcystis* to form colonies (Shen et al. 2011; Wang et al. 2016). Shi, Cai, Li, et al. (2009) have reported that bacterial strains isolated from the mucilage of *Microcystis* colonies influence the growth of unicellular *M. aeruginosa* positively or negatively. As *Microcystis* occurs mainly as colonial form in natural waters, it is essential to study the interaction between bacteria and colonial *Microcystis*. The aim of this study was to investigate effects of the bacteria attached to *Microcystis* colonies on the colony size and growth of colonial *M. aeruginosa*.

2. Materials and methods

2.1. Bacterial isolation

*Microcystis* colonies were collected from Lake Taihu (Jiangsu, China) in August, 2017. Healthy *M. aeruginosa* colonies were picked under an optical microscope according to the morphological classification (Yu et al. 2007). To remove free-living bacteria, *M. aeruginosa* colonies were washed 10 times on sterile 20-μm nylon screen with sterile BG11 medium (Rippka 1988). One drop of BG11 medium from the last washing step was inoculated on a R2A agar plate (Reasoner and Geldreich 1985), and no bacterial colonies were found after 96 hours of incubation at 28°C. *M. aeruginosa* colonies were then spread onto R2A agar plates, and incubated at 28°C. Bacterial colonies of distinct morphologies were picked and purified further on R2A agar plates.

2.2. DNA isolation and sequencing

Bacterial genomic DNA was isolated using the method of Berg et al. (2009). Microbial 16S rRNA gene was amplified with the universal primers pair 27F (5'-...
AGAGTTTGATCCTGGCTCAG-3’ and 1492 R (5’-TACGGCTACCTTGTTACGACTT-3’) (Weisburg et al. 1991). The amplification was performed with an initial denaturation step of 94°C for 1 min, followed by 30 cycles of 30 s at 94°C, 30 s at 50°C and 2 min at 72°C, plus a final extension at 72°C for 7 min. After being checked using 1% agarose gel electrophoresis, PCR products were purified using the SanPrep Column PCR Product Purification Kit (Sangon Biotech, Shanghai, China) and sequenced by GenScript Corporation Ltd. (Nanjing, China). The nucleotide sequences obtained in this study were deposited in GenBank under the accession numbers as shown in Table 1, and were compared to known 16S rRNA gene sequences in the GenBank database using BLAST to find the closest relatives.

2.3. Determination of the colony size and growth rate of M. aeruginosa

A colonial M. aeruginosa strain which was isolated from Lake Taihu in 2016 was grown in BG11 medium at 25°C under cool-white fluorescent light at 30 μmol photons m⁻² s⁻¹ with a 12:12 h light-dark cycle. Cyanobacterial cultures were manually shaken three times per day. Bacteria were grown in R2A medium at 28°C with shaking at 160 rpm. Bacterial cultures of exponential stage were harvested by centrifugation, and the cell pellets were rinsed and resuspended with sterile BG11 medium. After the bacterial isolates were inoculated into the cultures of M. aeruginosa, the co-cultures were incubated for 15 days. The control without inoculation of bacterial isolate was carried out at the same time. The density of the inoculated bacterial isolate was 1 × 10⁶ cells mL⁻¹ in the co-cultures, and the initial chlorophyll a (Chl-a) concentration was 0.85 mg L⁻¹.

The colony surface area of M. aeruginosa was used as an indicator for its volume and measured by a micrometer under the optical microscope (Eclipse E100, Nikon, Japan) (Wang et al. 2013). The colony was pressed into a thin-cake shape with a glass slide and cover slip before measurement. Fifty colonies were randomly chosen from each Microcystis culture. The growth rate (μ) of M. aeruginosa was measured according to equation: \( \mu = \frac{(X_2 - X_1)}{\Delta t} \) (Wang et al. 2014), where X2 and X1 represent the Chl-a content at the time t2 and time t1, respectively, and \( \Delta t = t_2 - t_1 \). The Chl-a content in cyanobacterial cultures were determined spectrophotometrically after extraction with 95% ethanol (Luo et al. 2013). All the experiments were replicated three times.

| Bacterial strain | Accession number | Closest relative (accession number) | Similarity (%) |
|------------------|------------------|-------------------------------------|----------------|
| QW20             | MK760077         | Sphingopyxis sp. strain YC-JH3 (MK217492) | 100            |
| QW04             | MK760068         | Sphingomonas wittichii strain 160 (EU730907) | 99             |
| QW07             | MK760070         | Delftia sp. TS40 (EU073106) | 100            |
| QW14             | MK760073         | Aeromonas caviae strain QSRB4 (MK007300) | 100            |
| QW15             | MK760074         | Pseudomonas chengduensis strain Hyper-1 (MK108035) | 100            |
| QW17             | MK760075         | Pseudomonas sp. JCM 28266 (LC133613) | 100            |
| QW19             | MK760076         | Stenotrophomonas maltophilia strain AOB9 (MK203000) | 100            |
| QW02             | MK760067         | Rheinheimera sp. TAPG2 (LT627667) | 99             |
| QW12             | MK760072         | Chryseobacterium sp. TDMA-2 (AB264125) | 99             |
| QW05             | MK760069         | Exiguobacterium indicum strain DSAM 62 (MH819520) | 100            |
| QW08             | MK760071         | Bacillus cereus strain US04 (MK696545) | 100            |
2.4. Detection of the inoculated bacterial isolates in the colonies of *M. aeruginosa* through denaturing gradient gel electrophoresis (DGGE)

10 mL of cyanobacterial cultures were sampled after 15 days of incubation. The samples were filtered through a 5.0 μm polycarbonate filter (Isopore Membrane Millipore), and followed by washing 10 times with sterile BG11 medium to remove free-living bacteria. One drop of BG11 medium from the last washing step was inoculated on an agar plate, and no bacterial colonies were found after 96 hours of incubation at 28°C. The filters were stored at −40°C for DNA extraction. Microbial DNA was extracted as described by Tillett and Neilan (2000). Partial bacterial 16S rRNA genes were amplified using primers 341f (5'-CCTACGGGAGGCAGCAG-3') with a 40 bp GC-clamp (5'-CGCCCGC CGCGCGGCGGGCCACGGGGG-3') attached to its 5' end and 907r (5'-CCGTCAAATTCMTTTRAGTTT-3') (Teske et al. 1996). A touchdown PCR was performed as described by Shi, Cai, Yang, et al. (2009). The touchdown thermocycling program was as follows: initial denaturation at 94°C for 5 min; 10 touchdown cycles of denaturation (at 94°C for 1 min), annealing (at 65-56°C for 1 min, decreasing 1°C every cycle) and extension (at 72°C for 1 min); 20 additional cycles of denaturation (at 94°C for 1 min), annealing (at 55°C for 1 min), and extension (at 72°C for 1 min), and a final extension at 72°C for 5 min. Amplicons were verified on a 1.2% agarose gel. DGGE was performed with a DGGE-2001 system (CBS Scientific Company, USA). PCR samples were loaded on 6% (wt/vol) polyacrylamide gels containing a 40%–70% linear gradient of denaturant (100% denaturant contains 40% deionized formamide and 7 M urea), and electrophoresis was performed in 1× TAE running buffer (40 mM Tris, pH 7.4, 20 mM sodium acetate, 1 mM EDTA) at 60°C for 16 h at 100 V. DGGE gels were stained in 1:10000 diluted GelRed (BIOTIUM, USA) for 30 min and photographed with a BioDoc-It® 220 Imaging System (UVP, USA).

2.5. Statistical analysis

The statistical analysis was carried out in the software IBM SPSS Statistics (version 19, IBM Company, Armonk, New York, USA) by using a one-way analysis of variance (ANOVA) with a Tukey post hoc test. Probability values of < 0.05 were considered statistically significant.

3. Results

3.1. Phylogenetic analysis of the isolated bacterial strains

Eleven bacterial strains were isolated from the mucilage of *M. aeruginosa* colonies collected from Lake Taihu. The bacterial isolates were assigned to *Alphaproteobacteria* (2/11), *Betaproteobacteria* (1/11), *Gammaproteobacteria* (5/11), *Bacteroidetes* (1/11) and *Firmicutes* (2/11) (Table 1).

3.2. Effects of the bacterial isolates on the colony size and growth of *M. aeruginosa*

Different responses of *M. aeruginosa* colony size were observed after inoculation of each bacterial isolate (Figures 1 and 2). At the end of the experiment, four isolates (QW05, QW07, QW08 and QW19) significantly decreased the colony size of *M. aeruginosa* by 36-51% ($P < 0.05$), and three isolates (QW04, QW12 and QW15) significantly increased the
M. aeruginosa colony size by 63-89% ($P < 0.05$), while four isolates (QW02, QW14, QW17 and QW20) had no effect on the M. aeruginosa colony size.

Figure 3 exhibits the effects of 11 bacterial isolates on the growth rate of M. aeruginosa. Three isolates (QW02, QW04 and QW17) significantly decreased the growth rate of M. aeruginosa by 21-47% ($P < 0.05$). Addition of one isolate (QW14) resulted in a significant increase of cyanobacterial growth rate by 22% ($P < 0.05$). Moreover, no significant
effect on M. aeruginosa growth rate was observed with the other seven isolates (QW05, QW07, QW08, QW12, QW15, QW19 and QW20) ($P > 0.05$).

Only one bacterial strain (QW04) was found to significantly influence both the growth rate and the colony size of M. aeruginosa (Figures 2 and 3). Six isolates (QW05, QW07, QW08, QW19, QW12 and QW15) had significant effect on the colony size of M. aeruginosa, while had no significant effect on cyanobacterial growth rate. Three isolates (QW14, QW02 and QW17) significantly influenced the cyanobacterial growth rate, while had no significant effect on the cyanobacterial colony size.

3.3. Detection of the inoculated bacterial isolates in the colonies of M. aeruginosa by DGGE

The DGGE profiles showed that each inoculated bacterial strain was present in M. aeruginosa colonies from the culture with addition of the bacterial isolate (Figure 4). A single DGGE band was observed for 10 isolates (QW02, QW04, QW05, QW07, QW08, QW12, QW14, QW15, QW17 and QW20), but four bands (S1, S2, S3 and S4) were observed for isolate QW19. The presence of 4 DGGE bands from strain QW19 was consistent with the previously reported four V3 region copies in the genome of some strains belonged to Stenotrophomonas maltophilia (the closest species of strain QW19) (Michon et al. 2012). Two isolates (QW02 and QW08) were found to be attached to M. aeruginosa colonies from both treatment and control cultures, indicating that they originally existed in the colonies of M. aeruginosa. Nine isolates (QW04, QW05, QW07, QW12, QW14, QW15, QW17, QW19 and QW20) were presented only in M. aeruginosa colonies from the treatment cultures. The PCR-DGGE analysis showed that each bacterial isolate was able to colonize M. aeruginosa colonies.

4. Discussion

Sequencing of 16S rRNA gene revealed that all bacterial isolates in this study belonged to Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Bacteroidetes and Firmicutes, which have been previously reported to be among the bacterial community.
attached to Microcystis colonies collected from Lake Taihu (Cai et al. 2014; Shi et al. 2010). Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria and Bacteroidetes have also been found in Microcystis colonies collected from a French lake (the Villerest reservoir) (Parveen et al. 2013). Sphingopyxis, Sphingomonas, Stenotrophomonas, Pseudomonas, Rheinheimera and Chryseobacterium (the closest genus of the bacterial isolates QW20, QW04, QW05, QW07, QW08, QW12, QW14, QW15, QW17, QW19 and QW20, respectively) have been previously detected in Microcystis colonies harvested from Lake Taihu (Cai et al. 2014; Shi, Cai, Li, et al. 2009; Shi et al. 2010). Aeromonas, Exiguobacterium and Bacillus (the closest genus of the bacterial isolates QW14, QW05 and QW08, respectively) are present in Microcystis colonies collected from Lake Taihu, Lake Dianchi and Lake Donghu (China) (Wang et al. 2016). Delftia (the closest genus of the bacterial isolate QW 07) is associated with Microcystis blooms occurred in water bodies of Germany, India, Portugal, Wisconsin (USA) and China (Dziallas and Grossart 2011). Sphingopyxis, Sphingomonas, Aeromonas, Bacillus, Stenotrophomonas, Pseudomonas, Rheinheimera and Chryseobacterium have also been found to be associated with cyanobacterial blooms occurring in Finnish lakes and rivers (Berg et al. 2009). Sphingopyxis and Sphingomonas are involved in degradation of microcystin (Dziallas and Grossart 2011; Wang et al. 2018). Stenotrophomonas maltophilia is able to fix nitrogen (Liba et al. 2006). Pseudomonas may be a provisional phosphorus bank for M. aeruginosa (Jiang et al. 2007).
It has been previously reported that the colony size affects the grazing of zooplankton on *Microcystis* and the colony buoyancy (Han et al. 2012; Wu and Kong 2009). In this study, seven isolates (QW05, QW07, QW08, QW19, QW04, QW12 and QW15) were found to significantly influence the colony size of *M. aeruginosa*, suggesting that they might influence zooplankton grazing and vertical migration of *Microcystis*, and in turn influence the formation and development of *Microcystis* blooms. Two isolates (QW15 related to *Pseudomonas* and QW04 assigned to *Sphingomonas*) increased the colony size of *M. aeruginosa*. *Pseudomonas* sp. CM-1 and *Sphingomonas* sp. CM-2 have been reported to be present in the laboratory-grown *Microcystis* colonies, but disappeared in the cyanobacterial cultures after the colonies of *Microcystis* separate into single cells, suggesting that they may be conducive to maintain the colonial form of *Microcystis* (Wang et al. 2015).

*Sphingomonas* is associated with *Microcystis* in all the samples from laboratory batch experiments, close-to-field experiments and water bodies of Germany, India, Portugal, Wisconsin (USA) and China, and thus is considered to be an integral element of *Microcystis* blooms (Dziallas and Grossart 2011). Extracellular polysaccharide (EPS) is considered to be involved in the formation of *Microcystis* colony (Wang et al. 2016). Two compounds tetradecamethylhexasiloxane and octamethylcyclotetrasiloxane which play a crucial role in polymer formation have been previously found in a *Microcystis*-associated bacterium *Shewanella putrefaciens* (Wang et al. 2016). It might be speculated that certain *Microcystis*-attached bacteria may stimulate the excretion of *Microcystis* EPS through producing bioactive compounds, and in turn increase the colony size of *Microcystis*. On the other hand, two isolates (QW19 belonged to *Stenotrophomonas maltophilia* and QW08 related to *Bacillus*) were observed to decrease the colony size of *M. aeruginosa* in this study. The abundance of *Bacillus* in Lake Xuanwu (China) is increased from the outbreak to the decline of a *Microcystis* bloom, and *Bacillus* dominates the bacterial community at the decline of the bloom (Zheng et al. 2008). *Stenotrophomonas maltophilia* and *Bacillus* are able to produce polysaccharide lyases (MacDonald et al. 2016; Ochiai et al. 2007). The colony size of *Microcystis* in Lake Taihu decreases at the decline of the bloom (Li et al. 2013). It might be speculated that certain *Microcystis*-attached bacteria may decrease the colony size of *Microcystis* through degrading EPS. However, it needed further investigation.

Similar to the report on unicellular *M. aeruginosa* (Shi, Cai, Li, et al. 2009), some colony-attached bacteria were found to stimulate or inhibit the growth of colonial *M. aeruginosa* in the present study. Bacterial strain QW14 related to *Aeromonas* stimulated the growth of colonial *M. aeruginosa*. A strain of *Aeromonas* associated with cyanobacterial bloom is reported to enhance the growth of unicellular *Microcystis* (Berg et al. 2009). Nutrient regeneration and production of growth-promoting factors (e.g. vitamins) by heterotrophic bacteria may be possible mechanisms through which bacteria stimulate *Microcystis* growth (Liu et al. 2008; Zhao et al. 2012). Consistent with the report on unicellular *M. aeruginosa* (Shi, Cai, Li, et al. 2009), no bacterial isolate with algicidal activity against colonial *M. aeruginosa* was found in this study, suggesting that no algicidal bacteria were attached to healthy colonies of *M. aeruginosa*. Two isolates (QW04 belonged to *Sphingomonas* and QW17 assigned to *Pseudomonas*) inhibited the growth of colonial *M. aeruginosa*. Two strains of *Sphingomonas* associated with cyanobacterial bloom and *Pseudomonas* A8 isolated from *M. aeruginosa* colonies have been found to inhibit the growth of unicellular *Microcystis* (Berg et al. 2009; Shi, Cai, Li, et al. 2009). It is assumed that the production of bioactive compounds possessing growth-inhibiting activities might result in the inhibition of the cyanobacterial growth (Salomon et al. 2003). It was possible that the bacterial isolate QW04 may inhibit the growth of *M. aeruginosa* and increase the
cyanobacterial colony size through producing bioactive compounds possessing growth-inhibiting and EPS excretion-stimulating activities.

5. Conclusions
Eleven bacterial strains were isolated from *M. aeruginosa* colonies harvested from Lake Taihu in this study. Among these bacteria, four bacterial isolates significantly influenced the growth rate of colonial *M. aeruginosa*, and seven bacterial isolates significantly influenced the colony size of *M. aeruginosa*. These data suggested that the bacteria attached to *Microcystis* colonies might influence the cyanobacterial colony size and growth, and in turn influence the formation and development of *Microcystis* blooms. In addition, three isolates (QW02, QW04 and QW17) significantly decreased the cyanobacterial growth rate, indicating that they might have the potential to control *Microcystis* growth in fresh water. To our knowledge, it is the first time to demonstrate that the bacteria attached to the colonies of *Microcystis* were able to influence the growth of colonial *Microcystis* and its colony size.

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Disclosure statement
No potential conflict of interest was reported by the authors.

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