Experimental evidence for the role of heterotrophic bacteria in the formation of *Microcystis* colonies

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Abstract *Microcystis*, a genus of cyanobacteria that is dominant in eutrophic lakes, occurs mainly as colonial morphs under natural conditions but as single cells in laboratory cultures. Recent studies have suggested that *Microcystis*–bacteria interactions significantly influence *Microcystis* morphology, but the underlying mechanism remains unclear. In this study, a total of 48 strains of heterotrophic bacteria were purified from *Microcystis* mucilage. Five bacteria, *Aeromonas veronii*, *Enterobacter aerogenes*, *Exiguobacterium acetylicum*, *Bacillus cereus* and *Shewanella putrefaciens*, can induce unicellular *Microcystis* to form colonies. Heterotrophic bacteria stimulated *Microcystis* growth and induced the production of extracellular polymeric substances in coculture treatments. Extracellular polymeric substances, such as extracellular polysaccharides (EPS), were responsible for the mucilage formation in colonial *Microcystis*. We analysed extracellular metabolic compounds produced by *Microcystis aeruginosa* and *Microcystis wesenbergii* using gas chromatography mass spectrometry. Filtrate extracts from coculture treatments indicated that some compounds, such as 2-dodecen-1-yl(-)

succinic anhydride and benzoic acid, 2,3-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester, might play a significant role in colonial *M. aeruginosa* or *M. wesenbergii* formation. Our data suggested that the interaction of *Microcystis* and heterotrophic bacteria was crucial for the formation of *Microcystis* colony and outbreak of *Microcystis* blooms.

Introduction

Large mucilaginous cyanobacterial blooms occur worldwide in freshwater lakes and oceans (Chen et al. 2009; Kurmayer et al. 2003). Species of *Microcystis*, such as *Microcystis aeruginosa*, *Microcystis wesenbergii*, *Microcystis flos-aquae* and *Microcystis viridis*, are dominant bloom-forming strains in China. Human activity and economic development are considered as main factors to promote the eutrophication and *Microcystis* blooms (Jancula and Marsalek 2011), but the mechanism of *Microcystis* as dominant species in eutrophic lakes is still unknown.

Microcystis includes colonial morph types frequently inhabiting eutrophic lakes. An important strategy for sustaining the competitive advantage of *Microcystis* is to maintain colonial morphology in eutrophic aquatic ecosystems (Reynolds et al. 1981). The colonial *Microcystis* has thick mucilage around *Microcystis* cells compared with their unicellular forms (Plude et al. 1991). The mucilage provides protective barriers for *Microcystis* and their attached bacteria from stress and adverse conditions (Pereira et al. 2009). Rippka (1988) and Wu and Song (2008) also show that colonial *Microcystis* have a competitive advantage compared with other

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phytoplankton in their life cycle, related to nutrients, grazer resistance, vertical migration and oxygen damage. However, colonial *Microcystis* cultures lose their mucilage and convert into individual cells gradually after some generations in the laboratory (Plude et al. 1991). In addition, the morphology of colonial *Microcystis* can be influenced by both abiotic and biotic factors, such as zooplankton predation (Burkert et al. 2001), microcystins stress (Sedmak and Eleršek 2006), nitrogen (N), phosphorus (P) and pH (Ma et al. 2014), and water residence time (Romo et al. 2013). Importantly, there are many bacteria living in the mucilage of colonial *Microcystis* (Doucette 1995; Brunberg 1999), but some bacteria disappear after colonies disaggregate into unicellular forms under axenic conditions in the laboratory (Shirai et al. 1989). Shen et al. (2011) also showed that heterotrophic bacteria were an important factor related to *Microcystis* colony formation, but the mechanism of how bacteria impact on *Microcystis* colony formation remains unclear.

The mucilage is the fundamental substance responsible for colonial *Microcystis* formation and development (Kessel and Eloff 1975; Plude et al. 1991). It consists primarily of extracellular polymeric substances (extracellular polysaccharides [EPS], proteins, nucleic acids and so on) secreted by *Microcystis* or associated bacteria (Xu et al. 2013). The mucilage is a mutual microenvironment for both algae and bacteria, and some interactions such as nutrient exchange inside the mucilage could stimulate the growth of both *Microcystis* and bacteria (Brunberg 1999; Imai et al. 1999). On the other hand, there are also some negative interactions between *Microcystis* and its associated bacteria. Some *Microcystis* species can produce hepatotoxins, which have a negative impact on the growth of *Escherichia coli* and *Streptoverticillium* sp. (Valdor and Aboal 2007). Conversely, bacteria, such as *Brevibacillus* sp. and *Alcaligenes denitrificans*, can produce metabolites to lyse of *Microcystis* cells directly or indirectly (Manage et al. 2001). These studies indicate that extracellular polymeric substances secreted by *Microcystis* and bacteria influence the interactions of *Microcystis* and their associated bacteria and may be responsible for *Microcystis* morphology change.

Despite the large number of field and laboratory studies that have been carried out, the interactions between colonial *Microcystis* formation and their attached bacteria are still not fully understood. To clarify the mechanism of colonial *Microcystis* formation and better understand the relationship between the alga and its associated bacteria, it is necessary to rebuild the *Microcystis* colonies in the laboratory. We hypothesise that heterotrophic bacteria present in the mucilage of *Microcystis* and their associated extracellular polymeric substances are crucial for the formation and maintenance of colonial *Microcystis*. In the present study, heterotrophic bacteria were isolated and purified from *Microcystis* mucilage, and the effects of monoclonal bacteria strains on morphology change of unicellular *Microcystis* were investigated. During the coculture of heterotrophic bacteria and *Microcystis*, the growth of *Microcystis* and bacteria, and the contents of extracellular compounds, including extracellular polysaccharides, proteins and nucleic acids, were also determined. Our data will help understand the role of heterotrophic bacteria in the dynamics of *Microcystis* blooms and the relationship between alga and its associated bacteria.

Methods

Isolation and cultivation of bacteria and algae

Microcystis samples were collected from hypertrophic areas of Lake Taihu (Jiangsu, China), Lake Dianchi (Yunnan, China) and Lake Donghu (Hubei, China) in July 2011. The *Microcystis* biomass was greater than 90 % of total algal biomass in the study lakes. The free-living bacteria were removed from *Microcystis* colonies according to Shi et al. (2010). Then, *Microcystis* colonies were washed three times with sterile deionised water to dissolve *Microcystis* mucilage and release attached bacteria (Shen et al. 2011). Aliquots of water containing the released attached bacteria were spread onto sterile nutrient agar plates containing 1 g L^{-1} peptone, 0.5 g L^{-1} NaCl, 0.5 g L^{-1} beef extract and 20 g L^{-1} agar. A total of 48 strains of the attached bacteria were isolated from the mucilage of *Microcystis* and frozen at −80 °C in 50 % glycerol for subsequent experiments. Seven unicellular *Microcystis* strains were used in the present study. *M. aeruginosa* PCC7806 and *M. aeruginosa* FACHB905 were obtained from Freshwater Algae Culture Collection at the Institute of Hydrobiology (Wuhan, China). *M. wesenbergii* CH, *M. wesenbergii* TH, *M. viridis*, *M. flos-aquae* and *Microcystis* sp. were isolated from Lake Chaohu in 2010. To reduce contaminants and obtain axenic strains of algae, unicellular algal cells were alternately cultured in solid and liquid culture media (Parker 1982). Prior to the experiment, 4′,6′-diamidino-2-phenolindole (DAPI) staining and fluorescence microscopy were used to detect whether or not *Microcystis* strains were contaminated with bacteria (Porter 1980). When contaminant bacteria presented in the algal culture, the alga was discarded.

Bacteria strains screening experiment

To select bacteria which can induce the aggregation of unicellular algae into colonies, a total of 48 strains of bacteria and seven strains of algae were cocultured respectively. Seven strains of unicellular algae at the exponential stage were collected and transferred into 1-L Erlenmeyer flasks containing 500-mL autoclaved BG11 standard medium (adjust pH to 7.1 with 1 M NaOH or HCl). The formula of BG11 medium was obtained from Freshwater Algae Culture Collection at the Institute of Hydrobiology (Wuhan, China). All treatments were

incubated in batch cultures of BG11 medium at 25 ± 1 °C and a 12:12-h light/dark cycle at an irradiance of 50 μmol photons $m^{-2} s^{-1}$. Erlenmeyer flasks were shaken three times every day. Following incubation, morphological changes in *Microcystis* cells were observed daily by microscopic examination. The phenomenon that obvious *Microcystis* mucilage appeared to link *Microcystis* cells into macroscopic groups in culture was used for the standard of colonial *Microcystis* formation (Plude et al. 1991). The colony size of colonial *Microcystis* formed in coculture was calculated according to Ishikawa et al. (2004) and Shen et al. (2011).

Extraction of bacterial genomic DNA

The whole genomic DNA of 48 strains of bacteria was extracted using a bacterial DNA Kit (Omega, Bio-Tek, USA) according to the manufacturer's recommendations. The 16S rDNA was amplified by PCR using the universal primer 27F, AGAGTTTGATCCTGGCTCAG, and 1492R, TACGGY TACCTTGTTACGACTT. The PCR products were submitted for sequencing using an automated ABI DNA sequencer at the Genomics Company (Beijing, China). Bacterial sequences were compared to known sequences in RDP database (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) to match the most similar sequences.

The colonial *Microcystis* cells which appeared in cocultures were termed *Microcystis* colony-inducing bacteria. These five bacteria were *Aeromonas veronii* (*Bac-AV*), *Enterobacter aerogenes* (*Bac-EN*), *Exiguobacterium acetylicum* (*Bac-EA*), *Bacillus cereus* (*Bac-BC*) and *Shewanella putrefaciens* (*Bac-SP*). The 16S rDNA sequences of these five bacteria have been submitted to NCBI with the following accession number KC166864-KC166868. Thus, in the present study, *M. aeruginosa* FACHB905, *M. wesenbergii* CH, *Bac-EA* and *Bac-SP* were selected for further investigation.

Microcystis and bacterial abundance

The screening experiment showed that both *Microcystis* and bacteria grew rapidly when the initial concentration of *Microcystis* and bacteria was 1×10^7 and 1×10^6 cells mL^{-1}, respectively. Therefore, the above concentrations of algae and bacteria were selected as initial concentrations on day 0. Unicellular *Microcystis* cells were harvested at the exponential phase and added to 500-mL Erlenmeyer flasks containing 400 mL of autoclaved BG11 medium. All treatments were incubated at 25 ± 1 °C and illuminated in a 12:12-h light/dark cycle at an irradiance of 50 μmol photons $m^{-2} s^{-1}$. Each treatment was replicated three times. Samples were collected daily, and the experiment lasted for 7 days.

Before counting the unicellular and colonial *Microcystis* in a haemocytometer using an Olympus BX50 light microscope (Tokyo, Japan), *Microcystis* cells were stained with Lugol's

iodine solution (Joung et al. 2006). For accurate counting, *Microcystis* cells were released from the colonial *Microcystis* using ultrasound. The size of colony area *Microcystis* was measured following the method of Ishikawa et al. (2004). Samples were preserved in 4 % (*v/v*) formaldehyde for determination of bacterial abundance. The fixed samples were then stained with DAPI, and cell amounts of bacteria were counted under an epifluorescence microscope (Zeiss Axioskop 20, Germany) (Porter 1980). A minimum of 10 replicates under microscope were counted for each sample.

Protein and nucleic acid determination

The samples were heated at 65 °C for 5 min to release proteins and nucleic acids from *Microcystis* or bacteria cells. Protein contents were measured according to Bradford (1976) using bovine serum albumin as a standard. Nucleic acid concentrations were measured by the diphenylamine colorimetric method using calf thymus DNA as a standard (Burton 1956).

Extractions and determination of EPS

EPS were classified into soluble EPS (SL-EPS), loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS). The samples were centrifuged at 2500×*g* for 15 min, and the supernatant was used to measure SL-EPS. The harvested precipitates were suspended in 0.05 % NaCl solution and centrifuged at 5000×*g* for 15 min. The supernatant was carefully collected for measurement of LB-EPS. The remaining precipitates were resuspended with 0.05 % NaCl solution and treated for TB-EPS extraction using the method of Xu et al. (2013). All solutions were filtered using 0.45-μm membrane filters (Millipore, USA). As a blank, BG11 medium was used for SL-EPS, and 0.05 % NaCl solutions were used for LB-EPS and TB-EPS. Polysaccharide contents were determined by the phenol–sulphuric acid method using glucose as a standard (Dubois et al. 1956).

Dichloromethane extraction

Cultures from control (*Microcystis* or bacteria) and coculture (*Microcystis* and bacteria) treatments were collected 4 days after inoculation. The filtrates were separated from *Microcystis* and bacteria cells using the 0.22-μm membrane filters (Millipore, USA). The extracellular compound extraction used the method described by Zhang et al. (2013). Dichloromethane (dichloromethane/culture filtrates = 2:1) was used to extract metabolites from the above filtrates. The dichloromethane extracts were analysed on a Gas Chromatograph Mass Spectrometer (GC/MS 7890A+5975C, Agilent). Chemical types of compounds were identified with NIST08.1 and Wiley 275 libraries. Authentic samples were also

compared with the mass spectra and retention times of chromatographic peaks.

Statistical analysis

Results are expressed as mean ± standard deviation. Statistical analysis was carried out using one-way ANOVA, followed by Tukey's post hoc test to determine any significant differences among different treatments. Standard statistical analysis and graphics were carried out using Microcal™ Origin 8.0 (Microcal Software Inc., USA).

Results

Bacterial identification by phylogenetic analysis

A total of 48 bacterial strains were isolated from the mucilage of *Microcystis* colonies. The affiliations of isolated bacteria originating from *Microcystis* mucilage were mainly dominated by Firmicutes (17/48) and Gammaproteobacteria (13/48), followed by Epsilonproteobacteria (7/48) (Fig. S1). When unicellular strains *Microcystis* spp. were exposed to the above 48 strains, five bacterial strains *Bac-AV*, *Bac-EN*, *Bac-EA*, *Bac-BC* and *Bac-SP* were observed to induce a change from unicellular *Microcystis* into colonies (Table 1). According to the molecular identification, *Bac-AV* was classified under Gammaproterobacteria, and *Bac-BC* was classified under Firmicutes, which were obtained from Lake Taihu. *Bac-EN* and *Bac-SP* were classified under Gammaproteobacteria, which were obtained from Lake Dianchi. *Bac-EA* was classified under Firmicutes and obtained from Lake Donghu.

Table 1 shows a possible species-specific relationship between *Microcystis* and bacteria. However, only four heterotrophic bacteria strains *Bac-AV*, *Bac-EN*, *Bac-EA* and *Bac-BC* could induce colony formation of unicellular *M. aeruginosa* FACHB905 and *M. aeruginosa* PCC7806. These bacteria

could not induce colony formation of other algae species. In addition, only *Bac-SP* induced colony formation of unicellular *M. wesenbergii* CH and *M. wesenbergii* TH. At the end of the experiment, most of unicellular *Microcystis* aggregated to colonies in the coculture treatments of *Microcystis* and bacteria (Fig. S2). No morphology changes of other algae were observed.

Interaction between bacteria and *Microcystis*

As shown in Fig. 1a, d, *Microcystis* cells in controls still maintained the unicellular form during the whole experiment. Upon exposure to *Bac-EA* and *Bac-SP*, unicellular *Microcystis* showed a tendency to aggregate. Colonial *M. aeruginosa* and *M. wesenbergii*, formed in the present experiment, did not show significant differences in morphology, and the morphology of colonial *Microcystis* was different from that of colonies under field conditions (Fig. 1b, e). Both of them were lobate and reticulated, with distinct colony margins and prominent slime composed of colloid substances around the colonies. The cell surfaces of *Microcystis* colonies turned blue after staining with Alcian Blue (Fig. 1c, f), indicating the presence of acidic polysaccharides in the colonies.

M. aeruginosa FACHB 905 started to aggregate 2 or 3 days after inoculation with the bacterium *Bac-EA*. The colony sizes reached a maximum aggregation size of 800 μm^2 after 5 days (Fig. 2). Other three bacteria (*Bac-AV*, *Bac-EN* and *Bac-BC*) also stimulated aggregation in *M. aeruginosa*, and the formed colonies exhibited similar morphological changes. Unicellular *M. wesenbergii* CH started to form colonies in the presence of *Bac-SP* and reached the maximum colony area 4 or 5 days after inoculation.

Growth of *Microcystis* and bacteria

The growth characteristics of *Microcystis* showed pronounced differences between control and coculture treatments during

Table 1 Impacts of bacteria on the colony formation in different *Microcystis* species on day 4

Strains	Dianchi Lake		Donghu Lake	Taihu Lake	
	Bac-SP	*Bac-EA*	*Bac-EN*	*Bac-AV*	*Bac-BC*
M. aeruginosa FACHB905	ND	+	+	+	+
M. aeruginosa PCC7806	ND	+	+	+	+
M. wesenbergii CH	+	ND	ND	ND	ND
M. wesenbergii TH	+	ND	ND	ND	ND
M. flos-aquae	ND	ND	ND	ND	ND
M. viridis	ND	ND	ND	ND	ND
Microcystis sp.	ND	ND	ND	ND	ND

Bac-SP Shewanella putrefaciens, *Bac-EA Exiguobacterium acetylicum*, *Bac-EN Enterobacter aerogenes*, *Bac-AV Aeromonas veronii*, *Bac-BC Bacillus cereus*, + colony formation, *ND* no colony formation

Unicellular *Microcystis* $\xrightarrow{\text{Bacteria}}$ *Microcystis* colonies $\xrightarrow{\text{Alcian Blue}}$ *Microcystis* colonies
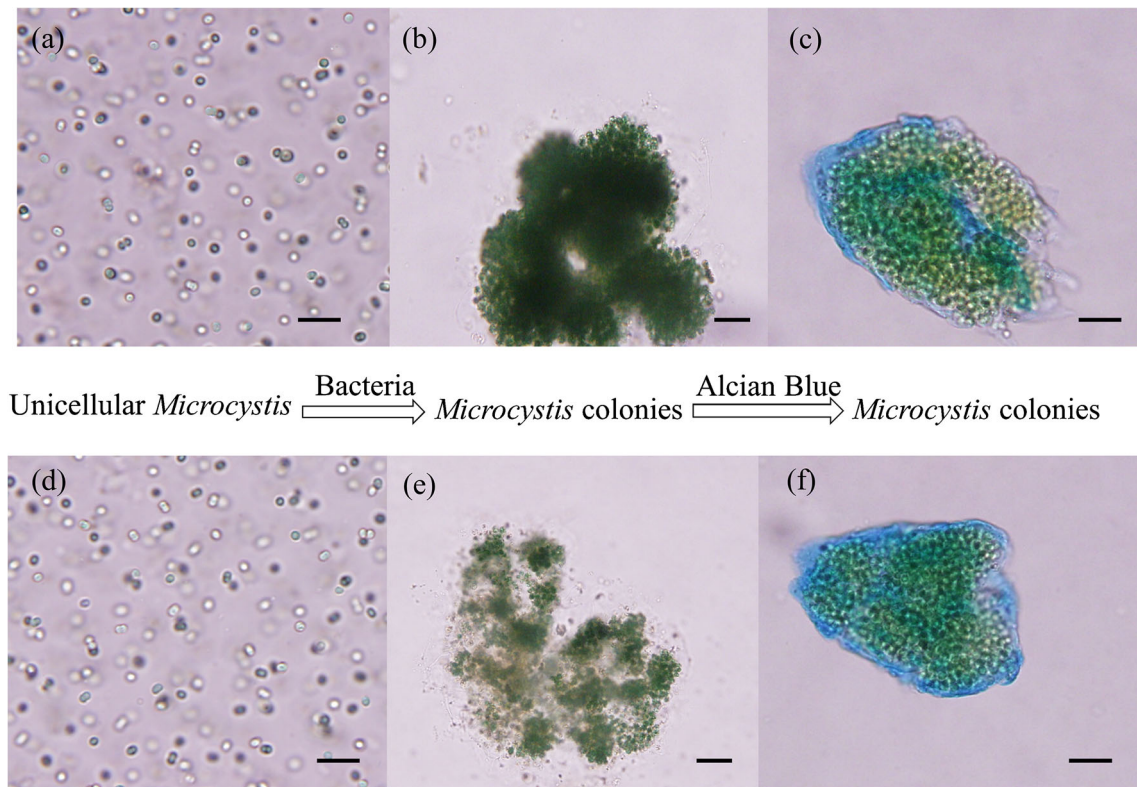
Fig. 1 Micrographs of *M. aeruginosa* and *M. wesenbergii* on day 4. **a** Control *M. aeruginosa*, **b** unicellular *M. aeruginosa* inoculated with *Bac-EA*, **c** colonial *M. aeruginosa* formed stained with Alcian Blue, **d** control *M. wesenbergii*, **e** unicellular *M. wesenbergii* inoculated with *Bac-SP*, and **f** colonial *M. wesenbergii* formed stained with Alcian Blue. *Scale bar* = 10 μm
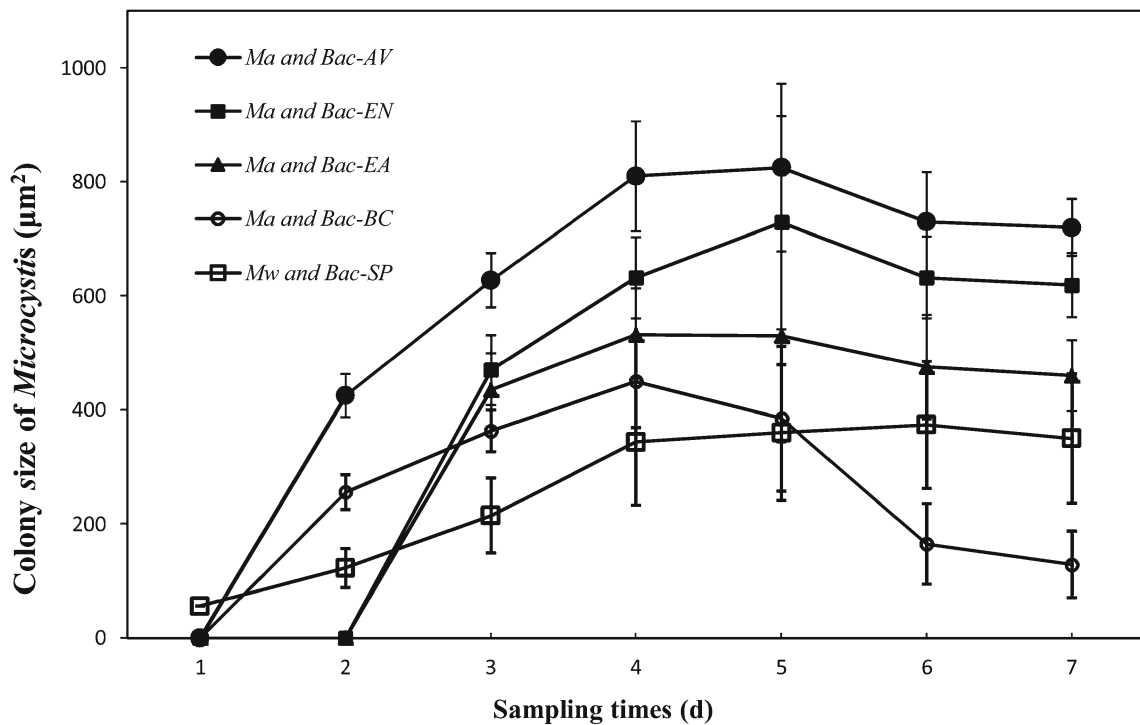

Fig. 2 Size of colonial *Microcystis* after incubating with associated bacteria. *Ma* and *Mw* represent *M. aeruginosa* FACHB905 and *M. wesenbergii* CH, respectively. *Bac-SP, Bac-EA, Bac-EN, Bac-AV* and *Bac-BC* represent *Shewanella putrefaciens, Exiguobacterium acetylicum, Enterobacter aerogenes, Aeromonas veronii* and *Bacillus cereus*, respectively. Mean ± std dev ($n = 3$)

the course of experiment. As shown in Fig. 3, the solid line represents the growth of *Microcystis* and bacteria in the coculture treatments, and the hollow line represents the growth of *Microcystis* and bacteria in controls. In Fig. 3a, the growth of *M. aeruginosa* in coculture increased remarkably under *Bac-EA* at the beginning of the experiments ($P < 0.05$), but cell numbers declined after day 4. The growth of *Bac-EA* in cocultures increased much more quickly than control *Bac-EA*. The growth of *M. wesenbergii* in coculture was significantly increased by *Bac-SP* ($P < 0.05$) compared to the control *M. wesenbergii* at 3 days and remained unchanged thereafter (Fig. 3b). The cell numbers of *Bac-SP* in coculture treatments also grew faster than in the control *Bac-SP*.

EPS, proteins and nucleic acids determination

Concentrations of proteins, total polysaccharides and nucleic acids are greatly increased in the coculture treatments of
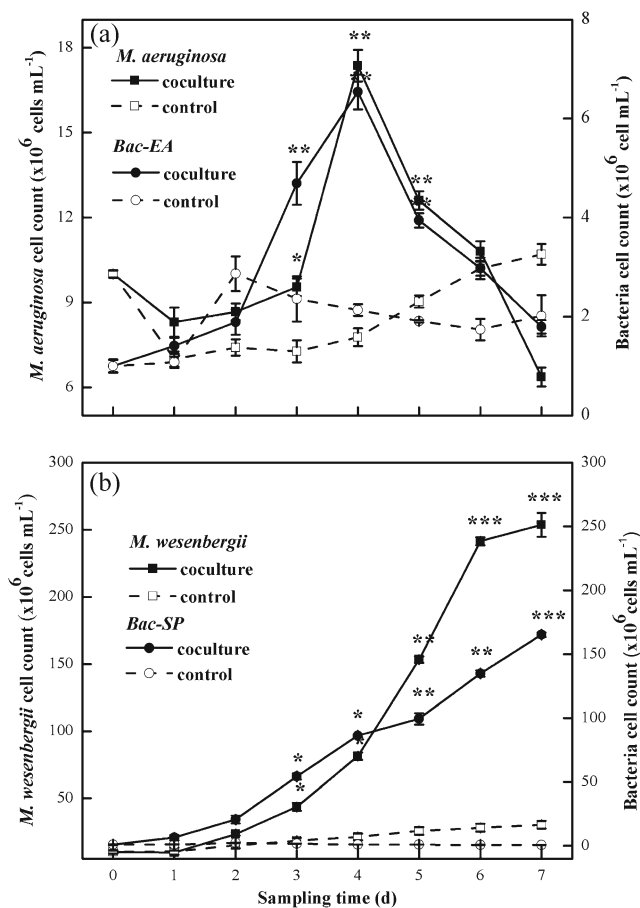

Fig. 3 Cell density of *Microcystis* and bacteria in control and coculture treatments. **a** Cell density of *M. aeruginosa* FACHB905 and *Exiguobacterium acetylicum* (*Bac-EA*). **b** Cell density of *M. wesenbergii* CH and *Shewanella putrefaciens* (*Bac-SP*). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Mean ± std dev ($n = 3$)

Microcystis and bacteria on day 4 ($P < 0.05$) and rose with prolonged incubation time (Table 2). Besides increased EPS concentration in the coculture treatments, the EPS compositions also varied after *M. aeruginosa* cells became aggregated. SL-EPS in control *M. aeruginosa* was the major component (>50 %) of total EPS throughout the experiment. However, when *Microcystis* was cocultured with heterotrophic bacteria, the percentage of TB-EPS in all coculture treatments quickly increased and became the major component (>60 %) (Fig. 4). There were no obvious differences between two coculture treatments (*M. aeruginosa* and *Bac-EA*, *M. wesenbergii* CH and *Bac-SP*).

Dichloromethane extraction

To explore the effects of potential compounds secreted by *Microcystis* and bacteria on colonial formation, the filtrates of the control and coculture treatments were extracted with CH_2Cl_2. For *M. aeruginosa* and *Bac-EA*, a total of 37 compounds were identified from the CH_2Cl_2 extract and grouped into 11 categories: alkane (12 compounds), ester (11), acetate (3), amine (3), amide (2), phenol (1), anhydride (1), anthracene (1), acid (1), alcohol (1) and alkali (1) (Table 3). A total of 28 compounds were identified in the coculture extracts, 15 compounds in the control *M. aeruginosa* extracts and 12 compounds in the control *Bac-EA* extracts. Only six compounds were observed in all the three filtrate extracts, seven compounds were observed in two extracts of control *M. aeruginosa* and their cocultures and nine compounds were observed in two extracts of control *Bac-EA* and their cocultures. Importantly, 18 compounds were identified in coculture extracts but not in two control extracts. In the two control extracts, we identified nine compounds which were not found in coculture treatments. Extracellular substances of a colonial *M. aeruginosa* FACHB939 obtained from Freshwater Algae Culture Collection at the Institute of Hydrobiology (Wuhan, China) were also extracted for extracellular substance detection. Six compounds were identified in control *M. aeruginosa*, *Bac-SP*, cocultures and colonial *M. aeruginosa* FACHB939. Only one compound was identified in cocultures and colonial *M. aeruginosa* FACHB939 but not others.

For *M. wesenbergii*, a total of 97 compounds from the control, cocultures and a colonial *M. wesenbergii* isolated from lakes filtrates were identified using GC/MS, which could be classified into eight categories, including alkane (10 compounds), ester (8), amine (2), phenol (2), nitrile (1), ketone (1), acetate (1) and other unknown compounds (72), not described in the subsequent article (Table 4). A total of 24 compounds were identified in the dichloromethane extraction of cocultures, 23 compounds were identified in the control *M. wesenbergii* CH and 25 compounds were identified in the control *Bac-EA*. Twelve compounds were identified in cocultures but not control *M. wesenbergii* and *Bac-SP*. Six

Table 2 Concentrations of proteins, total extracellular polysaccharides (TEPS) and nucleic acids in different treatments on day 4

Treatments	Concentrations (mg L^{-1})		
	Proteins	TEPS	Nucleic acids
Control *M. aeruginosa*	0.227 ± 0.0019	2.374 ± 0.0028	0.256 ± 0.0011
Coculture of *M. aeruginosa* and *Bac-EA*	0.621 ± 0.0022 a	8.503 ± 0.0028 a	0.726 ± 0.0037 a
Control *Bac-EA*	0.172 ± 0.0023	1.683 ± 0.0025	0.098 ± 0.0003
Control *M. wesenbergii CH*	0.162 ± 0.0009	2.452 ± 0.0072	0.258 ± 0.0008
Coculture of *M. wesenbergii* and *Bac-SP*	0.583 ± 0.0021 b	7.862 ± 0.0064 b	0.852 ± 0.0042 b
Control *Bac-SP*	0.123 ± 0.0010	1.732 ± 0.0029	1.323 ± 0.0014

Points represent the mean of three replicates and the error bars represent standard deviation. Numbers with lowercase letters are significantly different at $P < 0.05$ level among the different treatments

Bac-EA Exiguobacterium acetylicum, Bac-SP Shewanella putrefaciens

compounds were identified in cocultures and control *M. wesenbergii* but not in control *Bac-SP*. Six compounds were identified in cocultures and control *Bac-SP*. A colonial *M. wesenbergii* isolated from Lake Chaohu in 2014 was also extracted extracellular substance. There was no compound identified in all extracts (control *M. wesenbergii*, control *Bac-SP*, cocultures and colonial *M. wesenbergii*), but two compounds were identified in cocultures and colonial *M. wesenbergii* but not others.

Discussion

The interactions between bacteria and *Microcystis* can be better explained by coculture of *Microcystis* and monoclonal bacteria strains, compared with our previous experiment on *Microcystis* and complex bacteria community (Shen et al. 2011). In this study, five bacteria strains were found to stimulate colonial *M. aeruginosa* and *M. wesenbergii* formation. Four bacteria, *Aeromonas*

veronii, *Enterobacter aerogenes*, *Exiguobacterium acetylicum* and *Bacillus cereus* can induce unicellular *M. aeruginosa* into colonies, and *Shewanella putrefaciens* can induce unicellular *M. wesenbergii* into colonies. All of the five strains have been found in eutrophy lakes and were associated with cyanobacterial blooms. Liu et al. (2013) demonstrated that *Aeromonas* sp. can inhibit the growth of *Microcystis*. *Enterobacter* sp. related with *Microcystis* cells is a potential pathogenic member for humans and usually appears in the cyanobacterial blooms (Dziallas and Grossart 2011). *Exiguobacterium* sp. inhibits the growth of *Microcystis* in the cocultures (Tian et al. 2012). *Bacillus cereus* is a common cyanobacteriolytic bacterium (Nakamura et al. 2003). *Shewanella putrefaciens* strains can secret enzymes related with intermediary carbon metabolism and influence cyanobacteria growth (Scott and Nealson 1994). Thus, taken together with these studies, we speculated that species-specific relationships might exist in colonial *Microcystis* formation and their bacteria. Shi et al. (2012) also found a specific relationship between

Fig. 4 Extracellular polysaccharides (EPS) compositions in control and coculture treatments. Con and Co- represent control *M. aeruginosa* FACHB905, control *M. wesenbergii* CH, coculture of *M. aeruginosa* FACHB905 and *Exiguobacterium acetylicum*, and coculture of *M. wesenbergii* CH and *Shewanella putrefaciens*, respectively. TB-EPS, LB-EPS and SL-EPS represent tightly bound EPS, loosely bound EPS and soluble EPS, respectively. Mean ± std dev (*n* = 3)
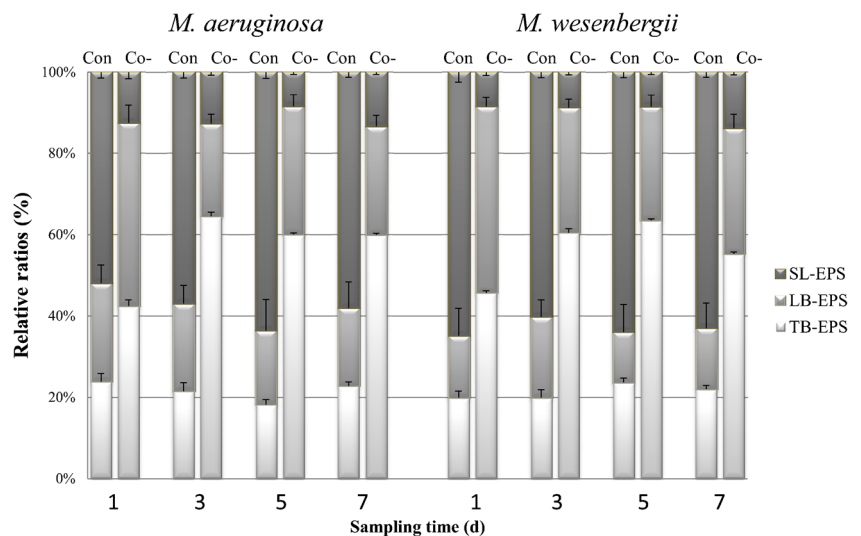
Table 3 Qualitative analysis of extracellular compounds in *M. aeruginosa* using GC/MS on day 4

Chemical types	No.	Compounds	Ma	EA	Ma + EA	CMa
Acetates	1	11,13-Dimethyl-12-tetradecen-1-ol acetate	ND	ND	+	ND
	2	Methyl 2-octylcyclopropene-1-octanoate	ND	ND	+	ND
	3	Z,E-7,11-Hexadecadien-1-yl acetate	ND	ND	+	ND
Acid	4	8,12-Tetradecadienoic acid	ND	ND	+	ND
Alcohol	5	Behenic alcohol	ND	ND	+	ND
Alkali	6	*d*-Glaucine	+	ND	+	ND
Alkanes	7	Octadecane	ND	+	+	ND
	8	1,15-Dihydrohexadecamethyloctasiloxane	+	ND	ND	ND
	9	1-chloro-Nonadecane	ND	ND	+	ND
	10	4x,10x-Cadinane	ND	ND	+	ND
	11	Decamethylcyclopentasiloxane	+	+	+	+
	12	Docosane	ND	+	+	ND
	13	Dodecamethylcyclohexasiloxane	+	+	+	+
	14	Eicosamethylcyclodecasiloxane	+	+	ND	+
	15	Heneicosane	+	ND	ND	ND
	16	Hexadecamethylcyclooctasiloxane	+	+	+	+
	17	Tetradecamethylcycloheptasiloxane	+	+	+	+
	18	Octadecamethylcyclononasiloxane	+	+	+	+
Amides	19	Benzo(*b*)thiophene-2-carboxamide	ND	ND	+	ND
	20	*N*-Benzyl-*N*-ethyl-*p*-isopropylbenzamide	ND	ND	+	+
Amines	21	2,7-Dimethyl-2,7-octadien-1-amine	ND	ND	+	ND
	22	Bis(2,7-dimethyl-2,7-octadien-1-yl)amine	ND	ND	+	ND
	23	Isomenthylamine	ND	ND	+	ND
Anhydride	24	2-Dodecen-1-yl(-)succinic anhydride	ND	ND	+	ND
Anthracene	25	9-(Methylaminomethyl) anthracene	ND	ND	+	ND
Esters	26	1,5-Dimethyl-1-vinyl-4-hexenyl ester	+	ND	ND	ND
	27	2,4-Bis[(trimethylsilyl)oxy]benzoic acid trimethylsilyl ester	+	+	ND	+
	28	2-Ethylhexyl-hexyl ester	ND	ND	+	ND
	29	2-Hydroxy- methyl ester	ND	ND	+	ND
	30	Butanoicacid, 3-methyl-, 3-phenyl-2-propenyl ester (9CI)	ND	ND	+	ND
	31	9-Undecenoic acid, ethyl ester	ND	+	+	ND
	32	Phthalic acid diiso butyl ester	ND	+	ND	ND
	33	Di(2-ethylhexyl) ester	ND	ND	+	ND
	34	Propionic acid, trimethylsilyl ester	+	ND	ND	ND
	35	Trimethylsilyl ester	+	ND	ND	ND
	36	2,4-Di-tert-butylphenol	+	ND	ND	ND
Phenols	37	2,2′-Methylenebis(6-tert-butyl-4-methylphenol)	+	+	+	+

Ma M. aeruginosa, *EA Exiguobacterium acetylicum*, *CMa* colonial *Microcystis* FACHB939, + compounds detected, *ND* compounds not detected

colonial *Microcystis* and their associated bacteria living in the mucilage. In addition, Parveen et al. (2013) showed a clear distinction between *Microcystis*-attached and free-living bacterial communities. These results provided evidence for specfic bacteria–*Microcystis* relationships. Specific coorpetation between bacteria and phytoplankton may enhance their survival in eutrophic ecosystems (Bratbak and Thingstad 1985). Wu and Song (2008) also found that the colonial *Microcystis* phenotype may possess

a higher ability to tolerate enhanced stress conditions compared to unicellular (noncolonial) phenotypes. Interestingly, one given bacterium could not induce all the unicellular *Microcystis* species to form colonies. For example, in the present study, *Enterobacter aerogenes* (Bac-EA) induced colonial formation of *M. aeruginosa* FACHB 905 and *M. aeruginosa* PCC7806 but could not induce *M. wesenbergii* CH, *M. wesenbergii* TH or other *Microcystis* species, to form colonies. The findings in this

Table 4 Qualitative analysis of extracellular compounds in *M. wesenbergii* using GC/MS on day 4

Chemical types	No.	Compounds	Mw	SP	Mw + SP	CMw
Acetate	1	1-Butanol, 3-methyl-, acetate	ND	ND	+	ND
Alkanes	2	1-(2-Methylallyl) azetidine	ND	ND	+	ND
	3	2,6,10,14-Tetramethylheptadecane	+	ND	+	ND
	4	2-Bromo dodecane	+	ND	+	ND
	5	3,5-Dimethyldodecane	+	ND	+	ND
	6	Docosane	+	ND	+	ND
	7	Hentriacontane	+	ND	+	ND
	8	Hexadecamethylheptasiloxane	ND	+	+	+
	9	Nonadecane	+	ND	+	+
	10	Octamethylcyclotetrasiloxane	ND	+	+	+
	11	Tetradecamethylhexasiloxane	ND	+	+	+
Amines	12	[2-Pyrrolidinyl] methylamine	ND	ND	+	ND
	13	Neomenthylamine	ND	+	+	ND
Esters	14	2-Bromopropionic acid, pentyl ester	ND	ND	+	ND
	15	Benzoic acid,2,3-bis [(trimethylsilyl)oxy]-, trimethylsilyl ester	ND	ND	+	+
	16	(-)-Epinephrine, tris(trimethylsilyl) ether	ND	ND	+	+
	17	Fumaric acid, butyl 3,4-dimethoxyphenyl ester	ND	ND	+	ND
	18	l-Proline, *N*-allyloxycarbonyl-, hexyl ester	ND	ND	+	ND
	19	l-Valine, *n*-propargyloxycarbonyl-, pentadecyl ester	ND	ND	+	ND
	20	L-Proline, *N*-valeryl-, octyl ester	ND	ND	+	ND
	21	Tetradecanedioic acid, bis (tert-butyldimethylsilyl) ester	ND	+	+	+
Nitrile	22	Undecanenitrile	ND	ND	+	ND
Ketone	23	1,1′-Dianthrimide	ND	ND	+	ND
Phenols	24	2,4-Di-tert-butylphenol	+	+	ND	ND
	25	3,5-Dimethoxyphenol	ND	+	+	+
Other compounds	26–41		+	ND	ND	ND
	42–59		ND	+	ND	ND
	60–97		ND	ND	ND	+

Mw M. wesenbergii, SP Shewanella putrefaciens, CMw colonial *Microcystis* isolated from Dianchi lake in China, *+* compounds detected, *ND* compounds not detected

Growth of *Microcystis* and bacteria

The present study clearly shows that *Microcystis* and their associated bacteria affected the growth significantly of *Microcystis* or bacteria growth in coculture treatments. The growth of *Microcystis* and bacteria displayed different interactions in different coculture treatments (*M. aeruginosa* and *Bac-EA*, *M. wesenbergii* and *Bac-SP*). For the coculture treatment of *M. aeruginosa* and *Bac-EA*, the cell amount of *Bac-EA* increased significantly in coculture treatments at the beginning of experiment compared with the controls (single culture of *M. aeruginosa* or *Bac-EA*). Extracellular organic matter released by photosynthesis from *Microcystis* cells can supply nutrients for bacteria growth (Bell 1984), and phytoplanktons are considered as bacterial 'incubator' to supply habitat and nutrients for bacteria (Worm and Sondergaard 1998). *M. aeruginosa* cells also increased at the beginning of experiment and may be stimulated by some substances secreted by bacteria (Brunberg 1999). However, in the later period, both the growth of *Microcystis* and bacteria were suppressed. This phenomenon could be explained by the combined effects of complex extracellular metabolic substances by *Microcystis* or bacteria (Table 3). These extracellular substances are secreted by bacteria or algae, and some compounds play a protective role in microbe growth (Armstrong et al. 2001), while others may have an algicidal role (Middelboe et al. 1995; Walker and Higginbotham 2000). For the coculture treatments of *M. wesenbergii* and *Bac-SP*, the cell count of *M. wesenbergii* and *Bac-SP* increased

The discussion in the preceding paragraph raises the question of interactions between bacteria and colonial *Microcystis* formation, and an understanding of these will facilitate the current understanding on the ecological success of *Microcystis* in eutrophic lakes.

throughout the experiment, and metabolic substances related with growth stimulation may account for the majority of the substances produced by *Microcystis* or *Bac-SP* in coculture. In any case, colony formation may have some connection with the concentration of *Microcystis* cells (Shen et al. 2011), and the growth of *Microcystis* provided enough cells for the formation colonial *Microcystis*. Furthermore, the bacteria present in colony *Microcystis* mucilage may be responsible for the growth of *Microcystis* cells in eutrophic lakes.

EPS, proteins and nucleic acids determination

Microcystis and bacteria in coculture treatments were enhanced to secrete extracellular substances compared with the controls in this study. Extracellular substances, especially EPS, play important roles in mucilage formation of colonial *M. aeruginosa* (Parveen et al. 2013), and mucilage that served to link *Microcystis* and their associated bacterial cells into colonies was rebuilt by *Microcystis* and/or bacteria in cocultures. In the present study, extracellular substances may guarantee the mucilage formation. These results accord with previous findings that total extracellular substances in the cells and sheaths of colonial *Microcystis* are significantly higher than in disaggregated cells (Zhang et al. 2007). Sundh (1992) and Giroldo et al. (2007) also show that EPS released by phytoplankton is a major component of dissolved organic carbon. The EPS secreted by *Microcystis* and bacteria contributes organic carbon to lakes and may play an important role in the freshwater ecosystems.

EPS was classified into three types, i.e. SL-EPS, LB-EPS and TB-EPS. TB-EPS may be the crucial extracellular substance which supported colonial *M. aeruginosa* mucilage compared with SL-EPS and LB-EPS (Klock et al. 2007; Xu et al. 2013). As shown in Table 2 and Fig. 4, TB-EPS in coculture treatments were higher than those in controls. Therefore, TB-EPS increased by interaction between *Microcystis* and *Bac-EA* and provided the material basis for colonial *Microcystis* mucilage. In addition, the polysaccharides produced by cyanobacteria reportedly form the hydrogen bonds with proteins, lipids and DNA to protect cyanobacterial cells from the harsh external environment. The levels of TB-EPS, proteins and nucleic acids significantly increased in cocultures of *Microcystis* and bacteria, implying that *Microcystis* cells reached the possibly condition for colonial *Microcystis* formation.

Extracellular metabolic substances

To clarify the mechanism of colonial *Microcystis* formation, filtrate extracts of different treatments were analysed using the GC/MS. Our results showed that *Microcystis* and bacteria in cocultures might secret some extracellular metabolites to induce colonial *Microcystis* formation. We identified 18

compounds in coculture treatments but not in their controls. Some of these compounds, such as 11,13-dimethyl-12-tetradecen-1-ol acetate, methyl 2-octylcyclopropene-1-octanoate, docosane, di(2-ethylhexyl) ester, 2,7-dimethyl-2,7-octadien-1-amine, isomenthylamine and 2-ethylhexyl-hexyl ester, are antioxidants (Adams 1978; Ivanovic et al. 2012; Liu et al. 2007; Ma et al. 2011; Malloy et al. 2011; Ngang et al. 2014; Sharman et al. 1994) and may protect *Microcystis* or *Bac-EA* cells from adverse environmental conditions. In particular, *N*-benzyl-*N*-ethy-*P*-isopropylbenzamide, 2-dodecen-1-yl(-) succinic anhydride and 9-(methylaminomethyl) anthracene were identified in coculture extracts but not from the control group (single culture of *M. aeruginosa* FACHB905 or *Bac-EA*). These compounds might play a significant role in colonial *Microcystis* formation due to their polymer function (Lee et al. 2005; Lin and Chen 2006). Wang et al. (2015) also reported that *N*-benzyl-*N*-ethy-*P*-isopropylbenzamide, detected in the extracts of a colonial *M. aeruginosa* FACHB939, was responsible for organic aerosols and mucilage formation. For *M. wesenbergii*, a total of 24 compounds were identified in coculture extractions. A total of 12 compounds, such as 1-(2-methylallyl) azetidine, [2-pyrrolidinyl] methylamine, 2-bromopropionic acid, pentyl ester, l-valine, *n*-propargyloxycarbonyl, pentadecyl ester and undecanenitrile, were only identified in cocultures but not in control *M. wesenbergii* and *Bac-SP*, and these compounds may provide help for *Microcystis* morphology change. We found that eight compounds, including octamethylcyclotetrasiloxane, tetradecamethylhexasiloxane, benzoic acid, 2,3-bis[(trimethylsilyl)oxy], trimethylsilyl ester, tetradecanedioic acid and bis(tert-butyldimethylsilyl) ester, appear in two extracts related with colonial *M. wesenbergii* formed in coculture and a colonial *M. wesenbergii* isolated from the lake. Among the above compounds, tetradecamethylhexasiloxane and octamethylcyclotetrasiloxane, which play a crucial role in polymer formation (Tajima and Yamamoto 1987), were also identified in extracts of control *Bac-SP*. Thus, *Bac-SP* was further confirmed to be responsible for *M. wesenbergii* colony formation. Two compounds, benzoic acid, 2,3-bis[(trimethylsilyl)oxy], trimethylsilyl ester and (-)-epinephrine, tris(trimethylsilyl) ether, identified in cocultures and a colonial *M. wesenbergii* but not in other extracts, were helpful to high plant growth (Mohamed et al. 2009). For other compounds, hentriacontane, identified in cocultures and control *M. wesenbergii*, is a biological promoter (Kolattukudy 1968) and may be crucial for *Microcystis* growth. Hence, these compounds, identified in the coculture, may be responsible for *Microcystis* morphology change.

Comparing the extracellular metabolic substances that only appeared in two cocultures (Table S1), we found 18 compounds, including ester (4 compounds), amine (3), acetate (3), alkane (2), amide (2), acid (1), alcohol (1), anhydride (1)

and anthracene (1), which were identified in the coculture of *M. aeruginosa* and *Bac-EA* (*Ma* and *EA*) but not their controls. Furthermore, 12 compounds, including esters (7), acetates (1), alkane (1), amine (1), nitrile (1) and ketone (1), were also identified in coculture of *M. wesenbergii* and *Bac-SP* (*Mw* and *SP*) but not their controls. Though the same chemical types, such as ester and acetate, were found in extracts of two cocultures (*Ma* and *EA*, and *Mw* and *SP*), there were no identical compounds in cocultures. This phenomenon can be explained by the study of Forni et al. (1997); the different polysaccharide compositions were confirmed in different *Microcystis* species. The different substance compositions in cocultures might be related to the different features of colonial *Microcystis*. Finding the extracellular metabolic of colonial *Microcystis* formation may contribute to a better understanding of the taxonomy and ecology of *Microcystis*. Moreover, variety compounds secreted by *Microcystis* or bacteria may release into freshwater to influence the biochemical cycle lakes.

Apart from associated bacteria and extracellular compounds, many studies reported that other factors (zooplankton, nutrients and so on) could also influence *Microcystis* colony formation and maintenance (Burkert et al. 2001; Ma et al. 2014). However, these factors influencing the morphological changes of *Microcystis* in laboratory cultures are often quite different from that in natural water bodies. Though pH and N/P ratio significantly affected the growth and colony size of *Microcystis* (Ma et al. 2014), the formation of colonies was independent of changes in N or P concentration and pH value in the present study. The N and P concentration in BG11 medium was sufficient for the growth of *Microcystis* and bacteria. The concentration of P in the medium was 7.13 and 6.77 mg L^{-1} before and after the experiment, respectively. The concentration of N in the medium was 247.06 and 241.42 mg L^{-1} before and after the experiment, respectively. The pH of each treatment did not change significantly, and pH value was approximately 6.95–7.10 during the course of experiment. In the present study, colonies rebuilt by the single bacteria strains isolated from the *Microcystis* mucilage. It is evident that heterotrophic bacteria play an important role in *Microcystis* colony formation. Anyhow, the morphological and physiological change of *Microcystis* may be as a result of regulating to the change of environmental conditions.

In conclusion, we obtained five key heterotrophic bacteria from the mucilage of *Microcystis* colonies and found five bacteria that can induce the aggregation of unicellular *M. aeruginosa* or *M. wesenbergii* into colonies for the first time. The results suggested that key heterotrophic bacteria played an important role in *Microcystis* colony formation. Heterotrophic bacteria and/or *Microcystis* produced and secreted EPS and organic/inorganic compounds change into

the culture, affecting the morphology and physiology of *Microcystis*. The aggregation of unicellular *Microcystis* into colonies may be an adaptive response for *Microcystis* to environmental stress, that is, a regulation mechanism that enables the *Microcystis* cells to overcome bacteria interference and other stimulation. Therefore, the interaction of *Microcystis* and heterotrophic bacteria is responsible for the formation of *Microcystis* colony and outbreak of *Microcystis* blooms. More work is required to elucidate the relationship between the alga and its associated bacteria and the mechanism of *Microcystis* bloom in eutrophic lakes.

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