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Formation of large colonies: a defense mechanism of *Microcystis aeruginosa* under continuous grazing pressure by flagellate *Ochromonas* sp.

Zhen YANG, and Fanxiang KONG*

State Key Laboratory of Lake Science and Environment, Nanjing Institute of Geography and Limnology, Chinese Academy of Sciences, 73 East Beijing Road, Nanjing 210008, China
*Corresponding author: fxkong@niglas.ac.cn

ABSTRACT

Induced colony formation under grazing pressure has been reported in our previous results. However, the colonies induced in these studies comprised only tens of cells which are far smaller than the naturally occurring colonies. In this work, unicellular Microcystis aeruginosa *Kützing were co-cultivated with flagellate* Ochromonas *sp. for 50 d to investigate colony formation in* M. aeruginosa *under continuous grazing pressure. Results revealed that colonial* M. aeruginosa *formed on the 10th d under the grazing pressure of flagellate. These algal colonies resulted from the daughter cells of freshly dividing cells that failed to separate during the reproductive process. The diameters and cell numbers of the colonies increased slowly with time. Under continuous grazing pressure by* Ochromonas *sp. for 50 d, the diameter of some colonies reached over 180 μm. Analysis showed that the extracellular polysaccharide (EPS) content and relative gas vesicle (RGV) of each cell increased significantly after colony formation. However, there was no significant difference on the monosaccharide composition between unicellular and colonial* M. aeruginosa*. The loose aggregation of cells in the floating colonies suggests that a correlation probably exists between cell compactness and colony buoyancy.*

Key words: morphological change, aggregation of algal cells, grazed-induced, relative gas vesicle, buoyancy.

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INTRODUCTION

Cyanobacterial blooms (blue-green algal scums) have been reported in freshwater environments world-wide. Some of these algae often lead to serious environmental problems, such as deoxygenation of underlying waters, subsequent fish kills, toxicity, foul odors, and lowered aesthetic value of affected waters (Falconer 1999; Ye et al. 2009; Zhang et al. 2011).

Microcystis aeruginosa, one of the most common species existent during cyanobacterial bloom, often occurs as large colonies with tens of thousands of cells under natural conditions. Colony formation plays an important role for the domination of this cyanobacterium in an aquatic system (Oliver 1994; Wu, and Song 2008). However, after isolation from the field and cultivation in axenic cultures in the laboratory for some generations, the colony disaggregates and develops into unicellular algae (Reynolds et al. 1981; Bolch, and Blackburn 1996), whose physicochemical properties are totally different from those of colonial algae. Thus, the results from laboratory studies using unicellular strain could not truly explain environmental phenomenon. Therefore, the mechanism of colony formation in *Microcystis* has to be examined to reveal the mechanism of cyanobacterial bloom.

Grazing pressure from cladocerans, a biotic factor, is an important trigger for the colony formation of green alga *Scenedesmus* sp. (Hessen, and Van donk 1993). The morphological change in *Scenedesmus* sp. is considered as an antipredator reaction. Moreover, the infochemicals released from zooplankton are apparently responsible for this reaction (Lampert et al. 1994; von Elert, and Franck 1999; Lurling 2003a, b).The effect of grazing pressure from cladocerans on colony formation in *M. aeruginosa* has also been investigated. Although colony formation was found occasionally in some previous studies when unicellular *M. aeruginosa* was co-cultivated with cladocerans(Jang et al. 2003; van Gremberghe et al. 2009), the ability of cladocerans to induce colony formation in *M. aeruginosa* is uncertain (Fulton, and Paerl 1987; Hessen, and Van donk 1993; Yang et al. 2006).

Impressive progress has been obtained recently using flagellate to study the mechanism of colony formation in aquatic bacteria and algae. Hahn et al. (2000, 2004) revealed that bacteria could form planktonic microcolonies under the strong grazing pressure of *Ochromonas* sp. These microcolonies exceeded the upper size limit for ingestion by flagellates protecting them from predation. The dialysis bag batch culture experiments indicated that colony formation in bacteria is probably controlled by infochemicals released by the flagellate (Blom et al. 2010). Burkert (2001) observed some *M. aeruginosa* colonies when *Ochromonas* sp. accidentally entered into the compartment of M. aeruginosa in a dialysis experiment. Our previous study also

demonstrated that the grazing pressure from Ochromonas sp. could induce the colony formation in M. aeruginosa (Yang et al. 2006). A filtration study showed that infochemicals released by flagellates apparently triggered the colony formation in *M. aeruginosa* (Yang et al. 2009a).

However, most colonies of *M. aeruginosa* induced in the previous studies consist of only tens of cells, which are far lesser than those occurring in the natural lake. In the present study, the unicellular *M. aeruginosa* is co-cultured with flagellate *Ochromonas* sp. for fifty days to search for large *M. aeruginosa* colonies under a continuous grazing pressure.

MATERIALS AND METHODS

Algae and Flagellate

M. aeruginosa (PCC 7806, 4.61±0.32 μm) were obtained from the Institute of Hydrobiology, the Chinese Academy of Sciences. The algae were grown in a BG-11 medium in batch culture in 250 mL flasks (Rippka et al. 1979) under an illumination intensity of 40 μmol quanta m^{-2} s^{-1} provided by cool white fluorescent lamps at 25°C with light–dark period of 12:12 h. Cultures were shaken manually once daily. Algae in its late exponential growth phase were used in the experiment.

Flagellates *Ochromonas* sp. (8.3±0.80 μm) were isolated from Lake Taihu, cultured in the flasks, and fed with autoclaved wheat seeds. The flagellate cultures were grown at 25°C under fluorescent light at an intensity of 40 μmol quanta m^{-2} s^{-1} with a light-dark period of 12:12 h.

Colony inducement experiment

To investigate the effect of flagellate grazing on *M. aeruginosa* density and colony formation, three grazing replicates with 100 mL unicellular *M. aeruginosa* (3×106 cell mL^{-1}) and *Ochromonas* sp. (20 000 individuals mL^{-1}) were cultivated in 250 mL flasks at 25°C under fluorescent light at an intensity of 40 μmol quanta m^{-2} s^{-1} with a light–dark period of 12:12 h for 50 d. Three bottles of unicellular *M. aeruginosa* with the same densities were cultivated under the same conditions as controls. The algal (colonies were disaggregated by sonication before counting) and flagellate densities, as well as the mean diameter of *M. aeruginosa* colonies (at least 40 colonies were measured for each flask), were determined using microscope after 2, 4, 6, 10, 14, 18, 22, 26, 30, 34, 38, 42, 46, and 50 d. The mean number of algal cell per colony was obtained from algal density divided by the number of colonies.

Polysaccharide and monosaccharide assay

To eliminate the error caused by the flagellates, *M. aeruginosa* colonies were leached using a strainer with a diameter of 200 mesh before analysis.

The extracellular polysaccharide (EPS) and intracellular polysaccharide (IPS) contents of unicellular *M. aeruginosa* in the control flasks and colonial *M. aeruginosa* under grazing treatment for 50 d were analyzed at the end of the induction experiment. Samples (10 mL) were adjusted to pH 10 and incubated at 45°C in a water bath for 4 h, then centrifuged at 27,500×g for 15 min. The supernates were filtered through 0.45 μm membrane filters and dialyzed against deionized water to remove the interference of the presence of ions in medium cultures to assay EPS content. The algae were resuspended in 10 mL distilled water. After sonicating and heating at 85°C in a waterbath for 1 h, they were treated with 1 mL 85% trichloracetic and centrifuged at 27,500×g for 15 min to remove the fragments and proteins. The supernates were filtered and dialyzed as described above and were used to assay IPS content. The polysaccharide content in the dialyzed supernate was determined using glucose solutions for calibration. All polysaccharides were measured using the phenolsulfuric acid assay (Dubois et al. 1956).

Algal culture samples were centrifuged and freeze dried to obtain lyophilized algal cells. The monosaccharide composition was determined after hydrolysis of lyophilized algal cells in 2 mol L^{-1} trifluoroacetic acid at 120°C for 3 h. Neutral sugars in the hydrolysate were converted into acetylated aldononitrile derivatives for gaschromatographic analysis (Li et al. 1982). Gas-liquid chromatography was performed on an HP6890 instrument equipped with an HP-5 5% phenyl methyl siloxane capillary column (30 m×0.25 mm ID) and a flame ionization detector.

Gas vesicle volume assay

The RGV of *M. aeruginosa* was estimated based on the method developed by Brookes and Ganf (2001). The mean RGV is calculated as the difference in side scatter of cells with intact gas vesicles and that of cells with all collapsed gas vesicles under 0.8 MPa pressure. Side scatter was measured on a single-cell basis using a flow cytometer (Benkman) with an argon laser (488 nm) as the excitation source, and RGV is measured in terms of relative side scatter unit (RSU).

Data analysis

All data were presented as mean±SD. Differences between the treatments and controls were analyzed by oneway ANOVA after testing for normality and homoscedasticity of the data. The level of statistical significance was considered at $p<0.05$.

RESULTS

Grazing and colony formation

Flagellate *Ochromonas* sp. exhibited a significant graz-

ing ability on unicellular *M. aeruginosa*. The *M. aeruginosa* density decreased rapidly from 3.0×10^6 to 2.2×10^5 cell mL^{-1} after they were cultivated with flagellates for 4 d (Fig. 1). Meanwhile, the flagellate density increased to 1.0×10^6 cell mL^{-1}. However, *M. aeruginosa* were not cleared completely by the flagellates, and its density in the culture increased from the 6th d. On the 30th d of mix–cultivation, the *M. aeruginosa* density recovered to 3.0×10^6 cell mL^{-1}, and reached to 8.05×10^6 cell mL^{-1} on the 50th d. By contrast, the flagellate density remained at approximately 1.0×10^6 cell mL^{-1} after the 6th day.

A few small colonies with several algal cell assemblages were observed on the 10th d of mix–cultivation. The diameter and cell number of colony increased with the extended mix–cultivation time (Fig. 2). On the 50th d, induced colonies had a mean diameter of 68.19 μm (varied from 16.45 187.24 μm) and a mean number of 377 algal cells per colony, indicating that some colonies consisted of thousands of algal cells. While *M. aeruginosa* in the control flasks were constantly dominated by unicells.

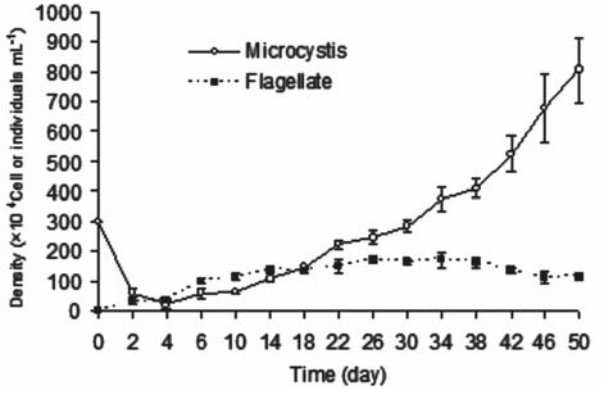

Fig. 1. Changes of cell density of *M. aeruginosa* and flagellate in the mix-cultivating system. Error bars represent 1 SD (n=3).

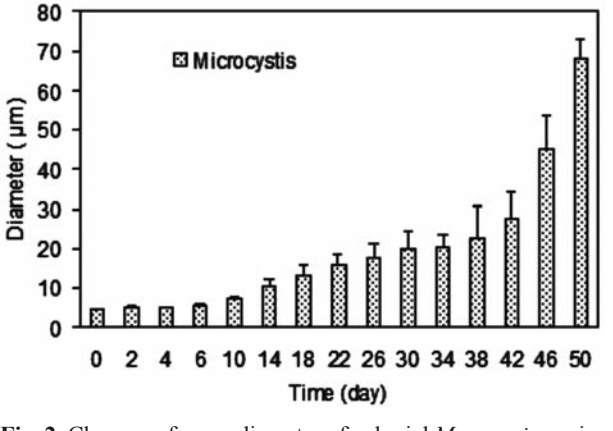

Fig. 2. Changes of mean diameter of colonial *M. aeruginosa* induced by flagellate. Error bars represent 1 SD (n=3).

Saccharides and buoyancy

The polysaccharide assay showed that colonial *M. aeruginosa* possess 3.36±0.33 pg EPS per cell, significantly higher ($p<0.05$) than that of the unicellular cells in the control flasks (Fig. 3). The IPS content in *M. aeruginosa* cells also increased after colony formation. However, no significant difference ($p>0.05$) was found using one-way ANOVA.

We analyzed eight main monosaccharides in *M. aeruginosa* including glucose, galactan, mannose, fucose, xylose, ribose, rhamnose, and arabinose, after hydrolysis of polysaccharide (Tab. 1). Although there was a considerable increase in the EPS of colonial *M. aeruginosa*, no significant difference was detected on the monosaccharide composition between unicellular and colonial *M. aeruginosa*.

Interestingly, aside from the colonies that sunk at the bottom of the bottle and suspended in the culture, we also found a number of colonies that floated on the surface. The side scatter and RGV of unicellular *M. aeruginosa* control flasks were 144.83±1.43 and 112.47±1.33 RSU, respectively, which were significantly lower ($p<0.05$) than those of the colonial cells (Tab. 2).

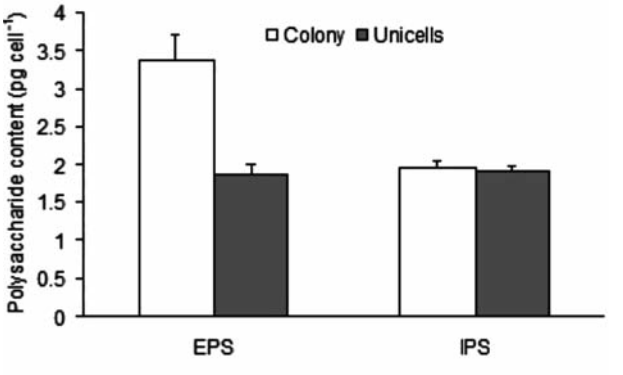

Fig. 3. Differentiation of extracellular polysaccharide (EPS) and intracellular polysaccharide (IPS) per cell between unicellular and colonial *M. aeruginosa*.

Tab. 1. Monosaccharides composition of unicellular and colonial *M. aeruginosa*.

Monosaccharides	Unicell (%)	Colony (%)
Glucose	80.12	79.65
Galactan	9.07	9.38
Mannose	3.89	4.02
Fucose	2.34	2.25
Xylose	1.63	1.50
Ribose	1.09	1.20
Rhamnose	1.02	1.03
Arabinose	0.84	0.97

Tab. 2. Side scatter and relative gas vesicles of unicellular, sinking and floating colonial *M. aeruginosa*.

	Side scatter		RGV
	With intact gas visicles	With gas visicle collapsed	
Unicell	144.83	32.37	112.47
Sinking colony	234.03	43.33	190.7
Floating colony	299.27	39.73	259.53

The side scatter and RGV in the settled colonial cells were 234.03±5.55 and 190.7±5.01 RSU, respectively, which were significantly less (p<0.05) than the side scatter (299.27±5.05) and RGV (259.53±6.22 RSU) of the floating colonial cells. Moreover, the floating colonies showed a higher mean diameter-to-mean cell number per colony ratio (0.26) than that (0.14) of the sinking colonies. This value, along with micrograph in Fig. 4, indicates that the aggregation of algal cells in the floating colonies was looser than that of the sinking colonies.

DISCUSSION

Similar to other flagellates (*e.g.*, *Collodictyon triciliatum, Diphylleia rotans*, and *Poterioochromonas* sp.) which have been reported in previous literature (Nishibe et al. 2002; Kim et al. 2006; Zhang et al. 2008), *Ochromonas* sp. exhibited a strong grazing ability on unicellular *M. aeruginosa* and high growth rate after grazing activaty in our experiment.

The hepatotoxic *Microcystis* strains are toxic to many potential grazers of *Microcystis*, which enable them to resist the grazing pressure from these zooplanktons (Demott et al. 1991; Jang et al. 2003). However, microcystins can not provide antiherbivore defense against mixotrophic flagellates (Wilken et al. 2010). Volume enlargement due to colony formation is the main mechanism of *M. aeruginosa* to resist the grazing pressure from *Ochromonas* sp. (Yang et al. 2009b). This defense mechanism entitled some of *M. aeruginosa* cells to survive in our co-cultivation system, even under strong grazing pressure. Due to the decrease in available food and growth space, growth of *Ochromonas* sp. became slower, and a cell concentration of approximately 1.0×10^6 cell mL^{-1} was maintained.

Two different mechanisms are involved in colony formation. The first mechanism is that colonies are formed when daughter cells of a freshly dividing cell fail to separate from mother cells during the reproductive process. The second mechanism involves the aggregation of already existing single cells or colonies (Lurling, and Van Donk 1997). Based on the continuous observation in our present experiment, the colony formation in *M. aeruginosa* was not emergent. Small colonies with several cells appeared on the 10^{th} d. These colonies evidently originated from the single cells which failed to separate during the cell division cycle. These undivided cells underwent multiple rounds of cell division that resulted in increased cell aggregation and eventually formed a colony. Therefore, the former mechanism describes the colony formation in *M. aeruginosa*. The *in situ* observation on colony formation and enlargement in *M. aeruginosa* from previous study in Taihu Lake also supports this mechanism (Cao, and Yang 2010).

Quantitative analysis of polysaccharide showed that colonial *M. aeruginosa* cells have higher EPS content compared with those of the unicellular cells. This finding is consistent to that of the previous studies on the biochemical composition of colonial and disaggregated *M. aeruginosa* cells in our laboratory (Zhang et al. 2007; Yang et al. 2008). EPS plays an important role on the conglutination of cells in colonial alga (De Philippis, and Vincenzini 1998; van Rijssel et al. 2000; Pajdak-Stos et al. 2001; Thornton 2002). The agglutinating ability of polysaccharides depends on their monosaccharide composition (Bahat-Samet et al. 2004). Our assay on mono-saccharides showed that there was no significant difference in the monosaccharide composition between unicellular and colonial *M. aeruginosa*. This result indicates that the enhancement of agglutination

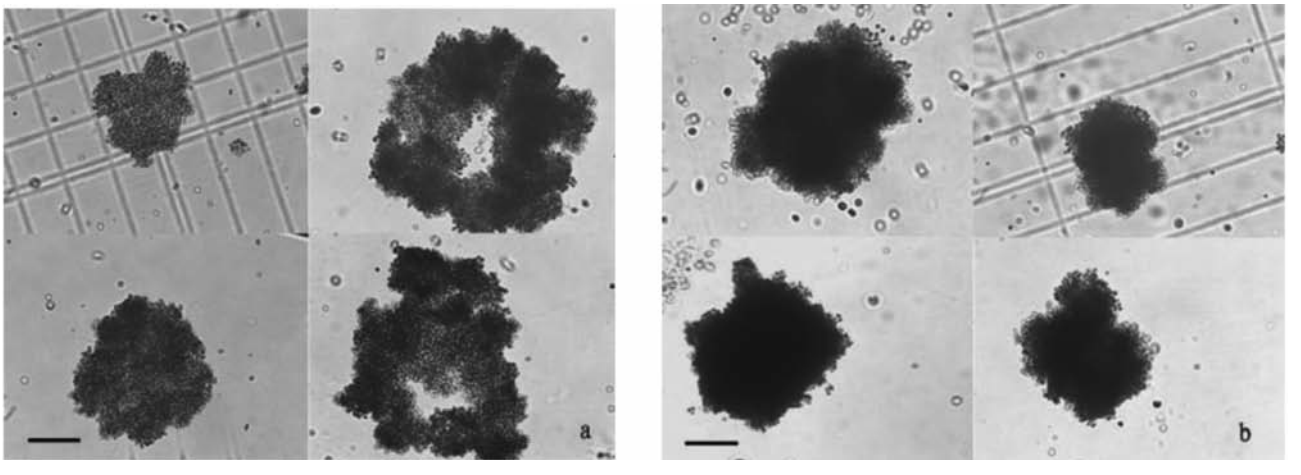

Fig. 4. Floating (a) and sinking (b) *M. aeruginosa* colonies induced by flagellate. Scale bars indicate 50 μm.

between algal cells of colonial *M. aeruginosa* is attributable to the increase of total polysaccharide content, not the alteration of polysaccharide composition.

The ability of *M. aeruginosa* to dominate in an aquatic system can be attributed to gas vesicles to some extent, providing buoyancy and reducing sedimentation losses (Reynolds, and Walsby 1975). When an artificial culture with abundant nutrition and equal light is used for cultivation, unicellular *M. aeruginosa* may not need to regulate buoyancy by gas vesicles to obtain additional light or nutrition. Consequently, the RGV in unicellular *M. aeruginosa* decreases. By contrast, the nutrient uptake and light absorption by colonial cells would decrease based on the "package-effect" (Kirk 1994), which generally has an opposite effect to gas vesicle accumulation (Reynolds, and Walsby 1975; Deacon 1990; Brookes, and Ganf 2001). These phenomena may be the reasons for the enhancement of RGV in colonial cells. Furthermore, we also observed that the aggregation of algal cells in floating colonies is looser than that in sinking colonies, signifying a probable correlation between cell compactness and colony buoyancy.

Given that *Ochromonas* sp. usually co-exist with *M. aeruginosa* in natural lakes (Van Donk et al. 2009), the grazing pressure from *Ochromonas* sp. could also be an important trigger for colony formation in *M. aeruginosa* in natural freshwater system. In the present study, the size and morphology of graze-induced *M. aeruginosa* colonies are similar to those of naturally occurring colonies as shown in previous studies. However, mucilage, which retains the typical shape of *M. aeruginosa* colony in natural lakes (Brunberg 1999), did not form on the periphery of graze-induced colonies. Thus mucilage formation probably requires other environmental factors, such as bacteria, light, and nutrients, to name a few.

Considering that the flagellate has a strong grazing ability on unicellular *M. aeruginosa*, *Ochromonas* sp. could be used as a potential biological control agent against *Microcystis* blooms (Zhang et al. 2008; Van Donk et al. 2009). However, for the phagotroph, prey size is an important selective factor for the grazing of *Ochromonas* who prefers food-size particles between 0.9 and 1.2 μm (Zhang, and Watanabe 2001; Pfandl et al. 2004). In our studies, *Ochromonas* sp. showed an extremely low clearance rate on colonial *M. aeruginosa*, even for a small colony with 16 μm diameter (Yang et al. 2009b). In natural lakes, *M. aeruginosa* usually exists as a colony with a diameter of hundreds or even thousands micrometers. Therefore, additional studies are needed regarding the application of flagellate as a biocontrol agent against *Microcystis* blooms.

CONCLUSIONS

Flagellate *Ochromonas* sp. exhibits a strong grazing ability on unicellular *M. aeruginosa*. Under a continuous grazing pressure by *Ochromonas* sp., unicellular *M. aeruginosa* could form a large colony with thousands of algal cells. EPS content and RGV of each cell increase significantly after colony formation. Based on the difference in morphology, as well as the mean diameter-to-mean cell number per colony ratio between floating and sinking colonies, a correlation probably exists between cell compactness and colony buoyancy.

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