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Ecological Dynamics of the Toxic Bloom-Forming Cyanobacterium *Microcystis aeruginosa* and Its Cyanophages in Freshwater[▽]

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The abundance of potentially *Microcystis aeruginosa*-infectious cyanophages in freshwater was studied using *g91* real-time PCR. A clear increase in cyanophage abundance was observed when *M. aeruginosa* numbers declined, showing that these factors were significantly negatively correlated. Furthermore, our data suggested that cyanophage dynamics may also affect shifts in microcystin-producing and non-microcystin-producing populations.

The major bloom-forming cyanobacterial species *Microcystis aeruginosa* forms noxious blooms in many eutrophic freshwater lakes, ponds, and reservoirs. A limited population (limited number of strains) of *M. aeruginosa* in the environment produces potent hepatotoxins called microcystins (7). These potent toxins in the *M. aeruginosa* blooms have caused many cases of animal and human poisoning (3, 8, 16).

Previously, most studies have focused on relationships among the cyanobacterial bloom dynamics and the changes in physicochemical factors (e.g., nutrient supply, light, and temperature) that influence cyanobacterial growth in the aquatic environment (28). Since the discovery that viruses are widespread in marine ecosystems (4), cyanophages that can infect cyanobacteria have been thought to be an alternative factor that may control the succession of cyanobacterial blooms (12, 14, 15, 18, 19). In addition, cyanophages can also influence the clonal composition of the host *Synechococcus* communities (14, 27) and could account for some of the cyanobacterial diversity observed in natural communities (22, 25, 30). Nevertheless, little is known about how freshwater cyanophages can affect the abundance and clonal composition of cyanobacterial blooms in lakes over time.

Our aim is to determine if the cyanophages have potential quantitative and qualitative effects on the *M. aeruginosa* communities in Lake Mikata in Japan. We performed two independent real-time PCR assays to monitor the dynamics of *M. aeruginosa* and its cyanophage communities. To quantify *M. aeruginosa*, we used the phycocyanin intergenic spacer (PC-IGS) that was previously used to examine total *M. aeruginosa* numbers (9, 32). A second real-time PCR assay was used to quantitatively detect potentially *M. aeruginosa*-infectious cyanophages using the primers targeting the viral sheath protein-encoding gene (*g91*) previously identified by Takashima et al. (21). To determine the effect of the cyanophages on the internal dynamics of the total *M. aeruginosa* communities, we examined the fluctuation in the abundance of potentially microcystin-producing *M. aeruginosa* populations using the real-time PCR and microcystin synthetase gene (*mcyA*)-specific primers (32) and monitored the relative size of the microcystin-producing subpopulation compared to the total population in relation to the cyanophage numbers using a field survey of *M. aeruginosa* blooms in a Japanese lake.

Water samples were collected from the surface layer (0.5 m) once per month from April to October in 2006 at a fixed point (35°33′N, 135°53′E) in Lake Mikata (Fig. 1). The cyanobacterial cells used for DNA extraction were sonicated gently and harvested by centrifugation at 14,400 × *g* for 10 min. DNA was extracted and purified using the previously described xanthogenate method (36). For phage DNA extraction, the samples were filtered through 0.2-μm polycarbonate filters (Advantec Toyo, Tokyo, Japan) and concentrated using ultracentrifugation at 111,000 × *g* for 1.5 h at 4°C; then the phage DNA was extracted as described previously (21). Each DNA extract was used as the PCR template for real-time PCR. The real-time PCR primer pairs 188F-254R, M1rF-M1rR, and SheathRTF-SheathRTR (Table 1) were used to amplify PC-IGS gene (66-bp), *mcyA* gene (107-bp), and *g91* gene (132-bp) fragments, respectively. The *mcyA* primers M1rF and M1rR were designed to be *M. aeruginosa* specific based on a comparison of cyanobacterial *mcyA* sequences of *M. aeruginosa* strains NIES298 and NIES102, *Planktothrix agardhii* strain NIVA-CYA126/8, and *Anabaena* sp. strain 90 (DDBJ/EMBL/GenBank accession numbers AB092804, AB092805, AJ441056, and AJ536156, respectively) (32), using BLAST (1) and Clustal W (24). The PC-IGS and *mcyA* PCR were conducted as previously described by Yoshida et al. (32), and the *g91* real-time PCR were conducted as previously described by Takashima et al. (21). Physicochemical parameters, temperature, the major dissolved inorganic nutrients, nitrate (NO_3-N), orthophosphate (PO_4-P), the number of cyanobacterial cells, and the total amounts of microcystins in the lake water samples were investigated as described previously (32, 33).

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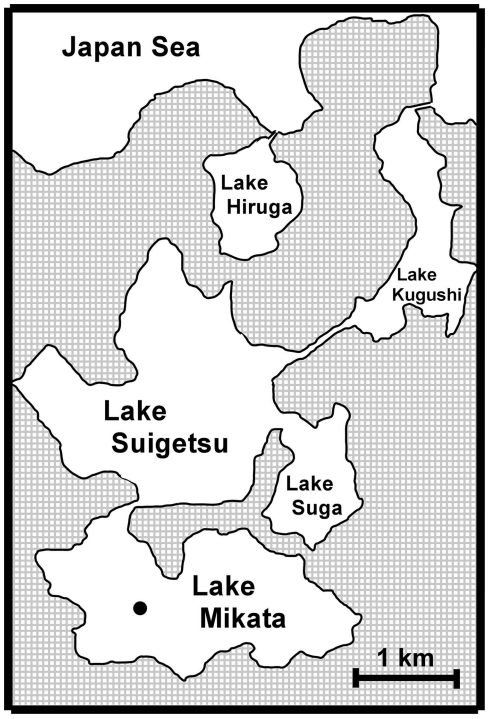

FIG. 1. Sampling site in Lake Mikata (35°33′N, 135°53′E).

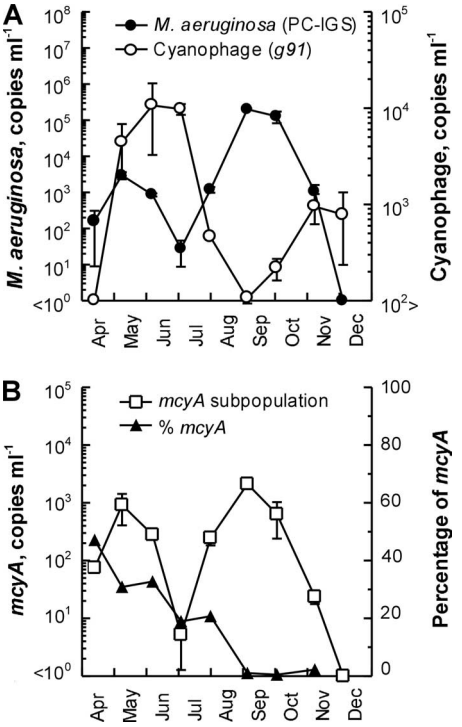

FIG. 2. (A) *M. aeruginosa* and cyanophage abundance in Lake Mikata from April to December 2006. The numbers of DNA copies per milliliter were determined using real-time PCR. The error bars indicate the standard deviations of three experiments. (B) *mcyA* subpopulation abundance and percentage of *mcyA* in Lake Mikata from April to December 2006. The numbers of *mcyA* gene copies per milliliter were determined using real-time PCR. The percentage of *mcyA* was determined by dividing the relative number of DNA copies in the *mcyA* subpopulation by the total number of copies in *M. aeruginosa* determined with the PC-IGS primer set.

The abundance of *M. aeruginosa* and the cyanophages in Lake Mikata was monitored from the spring through the early winter in 2006. The PC gene copy numbers of *M. aeruginosa* were low at the beginning of the study (Fig. 2A), increased from April (1.6×10^2 copies ml^{-1}) to May (3.0×10^3 copies ml^{-1}), and then declined gradually through June (2.7×10^1 copies ml^{-1}). During July, the number increased again and reached a maximum of 2.0×10^5 copies ml^{-1} on 5 September. The *M. aeruginosa* abundance decreased from then until November (1.0×10^3 copies ml^{-1}), and *M. aeruginosa* was not detected on 5 December. According to the microscopic analysis, *Planktothrix* and *Anabaena* were dominant genera in Lake Mikata (Table 2). The numbers of *M. aeruginosa* cells showed a positive correlation (Spearman's $r = 0.810$; $P = 0.015$; $n = 8$) with the *M. aeruginosa* PC gene copy numbers (Table 2 and Fig. 2A). However, the *M. aeruginosa* PC gene copy numbers determined on all sampling dates except 4 July were 3 to >200 times higher than the cell numbers observed by microscopy. A similar case has been reported by Vaitomaa et al. (26). This could have been due to the microscopic counting method, which depends on morphological characteristics as the number of non-colony-forming *M. aeruginosa* cells might not be included in any cell count (33, 36). Another possible explanation is that high PC gene copy numbers detected by real-time PCR resulted from multiple genome copies in a cell. In fact, laboratory-based physiological data revealed that *M. aeruginosa* cells have several genome copies (maximum, 11 copies) during the transition from the logarithmic growth phase to the stationary phase (9), as observed in another cyanobacterium, *Synechococcus* (5, 6, 11).

The cyanophage *g91* copy numbers detected were between 1.1×10^2 and 1.1×10^4 copies ml^{-1} throughout the sampling period, although the number was below the detection limit ($<1.0 \times 10^2$ copies ml^{-1}) on 13 April (Fig. 2A). The cyanophage abundance increased twice, with the first increase occurring from April ($<1.0 \times 10^2$ copies ml^{-1}) to June (1.1×10^4

TABLE 1. Primers used in this study

Primer	Target gene	Sequence (5′ to 3′)	Reference
188F	PC-IGS	GCT ACT TCG ACC GCG CC	9
254R	PC-IGS	TCC TAC GGT TTA ATT GAG ACT AGC C	9
M1rF	*mcyA*	AGC GGT AGT CAT TGC ATC GG	32
M1rR	*mcyA*	GCC CTT TTT CTG AAG TCG CC	32
SheathRTF	*g91*	ACA TCA GCG TTC GTT TCG G	21
SheathRTR	*g91*	CAA TCT GGT TAG GTA GGT CG	21

TABLE 2. Physical and chemical characteristics, numbers of cyanobacterial cells, and microcystin concentrations in Lake Mikata

Sampling date	Temp (°C)	NO_3-N concn (mg liter^{-1})	PO_4-P concn (mg liter^{-1})	No. (%) of cells (cells ml^{-1}) of[a]:			Total cyanobacterium concn (cells ml^{-1})[b]	Microcystin concn (ng liter^{-1})
				M. aeruginosa	*Anabaena* sp.	*Planktothrix* sp.		
13 April	13.1	0.3636	0.0078	1.6×10^1 (0.3)	ND	5.2×10^3 (99)	5.2×10^3	0.15
9 May	19.7	0.0032	0.0021	2.7×10^2 (0.4)	ND	6.3×10^4 (99)	6.3×10^4	3.25
6 June	24.3	0.0016	0.0022	2.2×10^2 (0.05)	2.7×10^5 (56)	2.1×10^5 (43)	4.8×10^5	1.34
4 July	26.1	0.0025	0.0031	2.4×10^2 (0.07)	1.5×10^5 (42)	2.0×10^5 (57)	3.5×10^5	0.75
1 August	27.9	0.0507	0.0029	3.7×10^2 (0.2)	2.1×10^4 (8)	2.3×10^5 (91)	2.5×10^5	3.34
5 September	27.3	0.0020	0.0019	6.3×10^3 (2)	2.6×10^5 (65)	1.3×10^5 (33)	4.0×10^5	5.03
3 October	23.7	0.0232	0.0017	6.6×10^2 (1)	4.7×10^2 (0.7)	6.2×10^4 (98)	6.3×10^4	1.86
8 November	16.0	0.0030	0.0012	3.2×10^1 (0.3)	1.0×10^4 (99)	ND	1.0×10^4	0.44
4 December	9.9	0.1602	0.0052	ND	ND	4.7×10^4 (100)	4.7×10^4	ND

[a] Percentages were determined as follows: number of cells of taxon/total number of cyanobacterial cells detected by microscopy × 100. ND, not detected.
[b] The total cyanobacterium concentration is the sum of the *M. aeruginosa*, *Anabaena*, and *Planktothrix* concentrations.

copies ml^{-1}) and the second increase occurring from September (1.1×10^2 copies ml^{-1}) to November (9.5×10^2 copies ml^{-1}). A clear increase in the phage abundance was observed when the host *M. aeruginosa* numbers declined. The cyanophage abundance was negatively correlated with the abundance of *M. aeruginosa* (Spearman's $r = -0.857$; $P = 0.014$; $n = 7$). The physicochemical factors, temperature, NO_3-N, and PO_4-P, which potentially controlled the growth of *M. aeruginosa* in the environment, were also considered in the correlation analysis (Table 2); however, we found no significant correlation (Spearman's $r = 0.333$, $r = -0.095$, and $r = -0.571$, respectively; $n = 8$). These observations suggest that the cyanophage is an important factor in determining the seasonal changes in *M. aeruginosa* abundance.

To evaluate the internal dynamics of *M. aeruginosa* blooms and to clarify the effect of the cyanophage on the community composition, the *mcyA* copy numbers of the potentially microcystin-producing populations were analyzed throughout the sampling period (Fig. 2B) and were found to be between 5.2×10^1 and 2.1×10^3 copies ml^{-1}; copies were not detected only in December. The microcystin concentration in Lake Mikata (Table 2) had a statistically significant positive correlation with the copy number of the *M. aeruginosa mcyA* gene (Spearman's $r = 0.762$; $P = 0.028$; $n = 8$). The ratio of the *mcyA*-containing subpopulation to the total *M. aeruginosa* population was 0.50 to 47.1% (Fig. 2B). The relative abundance of the *mcyA* subpopulation in April, May, June, July, and August was greater (>18%) than that in September, October, and November (0.50 to 2.25%), and there were clear differences in the relative abundance of the *mcyA* subpopulation between bloom stages. The data indicate that the relative abundance of the microcystin-producing subpopulation of the species *M. aeruginosa* changes over time and that microcystin-producing populations were outcompeted by non-microcystin-producing populations during the summer. As mentioned above, in the summer we observed a temporal decline in the *M. aeruginosa* abundance when the cyanophage abundance increased, showing that temporal changes in the cyanophage numbers affected the host *M. aeruginosa* (Fig. 2A). Together, these data suggest that the cyanophages may have been responsible for the fact that the microcystin-producing subpopulation was outcompeted in the summer and may have influenced the seasonal shift in the composition of the different *M. aeruginosa* populations.

The regulation of the internal dynamics of *M. aeruginosa* populations is thought to be complicated by a broad range of environment conditions, including the intraspecific selective lysis caused by cyanophages. We reported previously that the dynamics of microcystin-producing and non-microcystin-producing *M. aeruginosa* populations showed a significant correlation with nitrate concentrations in lake water (32). In this study, our data also suggest that high NO_3-N loading in the spring is a significant factor in increasing the growth of microcystin-producing *M. aeruginosa* populations at the beginning of the bloom (Table 2 and Fig. 2B).

The cyanophage communities in Lake Mikata determined using the *g91* real-time PCR products from the samples obtained on 9 May, 6 June, 4 July, 8 November, and 4 December were sequenced after construction of the clone libraries (Table 3). Clone libraries could not be produced from four samples (13 April, 1 August, 5 September, and 3 October) because the amounts of the *g91* PCR-amplified products were very small. A total of 108 sequences from five clone libraries (Table 3), which were almost identical at the amino acid level, were successfully generated (data not shown). At the nucleotide level, 10 differ-

TABLE 3. Sequence profiles from Lake Mikata clone libraries targeting the *g91* gene[a]

Sampling date	Genotype	No. of clones detected[b]
9 May	a	11
6 June	a	25
	b	1
	c	1
4 July	a	21
	d	1
	e	1
8 November	a	23
	f	1
	g	1
	h	1
4 December	a	19
	i	1
	j	1

[a] The PCR products from Lake Mikata samples obtained on 9 May, 6 June, 4 July, 8 November, and 4 December were sequenced.
[b] Number of clones detected in the PCR products from Lake Mikata.

ent genotypes (designated genotypes a to j) were identified for the cyanophage isolates and also for the Lake Mikata sample. Genotype a was the genotype most frequently encountered in the Lake Mikata sample and was identical to sequences of Ma-LMM01, Ma-LMM02, Ma-LMM03, and Ma-HPM05 that specifically infect only a microcystin-producing *M. aeruginosa* strain (21, 34, 35). Genotypes b to j were detected only once in the PCR products from Lake Mikata. All of the genotypes had 98.9% identity to genotype a, as shown by a single base substitution at different nucleotide positions. The clonal sequence diversity of the cyanophage communities suggests that the phage particles detected by the *g91* real-time PCR may be composed of different cyanophages (e.g., cyanophages having different host strain specificities) (2, 10, 13, 29, 37). A wide variety of host-phage systems in aquatic environments have been described by Sullivan et al. (17), Suttle and Chan (20), and Waterbury and Valois (27). We recently reported that *M. aeruginosa* populations have a high degree of genetic diversity at the intraspecies level (31), and their successional blooms revealed different dynamics during the bloom in Lake Mikata (33). We speculate there are various host-phage systems in the relationships between *M. aeruginosa* and its phages.

This report is the first report demonstrating that seasonal dynamics of the cyanophage community in freshwater was related to both the host *M. aeruginosa* abundance and shifts in the different populations (microcystin-producing and non-microcystin-producing populations). However, the results must be interpreted carefully. Our data also revealed the relatively high ratio of *M. aeruginosa* numbers to cyanophage numbers in late summer (Fig. 2A). This indicates that the cyanophage assemblage might have the ability to infect only a small percentage of the *M. aeruginosa* population present, and thus it is possible that the presence of the cyanophages results only in replacement of phage-sensitive populations by phage-insensitive populations ("killing the winner hypothesis") (23) rather than having a quantitative impact on the *M. aeruginosa* abundance. To develop a better understanding of the ecological impact of cyanophages on *M. aeruginosa* blooms, further investigations of the multiple host-phage interactions at the population (strain) level are required.

Nucleotide sequence accession numbers. The DDBJ/EMBL/GenBank accession numbers for the sequences reported here are AB361250 to AB361272 and AB375177 to AB375261.

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