Dynamics of Microcystin-Degrading Bacteria in Mucilage of Microcystis

Dynamics of microcystin-degrading bacteria in mucilage of *Microcystis*

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Abstract

To reveal the process of degradation of hepatotoxic microcystin produced in *Microcystis* cells during the *Microcystis* bloom period, we used fluorescence *in situ* hybridization (FISH) to analyze the population dynamics of microcystin-degrading bacteria in *Microcystis* mucilage. We designed and applied an oligonucleotide probe targeted to the 16S rRNA sequence of strain Y2 of a microcystin-degrading bacterium (MCD-bacterium), which was isolated from Lake Suwa, Japan. In both the 1998 and 1999 tests, FISH clearly showed that MCD-bacteria existed in the mucilage and that, when a high concentration of cell-bound microcystin was detected, MCD-bacteria exceeded 10% of the sum of bacteria hybridized with group-specific probes. The concentration of MCD-bacteria was highest in summer 1998, when a toxic species, *M. viridis*, was dominant. There was a high correlation between the number of MCD-bacteria in the mucilage and the concentration of cell-bound microcystin in the lake. Our results suggest that MCD-bacteria responded to changes in the concentration of microcystin and degraded the microcystin when it was released from *Microcystis* cells. We also analyzed changes in the bacterial community structure associated with the *Microcystis* colonies by using domain- and group-specific oligonucleotide probes. Changes in the concentrations of the *Cytophaga/Flavobacterium* group and δ-Proteobacteria, which can degrade macromolecules derived from *Microcystis* cells, were synchronized with changes in the concentration of *Microcystis*. The results not only suggest the significant role of MCD-bacteria in detoxification, but also demonstrate a possible sequence of degradation from *Microcystis* cells to microcystin maintained in the cell, which is then carried out by bacterial consortia in the mucilage.

Introduction

Microcystin, a cyclic hepatotoxin, causes serious disease, as reported by Jochimsen et al. [20]. In 1996, 54 out of 130 patients at a dialysis center in Caruaru, Brazil, died from water for dialysis contaminated by microcystins, which are produced by some cyanobacteria of the genera *Microcystis*, *Anabaena*, *Nostoc*, and *Oscillatoria.* Of these, *Microcystis*, which blooms in eutrophic tropical and temperate lakes and ponds, is the most common cyanobacterium [10].

Microcystin is produced and maintained within healthy cyanobacterial cells. Approximately 10% to 20% of microcystin is lost from healthy cyanobacterial cells in culture [40, 41]. When *Microcystis* cells are decayed, the concentration of dissolved microcystin may increase [44]. However, dissolved microcystin concentrations remain very low in lake water, even during the decay of *Microcystis* [36]. Jones and Orr [21] used high-performance liquid chromatography (HPLC) and a protein phosphatase inhibition assay to determine the loss of microcystin released to lake water following algicidal treatment. Furthermore, Christoffersen et al. [14] showed in laboratory and field experiments that the degradation of microcystin by indigenous bacteria in lake water was accelerated by the existence of dissolved organic carbon (DOC), and that the addition of cell lysates of toxic *Microcystis* elevated bacterial abundance, production and diversity. However, the mechanism of *in situ* degradation of microcystin remains to be clarified. Jones et al. [22] isolated a single strain of microcystin-degrading bacterium (MCD-bacterium) identified as *Sphingomonas* sp. from drainage. Bourne et al. [8] used this strain in laboratory experiments focused on the process of enzymatic degradation of microcystin. However, they did not examine the degradation of microcystin by MCD-bacteria in natural water. In 1996, we independently isolated a strain of MCD-bacterium, Y2, which belongs to a new genus related to *Sphingomonas*, from eutrophic Lake Suwa in central Honshu, Japan [37]. In this study, we examined the process of degradation of microcystin by bacteria in the natural environment, focusing on the population dynamics of MCD-bacteria.

Degradation of microcystin produced in *Microcystis* occurs during the 2-fold decomposition process of *Microcystis*: (a) the cell decays by autolysis, grazing by protozoa, zooplankton and fish [13], or viral [27] or bacterial activity [15, 46], and (b) the cellular products are then degraded by bacterial consortia. Daft et al. [15] showed that a myxobacterium isolated from fresh water was able to lyse *Microcystis*. Yamamoto et al. [46]

indicated that some bacteria of the genera *Alcaligenes* and *Pseudomonas* and the *Cytophaga/Flavobacterium* group also lysed *Microcystis*. Because these findings concerning cell decomposition of *Microcystis* were obtained by culture-dependent techniques, our knowledge is limited to the function of culturable bacteria. However, numerous non-culturable bacteria exist in the natural environment, exceeding 99% of the total bacterial count in various systems [11]. To complement this gap, the fluorescence *in situ* hybridization method (FISH, [2, 16]) was recently developed to identify individual cells, independent of culturability, at levels ranging from species to domain by using oligonucleotide probes specific to the 16S or 23S rRNA sequence. Modified FISH methods [12, 19, 25, 26, 34] have also been developed to visualize the metabolic function of a specific bacterial group.

The aim of this study is to elucidate the process of the degradation of microcystin by MCD-bacteria under the possible coexistence with various other bacteria with varying functions during the bloom of *Microcystis* in Lake Suwa. Based on the FISH method, we designed an oligonucleotide probe specific to the 16S rRNA sequence of strain Y2, and applied several probes specific to phylogenetic groups to examine a structural changes in the associated bacterial community with *Microcystis* colonies during the periods of a *Microcystis* bloom and its decline.

Materials and methods

Sampling of heterotrophic bacteria associated with Microcystis colony

Lake Suwa, located in Nagano Prefecture in Japan, is a eutrophic, temperate, shallow lake. The lake has a surface area of 13.3 km^2, a maximum depth of 6.4 m, and an average depth of 5 m.

Samples of heterotrophic bacteria associated with *Microcystis* colonies were collected from the surface water of the center of the lake during *Microcystis* blooms on 26 August, 9 September, and 7 October 1998, and on 11 August, 25 August, 8 September, and 6 October 1999. As the biomass of *Microcystis* was significantly lower in 1999, we slightly changed the collection procedure slightly. In 1999, water samples were prescreened with a nylon net with a mesh size of 40 μm and were kept in a glass bottle for a few hours. The floating *Microcystis* colonies and associated bacteria were then collected using a pipette. The isolated *Microcystis* colonies were filtered through a 3-μm-pore-size Nuclepore filter, and washed 3 to 5 times with phosphate-buffered saline (pH 7.2, Dulbecco's

PBS(–), Nissui, Japan) to eliminate any free-living bacteria. The washed colonies were sonicated 3 times (BRANSONIC B1200, 45 kHz, 30 W, 3 s mL^{-1}) to disrupt them and disperse the heterotrophic bacterial cells and *Microcystis* cells.

Fixation of heterotrophic bacteria for total count and fluorescence in situ hybridization

Heterotrophic bacterial samples for total bacterial count and FISH were fixed in paraformaldehyde solution (pH 7.4, final concentration 3%) for up to 24 h at 4 C. To avoid destruction of the *Microcystis* colonies in 1998 or the *Microcystis* cells and bacterial cells in 1999, fixed samples for FISH were filtered gently on a 0.22-μm Nuclepore filter (25 mm diameter) [23]. Cells on the filter were rinsed 3 times with PBS and dehydrated in 1 mL of 50%, 80%, and 99% ethanol for 3 min each, and the filter was then air-dried. Filters were stored at –20 C until hybridization.

Total count of associated bacteria

Fixed samples were stained with 4′,6′-diamidino-2-phenylindole (DAPI, final concentration 0.01 μg/mL [39]) and filtered gently on a 0.22-μm Nuclepore filter (25 mm diameter). A total of more than 1000 bacterial cells were enumerated by epifluorescence microscopy (universal epifluorescence microscopic system BX50-FLA, Olympus, Japan).

Whole-cell in situ hybridization

The 16S rRNA targeted oligonucleotide probes used in this study are shown in Table 1. They comprised a domain-specific probe for Bacteria (EUB338), phylogenetic-group-specific probes for α-, β-, γ-, and δ-Proteobacteria (ALF1b, BET42a, GAM42a, DEL) and the *Cytophaga/Flavobacterium* group (CF319a), and an MCD-probe designed specifically for microcystin-degrading bacteria ("strain Y2" in this study). These probes were labeled with rhodamine obtained from Takara Biotechnology (DALIAN), Japan.

Hybridization stringency was adjusted by varying the concentration of formamide in the hybridization buffer and NaCl in the washing solution. Hybridizations were performed at 46 C for 90 min on filters placed on slides

coated with gelatin, with hybridization buffer containing 0.9 M NaCl, 20 mM Tris•HCl (pH 7.4), 0.01% SDS, formamide (20% for EUB338, ALF1b, CF319a, and MCD, 35% for BET42a, GAM42a and DEL), and 5 ng μL $^{-1}$ of the respective labeled probe. Each filter was washed at 48 C for 15 min in pre-warmed washing buffer containing NaCl (0.225 M for EUB338, ALF1b, and MCD, 0.080 M for BET42a, GAM42a and DEL), 20 mM Tris•HCl (pH 7.4), 5 mM EDTA, and 0.01% SDS, rinsed with distilled water, and air-dried. The preparations, which were counterstained with 0.1 μg mL^{-1} DAPI [45] on glass slides for 5 min, were observed under a universal epifluorescence microscopic system, BX50-FLA with a 3CCD camera (C5810, Hamamatsu Photonics, Japan) with an image analysis system (SP500F, Olympus). More than 500 DAPI-stained bacterial cells were counted to determine the proportion of the probe-specific-labeled cells among the total of the associated bacteria.

The accuracy of the MCD-probe had previously been examined by using sequences of the 16S rRNA gene obtained from a GenBank: the MCD-probe was found to contain 1 mismatch for the 2 other known sequences of the 16S rRNA gene in the database; both sequences had been isolated from marine oligotrophic bacteria (AB021704, AB022713). These bacteria, which were obtained courtesy of Dr. I. Yoshinaga of Kyoto University, were not succeeded in hybridization of the designed MCD-probe with various condition of hybridization adjusted by concentration of formamide and temperature. There exist some arguments about the binding strength between the sequences of the designed probe and the target position of 16S rRNA. According to the reports of Fuchs et al. [17], the 16S rRNA position of the MCD-probe was not easily hybridized. However, we confirmed that Y2 strain was successfully visualized by using MCD-probe.

Determination of concentrations of Microcystis cells, chlorophyll a, and total cell-bound and extracellular microcystin in the lake

To determine the concentrations of *Microcystis*, chlorophyll *a*, and cell-bound and extracellular microcystin, surface water samples were collected from the center of the lake once two weeks between April and December in 1998 and 1999. Samples for cell counting of *Microcystis* were fixed in formaldehyde solution (final concentration, 1.5 % w/v). The concentration of *Microcystis* cells was estimated by using a Fuchs-Rosenthal hemocytometer (Kayagaki works, Japan) under a microscope (BH-2, Olympus).

To measure the chlorophyll *a* concentration, water samples were filtered through a glass fiber filter (GF/C, Whatman, UK), which was then soaked in 10 mL of methanol for 24 h at 4℃. After that, the residue was centrifuged at 3000 rpm for 15 min. Measurement of the chlorophyll *a* concentration from the supernatant was quantified spectrophotometrically by the method of Maker et al. [31].

Measurements of microcystin concentration and the clean-up of microcystin in preparation for high-performance liquid chromatography (HPLC) were carried out according to Park et al. [36]. For measurement of cell-bound microcystin, *Microcystis* cells were concentrated on a GF/C filter. The filter was then homogenized and extracted with 5% aqueous acetic acid, and, after centrifugation at 4000 rpm for 15 min, the supernatant was poured into an ODS cartridge (Bakerbond spe Octadecyl [C_{18}] 3 mL, USA). Microcystin extracted from the cartridge with 0.1% trifluoroacetic acid (TFA) – methanol was applied to the HPLC system (LC-9A S-I, Shimadzu, Japan), which was equipped with an ODS column (Cosmosil 5C18-AR 4.6 × 150 mm, Nacalai, Japan). The conditions of HPLC for analysis of microcystin were as follows: absorbance at 238 nm; methanol: 0.05 M phosphate buffer (58:42; pH 3.0) in the mobile phase; and a 1-mL-min^{-1} flow rate.

Extracellular microcystin was measured by 2 methods using a GF/C filtrate. In 1998, the GF/C filtrate sample was poured into an ODS cartridge (5 g, Chromatorex ODS, 100–200 mesh, Fuji Silysia Chemical, Kasugai, Japan). The cartridge was then rinsed with water and then with 20% methanol. Extracellular microcystin was eluted from the cartridge with 90% methanol and evaporated to dryness. After a silica gel cartridge (2 g, SepPak) had been preconditioned with methanol, the residue was dissolved in methanol and applied to the cartridge. After the cartridge was rinsed with methanol, extracellular microcystin was eluted with 70% methanol. The eluate was evaporated to dryness, and the residue was re-dissolved in methanol. The methanol solution was then analyzed by HPLC under the same conditions as above.

In 1999, extracellular microcystin was analyzed by enzyme-linked immunosorbent assay (ELISA), which determined the total concentration of microcystins without discriminating microcystin derivatives. Nagata et al. [33] showed very similar estimates from ELISA and a liquid chromatographic method for the analysis of microcystin, although the sensitivity of the ELISA method was higher. Thus, we used the ELISA method after Nagata et al. [32, 33].

Results

Concentrations of Microcystis cells, chlorophyll a, and cell-bound and extracellular-microcystin during Microcystis blooms

Changes in the concentrations of *Microcystis*, chlorophyll *a*, and cell-bound microcystin in water are given in Figure 1. In 1998 *Microcystis* appeared in the middle of June and bloomed, the dominant species being *M. ichthyoblabe* in July, and *M. viridis* from August to October. Relative proportion of *Microcystis* biomass formed more than 99% in total phytoplankton biomass during the blooming period of *Microcystis*. The concentration of *Microcystis* cells had 2 peaks: 2.4×10^9 cells L^{-1} on 29 July, and over 7×10^8 cells L^{-1} on 9 and 25 September, after which dates it decreased (Fig. 1A-1). The chlorophyll *a* concentration also showed 2 large peaks: 662 μg L^{-1} on 29 July and over 600 μg L^{-1} on 9 and 25 September. By 7 October it had decreased markedly to 180 μg L^{-1} (Fig. 1A-2). The cell-bound microcystin concentration increased exponentially in September up to 100 μg L^{-1}, then decreased to 50 μg L^{-1} or less in October (Fig. 1A-2). Although the changes in *Microcystis* cell concentration were paralleled by those of chlorophyll *a* and cell-bound microcystin, nonparametric Spearman statistical analysis showed significance only between the concentration of *Microcystis* cells and chlorophyll *a* ($r = 0.95$, $P < 0.01$, n = 12). The concentration of cell-bound microcystin fluctuated in parallel with that of chlorophyll *a* ($r = 0.81$, $P < 0.01$, n = 13). The extracellular microcystin concentration in the water was less than 0.5 μg L^{-1} throughout the 1998 study period (Table 2) – similar to the findings of Park et al. [36]. The concentration of extracellular microcystin was the highest on 29 July; by 3 December it had decreased to below the detection limit. Although its fluctuation was similar in pattern to that of the *Microcystis* cell concentration, no significant relationship between the two was detected by nonparametric Spearman statistical analysis.

In contrast to 1998, a minor bloom of *Microcystis* was observed in September 1999, showing that relative proportion of *Microcystis* biomass formed more than 99% in total phytoplankton biomass. It consisted of *M. ichthyoblabe*, *M. aeruginosa*, *M. novacekii*, and *M. wesenbergii*. However, none of those strains dominated strikingly. The abundance of *Microcystis* was 1 order of magnitude lower than that in 1998, ranging from 4.3×10^6 to 6.8×10^7 cells L^{-1} (Fig. 1B-1). The concentrations of chlorophyll *a* and cell-bound microcystin from 11 August to 6 October in 1999 ranged from 16 to 48 μg L^{-1} and from 0.54 to 4.49 μg L^{-1}, respectively (Fig. 1B-2).

Cell-bound microcystin was not analyzed between April and July, as the biomass of *Microcystis* was insignificant. The total concentration of extracellular microcystin in the water was less than 0.2 μg L^{-1} throughout the observation period (Table 2). Although the changes in the concentrations of cell-bound microcystin and extracellular microcystin appeared to parallel changes in the *Microcystis* cell concentration, nonparametric Spearman statistical analysis failed to show any significant relationships.

Population dynamics of bacteria associated with Microcystis

The density of bacteria associated with the *Microcystis* colonies ranged from 7.2×10^6 to 8.5×10^7 cells mL^{-1} of lake water in 1998 and from 1.1×10^4 to 3.2×10^5 cells mL^{-1} in 1999 (Table 3). The number of bacterial cells associated with each *Microcystis* cell increased from 25 on 26 August to 118 on 9 September 1998, but did not change markedly in 1999, ranging from 2.6 to 4.7 cells. The highest concentration of associated bacteria in the lake water was found on 9 September 1998 and on 8 September 1999, reaching 8.5×10^7 and 3.2×10^5 cells mL^{-1}, respectively. Whereas associated bacteria constituted 95% of the total bacteria in the water on 9 September 1998, associated bacteria in the 1999 study period constituted only 0.2% to 3.3% of the total bacteria. The difference in the concentration of associated bacteria between 1998 and 1999 was ascribed to the difference in the species composition of *Microcystis*.

Changes in community structure of associated bacteria

The community structure of bacteria associated with *Microcystis* was expressed on the basis of the number of each bacterial species, as determined by rRNA-targeted oligonucleotide probes, per 10^2 cells of *Microcystis* (Fig. 2), because an average colony of *Microcystis* in Lake Suwa consisted of at least 10^2 cells, although it fluctuated ranging from 127 to 529 cells. In 1998, the concentration of domain Bacteria visualized with the probe EUB338 ranged from 1.4×10^3 to 7.9×10^3 cells per 10^2 *Microcystis* cells, and the Bacteria were composed of between 56% and 69% DAPI-stained particles (data not shown). α-Proteobacteria were the second most common after β-Proteobacteria on 9 September and 7 October, increasing from 2.6×10^2 (26 August) to 2.0×10^3 (7 October) cells per 10^2 *Microcystis* cells (18% to 26% of the sum of the bacteria hybridized by the oligonucleotide probes

specific to α-, β-, γ-, and δ--Proteobacteria and the *Cytophaga/Flavobacterium* group) (Fig. 2A-1). β-Proteobacteria were dominant during the blooming of *Microcystis,* ranging from 5.1×10^2 to 2.7×10^3 cells per 10^2 *Microcystis* cells (28% to 36%). γ-Proteobacteria increased from 3.8×10^2 (26 August) to 1.3×10^3 (7 October) cells per 10^2 *Microcystis* cells (17% to 27%). δ-Proteobacteria made up the lowest percentage of the sum of bacteria hybridized with group-specific probes on 26 August, 9 September, and 7 October (5.3% to 13%). However, this group increased from only 75 (26 August) to 9.9×10^2 (9 September) cells per 10^2 *Microcystis* cells. This increase was about 2- to 4-fold higher than that of other groups in the period from August to September. The concentration of the *Cytophaga/Flavobacterium* group increased from 1.9×10^2 (26 August) to 1.5×10^3 (7 October) cells per 10^2 *Microcystis* cells (13% to 20%).

In 1999, the concentrations of domain Bacteria visualized with EUB338 ranged from 3.1×10^2 to 3.9×10^2 cells per 10^2 *Microcystis* cells, and the Bacteria were composed of between 78% and 94% DAPI-stained particles (data not shown). These numbers were higher than those in 1998, although the density of associated bacteria in 1999 was about 100 times less than in 1998. α-Proteobacteria existed at concentrations of between 1.2×10^2 cells and 1.4×10^2 cells per 10^2 *Microcystis* cells throughout the study period (Fig. 2B-1), and predominated in all samples, accounting for at least 30% of the sum of bacteria hybridized with group-specific probes. β-Proteobacteria also remained nearly constant at 1.0×10^2 cells per 10^2 *Microcystis* cells during the study period, constituting from 26% to 31% of the sum of bacteria hybridized with group-specific probes – a similar percentage to that in 1998. γ-Proteobacteria ranged from 49 to 57 cells per 10^2 *Microcystis* cells (13% to 18%). The densities of these 3 phylogenetic groups were similar to each other during the 1999 observations. δ-Proteobacteria and the *Cytophaga/Flavobacterium* group showed similar fluctuations with regard to relative abundance and cell density: both parameters for both groups had increased by 8 September and decreased by 6 October. The number of δ-Proteobacteria ranged from 11 to 50 cells per 10^2 *Microcystis* cells, reaching a peak on 8 September. This group made up only 3.5% to 13% of the sum of bacteria hybridized with group-specific probes. The *Cytophaga/Flavobacterium* group ranged from 13 to 69 cells per 10^2 *Microcystis* cells (4.0% to 18%), reaching a maximum on 8 September.

MCD-bacteria had increased remarkably in both relative abundance and density of associated bacteria per 10^2

Microcystis cells by 9 September 1998 (Fig. 2A-2). MCD-bacteria increased remarkably from 93 cells to 1.3 × 10^3 cells per 10^2 *Microcystis* cells between 26 August and 9 September, and by 7 October had decreased to 7.8 × 10^2 cells per 10^2 *Microcystis* cells, thus accounting for 6.6% to 17% of the total bacteria hybridized with group-specific probes. Their relative abundance among α-Proteobacteria was also high, ranging from 36% to 76% (data not shown). In 1999, MCD-bacteria ranged from 20 to 41 cells per 10^2 *Microcystis* cells and showed a tendency to increase in numbers in September, accounting for 6.1% to 11% of the sum of bacteria hybridized with group-specific probes (Fig. 2B-2). The relative abundance of MCD-bacteria among α-Proteobacteria was not as high as in 1998, ranging from 17% to 34%. The highest proportions of MCD-bacteria, in relation to both the sum of bacteria hybridized with group-specific probes and α-Proteobacteria, were observed on 8 September. These results from 1998 and 1999 indicated that the number of MCD-bacteria associated with 10^2 *Microcystis* cells increased in September and then decreased in October, closely paralleling changes in the microcystin concentration in both the water ($r = 1$, $P < 0.01$, n = 6) and *Microcystis* cells ($r = 0.89$, $P < 0.05$, n = 6).

Discussion

Strains of several species of the genus *Microcystis* produce 60 variants of hepatotoxic microcystin [38]. *M. viridis* is 1 such toxic species in Lake Suwa [35, 36]. The highest concentration of cell-bound microcystin was observed when *M. viridis* was dominant in September 1998.

Microcystis is surrounded by mucilage, which consists mainly of polysaccharide [3, 5] composed of glucose, mannose, fucose, xylose, galactose, and rhamnose. The thickness and solubility of the mucilage vary among *Microcystis* species [4, 24]: the mucilage of *M. viridis* is harder to dissolve in water than those of the other species [4]. During blooms, numerous bacteria are known to exist in the mucilage [9]. Furthermore, the abundance and community structure of the embedded bacteria might differ according to the *Microcystis* species.

When microcystin is released from a cell of *Microcystis,* it is trapped in the mucilage because of the mucilage's high viscosity. To reveal the process of degradation of microcystin, we therefore focused on the function of the bacteria embedded in the mucilage and tried to describe the population dynamics of the MCD-bacteria there. Our results revealed that the number of MCD-bacteria in the mucilage increased in September of both years and

correlated with the concentration of cell-bound microcystin, and the highest concentration of MCD-bacteria existed in 1998 when *M. viridis* was dominant. It is remarkable in natural systems that 1 gene specific clone of MCD-bacteria detected by FISH made up 1/10 of the whole bacterial community in September 1998 and 1999. These results suggest that MCD-bacteria responded to changes in the concentration of microcystin, and that MCD-bacteria were active in the mucilage of *Microcystis* when produced microcystin was present there. Jones et al. [22] reported that microcystin-degrading isolates require a lag time in the degradation of microcystin when they have not been exposed to microcystin *a priori* under experimental conditions. However, we assumed that MCD-bacteria in the mucilage were on 'stand-by' until the degradation of microcystin occurred: they could be directly exposed to any microcystin released from cells in the *Microcystis* colony. This suggests that MCD-bacteria could thus initiate the degradation of microcystin in the mucilage within 2 weeks so far we examined. These findings can explain why a bacterial species becomes predominant in a given system if it exerts a very specific function to degrade a specific compound, such as microcystin.

During the bloom of *Microcystis*, the concentrations of the *Cytophaga/Flavobacterium* group and δ-Proteobacteria were apparently synchronized with that of the *Microcystis* cells, with $r = 0.89$ ($P < 0.05$, n = 6) and $r = 0.94$ ($P < 0.05$, n = 6), respectively. To our knowledge, members of the *Cytophaga/Flavobacterium* group are able to degrade not only macromolecular compounds [12, 43], but also *Microcystis* cells [46]. Van Hannenn [43] suggested that Cytophagales, the related 16S rRNA sequence of which appeared in denaturing gradient gel electrophoresis after the lysis of cyanobacteria, could contribute to degradation of dissolved organic matter (DOM) released from this lysis. Recently, Cottrell and Kirchman [12] suggested from fluorescence *in situ* hybridization (MICRO-FISH) studies that the mode of bacterial utilization of DOM differs among phylogenetic groups: the *Cytophaga/Flavobacterium* group tends to prefer high-molecular-weight DOM such as proteins and chitin. Yamamoto et al. [46] showed by a culture-dependent method that some *Microcystis* were lysed specifically by some strains of this group isolated from the surface waters of Lake Suwa. These findings suggest that the *Cytophaga/Flavobacterium* group contributes to the lysis of *Microcystis* and degrades DOM derived from intracellular products of *Microcystis* in the mucilage. Grilli Caiola et al. [18] reported that *Bdellovibrio*-like bacteria, constituents of the δ-Proteobacteria, infect *Microcystis* cells and degrade peptidoglycan and the cell wall, although

Bdellovibrio is known to be a bacterial predator [6]. This suggests that δ-Proteobacteria might contribute to the lysis of *Microcystis*.

α-Proteobacteria and β-Proteobacteria tended to dominate in the mucilage of *Microcystis* during the bloom of *Microcystis*. Of the α-Proteobacteria, *Caulobacter* can attach to cyanobacteria and take up exudates of photosynthetic products [42]. *Alcaligenes* and *Pseudomonas*, which are β-Proteobacteria, are known to lyse *Microcystis* cells by attaching to them [28, 46].

We studied the process of degradation of microcystin in the light of changes in bacterial community structure in a natural environment, focusing particularly on strain Y2 of MCD-bacteria, which belongs to an undescribed genus [37]. We found that MCD-bacteria existed in a restricted space of the mucilage of *Microcystis,* and that the change in concentration of these bacteria was synchronized with the increase in the concentration of cell-bound microcystin. This suggests that MCD-bacteria in the mucilage responded to changes in the concentration of cell-bound microcystin; the microcystin was exuded from the cell of toxic *Microcystis* and degraded by the bacteria. The *Cytophaga/Flavobacterium* group and δ-Proteobacteria also changed their population densities in the mucilage, suggesting that they contributed to the degradation of *Microcystis* cells. Mucilage is revealed not only as a compound that binds *Microcystis* cells together, but also as a habitat for bacteria that exert their specific function to utilize and thus degrade *Microcystis* cellular materials.

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Table 1. Probe sequences and target sites

Probe name	Target organism	Sequence	Target site[a] rRNA Position	Reference
EUB338 :	domain Bacteria	5'-GCTGCCTCCCGTAGGAG T-3',	16S, 338-355	1
ALF1b :	a-Proteobacteria	5'-CGTTCG(C/T)TCTGAGCCAG-3'	16S, 19-35	29
BET42a :	b-Proteobacteria	5'-GCCTCCCCACTTCGTTT-3'	23S, 1027-1043	29
GAM42a :	g-Proteobacteria	5'-GCCTCCCCACATCGTTT-3'	23S, 1027-1043	29
DEL :	d-Proteobacteria	5'-CGGCGTCGCTGCGTCAGG-3'	16S, 385-402	1
CF319a :	**Cytophaga/Flavobacterium**group	5'-TGGTCCGTGTCTCAGTAC-3'	16S, 319-336	30
MCD :	MCD-bacteria	5'-CGCCACCAAAGCC TAAAAGG-3'	16S, 839-858	This study

[a] **Escherichia coli** numbering. [7]

Table 2. Concentration of extracellular microcystin in Lake Suwa, 1998 and 1999

Year	Date	extracellular microcystin concentration (μ g L^{-1})
1998[a]	Jun. 17	0.05
	Jul.29	0.47
	Aug. 26	0.24
	Sept. 25	0.17
	Oct. 21	0.09
	Dec. 3	N.D.[c]
1999[b]	May.20	0.09
	Jul. 28	0.03
	Aug. 11	0.04
	Aug. 25	0.10
	Sept. 8	0.12
	Sept. 22	0.08
	Oct. 6	0.06

[a]Microcystin determined with HPLC. Sum of microcystin-LR and -RR.

[b]Microcystin determined with ELISA.

[c]N.D., microcystin not detected.

Table 3. Number of **Microcystis** cells, free-living bacteria and bacteria associated with colonies of **Microcystis**

	Concentration of **Microcystis** cells (cells mL^{-1})	Associated bacteria (cells mL^{-1})[a]	Associated bacteria (cells **Microcystis** cell^{-1})	Free-living bacteria (cells mL^{-1})	Ratio of associated bacteria to total bacteria (%)
1998. Aug. 26	2.9× 10^{5}	7.2× 10^{6} ± 1.1× 10^{7}	25.1 ± 36.8	4.6× 10^{6} ± 1.1× 10^{6}	61
Sept. 9	7.4× 10^{5}	8.5× 10^{7} ± 1.8× 10^{8}	116 ± 248	4.3× 10^{6} ± 1.1× 10^{6}	95
Oct. 7	2.2× 10^{5}	2.6× 10^{7} ± 4.2× 10^{7}	118 ± 193	3.9× 10^{6} ± 9.2× 10^{5}	87
1999. Aug. 11	4.3× 10^{3}	1.1× 10^{4} ± 3.0× 10^{3}	2.6 ± 0.7	7.5× 10^{6} ± 1.5× 10^{6}	0.2
Aug. 25	3.9× 10^{4}	1.5× 10^{5} ± 7.7× 10^{4}	4.0 ± 2.0	7.8× 10^{6} ± 2.0× 10^{6}	1.9
Sept. 8	6.8× 10^{4}	3.2× 10^{5} ± 1.0× 10^{5}	4.7 ± 1.5	9.4× 10^{6} ± 1.5× 10^{6}	3.3
Oct. 6	1.1× 10^{4}	3.8× 10^{4} ± 1.2× 10^{4}	3.5 ± 1.1	5.7× 10^{6} ± 1.2× 10^{6}	0.7

[a] **Microcystis** cells per mL of lake water bacteria associated with **Microcystis** per **Microcystis** cell

Legends

Figure 1

Seasonal changes in the concentrations of *Microcystis* cells, chlorophyll *a*, and cell-bound microcystin at the center of Lake Suwa in 1998 (A) and 1999(B). A-1 and B-1 show the concentrations of *Microcystis* cells (triangles). A-2 and B-2 show the concentrations of chlorophyll *a* (open circles) and cell-bound microcystin (closed circles).

Figure 2

The composition of the bacterial assemblages in *Microcystis* colonies, as detected by rRNA-targeted oligonucleotide probes specific for α-, β-, γ-, and δ-Proteobacteria, the *Cytophaga/Flavobacterium* group, and microcystin-degrading bacteria. Samples were collected from the surface waters of Lake Suwa on 26 August, 9 September, and 7 October 1998 (A), and on 25 August, 8 September, and 6 October 1999 (B), when *Microcystis* was in bloom.

Figure 3

In situ hybridization of bacteria associated with colonies of *Microcystis*, viewed by epifluorescence microscopy. Bacteria in the colony of *Microcystis* stained by DAPI (A), and hybridized with a rhodamine-labeled probe specific to microcystin-degrading bacteria (B). Particles about 5 μm in diameter are cells of *Microcystis*.

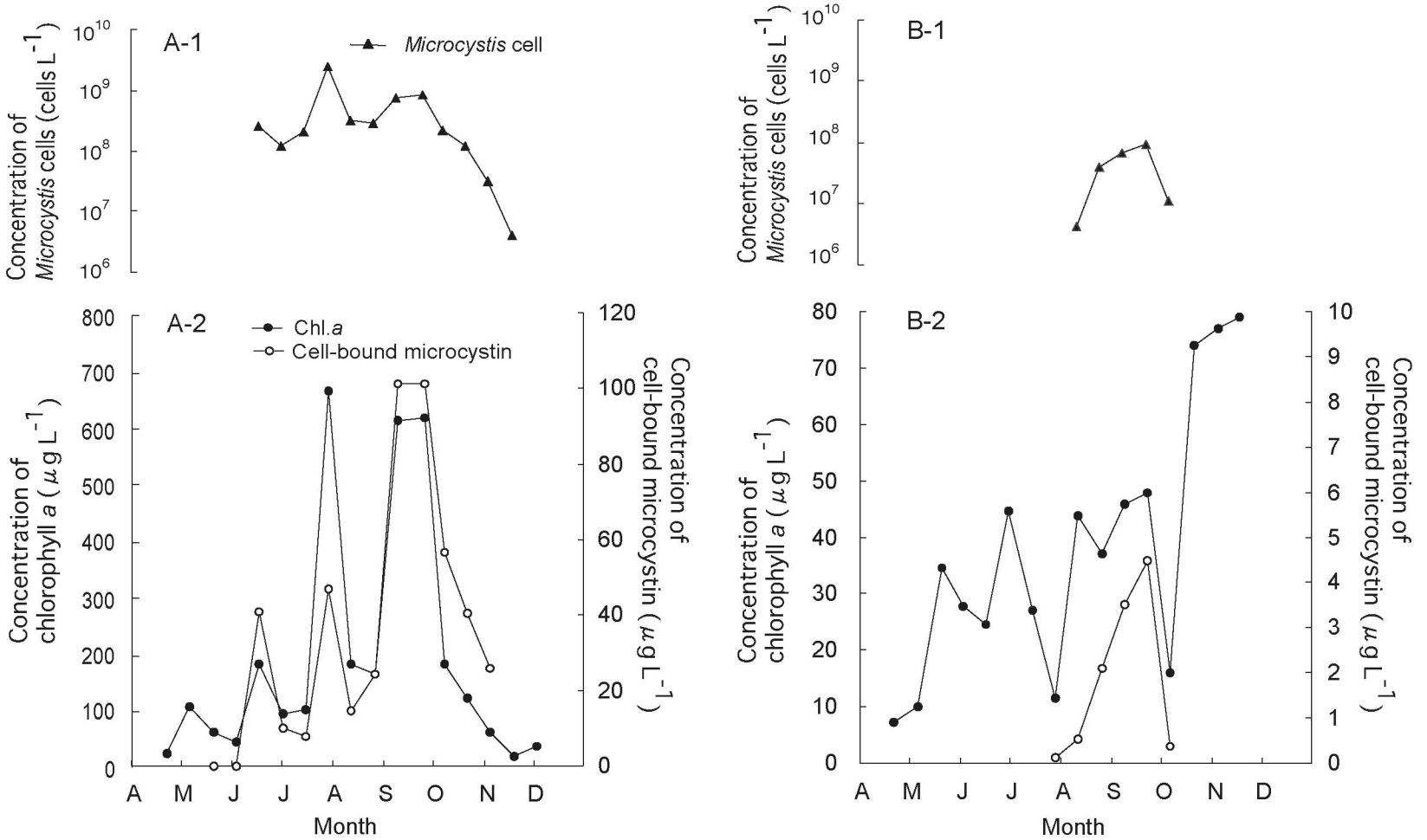
A-1
Microcystis cell
Concentration of Microcystis cells (cells L-1)
A-2
Chl.a
Cell-bound microcystin
Concentration of chlorophyll a (μg L-1)
Concentration of cell-bound microcystin (μg L-1)
Month
A M J J A S O N D
B-1
B-2

Fig. 1

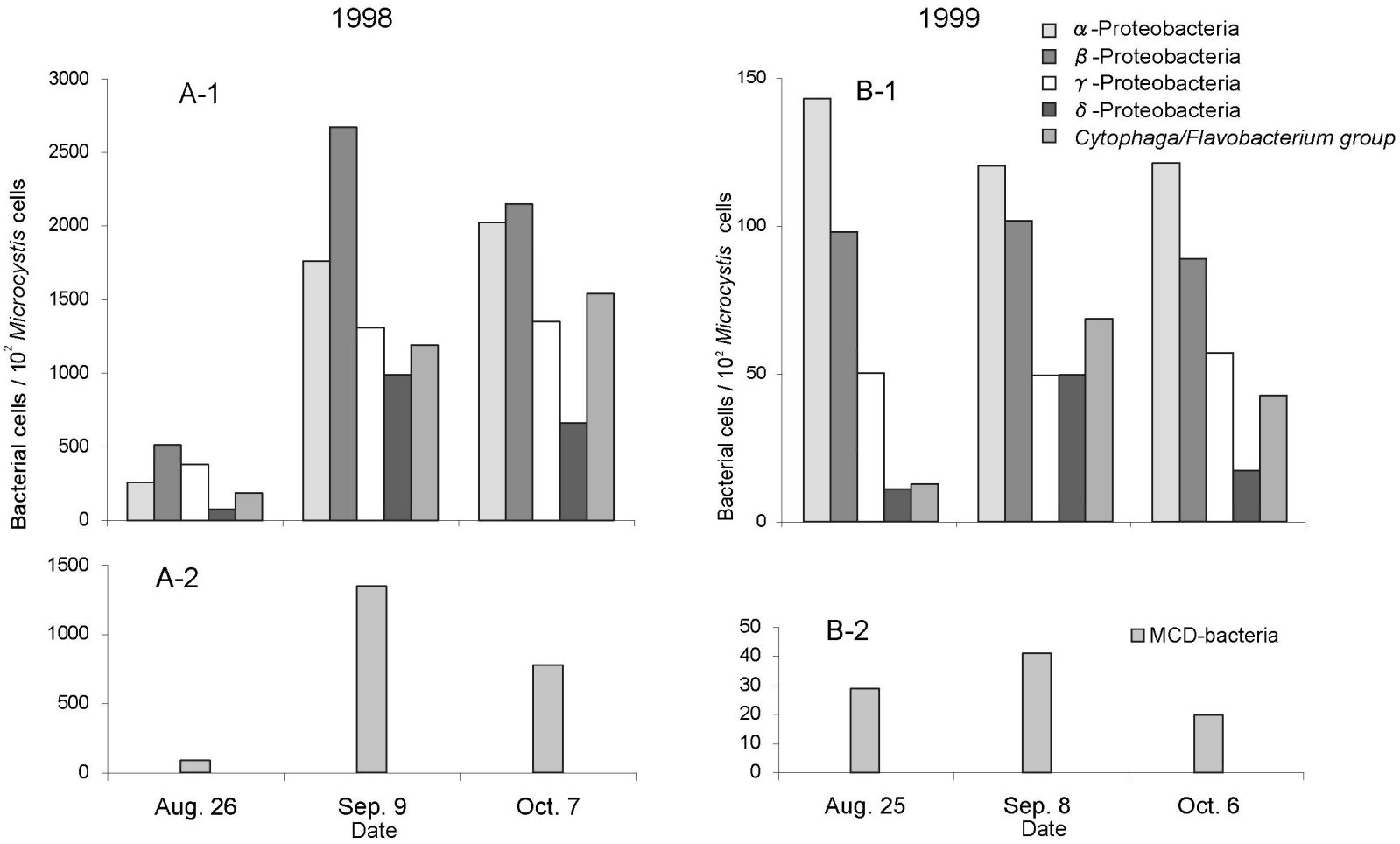
1998
1999
A-1
B-1
A-2
B-2
α -Proteobacteria
β -Proteobacteria
γ -Proteobacteria
δ -Proteobacteria
Cytophaga/Flavobacterium group
MCD-bacteria
Bacterial cells / 10² Microcystis cells
Bacterial cells / 10² Microcystis cells
3000
2500
2000
1500
1000
500
0
150
100
50
0
1500
1000
500
0
50
40
30
20
10
0
Aug. 26
Sep. 9
Oct. 7
Aug. 25
Sep. 8
Oct. 6
Date
Date

Fig.2

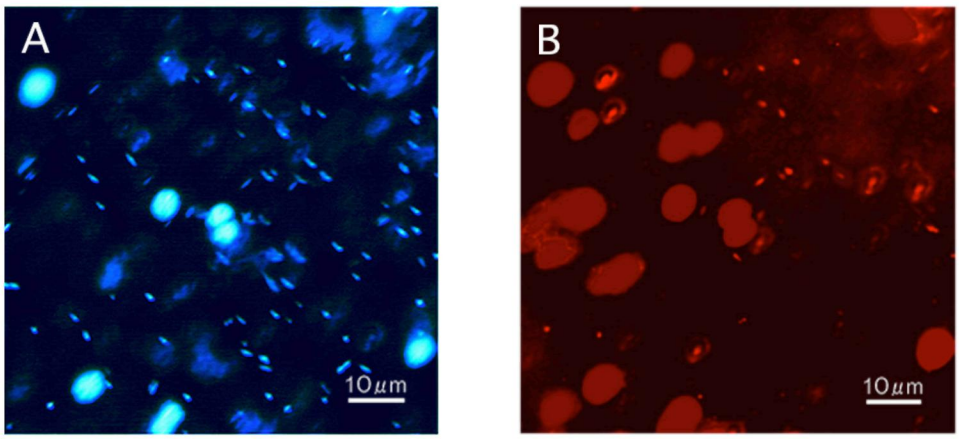

Fig. 3