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Population Dynamics of Free-Living Bacteria Related to the Microcystin-Degrading Strain Y2 in Lake Suwa and in Microcystin Amended Enrichments

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We investigated the population dynamics of the free-living microcystin-degrading isolate Y2 (MCD-isolate) in an eutrophic lake (Lake Suwa, Japan) using fluorescence *in situ* hybridization (FISH) with a specific probe. Free-living MCD-isolate was successfully visualized by direct viable count method combined with FISH analysis. The highest concentration of MCD-isolate existed in 1999 when high concentrations of dissolved microcystin (Mcyst) and *Microcystis* populations were present. Mcyst degradation experiments with free-living bacteria collected at different times during a *Microcystis* bloom in 2000 indicated that three samples of free-living bacteria completely degraded Mcyst-LR and its isomer. The lag time before the degradation was however different for the free-living bacteria. Free-living bacteria present during the mid-bloom of *Microcystis* completely degraded Mcysts with the shortest lag time. The number of free-living cells of MCD-isolate detected by the FISH method significantly increased when Mcyst was degraded with the fastest degradation rate in the mid-bloom sample. Other bacterial populations collected at mid- and late-bloom increased during the experiments with the exception of the δ-Proteobacteria. However, the community structure was stable. Our findings suggest that MCD-isolate exists with various bacterial consortia in water and degrades Mcysts, the function of which is considered to be induced by exposure to Mcyst.

Key words: *Microcystis*, microcystin, microbial degradation, free-living microcystin-degrading isolate, Fluorescence in situ hybridization

Hepatotoxin microcystin (Mcyst) produced by several cyanobacteria of the genera *Microcystis, Anabaena, Nostoc* and *Oscillatoria* causes serious disease in humans and animals[17,25]. Mcyst is a cyclic heptapeptide consisting of 7 amino acids, which consists of two unusual amino acids: 3-amino-9-methoxy-10-phenyl-2,6,8,-trimethyldeca-4,6-dienomic acid (Adda) and N-methyldehydroalanine, three D-amino acids: alanine, D-erythro-β-methylaspartic acid and r-linked glutamic acid and two variable L-amino acids. The type of Mcyst depends on the variation of two L-amino acids, e.g., leucine and arginine (Mcyst-LR), and the individual structural variations in all amino acids. Mcyst is known to be degraded in natural environments[12,19,22,32], although it is not easily degraded by chemical treatment[16]. We isolated a single strain of the microcystin-degrading bacterium, strain Y2 (AB084247)[30], from the eutrophic Lake Suwa, Japan. This isolate effectively degraded Mcyst *in vitro*[30]. The results of a 16S rDNA sequence analysis of strain Y2 indicated that it belonged to α-Proteobacteria and was related to the genus *Sphingomonas*[30], which includes another Mcyst-degrading bacterium, strain MJ-PV[18], isolated from a river in Australia.

Our previous study[28] described the population dynamics

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of the microcystin-degrading isolate strain Y2 (MCD-isolate) which was detected with a specific probe and the bacterial community structure in the mucilage of *Microcystis* during a bloom, using the fluorescence in situ hybridization (FISH) method. It was found that MCD-isolate existed in the mucilage of *Microcystis* and that its concentration was significantly synchronized with the increase in the concentration of cell-bound Mcyst. Population levels of the *Cytophaga/Flavobacterium* group and δ-Proteobacteria, which can degrade macromolecules in the structure of *Microcystis* cells[6,11,14,35,36], have also been shown to be parallel to changes in the concentration of *Microcystis* concentration in mucilage. This led us to suggest that MCD-isolate associated with *Microcystis* colonies can respond to concentration changes of cell-bound Mcyst, which is released from lysed *Microcystis* cells as a result of the activity of the *Cytophaga/Flavobacterium* group and δ-Proteobacteria.

Kenefick *et al.*[22] and Jones and Orr[19] reported that Mcyst was degraded in water, after its release from *Microcystis* cells treated with copper sulfate, an algicide. In addition, Rapala *et al.*[32] and Cousins *et al.*[12] showed that Mcyst-LR was degraded in a water sample taken from a reservoir during a *Microcystis* bloom. By contrast, our previous study[28] revealed specific degradation of Mcyst by MCD-isolate among various functional bacteria in the mucilage of *Microcystis*. Although the concentration of dissolved Mcyst was low, it seemed to be synchronized with the abundance of *Microcystis* cells in Lake Suwa, both in 1998 and 1999[28] (Fig. 1). We thus assumed that dissolved Mcyst was degraded by free-living MCD-isolate.

Free-living bacteria markedly differ from the bacteria associated with particles[1,2,13] in that their cells are smaller, rate of growth is slower, and affinity for dissolved nutrients is higher than those of the associated bacteria as shown by a low Km+Sn value (Km and Sn represent the half-saturation constant and the endogenous substance constant, respectively). We assumed that free-living MCD-isolate efficiently degraded dissolved Mcyst, as a result of their high affinity for dissolved Mcyst[28].

In the present study, we focused on the degradation of Mcyst by free-living MCD-isolate under the possible coexistence with various other bacteria in water. We tried to elucidate 1) the population dynamics of free-living MCD-isolate and 2) the process of degradation of Mcyst by free-living bacteria collected at different periods in the blooming of *Microcystis*. We employed the direct viable count (DVC) method[24] combined with FISH analysis (DVC-FISH)[29] to enhance the detectability of specific bacteria, because MCD-isolate was not detected in 1999 by FISH analysis, probably because of the low concentration of rRNA.

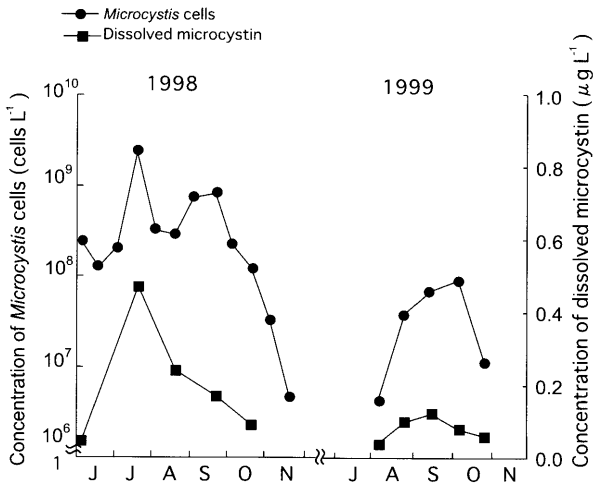

Fig. 1. Changes in the concentration of *Microcystis* cells (closed circles) and dissolved microcystin (closed squares) at the center of Lake Suwa in 1998 and 1999.

Materials and Methods

Sampling of free-living bacteria

Lake Suwa, located in the 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 free-living bacteria were collected from the surface at the center of the lake on May 20$_{th}$, August 26$_{th}$, September 9$_{th}$, October 7$_{th}$, and October 21$_{st}$ in 1998, on May 20$_{th}$, August 11$_{th}$, August 25$_{th}$, September 8$_{th}$, and October 6$_{th}$ in 1999, and on August 2$_{nd}$, August 30$_{th}$, and September 27$_{th}$ in 2000. Free-living bacteria were obtained by filtering the water samples with a 3 μm pore size Nuclepore filter (Whatman Ltd., UK) to eliminate *Microcystis* cells and particle-associated bacteria. The concentrations of chlorophyll *a*, cell-bound Mcyst, dissolved Mcyst, and *Microcystis* cells from the 1998 and 1999 sampling have been previously reported[28].

Fixation method for total count, direct viable count and fluorescence in situ hybridization

Bacterial samples used for total free-living bacterial count, direct viable count and FISH analysis were fixed with a paraformaldehyde solution (pH 7.4, 3% final concentration) for up to 24 hours at 4°C. Fixed samples for FISH analysis were gently filtered with a 0.22 μm pore size Nuclepore filter (25 mmϕ)[21]. After fixation, the cells present on the filters were rinsed 3 times with PBS and dehydrated

with 1 mL of 50%, 80% and 99% ethanol for 3 minutes each. The filters were then air-dried and stored at −20°C until hybridization.

Total free-living bacterial count method

The fixed samples were stained with 4',6-diamidino-2-phenylindole (DAPI, final concentration, 0.01 μg mL^{-1})[31] and more than 500 bacterial cells were enumerated by epifluorescence microscopy (a universal epifluorescence microscopic system BX50-FLA, Olympus Co., Ltd., Japan).

Whole cell in situ hybridization

The oligonucleotide probes used in this study are shown in Table 1. They comprised a domain-specific probe for domain Bacteria (EUB338), a group specific probe for α-, β-, γ-, and δ-Proteobacteria (ALF1b, BET42a, GAM42a, DEL), the *Cytophaga/Flavobacterium* group (CF319a), and the MCD-probe designed specifically for a Mcyst-degrading bacterium named "strain Y2" in our previous study[28]. These probes were labeled with rhodamine (Takara Biotechnology (DALIAN) Co., Ltd., Japan).

Hybridization stringency was adjusted by varying the concentration of formamide in the hybridization buffer and NaCl in the washing solution. Hybridization was performed on samples (filters) deposited on gelatin-coated slides at 46°C for 90 min with a 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. After the hybridization period, each filter was washed in a pre-warmed (48°C) washing buffer containing NaCl (0.225 M for EUB338, ALF1b, and MCD, 0.080 M for BET42a, GAM42a, DEL, and CF319a), 20 mM Tris·HCl (pH 7.4), 5 mM EDTA, and 0.01% SDS for 15 minutes, rinsed with distilled water and air-dried. The samples were then counterstained with 0.1 μg mL^{-1} of DAPI on glass slides for 5 minutes, and observed using a universal epifluorescence microscopic system BX50-FLA (Olympus Co., Ltd., Japan) with a 3CCD camera (C5810, Hamamatsu Photonics, Co., Ltd., Japan) with an image analysis system (SP500F, Olympus Co., Ltd., Japan). More than 500 DAPI-stained bacterial cells were counted to determine the contribution of the probe specific-labeled cells to the total number of free-living bacteria.

DVC—FISH method

To apply the DVC method[24] in combination with FISH analysis[29] to the study of the population dynamics of free-living MCD-isolate in 1999, we tried to determine the adequate concentration of nalidixic acid and yeast extract and the proper incubation period. Water samples were collected on May 20th 1999 and pre-filtered through a 3 μm pore size Nuclepore filter to collect free-living bacteria. The samples (100 mL) were incubated in the dark at 20°C with 0.001%, 0.002% and 0.003% nalidixic acid containing 0.0025% or 0.025% yeast extract. Samples were collected after different incubation periods, i.e., 0, 6, 12, 15, 18 and 21 hours, and then fixed with a paraformaldehyde solution as described before. In order to determine the effect of nalidixic acid on cell division, total bacterial counts were estimated by staining the fixed samples with acridine orange (AO; final concentration of 0.01 μg mL^{-1}). More than 500 bacterial cells were enumerated by epifluorescence microscopy (BH2, Olympus Co., Ltd., Japan). The concentration of free-living bacteria appeared to be inhibited by 0.003% nalidixic acid until 15 hours, which is longer than what has been previously reported by Kogure *et al.*[24]. Their study indicated that suitable conditions for DVC were a 6-hour incubation period at 20°C in the dark with 0.002% nalidixic acid and 0.025% yeast extract. On the other hand, Kalmbach *et al.*[20] used a higher concentration of pipemidic acid (i.e., 0.003%) in order to prevent cell division and incubated for 16 hours.

Table 1. Probe sequences and target sites

Probe name	Target organism	Sequence	Target site[a] rRNA Position	Reference
EUB338:	domain Bacteria	5'-GCTGCCTCCCGTAGGAGT-3'	16S, 338–355	4
ALF1b:	α-Proteobacteria	5'-CGTTCG(C/T)TCTGAGCCAG-3'	16S, 19–35	26
BET42a:	β-Proteobacteria	5'-GCCTTCCCACTTCGTTT-3'	23S, 1027–1043	26
GAM42a:	γ-Proteobacteria	5'-GCCTTCCCACATCGTTT-3'	23S, 1027–1043	26
DEL:	δ-Proteobacteria	5'-CGGCGTCGCTGCGTCAGG-3'	16S, 385–402	4
CF319a:	*Cytophaga/Flavobacterium* group	5'-TGGTCCGTGTCTCAGTAC-3'	16S, 319–336	3, 27
MCD:	MCD-bacteria	5'-CGCCACCAAAGCCTAAAAGG-3'	16S, 839–858	28

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

For our samples, the total number of bacterial cells treated with 0.003% nalidixic acid was not significantly different from those treated with 0.025% and 0.0025% yeast extract. Hence, the DVC method in combination with 0.003% nalidixic acid and 0.025% yeast extract was applied at 20°C for 15 hours.

Determination of the concentration of Microcystis cells

Water samples collected from June to November 1998, 1999 and 2000 were used to determine the concentration of *Microcystis* cells. The samples were fixed with a formaldehyde solution (final concentration, 1.5%). The abundance of *Microcystis* cells was estimated using an EKDS Haemacytometer (J.H.S., Bunkyo, Tokyo, Japan) under a microscope (BH-2, Olympus, Japan).

Purification and analysis of microcystin

Microcystis ichthyoblabe TAC95 (Tsukuba algal collection no. 95), which produces Mcyst-LR and 6(Z)-Adda-Mcyst-LR (stereoisomer of Adda of Mcyst-LR at the Δ6 double bond), was lyophilized and stored at −30°C until the purification of Mcyst. Mcyst was purified according to the method of Harada *et al.*[15]. Lyophilized cells were extracted with 5% aqueous acetic acid and the supernatant was introduced into an ODS silica gel cartridge (5 g, Chromotorex ODS 100–200 mesh, packed into a polypropylene cartridge). The cartridge was eluted with 90% methanol and the elution sample was concentrated by rotary evaporation at 30°C. The residue was used in the Mcyst degradation experiments. The concentration of Mcyst was measured by HPLC (High performance liquid chromatography) with an ODS column (Cosmosil 5C18-AR 4.6×150 mm, Nacalai, Japan). The HPLC system consisted of a Shimizu LC-9A pump (Shimazu, Japan) coupled to a SPD-10A set and a SPD-M10A photodiodearray detector and a C-R6A indicator. The parameters for the analysis of Mcyst were: absorbance at 238 nm, a methanol: 0.05 M phosphate buffer (pH 3.0, 58: 42) of moving phase, and a 1 mL min^{-1} flow rate. The concentration of Mcyst was quantified by comparing the peak area at 238 nm with a standard Mcyst according to Harada *et al.*[15].

Determination of microcystin-degrading activity of free-living microcystin-degrading bacteria

Samples containing free-living bacteria were collected from the lake during the early bloom (August 2_{nd} 2000), mid-bloom (August 30_{th} 2000) and late-bloom (September 27_{th} 2000) of *Microcystis*. These samples were collected according to the procedure described before. Mcyst-LR and 6(Z)-Adda-Mcyst-LR were spiked into each free-living bacterial sample at a final concentration of 5 μg mL^{-1}, which is higher than that of in situ to detect its decrease clearly. Incubation was performed in the dark on a rotary incubator at 20°C. Samples were collected at various time points for analysis of Mcyst (100 μL), which were stored at −20°C until HPLC analysis, for total bacterial count and for FISH analysis (3 mL).

Statistical analysis

Statistical analysis was performed with the *F*-test, Welch's *t*-test and Pearson's correlation coefficient test. The program package used was STATCELL Microsoft Excel add-in software[37]

Results

Changes of concentration of free-living microcystin-degrading bacteria in relation to the bloom of Microcystis

We already reported the concentrations of *Microcystis* cells, chlorophyll *a*, and cell-bound and dissolved microcystin in the lake[28]. The concentration of dissolved Mcyst was the highest on July 29_{th} and September 8_{th} in 1998 and 1999, respectively. The concentration started to decrease before the decay of the *Microcystis* bloom when Mcyst leaked from lysing *Microcystis*[28] (Fig. 1).

Population dynamics of free-living MCD-isolate in 1998 (Fig. 2-A) and 1999 (Fig. 2-B) were revealed by FISH analysis. the concentration of domain Bacteria increased from 4.9×10^5 cells mL^{-1} on May 20_{th}, 1998 to 2.4×10^6 cells mL^{-1} on October 21_{st}, 1998. The relative abundance of domain Bacteria increased from 30% on May 20_{th}, 1998 to 54% on October 7_{th}, 1998 of the total free-living bacteria. The concentration of α-Proteobacteria, which includes MCD-isolate, increased from 1.7×10^5 cells mL^{-1} on May 20_{th}, 1998 to 1.0×10^6 cells mL^{-1} on October 7_{th}, 1998, and decreased to 7.4×10^5 cells mL^{-1} on October 21_{st}, 1998. MCD-isolate was not detected by the FISH method in 1998.

Since a low rRNA concentration is the most ascribable reason for the low sensitivity of the conventional FISH method[5], we tried to increase the rRNA concentration through incubation with yeast extract and nalidixic acid which is known to prevent cell division. The combined DVC-FISH method indeed succeeded in detecting MCD-isolate that existed in the lake water in 1999 (Fig. 2-B). The concentration of domain Bacteria increased from 5.8×10^5 cells mL^{-1} on May 20_{th}, 1999 to 5.6×10^6 cells mL^{-1} on September 8_{th}, 1999, and decreased to 3.8×10^6 cells mL^{-1} on

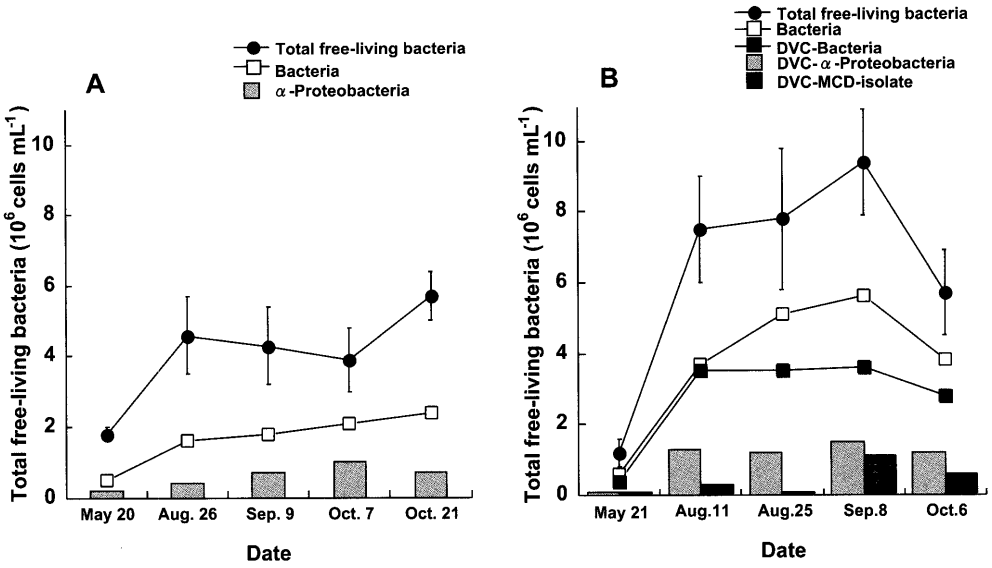

Fig. 2. Number of free-living bacteria detected by FISH and DVC-FISH with probes specific for domain Bacteria, α-Proteobacteria and MCD-isolate. Surface water samples were collected from Lake Suwa on May 20, August 26, September 9, October 7 and October 21, 1998 (A) and on May 20, August 25, September 8 and October 6, 1999 (B).

October 6th, 1999. The abundance ranged from 48% to 66% of the total number of free-living bacteria. The concentration of α-Proteobacteria increased from 1.1×10^5 cells mL^{-1} on May 20th, 1999 to 1.3×10^6 cells mL^{-1} on August 11th, 1999, and then ranged from 1.2 to 1.5×10^6 cells mL^{-1} from August 25th to October 6th 1999. The concentration of MCD-isolate drastically increased from 9.5×10^4 cells mL^{-1} on May 20th, 1999 to 1.1×10^6 cells mL^{-1} on September 8th, 1999, but the cell number dropped to 6.4×10^5 cells mL^{-1} on October 6th, 1999. The relative abundance of MCD-isolate ranged from 11% to 17% of the total number of free-living bacteria during the bloom of *Microcystis*. The ratio of MCD-isolate to α-Proteobacteria was apparently high during the bloom period, ranging from 70% to 86%, except on August 11th, 1999. Pearson's correlation test ($P<0.1$) did not show a significant relationship between MCD-isolate and *Microcystis* cells or dissolved Mcyst, and changes in the concentration of MCD-isolate fluctuated together with that of *Microcystis* cells ($r=0.72$, $n=5$) and dissolved Mcyst ($r=0.61$, $n=5$).

Degradation experiment using in situ free-living bacteria from early- to late-Microcystis blooms

Microcystis appeared in the middle of June in 2000 and the concentration apparently increased from 5.1×10^4 cells L^{-1} on July 19th to 1.2×10^6 cells L^{-1} on August 15th, then decreased from 3.8×10^5 cells L^{-1} on September 13th to 2.1×10^4 cells L^{-1} on September 27th (Fig. 3). The concentration of

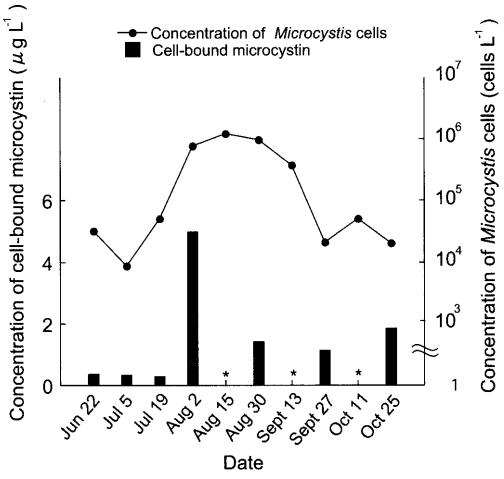

Fig. 3. Changes in the concentration of *Microcystis* cells (closed circles) and cell-bound microcystin (solid bars) at the center of Lake Suwa in 2000.

cell-bound Mcyst on August 2nd (early-bloom), August 30th (mid-bloom) and September 27th (late-bloom) in 2000 was 4.99, 1.43 and 3.25 μg L^{-1}, respectively (Fig. 3).

The degradation of Mcysts in above samples is shown in Fig. 4-A (early-bloom sample), 4-C (mid-bloom sample) and 4-E (late-bloom sample). Mcyst-LR and 6(Z)-Adda-Mcyst-LR present in all samples were degraded below the detection limit of the HPLC. The Mcysts present in the early-bloom samples were not degraded for at least 7 days, which is regarded as the lag time. Degradation did however

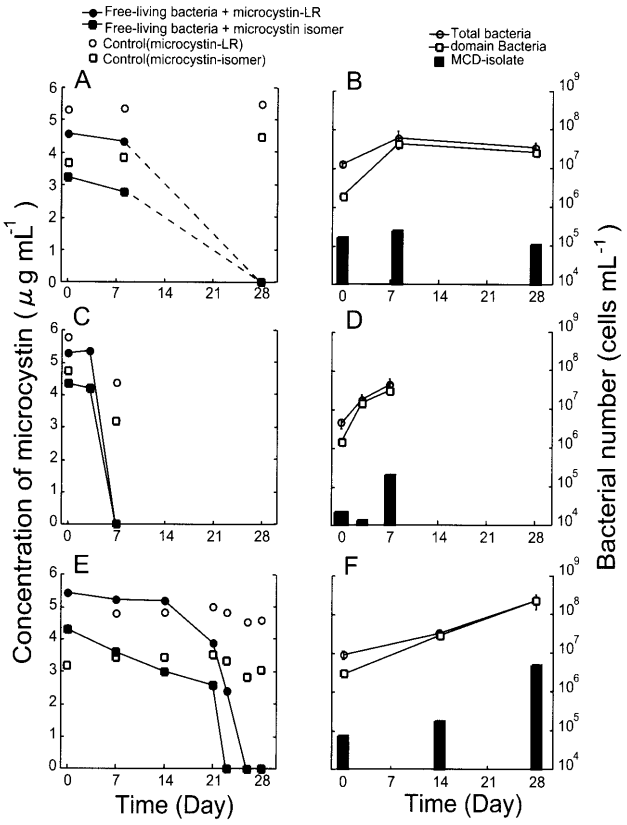

Fig. 4. Degradation of microcystin in filtered lake water (using a 3 μm pore size Nuclepore filter). Water samples were collected from Lake Suwa on August 2 (A, B), August 30 (C, D) and September 27 (E, F) 2000. A, C, E indicate changes in the concentration of microcystin-LR (circles) and its isomer (squares) in filtered lake water during incubation. Black and open points indicate with and without free-living bacteria, respectively. B, D, F show the change in the total number of bacteria (open circles), domain Bacteria (open squares) and MCD-isolate (solid bars) during incubation.

occur in 28 days (Fig. 4-A), but the analysis of Mcyst was not performed between day 7 and day 28. In mid-bloom sample, degradation of the Mcysts did not occur for the first 3 days, but was completed within 7 days (Fig. 4-C). The rate of degradation of Mcyst-LR from day 3 to day 7 was 1.34 μg mL^{-1} day^{-1}, the highest observed in the three experiments. In late-bloom sample, Mcyst-LR was not degraded for 14 days, but 20% of its initial concentration was degraded by day 21. It was then rapidly degraded between days 21 and 26 at a rate of 0.75 μg mL^{-1} day^{-1} (Fig. 4-E). 6(Z)-Adda-Mcyst-LR in late-bloom sample was gradually degraded until day 21, and then rapidly degraded between days 21 and 23. The degradation rates were 0.08 μg mL^{-1} day^{-1} for the first 21 days and 1.29 μg mL^{-1} day^{-1} between day 21 and day 23. In all experiments, 6(Z)-Adda-Mcyst-LR was degraded at a higher rate than Mcyst-LR. Neither Mcyst was not degraded in filter-sterilized lake water.

Behavior of microcystin-degrading isolate in early-, mid-, and late-Microcystis bloom samples during the Mcyst degradation experiment

The degradation activity of the free-living MCD-isolate differed among early-, mid- and late-*Microcystis* bloom samples. We employed both FISH and DVC-FISH to detect MCD-isolate to successfully elucidate the population dynamics. Concentrations of total free-living bacteria, domain Bacteria and MCD-isolate during the experiments are shown in Fig. 4-B, D, F. In early-bloom sample, The total free-living bacteria increased from 1.2×10^7 cells mL^{-1} on the initial day to 5.7×10^7 cells mL^{-1} on day 8 and then decreased to 3.4×10^7 cells mL^{-1} on day 28 (Fig. 4-B). Domain Bacteria increased from 1.8×10^6 to 4.0×10^7 cells mL^{-1} on day 8 and then decreased to 2.6×10^7 cells mL^{-1} on day 28. MCD-isolate increased from 1.6×10^5 to 2.4×10^5 cells mL^{-1} on day 8.

Total bacteria in the mid-bloom sample gradually increased from 4.1×10^6 to 4.3×10^7 cells mL^{-1} on day 7 (Fig. 4-D), whereas during the same period, domain Bacteria increased from 1.4×10^6 to 3.0×10^7 cells mL^{-1}. MCD-isolate populations hovered around $1–2\times10^4$ cells mL^{-1} for the first 3 days, which represents the lag phase for Mcyst degradation. This was followed by a rapid increase up to 2.0×10^5 cells mL^{-1} on day 7, corresponding to the degradation of Mcyst. From a statistical point of view, the increase was significant ($P>0.01$). The doubling time for that period was 1.0 day.

In late-bloom sample, total bacteria gradually increased from 8.9×10^6 to 2.3×10^8 cells mL^{-1} on day 28 (Fig. 4-F). Domain Bacteria increased from 2.8×10^6 to 2.2×10^8 cells mL^{-1} on day 28. MCD-isolate gradually increased from 7.5×10^4 to 1.7×10^5 cells mL^{-1} on day 14 ($P>0.01$) during the lag time for Mcyst degradation and then apparently increased to 4.9×10^6 cells mL^{-1} on day 28 ($P>0.01$), when Mcysts was already degraded. Doubling times of MCD-isolate from the initial day to day 14 and from day 14 to day 28 were 11.8 days and 2.7 days, respectively.

Changes in bacterial community structure in mid- and late-bloom samples during Mcyst degradation experiments were elucidated by FISH analysis (Fig. 5). In mid-bloom sample, α-, β-, and γ-Proteobacteria and the *Cytophaga/Flavobacterium* group increased by one order of magnitude from the initial day to day 3, with the exception of δ-Proteobacteria. α-Proteobacteria increased from 2.6×10^5 to 4.1×10^6 cells mL^{-1} on day 3 and then remained stable until

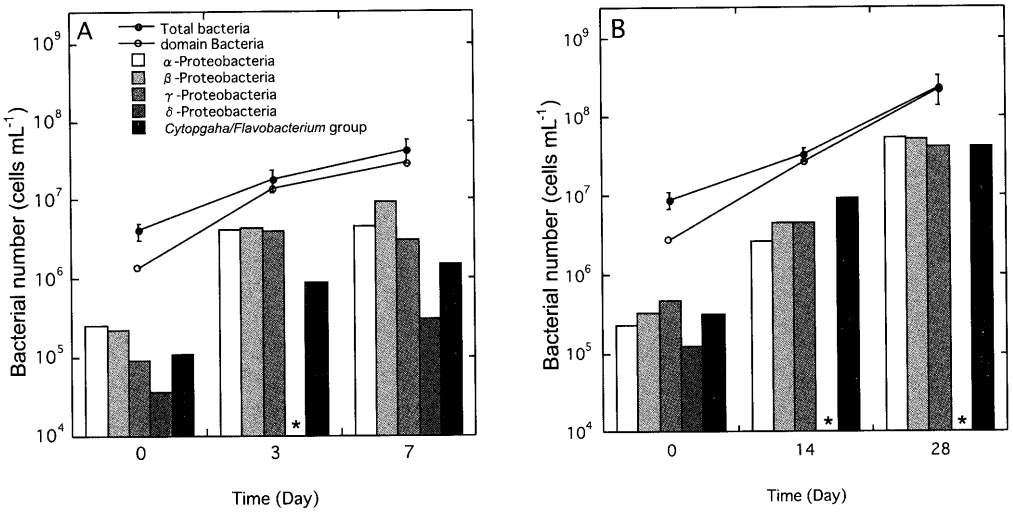

Fig. 5. Dynamics of free-living bacterial community structure estimated using rRNA-targetted oligonucleotide probes specific for α-, β-, γ-, δ-Proteobacteria and the *Cytophaga/Flavobacterium* group during microcystin degradation. Water samples were collected from Lake Suwa on August 30 (A) and on September 27(B), 2000. Black and open circles indicate total free-living bacteria and domain Bacteria, respectively. Asterisks indicate not detected.

day 7. β-Proteobacteria increased from 2.2×10^5 to 4.4×10^6 cells mL^{-1} on day 3, and continuously increased to 9.2×10^6 cells mL^{-1} until day 7. γ-Proteobacteria increased from 9.3×10^4 to 4.0×10^6 cells mL^{-1} on day 3. The *Cytophaga/Flavobacterium* group increased from 1.1×10^5 to 8.9×10^5 cells mL^{-1} on day 3 and then to 1.5×10^6 cells mL^{-1} on day 7. δ-Proteobacteria were present on the initial day at 3.6×10^4 cells mL^{-1}, but were absent on day 3 and then present again on day 7 (i.e., 3.0×10^5 cells mL^{-1}).

In late-bloom sample, α-, β-, and γ-Proteobacteria and the *Cytophaga/Flavobacterium* group constantly increased over 28 days, with the exception of δ-Proteobacteria. α-Proteobacteria increased from 2.3×10^5 to 2.6×10^6 cells mL^{-1} during the first 14 days, and then more continuously to 5.5×10^7 cells mL^{-1} until day 28. β-Proteobacteria increased from 3.2×10^5 to 4.4×10^6 cells mL^{-1} over 14 days and to 5.2×10^7 cells mL^{-1} until day 28. γ-Proteobacteria increased from 4.6×10^5 to 4.5×10^6 cells mL^{-1} over 14 days and then continuously to 4.1×10^7 cells mL^{-1} until day 28. The *Cytophaga/Flavobacterium* group increased from 3.1×10^5 to 9.1×10^6 cells mL^{-1} over 14 days and then to 4.2×10^7 cells mL^{-1} until day 28. δ-Proteobacteria were present on the initial day (i.e., 1.2×10^5 cells mL^{-1}), but were absent after 14 and 28 days. In both experimental periods, the bacterial community structure of day 3 to 7 and of day 14 to 28 incubation did not significantly differ from that of day 0. This suggests that the added high concentration of Mcyst did not strongly affect the bacterial community, but caused a clear increase in MCD-bacterium Y2.

Discussion

We have focused on the degradation process of Mcyst by differentiating the functional sites and functional bacteria into two phases, i.e., one is the mucilage of *Microcystis* where abundant bacteria are embedded and where excreted Mcyst is trapped for a while, and the other is the bulk water where indefinite free-living bacteria exist and where Mcyst might be quickly diluted. A high correlation exists between the MCD-isolate in the mucilage of *Microcystis* and the concentration of cell-bound Mcyst[28]. This suggests that the degradation of Mcyst in the mucilage of *Microcystis* occurs as a function of the bacteria embedded there. On the other hand, the concentration of dissolved Mcyst started to decrease before the decaying of the *Microcystis* bloom when Mcyst leaked from lysing *Microcystis*[28] (Fig. 1). The decrease of Mcyst in the water might be ascribable to the degradation of Mcyst by the activity of free-living bacteria. In the present study, it was elucidated that MCD-isolate existed in water as free-living forms and whether they degraded Mcyst in the bulk water.

The concentration of the free-living MCD-isolate found in water increased up to more than 10% of the total free-living bacteria when the highest concentration of dissolved Mcyst and a high concentration of *Microcystis* cells were present on September 8_{th}, 1999 (Fig. 2-B). It is remarkable that in a natural system, one gene-specific clone of MCD-isolate detected by the FISH method made up one tenth of the total free-living bacteria. In addition, the concentration

of dissolved Mcyst started to decrease before the decaying of the *Microcystis* bloom[28] (Fig. 1). These findings suggest that free-living MCD-isolate degraded Mcyst in the water. It is known that produced microcystin is maintained within healthy cyanobacterial cells, but approximately 10 to 20% of Mcyst is released from healthy cyanobacterial cells to the surrounding water[33,34]. It is then likely that Mcyst was supplied to free-living MCD-isolate even before *Microcystis* decayed. Bourne *et al.*[8,9] found three enzymes contributing to the degradation of Mcyst and one enzyme contributing to the uptake of small peptides. Strain Y2 degraded Mcyst four times faster in the medium without organic nutrients than in the medium with them[30]. Together with these results, our data implies that MCD-isolate utilizes Mcyst for growth producing Mcyst-degrading enzyme.

To obtain a more detailed knowledge of the degradation feature of free-living MCD-isolate, we carried out a similar experiment using in situ free-living bacteria taken from different *Microcystis* blooming periods, i.e., in early-, mid-, and late-bloom. The experiments clearly showed that Mcyst was completely degraded and that the lag time until the initiation of the degradation differed for free-living bacteria taken from the three different periods. In mid-bloom, MCD-isolate did not increase during the lag time but increased significantly when Mcyst was degraded completely, which suggests that free-living MCD-isolate contributed to the degradation of Mcyst utilizing it. The Mcyst degradation was the fastest in mid-bloom. Our results also showed that Mcyst was degraded in the water but the degradation rates and lag times varied among experiments. Jones and Orr[19] showed that the concentration of dissolved Mcyst remained high (i.e., 1.8 μg mL^{-1}) for 9 days after *Microcystis* cells were treated with algicide ($CuSO_4$), but that Mcyst was rapidly degraded within 3 days (90–95%). Rapala *et al.*[32], Cousins *et al.*[12] and Christoffersen *et al.*[10] carried out Mcyst degradation experiments using low concentrations of Mcyst (<0.2 μg mL^{-1}) and suggested that the degradation period varied from 4 days to 2 weeks. These findings indicate that Mcyst can be degraded with various periods in natural water and that it is irrespective of its concentration. The agent responsible for Mcyst degradation in water was however not addressed in the above studies.

Differences in lag time and Mcyst degradation periods by free-living bacteria taken from different times of the bloom period could be caused by temperature[12], exposure time to Mcyst[12,18,32] and constituents of the microbial community[12]. Our results suggest that temperature might not be the sole factor because two free-living bacterial populations taken from a similar temperature regime, i.e., 27.0°C (early-boom) and 26.1°C (mid-bloom), clearly showed different lag times. Rapala *et al.*[32] and Cousins *et al.*[12] suggested that indigenous microflora exposed to cyanobacterial blooms degraded Mcyst faster than when they were not exposed to it. It has indeed been shown that Mcyst is not degraded in water which is not exposed to *Microcystis* blooms[23], whereas Mcyst-degrading isolate can degrade Mcyst without a lag time, if it is exposed to Mcyst before the experiment[18]. We therefore suggest that the differences in lag time between free-living bacteria in early and mid-*Microcystis* blooms are caused by a longer exposure to Mcyst in the mid-bloom phase than in the early bloom phase. The high activity of MCD-isolate in mid-bloom sample was probably ascribable to a higher concentration of *Microcystis* cells. The free-living MCD-isolate was more easily exposed to Mcyst in mid-bloom period when a high concentration of Mcyst was produced by *Microcystis* cells (Fig. 3).

Our results did not show any remarkable changes in free-living bacterial community structure during the Mcyst-degrading experiments. Each phylogenetic bacterial group, with the exception of δ-Proteobacteria, increased during the experiments. The decrease of δ-Proteobacteria during the degradation periods suggests that this group was not involved in the degradation of Mcysts and that even their growth was suppressed by Mcysts. Christoffersen *et al.*[10] tried to elucidate the changes of indigenous bacterial community structure in Mcyst-degrading experiments using the DGGE technique. They showed that the bacterial diversity increased after the addition of Mcyst-LR, though clones of the bacterial community were not identified. With respect to the degradation steps of Mcyst, we previously reported that two characteristic peaks of intermediate products appeared during degradation experiments using the Y2 strain[30]. However, these products were not detected during the three degradation experiments in the present study. The degradation of Mcyst consists of several steps[30]. Thus we assume that the process is not only carried out by MCD-isolate but also by other free-living bacteria which degrade the intermediate products.

Conclusion

In order to elucidate the microbial degradation of Mcyst in water, we focused on the population dynamics of MCD-isolate in *in situ* free-living bacteria and on their degradation activity during different periods of *Microcystis* blooms. We found that high concentrations of free-living MCD-isolate occurred in the lake when high concentrations of dissolved Mcyst and *Microcystis* cells were present. Mcyst-

degradation experiments with free-living bacteria for different *Microcystis* bloom periods showed that Mcyst was degraded at a fastest rate during mid-bloom and that free-living MCD-isolate increased just after the degradation of Mcyst. These results strongly suggest that free-living MCD-isolate induced the production of Mcyst-degradation enzymes after the exposure to Mcyst, which was released from *Microcystis* cells even before the decay of the *Microcystis* bloom, and utilized for their growth. During the Mcyst-degradation experiments, other free-living bacterial community structure did not remarkably change and no intermediate products derived from the degradation were observed. These findings indicate that coexisting bacteria present in the water probably contributed to the degradation of the intermediate products.

We therefore conclude that free-living MCD-isolate carry out the degradation of dissolved Mcyst or at least the initiation of the degradation. The likely scenario for the degradation of hepatotoxin (Mcyst), in combination with the findings of the previous study[28]; is that once Mcyst is excreted from the cells, it is trapped in the mucilage surrounding *Microcystis* cells and attacked by MCD-isolate embedded in the mucilage matrix. Thereafter, the Mcyst released into the bulk water is degraded by MCD-isolate, but in the free-living form. Co-existing bacteria are also thought to play a role in the degradation, together with free-living and embedded MCD-isolates. As a result, the concentration of Mcyst in lake water is kept low.

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