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Toxicity and Toxins of Natural Blooms and Isolated Strains of *Microcystis* spp. (Cyanobacteria) and Improved Procedure for Purification of Cultures

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All samples of cyanobacterial blooms collected from 1986 to 1989 from Lake Kasumigaura, Ibaraki Prefecture, Japan, were hepatotoxic. The 50% lethal doses (LD_{50}s) of the blooms to mice ranged from 76 to 556 mg/kg of body weight. Sixty-eight *Microcystis* cell clones (67 *Microcystis aeruginosa* and 1 *M. viridis*) were isolated from the blooms. Twenty-three strains (including the *M. viridis* strain) were toxic. However, the ratio of toxic to nontoxic strains among the blooms varied (6 to 86%). Microcystins were examined in six toxic strains. Five toxic strains produced microcystin-RR, -YR, and -LR, with RR being the dominant toxin in these strains. Another strain produced 7-desmethylmicrocystin-LR and an unknown microcystin. This strain showed the highest toxicity. Establishment of axenic strains from the *Microcystis* cells exhibiting extracellularly mucilaginous materials was successful by using a combination of the agar plate technique and two-step centrifugation.

Cyanobacterial blooms are frequently observed in eutrophic waters (3). The occurrence of toxic cyanobacterial strains in these blooms has been reported worldwide, but the toxicity of cyanobacterial blooms varies widely (3, 8, 20). Death of animals after drinking fresh water containing toxic cyanobacteria has been reported (3, 8). Hepatotoxic cyclic heptapeptides produced by certain strains of *Microcystis aeruginosa* and *M. viridis* have been found (3, 20, 30). Ten different cyclic heptapeptide toxins, designated microcystin or cyanoginosin, have been isolated from *Microcystis* species. All contain 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid as a common component (1, 2, 3, 20). The distribution and types of toxin in bloom-forming *Microcystis* strains are poorly understood (20). In part this is due to the difficulties in isolating and culturing *Microcystis* clones. Furthermore, the ecology, physiology, genetics, and taxonomy of toxic and nontoxic *Microcystis* strains are unclear, since contaminants are associated intimately with the mucilaginous colonies. This has made the establishment of axenic strains difficult (7, 11). Efforts to obtain axenic strains have been carried out by UV irradiation, phenol treatment, addition of antibiotics to culture medium, and physical separation (4, 7, 21). Recently, we developed a technique for isolating axenic clones of *Microcystis* by using solid media (25), though some strains were still difficult to purify by this method.

Blooms are abundant, especially in summer, in Lake Kasumigaura (the second-largest lake in Japan); *M. aeruginosa* is the dominant species (20, 30). This paper describes the nature and activity of toxins among blooms in the lake, the clones of *Microcystis* spp. established from 1986 to 1989 from the lake, and the successful isolation of axenic strains by a combination of solid-medium cultivation and two-step centrifugation.

MATERIALS AND METHODS

Media and culture. Liquid CB medium and a solid form of the medium containing 0.4% agarose were used for culture (25). *Microcystis* strains were grown in standing culture under 2,000-lx continuous illumination from fluorescent (cool white) light at 30°C (25).

Water blooms. Water blooms were collected from 1986 to 1989 from Tsuchiura Bay in Lake Kasumigaura, Ibaraki Prefecture.

Isolation of axenic clones. The method for isolation of single cyanobacterial cells from water blooms, followed by purification, was described previously (25). However, the method failed to establish some axenic strains. Therefore, we added a two-step centrifugation to the previous method for these strains. In order to reduce contaminants, single cyanobacterial cells were cultured repeatedly in solid and liquid culture media alternately (25). The liquid culture containing mid-log-phase cells was then diluted 1:100 with distilled water and shaken by a vortex mixer to disperse the colonies. One milliliter of the cell suspension was centrifuged in a 1.5-ml microtube (C. A. Greiner und Sohne, GmbH & Co. KG, Frickenhausen, Germany) at 150 × *g* for 30 min at 20°C, followed by higher-speed centrifugation (1,000 to 4,000 × *g*) for 5 min at 20°C. Centrifugation was performed with a microcentrifuge (MR-150; Tomy Seiko Co., Tokyo, Japan) fitted with a fixed-angle rotor (TMA-2). After the two-step centrifugation, the surface layer (1 μl) was withdrawn with a micropipette (Drummond Scientific Co., Broomall, Pa.) and plated on CB-agarose medium. When necessary, the sample was diluted in water before being plated. After incubation, each strain was selected from a contaminant-free individual colony.

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Test for contamination and cell identification. Microbial contamination was tested for by phase-contrast microscopy and cultivation (4, 25). For the latter procedure, samples were inoculated into the following media by using a 10% (vol/vol) inoculation: diluted nutrient agar (25), thioglycolate medium (Nissui Seiyaku Co., Tokyo, Japan), potato dextrose agar (Nissui Seiyaku Co.), CB-peptone medium (5 g of peptone and 120 g of gelatin per liter of CB medium), tryptone broth (5 g of Bacto-Tryptone and 15 g of Bacto-Agar per liter) and brain heart infusion broth (Eiken Chemical Co., Tokyo, Japan). The strains isolated from the blooms were identified by following the procedure of Komárek (16).

Cell counts. The total number of cells was counted microscopically by using a bacterial counting chamber (Kayaba Seisakusho Co., Tokyo, Japan). Viable counts were made by plating cells on CB-agarose medium (25).

Assay of *Microcystis* sp. cell toxicity. The bioassay for *Microcystis* sp. cell toxicity has been described previously (20). Naturally occurring or cultured cells grown in CB medium were washed by centrifugation. After three consecutive cycles of freeze-thaw, cells were disrupted by ultrasonication (model 185 sonifier; Branson) at 50 W for 3 min. The resultant cell suspension was centrifuged at 30,000 × *g* for 15 min. The supernatant was filtered through a membrane filter (pore size, 0.45 μm; Acrodisc; Gelman Sciences Inc., Ann Arbor, Mich.) and lyophilized. When tested, the lyophilized material (cell extract) was dissolved in saline and injected intraperitoneally into male C57BL/6 mice (11 to 16 weeks old) or male C3H/HeJ mice (16 weeks old). The cells (i.e., cell extracts) were scored as toxic if one or more in a group of three mice were killed within 3 h after an injection with 6.5 mg (dry weight) of cells per mouse. The 50% lethal dose (LD_{50}; milligrams [dry weight] per kilogram of body weight) was calculated by the method of Reed and Muench (22) from the number of dead mice within 3 h after the injection of twofold-diluted cell extracts into a group of five mice.

Assay of lake water toxicity. Lake water was filtered through no. 5A filter paper (Toyo Rosi Co., Tokyo, Japan) followed by Whatman GF/C paper (Whatman Ltd., Maidstone, England) to remove cells of *Microcystis* spp. and other microorganisms. Each 100 ml of filtrate was passed through an octyldecyl silane cartridge, which was then washed with distilled water and eluted with 80% methanol in water (20). The eluate was evaporated to dryness, and the resulting sample was solubilized in 1 ml of physiological saline. The sample (0.2 ml) was then injected intraperitoneally into C57BL/6 mice.

Analysis of microcystins. The identification and determination of microcystins were carried out by using high-performance liquid chromatography (HPLC) (20). Standard microcystins LR, YR, RR (20), 7-desmethylmicrocystin-LR (7-DMLR) (13a), and 3-DMLR (13) were prepared from *M. aeruginosa* M-228, *M. viridis* TAC-44, *M. aeruginosa* K-139, and water blooms in the lake. The retention times of toxins were determined compared with those of standard toxins by using several different mobile phases (12, 13, 14).

RESULTS

Constituents and toxicity of water blooms. Microscopically, *M. aeruginosa* was the most abundant (>90%) cyanobacterium in every water bloom collected from Lake Kasumigaura. *M. viridis* and *M. wesenbergii* were also observed, but *Anabaena* species were scarce. Every water bloom tested was toxic to mice; acute toxic death occurred within 3 h after intraperitoneal injection. Severe necrosis was observed by histopathological examination in the livers, but not other organs, of the dead mice. The LD_{50} ranged from 76 to 556 mg/kg (Table 1). LD_{50}s of the samples in 1988 varied greatly according to collection time. When toxins of some blooms were chromatographically analyzed, three different toxins, microcystin-RR, -YR, and -LR, were detected. The proportions of the three toxins varied (Table 1).

TABLE 1. Toxicity and ratio of toxic *Microcystis* strains in natural blooms

Date (mo/day/yr)	LD_{50} (mg/kg)	Microcystin (μg/100 mg of cells)			No. of toxic strains[a]/no. of strains tested
		-RR	-YR	-LR	
9/2/1986	84	47.3	14.6	24.0	1/6
9/24/1987	122	3.2	6.2	14.9	12/14
7/15/1988	173		NT[b]		8/17
8/15/1988	285	2.2	2.9	7.3	1/13
9/14/1988	556	2.4	3.8	3.7	1/18
8/21/1989	76		NT		NT
9/12/1989	99[c]		NT		NT
9/12/1989	106[c]		NT		NT
9/12/1989	124[c]		NT		NT

[a] Strains were scored as toxic if one or more in a group of three mice was killed after injection of 6.25 mg (dry weight) of cells per mouse.
[b] NT, Not tested.
[c] The samples collected on 12 September 1989 were from stations at least 15 m apart.

Toxicity of lake water. To determine if the toxin(s) was detectable in lake water, water was collected from two locations (S-1 and S-2) in a bloom-rich area on 12 September 1989. Sample S-1 contained 12.85 mg (dry weight) of substances per ml, and the LD_{50} was 99 mg/kg. S-2 contained 12.42 mg (dry weight) of substances per ml, and the LD_{50} was 106 mg/kg. After filtration, each sample was injected intraperitoneally into mice. No deaths occurred within 5 h, and no gross pathologic changes were seen in the liver. Furthermore, no microcystin-RR, -YR, or -LR was detected in the samples, even when the samples were concentrated several times.

Toxicity of isolated *Microcystis* strains. By single-colony isolation, 68 strains (67 *M. aeruginosa* and 1 *M. viridis*) were isolated from the blooms that had been collected between 1986 and 1988. We examined the toxicity of the mid-log-phase cells of isolates (Table 1), though the toxicity of the cells in culture varies according to the growth phase (20). Of the 68 strains, 23 (22 *M. aeruginosa* and 1 *M. viridis*) were toxic and 45 were nontoxic. The proportion of toxic strains isolated from the blooms varied (6 to 86%). Except for one strain (*M. aeruginosa* B-47, isolated in 1987), these toxic strains, including *M. viridis*, produced microcystin-RR, -YR, and -LR.

A typical elution profile of the high-performance liquid chromatogram of the cell extract prepared from *M. aeruginosa* M-20 is shown in Fig. 1A. Besides microcystin-RR, -YR, and -LR, 3-DMLR was detected in this strain. In other toxic strains unidentified small peaks were observed, but the toxicities of these peaks were not examined. On the other hand, the extract of *M. aeruginosa* B-47 did not show any detectable RR, YR, or LR, but two other toxic peaks were observed (7-DMLR and X in Fig. 1B). One of the peaks coincided with 7-DMLR, and another was the same as microcystin, which was deduced to be 3,7-didesmethylmi-

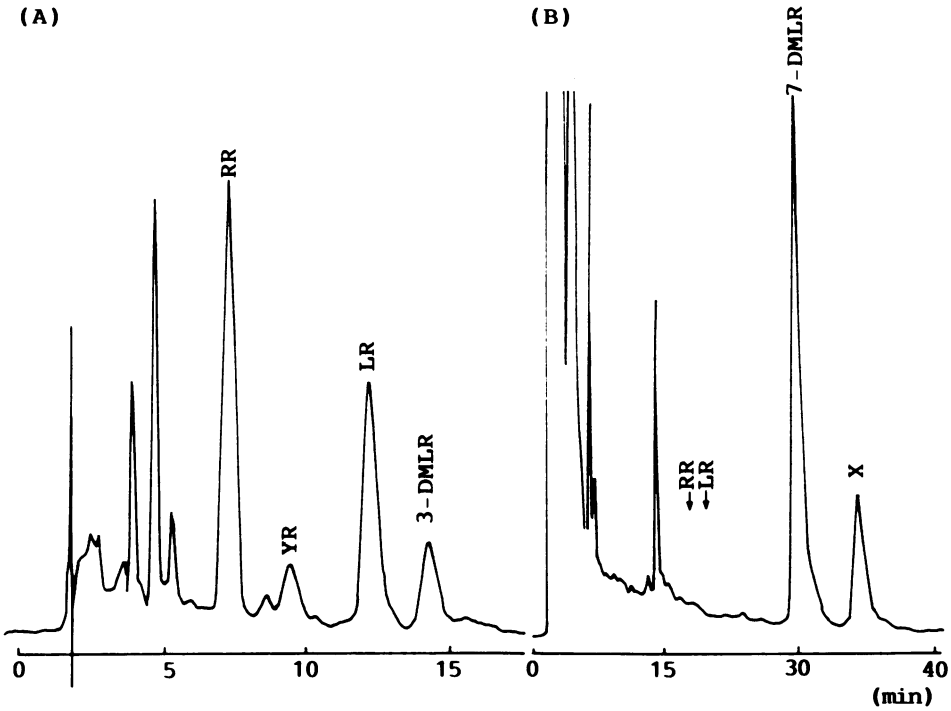

FIG. 1. High-performance liquid chromatograms of *M. aeruginosa* M-20 (A) and *M. aeruginosa* B-47 (B) extracts. Columns, Nucleosil $5C_{18}$ (4.6 by 150 mm) (A) and Inertsil C_{18} (4.6 by 250 mm) (B); mobile phases, methanol–0.05 M phosphate buffer (pH 3.0; 58:42) (A) and 26% acetonitrile in 10 mM ammonium acetate (B); detection UV, 238 nm. RR, Microcystin-RR; YR, microcystin-YR; LR, microcystin-LR; X, unidentified toxic peak.

crocystin-LR, and was detected in *M. aeruginosa* K-139 (13a, 20).

The LD_{50}s and the microcystin content of these toxic strains were examined (Table 2). The cell extract of *M. aeruginosa* B-47 showed the highest toxicity (LD_{50}, 22 mg/kg). Proportions of RR, YR, and LR differed among the other strains; RR was the dominant toxin.

Establishment of axenic strains. Establishment of axenic strains was attempted from the strains isolated by the single-colony isolation procedure. Pure cultures of strains from *M. aeruginosa* M-20, B-47, and S-77 were obtained by repeated isolation in alternate cultures by using solid or liquid medium (25). However, *M. aeruginosa* B-35 and *M. viridis* S-70 could not be purified by this method. For these two strains, two-step centrifugation was applied after repeated isolation. The first step of centrifugation (150 × *g* for 30 min) allowed gas-vesiculate cells to float in the surface layer of the medium, while contaminants were reduced by two-thirds (Table 3). After the second step of centrifugation (1,000 to 4,000 × *g* for 5 min), the contaminants were further reduced in the surface layer. Contaminants were eliminated most effectively when the second step was carried out at 2,000 or 3,000 × *g* for 5 min (contaminant-to-*Microcystis* cell

TABLE 2. LD_{50} and amount of microcystin of isolated *Microcystis* strains

Strain	LD_{50} (mg/kg)	Microcystin (μg/100 mg of cells)			
		−RR	−YR	−LR	Other
M. aeruginosa					
M-20[a]	49	87.2	17.2	62.5	+[b]
B-35[c]	78	176.8	35.3	72.9	−[d]
B-47[c]	22	−	−	−	+[e]
B-56[c]	107	113.0	9.8	27.2	−
S-77[f]	113	39.2	23.6	31.2	−
M. viridis					
S-70[f]	125	134.6	37.3	29.0	−

[a] Date of collection, 2 September 1986.
[b] 3-DMLR was detected, but the amount was not determined.
[c] Date of collection, 24 September 1987.
[d] −, Not detected.
[e] 7-DMLR and an unknown microcystin were detected, but the amount of neither toxin was determined.
[f] Date of collection, 15 July 1988.

TABLE 3. *Microcystis* sp. and contaminant in the surface layer after centrifugation

Centrifugation[a] (× *g*)	*Microcystis* sp.[b]/contaminant[c]	Ratio[d]
Precentrifugation	448/904	2.02
Step 1, 150	1,990/370	0.19
Step 2		
1,000	1,324/154	0.12
2,000	478/22	0.05
3,000	324/20	0.06
4,000	56/8	0.14

[a] Step 1 was conducted for 30 min; step 2 was conducted for 5 min. The various second steps followed step 1.
[b] *M. aeruginosa* B-35.
[c] Both values in each ratio are 10^5 cells per milliliter. Cells were counted microscopically by using a counting chamber for bacteria.
[d] Ratio of contaminant cells to *Microcystis* cells.

TABLE 4. CFU of surface layer after centrifugation

Centrifugation	CFU/μl			
	M. aeruginosa B-35		*M. viridis* S-70	
	Contaminant	*Microcystis* sp.	Contaminant	*Microcystis* sp.
Precentrifugation	10^4	10^4	1×10^3	1×10^3
Step 2	8	10^3	3×10^2	5×10^3

ratio, 0.05 to 0.06). Higher-speed centrifugation (2,000 or 3,000 × *g* for 5 min) without prior low-speed centrifugation was ineffective for isolating axenic strains, because it decreased the number of *Microcystis* cells in the surface layer without a similar decrease in contaminants (data not shown).

The CFU from the surface layer after two-step centrifugation of strains B-35 and S-70 are shown in Table 4. The initial ratio of *Microcystis* cells to contaminant was 1:1. After centrifugation, the ratio of strain B-35 to contaminant was 1:0.008 and that of strain S-70 to contaminant was 1:0.06. Then, 1 μl of the surface layer was diluted 1:100 with sterilized water and plated on CB-agarose medium. Microscopic observations showed that many resulting colonies of both strains were contaminant free. Cells shown to be contaminant free by microscopy were isolated and inoculated at high densities into the culture medium in order to confirm their purity. Thus, axenic clones of *M. aeruginosa* B-35 and *M. viridis* S-70 were obtained.

DISCUSSION

The toxicity of blooms in Lake Kasumigaura seems variable, since it has been reported that individual blooms collected in 1978 were nontoxic (28), that those collected in 1984 were both toxic and nontoxic (26, 30), and that those collected in 1985 were toxic (20). All of the blooms examined in this study (1986 to 1989) were toxic. Carmichael and Gorham have already reported that toxicity varied greatly, depending on where and when blooms were collected (5). Cyanobacterial toxin levels per unit of cyanobacterial biomass varied widely from week to week at individual locations (8). These observations indicate that the toxicity of blooms is not constant and is presumably influenced by environmental factors.

Marked differences in toxicity (LD_{50}) to mice were observed among the blooms (Table 1). The blooms were composed of both toxic and nontoxic strains, and the toxic-to-nontoxic strain ratio obviously differed from bloom to bloom (Table 1). The ratio of toxic to nontoxic strains in the blooms and the content of toxins in individual cells are presumably the key factors influencing the toxicity of the blooms. The toxicity of *M. aeruginosa* depends on the growth phase, light intensity, temperature, and nutrients (8, 15, 20, 29, 32). Moreover, nontoxic cells of *Microcystis* spp. in the blooms may participate in the toxicities of the blooms. Shirai et al. observed that nontoxic *M. aeruginosa* was capable of inducing delayed-type hypersensitivity in mice (26). Such influence on the host response by nontoxic cyanobacteria may change the susceptibility of the host to toxic cells in water blooms. Furthermore, it can be assumed that contaminant microbes, other than toxic *Microcystis* cells, influence the toxicity of water blooms. Collins reported toxicity of bacteria associated with *M. aeruginosa* (9).

It has been considered that microcystin is not secreted by an active transport system or released by destruction of the cells (6). Degradation of microcystin might have occurred inside the cells, since toxicity of stationary-phase cells is lower than that of mid-log-phase cells (15, 20). We detected no toxicity or microcystins in filtrates from water bloom-rich water in this study. However, Hughes et al. reported that a toxic substance was detected in the culture filtrate even in the early stages of growth, indicating that leakage and/or lysis was occurring (15). These findings suggest that some microcystin may be released from degraded cells in water blooms into the water, but the amount in the lake water will usually be undetectable.

Analysis by HPLC revealed several different microcystins in the blooms (Table 1) and in the established strains (Table 2 and Fig. 1). All the blooms tested contained microcystin-RR, -YR, and -LR, though the contents varied among blooms. The cells of all but one of the established strains showed all three kinds of toxin, although microcystin-RR was predominant. Watanabe et al. reported that high amounts of microcystins were generally observed in highly toxic blooms and cells and that the LD_{50}s of microcystin-RR, -YR, and -LR to mice were 600, 68, and 73 μg/kg, respectively (31). *M. aeruginosa* B-47 did not have these microcystins but did contain another, 7-DMLR (Fig. 1B). Perhaps this strain was simply responding to a set of environmental variables in a different manner in space and time. Although the LD_{50} of 7-DMLR has yet to be determined, it should be highly toxic, since B-47 cells showed the highest toxicity (Table 2).

Establishment of axenic clones of *Microcystis* spp. is required in order to study the ecology, physiology, genetics, and taxonomy of non-toxin- and toxin-producing strains. It has long been thought that *Microcystis* species cannot grow properly on agar medium (23), but we have developed a solid medium for growth of *Microcystis* spp. and succeeded in isolating axenic strains by a single-colony isolation procedure using solid medium (25). However, this procedure was insufficient for isolating axenic cells from some strains, such as B-35 and S-70, with sticky contaminants. Parker reported the selective growth of *Microcystis* spp. in agar media containing Na_2S, which inhibited or killed associated contaminants (21). However, strains B-35 and S-70 were not purified by Parker's method. Walsby reported the use of low-speed centrifugation to isolate a green alga from cultures of a *Spirulina* sp. and flagellates from cultures of an *Anabaena* sp. (27). His method was applied to the isolation of an axenic clone of these species (7). We also tried to isolate axenic strains from B-35 and S-70 strains by low-speed centrifugation. However, it was insufficient for isolating the cells from contaminants. The addition of a two-step centrifugation to the solid-agar cultivation resulted in successful isolation of the axenic strains. The two-step centrifugation left a large number of *Microcystis* cells in the surface layer and spun down the contaminants (Tables 3 and 4), because positive buoyancy is a general feature of *Microcystis* species (7).

Microcystin induces rapid and extensive centrilobular necrosis of the liver with loss of characteristic architecture of the hepatic cords (10, 20). Runnegar and Falconer suggest that microcystin probably affects a cytoskeletal component (24). Recently, MacKintosh et al. (17) and Yoshizawa et al. (33) have reported that microcystins are inhibitors of protein phosphatases. Inhibition of protein phosphatases by microcystins may cause hepatic cell damage (33). On the other hand, Nakano et al. reported that toxic *M. aeruginosa* cells or the cell extract was capable of inducing interleukin 1 or

tumor necrosis factor production by macrophages in mice (18, 19). A complicated effect of the toxin may be one of the reasons for differences in toxicity among water blooms.

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