Degradation of the Cyanobacterial Hepatotoxin Microcystin by a New Bacterium Isolated from a Hypertrophic Lake

Ho-Dong Park,[1] Youhei Sasaki,[1] Tomoko Maruyama,[1] Eiji Yanagisawa,[2] Akira Hiraishi,[3] Kenji Kato[4]

[1]Department of Environmental Sciences, Faculty of Science, Shinshu University, Matsumoto 390-8621, Japan

[2]Miroku Medical Co. Ltd., Mochizuki-machi, 384-2201, Japan

[3]Department of Ecological Engineering, Toyohashi University of Technology, Tenpaku-cho, Toyohashi 441-8580, Japan

[4]Institute of Geosciences, Shizuoka University, Shizuoka 422-8529, Japan

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ABSTRACT: A bacterium capable of degrading microcystins-RR, -YR, and -LR was isolated from a hypertrophic lake. The bacterium, designated Y2 and classified phenotypically as a member of the genus *Sphingomonas*, was shown to be distinct phylogenetically from any established species of *Sphingomonas* on the basis of 16S rDNA sequencing. The bacterium was tentatively identified as *Sphingomonas* by manual chemotaxonomy, but 16S rRNA sequencing analysis suggests that it is in fact a new species or even a new genus. When the Y2 bacterium was added to microcystins present in culture medium, the microcystins were degraded thoroughly in 4 days. The highest degradation rates of microcystins-RR and -LR were 13 and 5.4 mg L^{-1} day^{-1}, respectively. The degradation rates were strongly dependent on temperature and the maximum rate was at 30°C.
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Keywords: microcystin; biodegradation; degradation rate; Lake Suwa

INTRODUCTION

Cyanotoxins and toxic cyanobacterial blooms in eutrophic lakes, rivers, and reservoirs have been reported during the last two decades all over the world (Skulberg et al., 1984; Gorham and Carmichael, 1988; Carmichael, 1992; Park et al., 1998b). These toxic blooms have caused the death of livestock and wildlife and illness and even death in humans (Billings, 1981; Falconer, 1989). For example, over 50 patients at a dialysis center in Caruaru, Brazil, died from February to September 1996. Although the cyanobacterial species responsible have not yet been identified completely, microcystins produced by cyanobacteria were detected in water from the reservoir and the dialysis center, and in serum and liver tissue of affected patients (Jochimsen et al., 1998).

Cyanotoxins are classified functionally into two groups, cytotoxins and biotoxins. Biotoxins are subdivided into hepatotoxins and neurotoxins (Carmichael, 1997). *Microcystis aeruginosa* is the most common toxic cyanobacterium found worldwide, and it produces potent cyclic peptide hepatotoxins, termed microcystins

Correspondence to: Ho-Dong Park; e-mail: pparkhd@gipac.shinshu-u.ac.jp.

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(Carmichael, 1988, 1992; Carmichael et al., 1988; Park and Watanabe, 1996) of which almost 60 variants have been isolated (Rinehart et al., 1994). Microcystin is produced not only by *M. aeruginosa*, but also by *M. viridis* (Watanabe et al., 1986; Kusumi et al., 1987), *Anabaena flos-aquae* (Krishnamurthy et al., 1986), *Oscillatoria agardhii* (Meriluoto et al., 1989), and *Nostoc* sp. (Sivonen et al., 1990). The chemical structures of the hepatotoxins contained in *M. aeruginosa* have been elucidated by Botes et al. (1984, 1985).

Although the cyanobacterial production of microcystins in aquatic environments has been well investigated, studies on microbial degradation of microcystin have been very limited. A strain of bacterium (strain MJ-PV) was isolated from irrigation drainage water in Australia that participated in the degradation of microcystins. This strain was identified chemotaxonomically as a new *Sphingomonas* species (Jones et al., 1994; Bourne et al., 1996). Degradation of microcystin-LR was mediated by at least three intracellular hydrolytic enzymes, and two intermediates of microcystin-LR enzymatic degradation were identified as the linearized (acyclo-) microcystin-LR and tetrapeptide NH_2-Adda-Glu(iso)-methyldehydroalanine-Ala-OH (Bourne et al., 1996). In contrast, Jones et al. (1994) suggested that biphasic degradation was due to the sequential induction of two separate bacterial populations: One that could rapidly utilize microcystin-LR as a source of carbon and energy, and a second that co-metabolized the remaining low concentration of microcystin-LR (Jones and Orr, 1994). Since removing toxins from water supplies is highly important, it is desirable to identify bacteria capable of microcystin degradation. We isolated a strain that was able to degrade microcystin from a hypertrophic lake, Lake Suwa, Japan, during a *Microcystis* bloom. Degradation of microcystin by this strain was examined under various conditions. 16S rRNA sequencing suggested that this isolate is a new genus and species.

MATERIALS AND METHODS

Isolation and Identification of Degradative Bacterium

Water samples were taken from a hypertrophic lake, Lake Suwa, Japan, during the bloom of *Microcystis* in 1995. Diluted samples of lake water (10^{-7}–10^{-5}) were inoculated onto a nutrient agar (NA medium, 1% agar plate) plates. Single colonies from these plates were transferred to liquid nutrient broth medium (NB medium). Ten bacterial strains were inoculated into the NB medium, which contained microcystins to investigate degradation of microcystin. When the strains were isolated from Lake Suwa, the water temperature of the lake was 27°C. All cultures of strains were subsequently maintained at 27°C in the dark. The strains were identified with ID-Test-SP18 and ID-Test-NF18 test kits, Candida check, and manual method. The strain Y2 was identified using a chemotaxonomic method and 16S RNA sequencing (Brosius et al., 1978). Cell size was measured using an image analyzer (Olympus, XL-500) equipped for epifluorescence microscopy (Olympus, BHS-2).

Purification and Analysis of Microcystin

Microcystis cells for purification of microcystin were collected from Lake Suwa during the summer. Samples were concentrated by plankton net and lyophilized and stored at −30°C until purification of microcystin was performed. Purification of microcystin was carried out according to the method of Harada et al. (1988). Lyophilized cells were extracted with 5% aqueous acetic acid and the supernatant was applied to an ODS silica gel cartridge (5g, Chromatorex ODS, 100–200 mesh, packed into a polypropylene cartridge). The 90% methanol-extracted eluate from the cartridge was applied to a silica gel cartridge (2g, Sep-Pak), which was preconditioned with methanol, and the cartridge rinsed with methanol. Microcystins were eluted with 50% methanol–water. The eluate was then concentrated by rotary evaporation at 30°C. The residue was used in the microcystin biodegradation experiments. The residual microcystins showed over 90% purity by high pressure liquid chromatography (HPLC) analysis compared with standard microcystins, which were provided by Harada et al. (1988).

The concentrations of microcystins were measured by an HPLC system equipped with an ODS column (Cosmosil 5C18-AR, 4.6×150 mm, Nakarai, Japan). The HPLC system consisted of a Shimadzu (Kyoto, Japan) LC-9A pump coupled to a SPD-10A set at 238 nm and a SPD-M10A photodiodearray detector and a C-R6A integrator. The concentration of microcystin was quantified by comparing the peak area at 238 nm, after separation with a methanol : 0.05 *M* phosphate buffer (pH 3.0, 58 : 42), with the standard microcystins. The flow rate was 1 ml/min. All chemicals were of analytical grade.

Biodegradation of Microcystin

The ability of the 10 bacterial strains to degrade microcystins isolated from Lake Suwa was examined by inoculating them into 10-ml NB medium, which contained either microcystins-RR (20 mg L^{-1}) or -LR (20 mg L^{-1}), for 20 days at 27°C in the dark. Subsamples for microcystin analysis were taken by removing super-

natant following centrifugation of 2 ml after 0, 1, 2, 3, 6, 10, and 20 days of incubation. The supernatant was stored at 4°C until HPLC analysis.

Degradation of microcystins-RR and -LR by strain Y2 was examined at initial concentrations of 4, 7, 18, or 37 mg L^{-1} for microcystin-RR, and 3, 5, 10, and 20 mg L^{-1} for microcystin-LR. Strain Y2 was inoculated at a density of 2.5×10^6 cells ml^{-1}. Subsamples for microcystin analysis were taken by removing the supernatant following centrifugation of 2 ml after incubation for 0, 3, 6, 9, and 12 days.

The degradation rate of microcystins-RR and -LR by strain Y2 at various incubation temperatures was tested at 5, 10, 20, and 30°C. Subsamples for microcystin analysis were taken every day during 7 days of incubation by removing supernatant following centrifugation.

The free organic DM medium, which contained only microcystin and inorganic nutrients, was employed for the degradation experiment. The incubation temperature was 30°C. In every experiment bacterial-free medium was employed as a control.

16S rDNA Sequencing and Phylogenetic Analysis

A 16S rDNA fragment that corresponded to positions 8 to 1543 of *Escherichia coli* 16S rRNA (Brosius et al., 1978) was amplified directly from cell lysates by PCR and purified by the polyethylene glycol precipitation method, as described previously (Hiraishi et al., 1994, 1995). The PCR product was sequenced directly with a SequiTherm Long-Read Cycle sequencing kit (Epicentre Technologies, Madison, MA) and fluorescence-labeled sequencing primers and analyzed with a Pharmacia ALF DNA sequencer. Sequence data were compiled with the GENETYX-MAC program (Software Development, Tokyo, Japan) and subjected to phylogenetic analysis with the Ribosomal Data Project II (Maidak et al., 1999). Multiple alignment of sequence, calculation of the corrected evolutionary distance (Kimura, 1980), and construction of a phylogenetic tree by the neighbor-joining method (Saitou and Nei, 1987) were performed with the CLUSTAL W program (Thompson et al., 1994). The topology of trees was evaluated by bootstrapping with 1000 resamplings (Felsenstein, 1985). Alignment positions with gaps and unidentified bases were excluded for the calculations.

RESULTS

Isolation of Microcystin-Degrading Bacteria

Among 10 strains isolated from Lake Suwa during the bloom of *Microcystis*, only one, strain Y2, was shown to degrade microcystin-RR, -YR, and -LR. This strain was strictly aerobic, chemoorganotrophic, and gram-negative. The cell was rod-shaped, 0.79 ± 0.23 μm in length and 0.49 ± 0.08 μm in width, and produced yellow-colored colonies on agar media. The results of phenotypic tests with a ID-Test-NF18 kit (Code No. 527400) showed that this bacterium could be classified as *Sphingomonas paueimobilis*, although the probability was very low (0.17%).

Degradation of microcystins by strain Y2 commenced within 1 day and the remaining microcystins-RR and -LR were only 5.4 and 10.2%, respectively, of the initial concentration at day 3 (Fig. 1). Both microcystins were completely degraded within 6 days. Degradation of microcystin was observed in HPLC chromatograms, and two new peaks, which have very similar ultraviolet (UV) spectra between 200 and 300 nm to microcystin-LR, but different retention times were observed during the degradation. The two peaks must be degradation products of microcystin because the HPLC chromatograms were very similar to the linearized (acyclo-) microcystin-LR and tetrapeptideNH_2-Adda-Glu(iso)-methyldehydroalanine-Ala-OH (Bourne et al.,

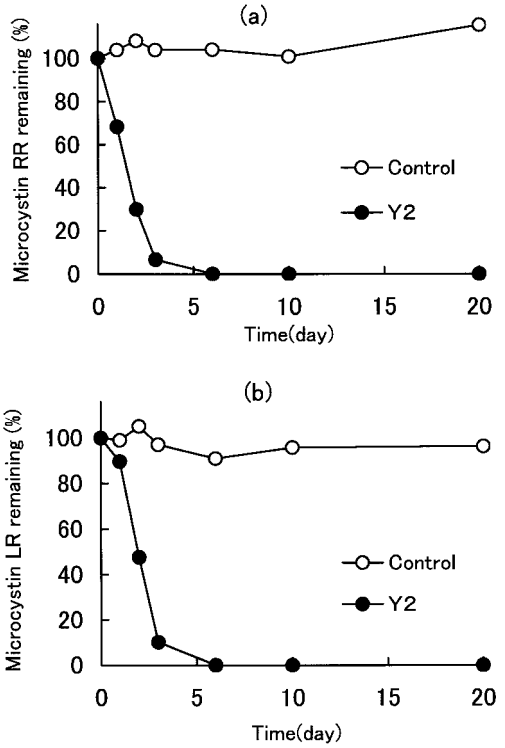

Fig. 1. Degradation of microcystins-RR(a) and -LR(b) over a 20-day period with the strain Y2 isolated from Lake Suwa. Control indicates culture medium without bacteria. The initial concentration of microcystins was adjusted to 20 mg L^{-1} of the NB medium at 27°C in the dark. Initial cell density was 2.5×10^6 cells mL^{-1}.

1996). The degradation products disappeared within 6 days. Other strains and the control did not show any significant degradation of microcystins. This tendency was confirmed by repeats of the same experiments (data not shown).

Phylogenetic Analysis

To obtain more definitive information on the taxonomic and phylogenetic position of strain Y2, we determined its 16S rRNA gene sequence and compared this with the sequences available from the Ribosomal Data Project. On-line homology search showed that, among established species, the 16S rDNA sequence of strain Y2 was most similar to the sequence of *Rhizomonas subrifaciens* (94.6% similarity) among the sequences of the established species.

Evolutionary distances were calculated for a dataset that consisted of the sequence of strain Y2 and 28 other sequences of the a-4 group of the alpha subclass of the Proteobacteria with *Rhodospirillum rubrum* as an outgroup. A neighbor-joining phylogenetic tree was reconstructed on the basis of the distance matrix data thus obtained (Fig. 2). Strain Y2 branched deeply from a major cluster, which consisted of *Rhizomonas subrifaciens*, *Blastomonas natatoria*, and some members of the

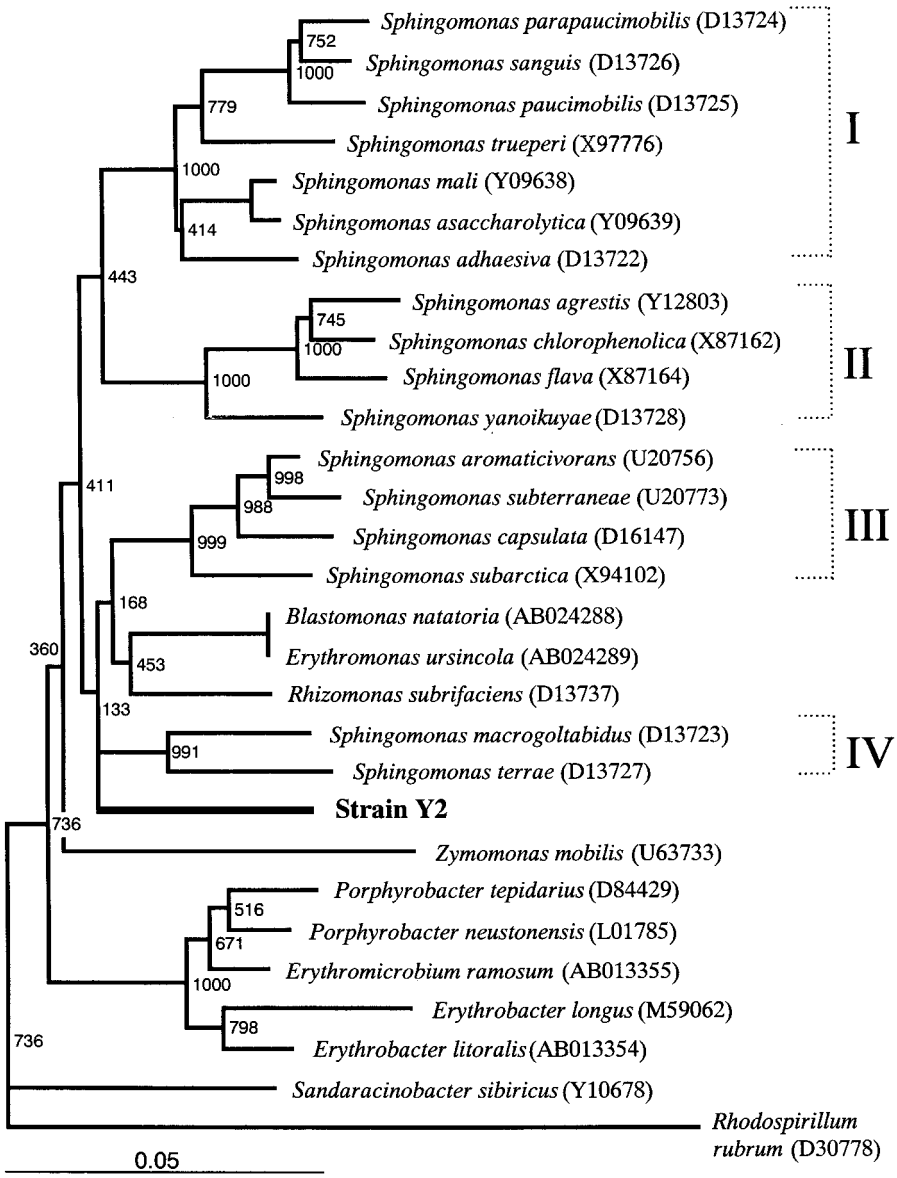

Fig. 2. Distance matrix tree showing phylogenetic relationship between strain Y2 and other members of the alpha-4 group of Proteobacteria. The sequence of *Rhodospirillum rubrum* was used as an outgroup to root the tree. Bootstrap values with 1000 trials are shown at branching points of interest. Scale bar = 5% nucleotide substitution.

genus *Sphingomonas*. Established species of the genus *Sphingomonas* were phylogenetically diverse and classified into four major subgroups designated here I to IV. Therefore, it is more appropriate to classify strain Y2 as a new genus and species than to include it as a member of the genus *Sphingomonas*.

Degradation Rate of Microcystin

Figure 3 shows the degradation rate of microcystin-RR and -LR from various initial concentrations. The degradation rate increased proportionally to the initial concentration for 4–18 mg L^{-1} microcystin-RR, but the change was less marked between 18 and 37 mg L^{-1}. The rate was slightly lower at 37 mg L^{-1}. The degradation rate of microcystin became large in accordance with concentration from several mg L^{-1} day^{-1} to a maximum of ca. 13 mg L^{-1} day^{-1} [Fig. 3(a)]. The degradation rates differed between several mg L^{-1} day^{-1} and ca. 20 mg L^{-1} day^{-1} by a factor of 2 or 3 when strain Y2 was inoculated at 2.5×10^6 cells mL^{-1}. The degradation rate of microcystin showed almost the same pattern with microcystin-LR as with microcystin-RR, but the rate for microcystin-RR was about twice as high as that for microcystin-LR for each initial concentration. The highest degradation rate of microcystin-LR was 5.4 mg L^{-1} day^{-1} and that for microcystin-RR was 13 mg L^{-1} day^{-1}, when incubated with microcystin as an initial concentration of 18 mg L^{-1} . Strain Y2 was also tested on degradation of microcystin-YR, but at only one initial concentration: The degradation rate was 61 mg L^{-1} day^{-1} for the initial concentrations of 22 mg L^{-1} (figure not shown). Degradation rate showed almost the same pattern for microcystin-YR as for microcystin-RR and -LR, but the degradation rate of microcystin-YR was about 10 times higher than that of microcystin-LR.

Figure 4 shows the growth of strain Y2 with the various initial concentrations of microcystin-RR. The density of strain Y2 was 2.5×10^6 cells mL^{-1} at day 0 in each case. The density was $4–6 \times 10^8$ cells mL^{-1} after incubation for 3 days, when almost all the microcystin ($> 95\%$) was degraded.

The degradation rates were strongly dependent on the incubation temperature. Figure 5 shows degradation rates for microcystins-RR and -LR by strain Y2 at various incubation temperatures ranging from 5 to 30°C. Degradation of microcystin began within 1 day when incubated at temperatures between 10 and 30°C. Microcystin was consumed to below the detection limits after incubation for 4 days. On the other hand, degradation at 5°C was very slow and microcystin remained at over 30% of the initial concentration after 7 days. The highest degradation rate occurred at 30°C. The influence of temperature on the rate of microcystin degradation was almost the same for microcystin-LR and microcystin-RR. The control showed no

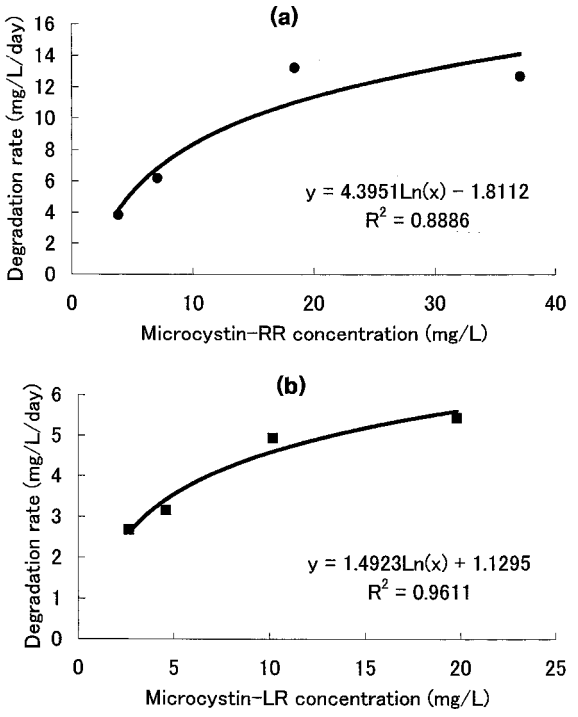

Fig. 3. Degradation rate of microcystins-RR(a) and -LR(b) with Y2 strain and various initial concentrations in NB medium at 27°C in the dark: (a) 4, 7, 18, 37 mg L^{-1}; (b) 3, 5, 10, 20 mg L^{-1}. Initial cell density was 2.5×10^6 cells mL^{-1}.

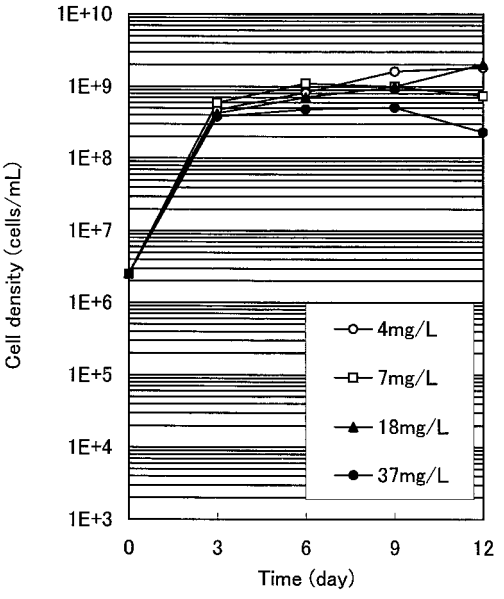

Fig. 4. Growth of strain Y2 on various initial concentration of microcystin-RR (4, 7, 18 and 37 mg L^{-1}) in NB medium at 27°C in the dark. Initial cell density was 2.5×10^6 cells mL^{-1}.

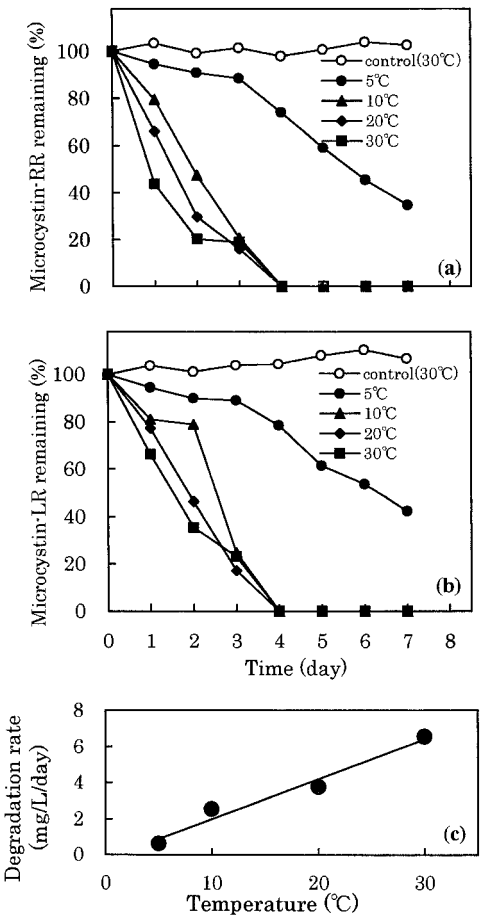

Fig. 5. Degradation of microcystins-RR and -LR with strain Y2 on the various incubation temperature, 5, 10, 20, and 30°C. (a) Degradation of microcystin-RR. The control (culture medium without bacteria) was incubated at 30°C. (b) Degradation of microcystin-LR. (c) Degradation rate of microcystin-RR (mg L^{-1} day^{-1}). Initial concentration of microcystins was adjusted to 20 mg L^{-1} of the NB medium in the dark. Initial cell density was 2.5×10^6 cells mL^{-1}.

degradation of microcystins after incubation for 7 days. The degradation rate of microcystin-RR increased with increase in temperature from 5 to 30°C [Fig.5(c)]. The degradation time in the organic-free medium, which contained only microcystin and inorganic nutrients, was four times faster than with organic nutrient medium (figure not shown).

DISCUSSION

While cyanobacterial production of microcystin in aquatic environments has been well investigated, very limited research has been carried out on microbial degradation of microcystin. We isolated a microcystin-degrading bacterium, strain Y2, from the surface water of the hypertrophic Lake Suwa, Japan. This bacterium was phenotypically similar to members of the genus *Sphingomonas*, but was shown to be phylogenetically distinct from any established species of this genus based on the 16S rDNA sequence data. When the strain was grown in the presence of microcystins, the peptide was decomposed completely in 6 days. Strain Y2 showed a higher rate of degradation of microcystin than strain MJ-PV studied by Jones et al. (1994) and did not show any lag phase during the degradation of microcystin. Jones et al. (1994) reported that the lag phase disappeared upon readdition of microcystin-LR to the water and suggested that biphasic degradation was provided by the sequential induction of two separate bacterial populations: one that could utilize microcystin-LR as a source of carbon and energy, and a second that co-metabolized the remaining low concentration of microcystin-LR (Jones and Orr, 1994).

In contrast, Y2 alone showed a distinct degradation pattern. Degradation of microcystin by the strain Y2 was also examined with an organic nutrient-free medium containing only microcystin and inorganic nutrients: Degradation was four times faster than in medium containing organic nutrients. This suggests that strain Y2 can use microcystin as a carbon and energy source. Furthermore, strain Y2 showed very high degrading activity to microcystin-YR. Microcystin-YR contains seven peptide-linked amino acids, consisting of D-alanine, β-linked erythro-β-methylaspartic acid, γ-linked glutamic acid, tyrosine of L-amino acid, arginine of L-amino acid, *N*-methyldehydroalanine, and 3-amine-9-methoxy-10-phenyl-2,6,8,-trimethyldeca-4,6-dienoic acid. Microcystins-RR and -LR were the main components of the cyanotoxins contained in Lake Suwa's *Microcystis* blooms, but microcystin-YR was either present in very small quantities or undetectable (Park et al., 1998a). The degradation rate of microcystin-YR was 61 mg L^{-1} day^{-1} for the initial concentration of 22 mg L^{-1}.

Microcystin predominantly exists inside the cyanobacterial cells (intracellular microcystin) in lake water (Park et al., 1996), while only a small quantity exists in filtered lake water (extracellular microcystin). In Lake Suwa, a high concentration of intracellular microcystin was found during the exponential growth phase of *Microcystis* bloom, with the highest value being 184 μg L^{-1} on 10 October 1994. In contrast, the highest concentration of microcystin in filtered lake water was found at the end of the bloom, amounting to < 4 μg L^{-1} during the period of the study. The high percentage of extracellular microcystin in filtered lake water at the end of blooms might indicate that release of microcystin from cells occurred predominantly during senescence through decomposition of the *Microcystis* (Park et al., 1998a). It is possible that extracellularly released microcystin was decomposed swiftly, if a mi-

crocystin-degrading bacteria was present, as the observed concentration of microcystin in filtered lake water was not high compared with the amount of intracellular microcystin.

The fate of microcystin in the aquatic ecosystem has been investigated by many researchers throughout the world. Possible pathways of microcystins in a lake ecosystem were studied from five perspectives: production, adsorption, physiochemical decomposition, bioaccumulation, and biodegradation. Of these, only microcystin production has been fully clarified. The presence of a cyanobacterial toxin can be a potential threat, and therefore water treatment requires more attention. A complete understanding of the mechanism of microcystin degradation in the lake ecosystem requires more intensive study, with quantitative enumeration of microcystin-degrading bacteria, which should be done in conjunction with a study on the microbial ecological mechanism of the degradation of cyanobacteria itself.

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