Degradation characteristics of microcystins by isolated bacteria from Lake Kasumigaura

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ABSTRACT

A microcystin degradative bacteria was isolated from natural lakes and its phylogenetic position was confirmed. The 16 s rRNA of the bacteria exhibited 98.5% homology with the 16 s rRNA of *Sphingomonas stygia* belonging to the alpha subdivision on proteobacteria. The bacteria could degrade microcystins as a sole carbon source, therefore the bacteria utilizes microcystin. The bacteria could degrade microcystins LR, YR and RR, and their specific degradation rates were 0.66 h^{-1}, 1.25 h^{-1} and 1.90 h^{-1}, respectively. The degradation activity and growth rate of the bacteria was decreased under alkaline pH conditions.

Key words | 16 s rRNA, microcystin, *Sphingomonas*

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INTRODUCTION

Worldwide, massive accumulations of cyanobacteria called water blooms have frequently occurred in eutrophic lakes and reservoirs during the long period from summer to autumn (Skulberg *et al.* 1984; Ohkubo *et al.* 1993). Although water bloom is often formed by *Microcystis*, *Oscillatoria*, *Anabaena* and other cyanobacteria, most cases are due to *Microcystis* sp. (Carmichael *et al.* 1988; Harada *et al.* 1991*a*,*b*). The proliferation of *Microcystis* causes serious problems such as high rise of pH value, depletion of dissolved oxygen and an offensive odour. Moreover, some cyanobacteria produce toxic compounds such as microcystins.

Microcystins are haepatotoxic compounds with cyclic heptapeptides resulting in the death of fishes, birds, many kinds of domestic animals and even humans. In 1996 in Brazil, many people died from using water containing microcystins introduced during treatment. Moreover, recent studies have reported that microcystins are related to cancer. The World Health Organization (WHO) has established a provisional guideline value (1 μg l^{-1}) of microcystin LR for drinking water.

As microcystins are stable against physico-chemical factors, biodegradation is the main factor of decrease in microcystins in natural water (Jones & Orr 1994; Lam

et al. 1995; Cousin *et al.* 1996). There have been some reports on the degradation of microcystins by aquatic bacteria, but there has been no information about the phylogenetic positions of microcystin degradative bacteria and their fundamental physiological characteristics until now (Jones & Bourne 1994; Takenaka & Watanabe 1997). Such information would be very useful to know the diversity of organisms contributing to the microcystin degradation in aquatic environments.

In this paper the phylogenetic position and physiological characteristics of microcystin degradative bacteria isolated from Lake Kasumigaura were examined. Furthermore, as the pH of lake water changes widely according to season, it includes information on the effect of pH on the degradation of microcystins by the bacteria.

MATERIALS AND METHODS

Isolation of microcystin degradative bacteria

Surface water as an isolation source was collected from Lake Kasumigaura in summer in 1999. The water sample was filtered with a glass filter (1.2 μm pore size). One mg l^{-1} of microcystin LR was added in 10 ml of filtered water in test tubes to the enrichment culture of microcystin-degrading bacteria. Microcystin removal was monitored by HPLC. To isolate single colonies, samples from the enrichment culture were streaked onto solidified PGY medium (5 g peptone, 2.5 g yeast extract, 1 g glucose, 15 g agar per litre). Fifty visible colonies which appeared on these medium plates were picked up at random and transferred to a liquid medium containing mirocystin LR as a sole carbon source (1 mg microcystin, 5 mg $Ca(NO_3)_2 \cdot 4H_2O$, 10 mg KNO_3, 5 mg $NaNO_3$, 4 mg Na_2SO_4, 5 mg $MgCl_2$ $6H_2O$, 0.5 mg $NaEDTA_2$ H_2O, 0.05 mg $FeCl_3$ $6H_2O$, 0.5 mg $MnCl_2 \cdot 4H_2O$, 0.05 mg $ZnCl_2$, 0.5 mg $CoCl_2$ $6H_2O$, 0.5 mg Na_2MoO_4 $2H_2O$, 2 mg H_3BO_3 50 mg per litre) to check the microcystin LR degradation activity. Each test sample was incubated for 5 days at 30°C with shaking (250 rpm) and the amount of remaining microcystin LR in the sample liquid was analysed by HPLC.

We chose one stain which degraded microcystin LR remarkably faster than the other strains for the microcystin degradation experiments.

The isolated bacteria were grown in liquid PY medium (5 g peptone, 2.5 g yeast extract per litre) and stored frozen at −30°C.

Identification of microcystin degradative bacteria by biochemical and molecular methods

A gram stain test was performed by standard procedures. The biochemical characteristics of isolated bacteria were examined by BIOTEST (Eiken Co. Ltd, Japan) and *Bergey's Manual* was referred to regarding the genus.

We entrusted NCIMB Japan with the analysis of 16 s rRNA. Related sequences were obtained from the DNA data bank of Japan (DDBJ) on the web site. Phylogenetic analysis was performed by using the BLAST search program in order to know the genetic distance of the isolated bacteria and other species. The sequence alignments and phylogenetic distance were calculated by using the CLUSTAL W algorithm, and the phylogenetic tree was drawn by using the Tree View program (free software, Division of Environmental and Evolutionary Biology, Institute of Biological and Life Science, University of Glasgow).

Test of degradation activities for microcystin LR, RR and YR of isolated bacteria

Bacteria were added to test tubes containing 5 ml of inorganic medium and 1 mg l^{-1} of each microcystin (LR, RR and YR) as a sole carbon source. Bacteria concentration was adjusted to 0.3 optical density (OD_{660}).

The bacteria were pre-incubated with PY medium for 40 h. Bacteria in the late logarithmic growth phase were removed and centrifuged at 12,000 g for 5 min. The supernatant was decanted and settled bacteria were resuspended with 0.05 M potassium phosphate buffer (pH 7.0). The washing procedures were repeated three times.

The test tubes with microcystin and washed bacteria were incubated at 30°C on a shaker at 250 rpm. The degradation test was performed three times under the same conditions.

Conditions of HPLC

Microcystins were analysed by HPLC (Simadzu) using reverse phase column Cosmosil C 18 4.6 × 250 mm

Table 1 | Physiological and morphological characteristics of isolated bacteria MD-1*

Colony colour	Yellow	ß Galactosidase	–	Utilization: Glucose	–
Cell form	Rod	H2S production	–	Xylose	+
		Escrine	+	Mannose	–
Cell size (μm)					
Width	0.3–0.5	Citrate utilization	–	Arabinose	+
Length	0.7–1.0	Arginine dehydrogenase	+	Fuluctose	–
Gram stain	–	Urease	–	Martose	–
				Ramnose	–
Oxidase	+	Acetoamido degradation	–	Mannitol	–
Malonic acid utilization	–	Gelatinase	+	Saccharose	+

* + and – mean positive and negative, respectively. Bacteria seeded in each well were incubated for 48 h at 30°C.

(nacalai tesque, Japan). The mobile phase was 60% methanol controlled at pH 3 with 0.05 M phosphate buffer. The flow rate was 1 ml/min. The column oven was set at 40°C and the wavelength was set at 238 nm.

Test of pH effect on microcystin LR degradation activity of isolated bacteria

Bacteria were added to test tubes containing 5 ml of inorganic medium and 1 mg l^{-1} microcystin LR as a sole carbon source. The bacteria concentration was adjusted to 0.3 optical density (OD_{660}). pH was adjusted with three buffers. pH 6.80, 7.02, 7.65 and 8.1 were adjusted with potassium phosphate buffer, pH 8.05, 8.50, 9.0, 9.45 and 10.00 with boric acid buffer and pH 9.90 and 10.40 with NaOH. The test tubes were incubated at 30°C on a shaker at 250 rpm. When the test was finished, the pH value in each test tube was measured in order to check stability.

Test of pH effect on growth of isolated bacteria

One ml of stored MD strain was added to 100 ml of PY medium in flasks. The pH of the PY medium was adjusted to 6.9, 8.7 and 9.8 with potassium phosphate buffer, boric acid buffer and NaOH. The growth of MD was measured by optical density (OD_{660}).

RESULTS AND DISCUSSION

Isolation and identification

Five colonies among 50 isolated colonies from the enrichment culture could degrade microcystin LR. In particular, strains No. 14 and No. 16 of the five colonies were able to degrade microcystin LR very rapidly at 95% and 100% within five days. It is thought that these bacteria utilize microcystin LR as a carbon source because microcystin LR was the sole carbon source in this test. Strain No. 16 (MD) had the highest degradation activity. We named the strain as MD and it was identified by biochemical and genetic methods.

Table 1 shows the results of biochemical and morphological tests on the MD strain. The colony colour of the MD strain was yellow, the cell was rod-shaped, of 1.0–1.2 μm length and 0.5–0.7 μm width, and the gram stain was negative. The MD strain was positive for oxidase, gelatinase, arginine dehydrogenase and escrine, and could

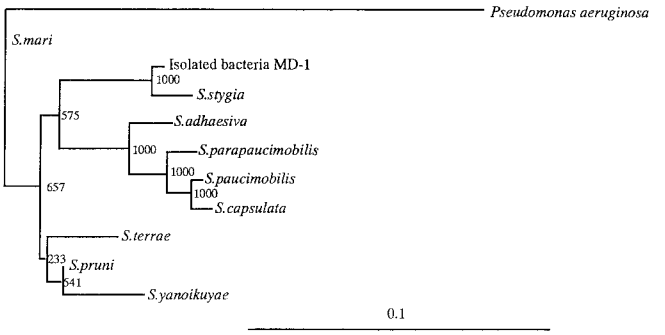

Figure 1 | Phylogenetic tree of 16 s rRNA from isolated bacteria MD-1 and the related members of bacteria. The tree was constructed by using a neighbour-joining tree and a 1000 bootstrap for the confidence level. The sequences used were from DDBJ and GenBank under the accession number: Pseudomonas aeruginosa, AB037545; Sphingomonas mari, D28574; Sphingomonas stygia, AB025013; Sphingomonas adhaesiva, D84527; Sphingomonas parapaucimobilis, D84525; Sphingomonas paucimobilis, D84528; Sphingomonas capsulata, D84532; Sphingomonas terrae, D84531; Sphingomonas pruni, D28568; Sphingomonas yanoikuyae, D84536.

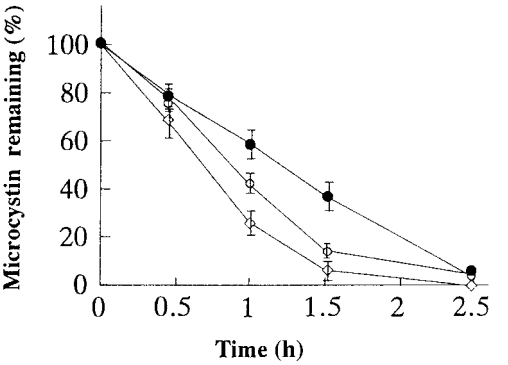

Figure 2 | Degradation of microcystin LR, microcystin YR and microcystin RR by isolated bacteria MD-1. Symbols: ●, microcystin LR; ○, microcystin YR; ◇, microcystin RR.

utilize aribinose, xylose and saccharose. Referring to *Bergey's Manual*, these characteristics of the MD strain placed it in the aerobic *Pseudomonadaceae* group.

Sequences of the 16 s rRNA of the MD strain (501 bp) were determined. The 16 s rRNA of the MD strain exhibited 98.5% homology with the 16 s rRNA of *Sphingomonas stygia* (accession number AB025013) belonging to the alpha subdivision on proteobacteria. The phylogenic tree based on the sequences of *Sphingomonas* group and *Pseudomonas aeruginosa* is shown in Figure 1.

There have been few reports about microcystin degradative organisms. *Sphingomonas* sp. and *Pseudomonas aeruginosa* were isolated and their microcystin degradation abilities were revealed in previous studies (Bourne & Jones 1996; Takenaka & Watanabe 1997). The MD strain was also identified as *Sphingomonas* sp. by sequences of 16 s rRNA. It is known that many species in the *Pseudomonadaceae* group, including *Sphingomonas*, have the ability to degrade various kinds of chemicals which are hard to degrade, and can produce several specific enzymes adapted to them (Higson & Focht 1990; Folson *et al.* 1990; Nagata *et al.* 1999). On the other hand, microcystin was hard to degrade for general enzymes. We research the stability of microcystin LR for some general proteases such as trypsin, chimotrypsin, collagenase and pepsin, which are low in substrate specificity and have their specific activation sites in the microcystin LR structure. None of the examined proteases degraded microcystin LR (unpublished). Microcystins would be hard to be degraded by proteases because they are cyclic heptapeptide structures that are low in flexibility. So it is suggested that, in lake water, the biodegradation of microcystins is performed by a certain specific enzyme produced by some bacteria which have a high adaptation ability, such as those in the *Pseudomonad* group.

Degradation of microcystin by the MD strain

Figure 2 shows the degradation of microcystin by the MD strain. One mg l^{-1} of microcystin LR was 95% degraded in 2.5 h. Microcystin RR and microcystin YR were degraded by the MD strain too, and their degradation ratios were 96% and 98% in 2.5 h. In order to understand the degradation extent of microcystin RR, YR and LR at the *in situ* level, firstly it was expressed as a specific degradation rate in *in vitro* studies. Specific degradation rates of the three microcystin types, LR, RR and YR, were calculated as 0.66 h^{-1}, 1.25 h^{-1} and 1.90 h^{-1}, thus the bacterium has the highest degradation activity on microcystin RR (Table 2).

The MD strain was able to degrade microcystin RR and microcystin YR, in spite of using microcystin LR for the enrichment culture or the induction of degradation activity. Jones & Bourne (1994) reported that *Sphingomonas* sp. isolated from a natural water body in Australia could degrade both microcystin RR and microcystin LR, and the degradation rate of microcystin LR was higher than that of microcystin RR. Conversely, the

Table 2 | Comparison of the specific degradation rate of each microcystin with *Sphingomonas* sp. MD-1

Substrate	Specific rate (h^{-1})
Microcystin LR	0.66
Microcystin YR	1.25
Microcystin RR	1.90

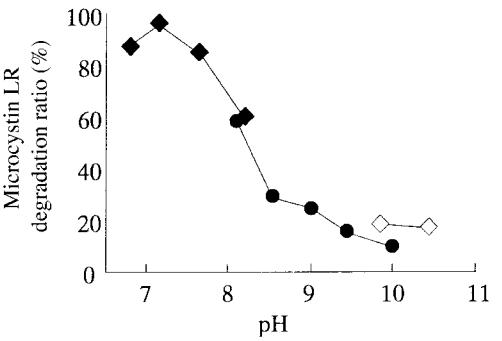

Figure 3 | Effect of pH on degradation of microcystin by isolated bacteria MD-1. Symbols: ◆, phosphate buffer; ●, boric buffer; ◇, NaOH.

MD strain degraded microcystin RR most rapidly among the three types of microcystin. From these results, it is suggested that the degradation rates for microcystins vary according to the different species of bacteria or living environments. We also surveyed the degradation of three types of microcystins by indigenous mixed bacteria in a lake. In this experiment, microcystin LR was the most rapidly degraded of the three types by indigenous mixed bacteria. The ratios of degradation rates of the three types of microcystins, LR, RR and YR were 1:0.6:0.9. On the other hand, the ratio in the case of *Sphingomonas* sp. in this paper was 1:2:2.8 (Table 2). The difference in degradation ratio pattern among three types of microcystin reveals the diversity of microcystins degrading bacteria and degradation mechanisms.

pH effect on the degradation of microcystin LR by the MD strain, and the growth of the bacteria

Figure 3 shows the effect of pH on the degradation of microcystin LR by the MD strain. The degradation activity for microcystin LR by the MD strain was the highest at

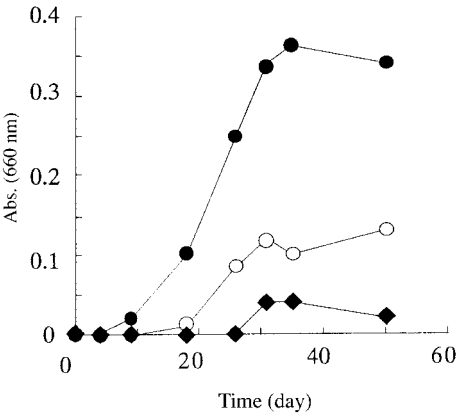

Figure 4 | Effect of initial pH on growth of MD-1. Symbols: ●, pH 6.9; ○, pH 8.7; ◆, pH 9.8.

pH 7.2. One mg l^{-1} of microcystin LR was degraded more than 98% in 3 h. In alkaline media, the degradation activities of the MD strain were lower than in the neutral pH condition, the activities at pH 8.1 and pH 9.5 were 50% and 15% of that at pH 7·2.

The growth of the MD strain under several pH conditions was examined (Figure 4). The bacteria grew slowly at the initial pH conditions of 8.7 and 9.8. In contrast, the bacteria grew rapidly under the initial pH condition of 6.9. From these results, it was found that the MD strain lives in a neutral pH condition and has the highest degradation activities for microcystin LR in that condition.

There have been no reports on the effect of pH on biodegradation of microcystins. We surveyed the microcystin LR degradability of the MD strain under several pH conditions, and found the degradability was lower under the alkaline condition than the neutral condition. In general, waters of eutrophic lakes are alkaline in summer owing to photosynthesis by algae or aquatic plants. In Lake Kasumigaura, which is an isolation source of the MD strain, the pH value reaches over 10.0 in mid-summer and massive water bloom occurs. It was supposed that the activity of the MD strain was repressed owing to the high pH in natural water. The pH value returns to neutral condition in autumn, so the MD strain may grow in the lake water and degrade microcystins produced by cyanobacterial cells in that season. In our previous studies, microcystin LR could be degraded by indigenous mixed bacteria even in the alkaline condition, similar to the

neutral pH condition. Accordingly, other alkaliphilic bacteria may strongly affect the degradation of microcystins in the summer season.

We can conclude that various organisms are concerned in the degradation of microcystin from two view points, such as the difference of degradation ratio patterns of three types of microcystins and the variation of favourable pH condition among the organisms.

The discovery of various microcystin-degrading bacteria, and recognition of the diversity, can construct the foundation of the understanding of the dynamics of microcystin in natural water.

CONCLUSIONS

The bacterium which can degrade microcystin was isolated from a natural lake, and some characteristics were clarified as followed:

1. The bacterium could degrade microcystin as a sole carbon source, therefore the utilization of microcystin by the bacterium as a food source is indicated.
2. From the results of genetic and biochemical analysis, the bacterium was identified as *Sphingomonas* sp. The same genus (not the same species) had been isolated from a lake in Australia, but the degradation ratios of microcystin RR, YR and LR of the two species were not the same. It is expected that various kinds of degradation mechanisms exist, and they are different in the two kinds of organisms in spite of the fact that they are in the same genus.
3. Many kinds of species which can produce several enzymes that have specific functions belong to the *Pseudomonas* group; the bacterium isolated in this study may have a unique enzyme for the degradation of microcystin. To advance the studies for microcystin degradation, the information about the specific enzyme is needed.
4. *Sphingomonas* sp. was weak under alkaline conditions. In a eutrophic lake, the water becomes alkaline in condition in summer, therefore the degradation abilities would be repressed in the summer season.

REFERENCES

Bourne, D. G. & Jones, G. J. 1996 Enzymatic pathway for the bacterial degradation of the cyanobacteria cyclic peptide toxin microcystin LR. *Appl. Environ. Microbiol.* **62**(11), 4086–4094.

Carmichael, W. W., Beasley, V. R., Bunner, D. L., Eloff, J. N., Falconer, I., Gorham, P., Harada, K., Krishnamurthy, T., Yu, M.-J., Moore, R. E., Rinehart, K., Runnegar, M., Skulberg, O. M. & Watanabe, M. F. 1988 Naming of cyclic heptapeptide toxins of cyanobacteria (blue-green algae). *Toxicon* **26**, 971–973.

Cousin, I. T., Bealing, D. J., James, H. A. & Sutton, A. 1996 Biodegradation of microcystin LR by indigenous mixed bacterial populations. *Wat. Res.* **30**(2), 481–485.

Folson, B. R., Chapman, P. J. & Prichard, P. H. 1990 Phenol and trichloroethylene degradation by *Pseudomonas cepacia* G4: kinetics and interactions between substrates. *Appl. Environ. Microbiol.* **56**(5), 1279–1285.

Harada, K., Ogawa, K., Kimura, Y., Murata, H., Suzuki, M., Thorn, P. M., Evans, W. R. & Carmichael, W. W. 1991*a* Microcystins from *Anabaena flos aquae* NRC 525-17. *Chem. Res. Toxicol.* **4**, 534–540.

Harada, K., Ogawa, K., Matuuira, K., Nagai, H., Murata, H., Suzuki, M., Itezono, Y., Nakayama, J., Shirai, M. & Nakano, M. 1991*b* Isolation of the toxic heptapeptide microcystins from an axenic strain of *Microcystis aeruginosa* K-139. *Toxicon* **29**, 479–489.

Higson, F. K. & Focht, D. D. 1990 Degradation of 2-bromobenzoic acid by a strain of *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.* **65**(6) 1615–1619.

Jones, G. J. & Bourne, D. G. 1994 Degradation of the cyanobacterial hepatotoxin microcystin by aquatic bacteria. *Natural Toxins* **2**(4), 228–235.

Jones, G. J. & Orr, P. T. 1994 Release and degradation of microcystin following algicide treatment of a *Microcystis aeruginosa* bloom in a recreational lake, as determined by HPLC and protein phosphatase inhibition assay. *Wat. Res.* **30**(2), 481–485.

Lam, A. K. Y., Fedrak, P. M. & Prepas, E. E. 1995 Biotransformation of the cyanobacterial hepatotoxin microcystin-LR, as determined by HPLC and protein phosphatase bioassay. *Environ. Sci. Technol.* **29**, 242–246.

Nagata, Y., Miyauchi, K. & Takagi, M. 1999 Complete analysis of genes and enzymes for g-hexachlorocyclohexane degradation in *Sphingomonas paucimobilis* UT26. *J. Industrial Microbiol. & Biotechnol.* **23**, 380–390.

Ohkubo, N., Yagi, O. & Okada, M. 1993 Studies on the succession of blue-green algae, *Microcystis*, *Anabaena*, *Oscillatoria* and *Phormidium* in Lake Kasumigaura. *Environ. Technol.* **14**, 433–442.

Skulberg, O. M., Codd, G. A. & Carmichael, W. W. 1984 Toxic blue-green algae blooms in Europe. A growing problem. *Ambio* **13**, 244–247.

Takenaka, S., & Watanabe, F. M. 1997 Microcystin LR degradation by *Pseudomonas aeruginosa* alkaline protease. *Chemosphere* **34**(4), 749–757.

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