Quantification of Microcystin-degrading Bacteria in a Biofilm from a Practical Biological Treatment Facility by Real-time PCR

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ABSTRACT
A rapid decrease in the concentration of microcystin due to the decline of Microcystis spp. cells has been observed during fall in Japan. Past researches have shown the involvement of microcystin-degrading bacteria in this phenomenon, but the process by which it occurs has not yet been elucidated. In this research, microcystin-degrading bacteria were quantified using real-time TaqMan polymerase chain reaction. The new TaqMan probe was based on the sequence of the mlrA gene that is conserved in microcystin-degrading bacteria; new primers were similarly developed. These new primers and probe enabled the precise examination of microcystin-degrading bacteria in a biofilm. Moreover, the bacteria present in a biofilm from a practical biological treatment facility could be detected and quantified. The results showed that microcystin-degrading bacteria existed in the biofilm throughout the year, and the number of bacterial cells increased in fall.

Keywords: microcystin, microcystin-degrading bacteria, Microcystis spp., Sphingomonas spp., water bloom

INTRODUCTION
Hepatotoxic microcystins produced by cyanobacteria belonging to the genera Microcystis, Anabaena, Oscillatoria, and Nostoc are frequently observed in Japanese lakes and reservoirs (Ohkubo et al., 1993). The proliferation of water bloom-forming Microcystis spp. is fatal to fish, wild birds, and mammals, since ingestion of microcystins that are produced by Microcystis spp. is toxic to these organisms (Magalhaes et al., 2001). In 1996, microcystins presented a public health threat; the most serious incident involving humans occurred in Brazil, when 76 patients of a hemodialysis clinic died (Jochimsen et al., 1998). Moreover, past studies have reported the carcinogenicity of microcystins through tumor promotion (Carmichael et al., 1988).

Microcystins are physicochemically stable and they could continue to remain in lake water, even if Microcystis spp. cells disappeared. However, Sugiura et al. (2002) reported a decrease in the concentration of microcystins along with a decrease in the number of Microcystis spp. cells during autumn in a Japanese lake (Sugiura et al., 2002). For this reason, biodegradation is assumed to be the main factor responsible for the decrease of microcystins in the environment (Tsuji et al., 1995; Saito et al., 2003b).

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Some microcystin-degrading bacteria have been previously detected in several different areas of the world. In 1994, Jones et al. (1994) isolated for the first time a microcystin-degrading bacterium, MJ-PV, from an Australian water body. The bacterium was identified as *Sphingomonas* sp. based on the 16S rRNA gene sequence (Jones et al., 1994). The microcystin-degrading gene cluster (*mlrA, B, C, and D*) was detected in the bacteria by Bourne et al. (2001). The enzyme encoded by the *mlrA* gene can break open the cyclic structure of microcystin. After opening the ring structure, linear microcystin-LR is degraded by the peptidases encoded by *mlrB* and *mlrC*, and divided into its constituent amino acids. It is known that *mlrD* encodes the transporter protein that allows the uptake of microcystins into the cell (Bourne et al., 1996; Bourne et al., 2001).

Saito et al., (2003b) reported that suspension of a biofilm from a practical biological treatment facility, which removed organic matter, nutrients, and odors using contact filters in water purification process, degraded microcystins promptly. Their report indicated that microcystin-degrading bacteria exist in the biofilms from practical biological treatment facilities. In addition, they designed a new primer set for detecting microcystin-degrading bacteria (Saito et al., 2003a). These primers are able to detect the conserved sequence of *mlrA*, which breaks open the cyclic structure of microcystins, and have been used for detection and identification of microcystin-degrading bacteria in some reports. However, the bacteria have not been quantified using polymerase chain reaction (PCR), and the changes and dynamics of microcystin-degrading bacteria have not been elucidated. To address this, real-time PCR, which can detect and quantify with high sensitivity, was used in this study.

The aim of this study was to develop a method of quantification of microcystin-degrading bacteria in water environment. We estimated microcystin-degrading bacteria in a biofilm from a practical biological treatment facility by real-time PCR using the newly designed primer set and a TaqMan probe. The specificity of new primers was investigated using PCR from six microcystin-degrading bacteria, MD-1, C-1, Y2, MG-15, MG-22, and *Paucibacter*. Moreover, we quantified and monitored the population dynamics of the microcystin-degrading bacteria in the biofilm throughout the year. This method will clarify the mechanism of degradation of microcystins in lakes with water blooms.

**MATERIAL AND METHODS**

**Bacterial strains and cultivation**

Table 1 shows the strains examined in this study. MD-1 strain was isolated from Lake Kasumigaura in Japan (Saito et al., 2003a). MD-1 strain can degrade the microcystin analogs, microcystin-RR, -YR, and -LR. This bacterium is included in the genus *Sphingomonas* (accession number AB110635). The strain C-1 was isolated from Lake Hongfeng in China and this strain is included in the genus *Sphingopyxis* (AB161684). The strain Y2 was provided by Dr. Park from Shinshu University. This strain was isolated in 1997 from Lake Suwa (Park et al., 2001) and is included in the genus *Sphingomonas* (AB084247). The strains MG-15 and MG-22 were isolated from *Monas guttula* that preys on *Microcystis* cells. These strains are included in the genus *Sphingopyxis* by gene analysis. A *Paucibacter* strain was isolated from the sediment of a
eutrophic lake in southern Finland (Rapala et al., 2005). These six strains were cultivated in 1/5 PY medium (1 g of peptone and 0.5 g of yeast extract per 1,000 mL).

<table>
<thead>
<tr>
<th>Strains</th>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MD-1</td>
<td><em>Sphingomonas</em> sp.</td>
<td>Saito et al., 2003a</td>
</tr>
<tr>
<td>C-1</td>
<td><em>Sphingopyxis</em> sp.</td>
<td>no reference*</td>
</tr>
<tr>
<td>Y2</td>
<td><em>Sphingosinicella microcystinivorans</em></td>
<td>Park et al., 2001</td>
</tr>
<tr>
<td>MG-15</td>
<td><em>Sphingopyxis</em> sp.</td>
<td>no reference**</td>
</tr>
<tr>
<td>MG-22</td>
<td><em>Sphingopyxis</em> sp.</td>
<td>no reference**</td>
</tr>
<tr>
<td>Paucibacter</td>
<td><em>Paucibacter</em> sp.</td>
<td>Rapala et al., 2005</td>
</tr>
</tbody>
</table>

*Isolated by our research group
**Provided by Dr. Fujimoto from Tokyo University of Agriculture

**Biofilm samples**
Samples of biofilm (10 g) were taken from a biological contact material, honeycomb catalyst, of a practical biological treatment facility—a drinking water treatment plant influent from Lake Kasumigaura—every month from September 2005 to September 2006. Water blooms were not observed in this period.

**DNA extraction**
Total DNA was extracted from the biofilm of the practical treatment facility using an ISOIL for Beads Beating kit, according to the manufacturer’s instruction (Nippon Gene, Japan). A 200 µL axenic sample and 200 µL biofilm sample were used for DNA extraction. The samples were homogenized with 450 µL Lysis Solution BB and 50 µL Lysis Solution 20S. After homogenizing, the samples were incubated at 65°C for 30 min to increase the DNA yield. A volume of 600 µL of supernatant was placed in a new tube, and mixed with purifying solution. After vortexing with chloroform, the sample was centrifuged at 12,000 g for 15 min. A volume of 800 µL supernatant was placed in a new tube with 800 µL precipitation solution, mixed, and centrifuged at 20,000 g for 15 min at a temperature of 4°C. The supernatant was removed, and 1 mL of wash solution was added. The sample was lightly mixed and centrifuged (20,000 g, 10 min, 4°C). The supernatant was removed, lightly mixed with 1 mL of 70% ethanol, and then centrifuged (20,000 g, 5 min, 4°C). After removing the supernatant, the sample was allowed to dry and the resulting pellet was dissolved in 100 µL of sterilized water.

**PCR amplification**
PCR amplifications were conducted using modified protocols and the MF-MR and QMF-QMR primer sets described in Table 2. New primers, QMF and QMR, were created based on high homology positions of sequences of *Sphingomonas* sp. MD-1, and designed to assure product size under 350 bps. New probe QMT was designed based on the amplification products from QMF and QMR by Applied Biosystems Co. USA. Thermal cycling was carried out as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95 °C for 10 s, annealing at 55°C for 10 s, and polymerization at 72°C for 30 s. The amplification reactions contained 1 µL of extracted DNA, 10 pmol of each primer, 2.5 U of *Ex Taq* polymerase (Takara, Japan), 5 µL of 10× *Ex Taq* buffer, and 4 µL of dNTP mixture, made up to a final volume of 50
µL using pure water. PCR was performed using a GeneAmp 2400 Thermocycler (Applied Biosystems Co., USA). The PCR reaction products and SYBR Green for DNA staining were electrophoresed on a 2% agarose gel. The DNA of C-1 strain was used as a positive control, and pure water was used as a negative control.

<table>
<thead>
<tr>
<th>Primer and Probe</th>
<th>Sequence (5’-3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MF</td>
<td>gacccgatgttcaagatact</td>
<td>Saito et al., 2003b</td>
</tr>
<tr>
<td>MR</td>
<td>ctctceccacaatcaggac</td>
<td></td>
</tr>
<tr>
<td>QMF</td>
<td>agacgcacgctcacctcaa</td>
<td>in this study</td>
</tr>
<tr>
<td>QMR</td>
<td>gagcagttcacgaaatcc</td>
<td></td>
</tr>
<tr>
<td>QMT (Probe)</td>
<td>atagcctctactgtttccgccgcc</td>
<td></td>
</tr>
<tr>
<td>BACT1369F</td>
<td>cggtgaatacgttcyggg</td>
<td>Suzuki et al., 2000</td>
</tr>
<tr>
<td>PROK1492R</td>
<td>ggwtaccttgttacgactt</td>
<td></td>
</tr>
<tr>
<td>TM1389BACT2 (Probe)</td>
<td>cttgtacacaccgccgcgccgct</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2 - Primers and TaqMan Probes used**

**Real-time PCR**
The microcystin-degrading bacteria were quantified by real-time PCR using QMF and QMR oligonucleotide primers, and a QMT TaqMan probe. BACT1369F and PROK1492R primers and a TM1389BACT2 probe were used to quantify the total bacterial count (Suzuki et al., 2000) (Table 2). Real-time PCR was conducted by first performing the initial denaturation and activation of “hot start” type DNA polymerase at 95°C for 10 min. This was then followed by 50 cycles of denaturation at 95°C for 10 s. Annealing was then performed for QMF, QMR, and QMT at 65°C for 35 s; and for BACT1369F, PROK1492R, and TM1389BACT2 at 56°C for 35 s. The amplification reactions required 1 µL of extracted DNA, 10 pmol of each primer, 30 pmol of TaqMan probe, 25 µL of TaqMan Gene Expression Master Mix (Applied Biosystems Co. USA), made up to a final volume of 50 µL using pure water. Real-time PCR was performed using a 7,500 real-time PCR system (Applied Biosystems, USA). The DNA of C-1 strain was used as a positive control, and pure water was used as a negative control.

**RESULTS AND DISCUSSION**

**Investigation of new primers**
To investigate the effectiveness of the newly designed primers used in this study, they were used for detecting the mlrA gene conserved in microcystin-degrading bacteria. The strains MD-1, C-1, Y2, MG-15, MG-22, and Paucibacter were used as samples. The amplification products were obtained from MD-1, C-1, and Y2 having the mlrA sequence, but no amplification products were obtained from MG-15, MG-22, and Paucibacter strains (Fig. 1A). These results suggested that the strains without amplification products have an unknown mechanism for degrading microcystins. The existence of the mlrA gene was not confirmed in some microcystin-degrading bacteria, and therefore, further studies are required to elucidate the relationship between microcystin-degrading bacteria and the degradative mechanism of microcystins (Meriluoto et al., 2005; Rapala et al., 2005). In addition, the detection procedure using the new primers was compared to that using MF and MR primers that have been used to
detect the *mlrA* gene in previous studies. The amplification products could be detected with higher sensitivity using the new primers than using MF and MR primers (the amplification products showed no increase, or a slight increase for the Y2 strain) (Fig. 1B).

![Figure 1](image)

**Fig. 1** - (A) Comparison of detection of *mlrA* gene using QMF and QMR. (B) Comparison of detection of *mlrA* gene using MF and MR primers. Arrows indicate the DNA product size. The lanes M, 1, 2, 3, 4, 5, 6, and N correspond to DNA marker, MD-1, C-1, Y2, MG-15, MG-22, *Paucibacter*, and negative control, respectively.

**Quantification of microcystin-degrading bacteria by real-time PCR**

The standard curve was generated from serial 10-fold dilutions of DNA carrying the *mlrA* of strain C-1. The quantification of microcystin-degrading bacteria was clear and could be quantified at concentrations as low as $10^2$ cells/mL. Moreover, the microcystin-degrading bacteria in the biofilm of the biological treatment facility were quantified. The bacteria were present in the biofilm throughout the year, suggesting that the biofilm of the biological treatment facility was able to degrade microcystins (Fig. 2). The increase in the concentration of cell numbers was observed in fall when the water bloom typically ends (Park *et al.*, 1998; Sugiura *et al.*, 2002). The increase of cell number was also confirmed in May although the field data was not taken. According to the annual report of the water quality of Ibaraki Prefectural Public Enterprise Bureau, the concentrations of phytoplankton were increased from autumn to spring of 2005 to 2006. The abundance of microcystin – degrading bacteria was positively related to the chlorophyll concentration (Bird and Kalff, 1984), and the phenomena as described previously, suggest that the increase in the number of degrading bacteria in May might be responsible for the increase in the concentration of phytoplankton in Lake Kasumigaura.
Fig. 2 - Seasonal changes in the cell number of microcystin-degrading bacteria in the biofilm from a practical biological treatment facility from September 2005 to September 2006.

To investigate the abundance ratio of microcystin-degrading bacteria in the biofilm, the concentration of total bacteria was quantified by real-time PCR using BACT1369F and PROK1492R primers and a TM1389BACT2 probe. Microcystin-degrading bacteria comprised approximately 0.005% of the total bacteria present in the biofilm, and their cell number increased during fall (Fig. 3).

Fig. 3 - Seasonal changes in the abundance ratio of microcystin-degrading bacteria in the biofilm from a practical biological treatment facility from September 2005 to September 2006.
Recent studies have reported that *Sphingomonas* sp. degrade dioxin, nonylphenol, polycyclic aromatic hydrocarbon (PAH), and other persistent substances (Wilkes *et al.*, 1996; Tanghe *et al.*, 1999; Shi *et al.*, 2001; Sakai *et al.*, 2007). We expect this study to promote the research on the dynamics of the bacteria involved in the degradation of persistent substances *in situ*. We showed that microcystin-degrading bacteria were present in a biofilm from a practical biological treatment facility. However, microcystin-degrading bacteria that do not possess the *mlrA* gene might exist, so, researches on the quantification of microcystin-degrading bacteria, including bacteria without *mlrA* gene are required. Moreover, an increase in the cell number of microcystin-degrading bacteria was observed in the biofilm in autumn at the practical biological treatment facility, but there is little information about the dynamics of the bacteria in lake water and other water bodies. Therefore, further investigation of the dynamics of the bacteria in lakes with cyanobacteria is required.

**CONCLUSION**

We successfully quantified the microcystin-degrading bacteria in a biofilm from a practical treatment facility sensitively and specifically by real-time PCR using newly designed primers and a TaqMan probe. The microcystin-degrading bacteria were detected using a new probe and primer sets, and quantified specifically in the biofilm by real-time PCR. It was found out that microcystin-degrading bacteria were present in the biofilm of the practical biological treatment facility throughout the year, and the abundance ratio of the microcystin-degrading bacteria increased to approximately 0.005% of the total bacteria. The number of cells increased in fall, and the number of microcystin-degrading bacteria increased with the increase in the concentration of phytoplankton.

By using methods described in this paper, the behavior of microcystin-degrading bacteria in water environment can be determined, and moreover, this method might be able to clarify the mechanism of the degradation of microcystin. Further studies, like the degradation of microcystins by bacteria without *mlrA* gene or analysis of the relationship between cyanobacteria and microcystin-degrading bacteria, are required to elucidate the mechanism. However, this result will be the first step for the clarification of microcystin degradation.

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