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Dynamics of the functional gene copy number and overall bacterial community during microcystin-LR degradation by a biological treatment facility in a drinking water treatment plant

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Information is limited on the potential for microcystins (MCs) degradation by carrier-attached biofilms obtained in winter that were not exposed to detectable levels of MCs in the preceding months. Under controlled laboratory conditions, we confirmed that microcystin-LR (MCLR) was effectively biodegraded within 5.5 days in cultures of the biofilm sampled in winter. Quantitative polymerase chain reaction (qPCR) assays revealed that seasonal variations in the MCLR-degradation potential of the biofilm were closely related to the initial MCLR-degrader population in the biofilm. Indigenous MCLR-degraders in the biofilm could accumulate by exposure to natural MCLR in the water column, accelerating MCLR-degradation. The qPCR assay suggested that MCLR may be a primary substrate for the degraders in the presence of another labile organic carbon associated with the biofilm under the present study conditions. qPCR and PCR-denaturing gradient gel electrophoresis (DGGE) for 16 S rDNA demonstrated that the overall bacterial population from the winter biofilm rapidly increased with the MCLR-degrader population and remained stable after day 3.5, while the overall bacterial community structure shifted throughout the entire biodegradation period. This study is important to the in-depth understanding of microbial degradation of MCs and could facilitate the bioremediation of MCs in polluted habitats.

[**Key words:** Biofilm; Degradation; Denaturing gradient gel electrophoresis (DGGE); Microcystins (MCs); *mlrA* gene; Seasonal variation; TaqMan quantitative polymerase chain reaction (qPCR)]

Climate change has increased cyanobacterial blooms in lakes and reservoirs worldwide (1,2). Blooms of species belonging to the genera *Microcystis*, *Anabaena*, *Planktothrix* (*Oscillatoria*), *Nostoc*, and *Hapalosiphon* (3,4), which produce toxic microcystins (MCs) in potable water sources, are of particular concern. MCs are a family of cyclic heptapeptide hepatotoxins that can cause liver failure in a variety of organisms (5) and severely compromise human health (6). As a consequence, a guideline level of 1.0 μg L^{-1} microcystin-LR (MCLR) equivalents has been set for MCs by the World Health Organization (7). The effective removal of MCs is therefore a major effort in the drinking water industry.

Microbial degradation is the most important mechanism for elimination of MCs in the natural environment (8) and qualifies as an alternative water treatment strategy (9,10). MCs biodegradation by heterotrophic bacteria has been investigated in various media including water columns (11–13), sediments (10,14), and soils (15,16).

Microorganisms accumulate in polysaccharide matrices and form structural and functional microbial assemblies on surfaces submerged in water that are commonly known as biofilms (17). Biofilms play a crucial role in the assimilation, retention and transformation of organic materials (18) and also influence the fate of environmental contaminants in aquatic environments through sorption and biodegradation (19). Naturally-originated submerged biofilms attached to carriers have been used for effective bloom removal and MCs biodegradation under controlled laboratory conditions. Most studies have focused on biofilms sampled in summer and/or autumn (20–22), when MCs concentrations reach high levels in water bodies (1) and there is a consequent increase in the MC-degrading bacterial populations in submerged biofilms. However, the quality of the water column varies with the season. This may influence the population and composition of the microorganism matrix in the biofilm attached to the carrier in a biological treatment facility, further affecting the MC-degradation ability and behavior of the indigenous degraders. In particular, microorganisms in biofilms obtained in winter have experienced a prolonged break from MCs exposure and probably have a low abundance of indigenous MC-degraders (23). However, the MC-degradation potential and characteristics of microorganisms in

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winter biofilms have not been well-studied. Exploration of the capabilities of winter biofilms would provide water authorities with more comprehensive monitoring data and enable them to manage water security more effectively, with consideration of different environment factors.

The *mlrA* gene encodes the enzyme responsible for the initial hydrolytic cleavage of the cyclic MCs structure during MCs biodegradation (24,25). Conventional *mlrA* gene-targeted polymerase chain reaction (PCR) assays have been successfully employed to qualitatively detect the presence of indigenous MC-degrading bacteria in lakes (26) and biofilms (11,27), but quantitative analyses of indigenous MC-degrading bacterial populations are still limited. Quantitative real-time PCR (qPCR) assays have recently been developed for the *mlrA* gene (23,28) that could be used to detect shifts in the population of MC-degraders during the process of MC-degradation.

Effective MCs bioremediation depends on the identification of seasonal variations in MC-degradation potential of the biofilms and the factors responsible for these variations, as well as how the dynamics of the MC-degrader population in winter biofilms link to those of biofilms in other seasons. Thus, there is an urgent need for quantitative analysis of indigenous MC-degraders throughout these MC-degradation processes.

As one of the most toxic analogs of MCs, MCLR was used as the target toxin. First, the MCLR-biodegradation ability of a carrier-attached biofilm obtained in winter and the seasonal variation in the MCLR-degradation characteristics of the biofilm were analysed under controlled laboratory conditions. MCLR-degraders were quantified during MCLR-degradation processes by the microorganisms in biofilms from different seasons using TaqMan qPCR. From these results, a link was established between the dynamics of the MCLR-degrader population of the biofilm in winter and those in subsequent seasons; factors that correlate closely with these variations are discussed. qPCR and PCR-denaturing gradient gel electrophoresis (DGGE) for 16 S rDNA were then used to probe the development of the overall bacterial community during MCLR-degradation by microorganisms in the winter biofilm. These data enhance our understanding of the microbial MC-degradation process and will promote the successful application of bioremediation technologies.

MATERIALS AND METHODS

Materials and chemicals Lake Kasumigaura, Japan, is a source of water for drinking and other purposes and receives a large nutrient load (nitrogen and phosphorus) from domestic, municipal and agricultural effluent. Consequently, the lake is a frequent site of toxic MC-producing cyanobacterial blooms with a transient nature, especially during summer and/or autumn (1,29). To reduce the risk associated with toxic MCs, a water treatment plant (WTP) near the lake employs a biological treatment facility packed with vinyl chloride board as a carrier for biofilms. The carrier is kept submerged in the biological treatment (BT) tank. For sampling, the biofilm matrix (active biofilm) coating the surface of the carrier was scraped into a sterile centrifuge tube in January (winter), June (summer; before occurrence of bloom), and September (autumn; after occurrence of bloom) 2010, respectively.

The apparatus upstream of the BT tank includes an intake tower, a pumping station, and a receiving well (RW). The RW receives original lake water via pumping. The original lake water and bio-treated water were sampled from the RW and the BT tank, respectively. Neither water source contained detectable levels of MCs in January and June 2010, or during the previous months, as detected using high performance liquid chromatography (HPLC). However, MCLR concentrations in both water sources were detectable in August and September 2010 (data not shown). Some of the quality characteristics of these water sources during the study period can be found in the Supplementary Material (Table S1). All of the sampled materials were transported on ice to the laboratory at the University of Tsukuba within 1 h and used for experiments immediately.

MCLR standard compound (≥90% purity) was purchased from Wako Pure Chemical Industries, Ltd., Japan, and dissolved in methanol (HPLC-grade) to prepare a stock solution with a concentration of 25 μg mL^{-1}.

MCLR biodegradation MCLR-degradation test was performed in a series of 50-mL glass test tubes. To obtain a fresh suspension of the active biofilm, 0.2 g (fresh weight, FW) of active biofilm was immediately added aseptically to 30 mL of sterile liquid medium (30) (5 mg L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$, 10 mg L^{-1} KNO_3, 5 mg L^{-1} $NaNO_3$, 5 mg L^{-1} Na_2SO_4, 5 mg L^{-1} $MgCl_2 \cdot 6H_2O$, 0.5 mg L^{-1} $Na_2EDTA \cdot 2H_2O$, 0.05 mg L^{-1} $FeCl_3 \cdot 6H_2O$, 0.05 mg L^{-1} $MnCl_2 \cdot 4H_2O$, 0.05 mg L^{-1} $ZnCl_2$, 0.5 mg L^{-1} $CoCl_2 \cdot 6H_2O$, 0.5 mg L^{-1} $Na_2MoO_4 \cdot 2H_2O$, and 2 mg L^{-1} H_3BO_3, adjusted to pH 7.0) within 3 h after sampling. The biofilm suspension was thoroughly mixed, and 1 mL of the suspension was aseptically inoculated into 9 mL of liquid medium for a resulting biofilm culture volume of 10 mL in a tube. The MCLR solution was then added to the tube at a final concentration of 100 μg L^{-1}. Two control cultures were set up in parallel to account for any loss of MCLR due to abiotic factors: one that contained an equivalent portion of inactivated biofilm (autoclaved at 121°C for 20 min) inoculated into liquid medium spiked with 100 μg L^{-1} of MCLR and one that contained the MCLR-spiked medium without biofilm. All of the samples were capped with sterile stoppers and incubated (EYELA MTI-203) under a 12 h:12 h light–dark cycle at 28°C. Sampling was conducted periodically in duplicate on days 0, 0.5, 1.5, 2.5, 3.5, 5.5, and 7. At each sub-sampling, 1.0 mL and 8.0 mL aliquots of culture were removed from the same test tube for MCLR analysis and DNA extraction. All glassware was autoclaved at 121°C for 20 min before use.

Pretreatment and MCLR analysis Prior to MCLR analysis, the 1.0 mL aliquot was filtered (pore size: 0.22 μm, PTFE Hydrophilic, Millipore). To remove any residual fraction of MCLR adsorbed on cells within the biofilm, 0.5 mL of methanol was passed slowly through the membrane to rinse cells trapped on the membrane. The two filtrates were thoroughly mixed, and the MCLR concentration was immediately analysed by HPLC (Shimadzu 10A series, Shimadzu, Kyoto, Japan) using the following conditions: 50 μL of sample was injected into a 3.0×250 mm Waters SunFire™ C_{18} column (5 μm, Ireland) with an oven temperature of 40°C. The mobile phase was 50% methanol in 0.05 M phosphate buffer (pH 2.5), with a flow rate of 0.58 mL min^{-1}. The MCLR concentration was measured by calibrating the peak areas (at wavelength of 238 nm) with an external standard. The HPLC system had a detection limit of 0.1 μg L^{-1}.

DNA extraction and *mlrA* gene detection The microbial cells in the culture were vacuum-collected on 0.22 μm-pore membranes (Nitrocellulose, Millipore). The total DNA was extracted with a bead beating kit according to the manufacturer's instructions (ISOIL Bead Beating; Nippon Gene, Japan). The total DNA was suspended in 50 μL of TE buffer (10 mM Tris–HCl, 1.0 mM EDTA, pH 8.0) and stored at −20°C. The total DNA concentration was determined spectrophotometrically at 260 nm by GeneQuant (GE Healthcare).

Prior to quantitative analysis, conventional *mlrA* gene-targeted PCR was performed to qualitatively determine if bacteria possessing the *mlrA* gene were present during the biodegradation test period. The conventional PCR assay was conducted on a GeneAmp® 9700 PCR system (Applied Biosystems, Foster City, CA, USA), using primer sets MF-MR (26) with the isolated MC-degrading bacteria *Sphingomonas* sp. MD-1 (30), Y2 (31,32), and *Sphingopyxis* sp. C-1 (33) as positive controls. Each 20 μL reaction mixture contained 1×*Ex Taq* buffer, 2.0 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate, 0.5 U *Takara Ex Taq* DNA polymerase (Takara Bio Inc., Japan), 5.0 pmol of each primer, and 2.0 μL of either a control sample or DNA template. Thermal cycling commenced with an initial denaturation at 94°C for 5 min, followed by denaturation at 94°C for 1 min, annealing at 59°C for 30 s, and primer extension at 72°C for 1 min.

TaqMan qPCR analysis The *mlrA* and bacterial 16 S rDNA genes were quantified during the entire period of MCLR degradation by a TaqMan qPCR assay on a 7500 Real-time PCR System (Applied Biosystems) with the primer sets and TaqMan probes described previously (28,34), except that some probes were labeled with TAMRA quencher at the 3′ end instead of BHQ1. The target groups and the sequences of the primers and probes in qPCR analyses are summarized in Table 1. For calibration, a DNA standard curve for the corresponding gene was prepared from a serial dilution of either a purified 807-bp *mlrA* or 1465-bp 16 S rDNA gene fragment from the MC-degrading bacteria *Sphingomonas* sp. MD-1. Results were linear between 3.29×10^0 and 3.29×10^6 copies μL^{-1} with a linear correlation coefficient (R^2) and reaction efficiency (e) of 0.99 and 97.61%, respectively, for the *mlrA* gene; results were linear between 2.67×10^1 and 2.67×10^6 copies μL^{-1} with R^2 and e values of 0.99 and 97.56%, respectively, for the 16 S rDNA gene.

Duplicate total DNA extracts from each sampling were pooled before the qPCR assay. qPCR reactions were performed in triplicate, and each 20 μL reaction mixture contained the corresponding primer and probe at final concentrations of 0.3 μM and 0.2 μM, respectively, 10 μL of THUNDERBIRD Probe qPCR Mix, 0.04 μL of 50×ROX reference dye (TOYOBO CO., LTD. Osaka, Japan), and 1.0 μL of either a DNA standard or sample template. The thermal profile consisted of an initial denaturation at 95°C for 1 min, followed by 45 cycles of denaturation at 95°C for 15 s and annealing/extension at 62°C (for the *mlrA* gene) or 56°C (for the 16 S rDNA gene) for 45 s. The increase in fluorescent signal was measured at the end of each annealing/extension step. Data acquisition and analysis were performed using the 7500 System SDS Software Version 1.4 (Applied Biosystems).

PCR-DGGE assay For DGGE analysis, a 193-bp 16 S rDNA fragment was PCR-amplified with the primer set as described previously (35) (Table 1), using a GeneAmp® 9700 PCR system (Applied Biosystems). Each 20 μL reaction mixture contained 1×*Ex Taq* buffer, 2.0 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate, 0.5 U *Takara Ex Taq* DNA polymerase (Takara Bio Inc., Japan), 5.0 pmol of each primer, and 1.0 μL of DNA template. The lowest dilution of respective template that did not give non-specific products was used. Thermal cycling commenced with an initial denaturation at 94°C for 5 min, followed by a touchdown procedure consisting of denaturation at 94°C, annealing, and primer extension at 72°C; each step was conducted for 30 s and the initial annealing temperature of 65°C was subsequently decreased by 0.5°C per cycle until the touchdown temperature of 55.5°C was reached. Ten additional cycles were