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Comparative study for the effects of variable nutrient conditions on the biodegradation of microcystin-LR and concurrent dynamics in microcystin-degrading gene abundance

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ABSTRACT

Microcystin-LR (MCLR) degradation capability of biofilm was investigated with and without additional nutrients (nitrate, ammonium, peptone and glucose) at concentrations of 100 and 1000 mg L^{-1}. The MCLR-degradation was stimulated with nitrate and inhibited with other nutrients, except for that glucose of low concentration had no obvious effect. Both stimulatory and inhibitory effects enhanced with increasing concentration of corresponding nutrient. Quantitative polymerase chain reaction (qPCR) indicated that enhanced inhibition in biodegradation correlated to increased inhibition in functional gene (*mlrA*) abundance, as nutrient concentration increased. Stimulated biodegradation under low nitrate concentration may result from more rapid increase in *mlrA* gene abundance. These suggested that MCLR-degradation largely depended upon responsible bacterial population, which was affected by population of other bacteria in biofilm according to 16S rDNA-targeting qPCR. However, inhibited *mlrA* gene abundance implied that the stimulated biodegradation under high nitrate concentration might be involved in the mechanisms not related to MCLRDB population.

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1. Introduction

Cyanobacterial blooms frequently occur under eutrophic conditions in lakes and reservoirs worldwide (Tsuji et al., 2006; Wu et al., 2010). Blooms of species belonging to *Microcystis*, *Anabaena*, *Planktothrix* and *Nostoc* are more harmful as they can produce microcystins (MCs) in water bodies (Watanabe et al., 1992). MCs are a large group of cyclic heptapeptide hepatotoxins with nearly 90 structural variants have been described (Welker and von Döhren, 2006). It has been documented that MCs pose serious threat to biodiversity and the equilibrium of aquatic ecosystems, and even elicit lethal effects on humans exposed to them (Azevedo et al., 2002; Carmichael et al., 2001; Wang et al., 2011). Therefore, the effective removal of MCs is imperative for water utilities.

Biodegradation is proposed as a major pathway for natural elimination of MCs (Welker et al., 2001). Microorganisms always accumulate in polysaccharide matrices and form structural and functional microbial assemblages on submerged surfaces that are commonly known as biofilms (Grützmacher et al., 2002). Naturally-originated biofilms affect the fate of water contaminants through their sorption and biodegradation capacities (Pusch et al., 1998). Biofilm-mediated degradation of MCs has been proven as a cost-efficient strategy for MC-remediation and is routinely practiced in potable water treatment process (Bourne et al., 2006; Ho et al., 2006a,b). However, the MC-degradation potential of the microbial assemblages in a biofilm matrix is susceptible to the fluctuation in environmental conditions. Particularly, it is noteworthy that available nutrient substrates frequently enter the aquatic environment, together with MCs released during occurrence of water blooms. Most studies on MC-biodegradation have been performed with carbon source addition, but few have dealt with the MC-biodegradation with other nutrient substrates addition (Ho et al., 2006a; Ho et al., 2010; Ishii et al., 2004; Jones and Orr, 1994; Park et al., 2001; Rapala et al., 1994; Saito et al., 2003b). Moreover, relatively little attention has been paid to compare MC-degradation with and without addition of nutrients (Eleuterio and Batista, 2010), especially for the MC-degradation by the microorganisms in environmental samples, which is of more practical significance than that by pure isolate in nature. Lack of such information may inhibit better understanding on natural MC-biodegradation.

Abbreviations: MCs, microcystins; MCLR, microcystin-LR; MCDB, MC-degrading bacteria; MCLRDB, MCLR-degrading bacteria; PCR, polymerase chain reaction; qPCR, quantitative real-time PCR; HPLC, high performance liquid chromatography; WTP, water treatment plant; BT tank, biological treatment tank; RW, receiving well; TOC, total organic carbon; DOC, dissolved organic carbon.

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The *mlrA* gene encodes the enzyme responsible for initial hydrolytic cleavage of the cyclic MCs structure during MC-biodegradation (Bourne et al., 2001). Targeting such gene in polymerase chain reaction (PCR) allows qualitative detection of indigenous MC-degrading bacteria (MCDB) (Bourne et al., 2006; Ho et al., 2006b; Saito et al., 2003a). Until recently, a quantitative real-time PCR (qPCR) assay targeting *mlrA* gene has been developed (Hoefel et al., 2009), which offers a powerful tool for detecting the MCDB population. However, to the best of our knowledge, so far no study has quantitated *mlrA* gene to analyze and compare the concurrent dynamics of MCDB population during MC-biodegradation processes under various nutrient conditions. Besides, the relationship between the changing nutrient conditions and MCDB population has never been elucidated.

Considering variable environmental factors, knowledge on how the changes in nutrient conditions influence MC-biodegradation and the MCDB population within microbial community is necessary for successful application of MC-bioremediation. Microcystin-LR (MCLR) is one of the most toxic variants of MCs (Imanishi et al., 2005), and used as the targeted toxin in this study. To identify the effects of different nutrients conditions on MCLR-biodegradation, degradation tests were performed in the absence and presence of carbon source (glucose), or organic and inorganic nitrogen source (peptone, ammonia chloride, sodium nitrate) at different concentrations, using naturally-originated biofilm as inocula. Furthermore, the *mlrA* and bacterial 16S rDNA genes were quantified as surrogates for indigenous MCLR-degrading bacteria (MCLRDB) and overall bacterial population, respectively, during the biodegradation processes under different nutrient conditions. From these results, the relationships between nutrient concentration, MCLR-biodegradation and *mlrA* gene abundance were established. Additionally, possible mechanisms for the effects of nutrients on MCLR-degradation by microorganisms in the biofilm were discussed.

2. Methods

2.1. Standard and chemicals

MCLR standard ($\geqslant$90% purity, Wako Pure Chemical Industries, Ltd., Japan) was stored at −20 °C as stock solution prepared with a concentration of 25 μg mL^{-1}. High performance liquid chromatography (HPLC)-grade methanol was used to prepare HPLC mobile phase. All other chemicals were of reagent grade.

2.2. Sampling

Lake Kasumigaura (Ibaraki, Japan) serves as a water source for drinking and other purposes and receives a large nutrient load from domestic, municipal and agricultural effluent. Over decades toxic cyanobacterial blooms have frequently occurred in the lake (Sugiura et al., 2002, 2004). To reduce the risk associated with MCs, the water treatment plant (WTP) nearby the lake set up a biological treatment facility packed with vinyl chloride board as a carrier for biofilm habitat. The facility is kept submerged in the biological treatment (BT) tank. The biofilm matrix (active biofilm) coating the carrier was scraped into a sterile capped tube in October 2010.

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 by pumping. The original lake water and bio-treated water were sampled from the RW and BT tank, respectively. MCLR concentrations in both water sources were detectable in August and September 2010 (unpublished data), as detected by HPLC. This suggested that the MCLRDB in the biofilm of October 2010 had been exposed to natural MCLR during the preceding months. All of the sampled materials were transported on ice to the laboratory within 1 h and used for experiments immediately.

2.3. MCLR-biodegradation

To obtain biofilm suspension, 5 g (fresh weight) of active biofilm was aseptically added into 150 mL of sterile distilled water. The biofilm suspension was thoroughly homogenized. MCLR-biodegradation tests were conducted in a series of 30-mL glass test tubes. In each tube, 1.0 mL of aliquot biofilm suspension was aseptically inoculated into 9.0 mL of sterile distilled water to set a resulting culture volume of 10 mL. The cultures were then spiked with MCLR to establish a final concentration of 100 μg L^{-1}, which is within the typical range of concentration during blooms (Eleuterio and Batista, 2010). The contents of ammonia nitrogen (NH_4-N), nitrate (NO_3-N), total organic carbon (TOC) and dissolved organic carbon (DOC) in this resulting culture were 0.249, 0.02, 17.9 and 14 mg L^{-1}, respectively, before MCLR addition. The tubes containing culture were capped with sterile stoppers and incubated in 12:12 h light–dark cycle at 28 °C. Sampling was conducted periodically in duplicate. At each sampling, 1.0 and 8.0 mL aliquots of culture were removed from the same test tube for MCLR analysis and DNA extraction, respectively. The residual culture in tube was used for pH detection with a pH meter (Mettler Toledo, MP 220, UK). Two control cultures were set up in parallel to account for any abiotic loss of MCLR, including (i) an equivalent portion of autoclaved biofilm inoculated into water spiked with 100 μg L^{-1} of MCLR and (ii) the MCLR-spiked water without biofilm. All glassware was autoclaved at 121 °C for 20 min before use.

The effect of nutrients on MCLR-degradation characteristics of biofilm was investigated by addition of sodium nitrate (100, 1000 mg L^{-1}), ammonium chloride (100, 1000 mg L^{-1}), peptone (100, 1000 mg L^{-1}) or glucose (100, 1000 mg L^{-1}). These tests were carried out with the identical protocol and experimental conditions as those described above except those specified.

2.4. MCLR analysis

Before MCLR analysis, the 1.0 mL aliquot was filtered (pore size: 0.22 μm, PTFE Hydrophilic, Millipore, USA). 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 analyzed by HPLC (Shimadzu 10A series, Shimadzu, Japan) with the following conditions: 50 μL of sample was injected into a Waters SunFire™ C_{18} column (3.0 × 250 mm, 5 μm, Ireland) with an oven temperature of 40 °C. Fifty percent methanol with 0.05 M phosphate buffer (pH 2.5) was employed as mobile phase at 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}.

2.5. DNA extraction

The microbial cells in culture were vacuum-collected on 0.22 μm-pore membranes (Nitrocellulose, Millipore, USA). Total DNA was extracted with a bead beating kit according to the manufacturer's instructions (ISOIL Bead Beating, Nippon Gene, Japan). The resulting DNA was suspended in 50 μL of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and stored at −20 °C. Extracted DNA concentration was determined spectrophotometrically at 260 nm with a GeneQuant pro analyzer (Amersham Pharmacia, Cambridge, England).

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