Effect of Microcystin and Its Degradation Products on the Transcription of Genes Encoding Microcystin Degrading Enzymes

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Abstract

It is known that mlrA, mlrB and mlrC encode microcystin (MC)-degrading enzymes in MC-degrading bacteria. We discovered the mechanism by which transcription of these genes was induced in MC-degrading bacterium Sphingopyxis sp. C-1. We showed induction of the transcription of mlrA, mlrB and mlrC by microcystin–LR (MCLR) and that of mlrA and mlrB by MCLR degradation products (linear microcystin, H–Adda–Glu–Mdha–Ala–OH (tetra peptide) and 2S, 3S, 8S, 9S–3–amino–9–methoxy–2, 6, 8–trimethyl–10–phenyleneca–4E, 6E–dienoic acid (Adda)). Adda played a key role in the induction of transcription of mlrA and mlrB, while the cyclic structure of MCLR was important for the induction of transcription of mlrC. It was found that the MC-degrading bacteria responded to MCLR and its degradation products by degrading microcystin through a sequential chain reaction for expression of MlrA, MlrB and MlrC.

Key words: microcystin, microcystin-degrading bacterium, the genes encoding microcystin-degrading enzymes (mlrA, mlrB, mlrC), transcription of mlrA, mlrB, mlrC

INTRODUCTION

Toxic blooms of cyanobacteria frequently occur in eutrophic lakes, ponds and reservoirs throughout the world4–9. It has been reported that blooms of up to 70% are potentially toxic6–7. The toxic blooms of cyanobacteria, such as Microcystis, Anabaena, Planktothrix (Oscillatoria), Nostoc and Hapalosiphon species, produce a family of cyclic heptapeptide hepatotoxins called microcystins (MCs)8–9. The most common MC is microcystin–LR (MCLR), which has the structure cyclo (D–Ala–L–Leu–D–MeAsp–L–Arg–Adda–D–Glu–Mdha). MeAsp is D–erythro–β–methylasparatic acid and Mdha is N–methyl–dehydroalanine.


MCs inhibit protein serine/threonine phosphatases 1 and 2A and have a tumour-promoting activity5–12. An important concern is the possibility that chronic exposure to low concentrations of MCs in drinking water supplies may promote tumour growth in the human liver5–12. Thus, currently, there is a significant risk that MCs may contaminate drinking water supplies and pose a threat to public health. For example, in 1996, more than 50 haemodialysis patients died from using water contaminated with MCs in Caruaru, Brazil14, 15.
MCs are structurally stable against physicochemical and biological effects, such as pH, temperature, sunlight and general enzymes, under natural conditions. However, in the aquatic environment, the dynamics of MCs depend on the population density of *Microcystis* sp. The population density has been shown to dramatically decrease in a short period of time, and MCs in the water column simultaneously decrease during the fading out of a bloom. Okano *et al.* noted that the degradation of MCs by microorganisms requires specific enzymes and biodegradation trials of MCs have been conducted. In 1994, Jones *et al.* isolated the MC–degrading bacterium, *Novosphingobium* sp. (synonymous with *Sphingomonas* sp.) for the first time. MC–degrading bacteria in aquatic environments have been well studied. Bourne *et al.* suggested an enzymatic pathway of MCLR degradation by MC–degrading enzymes including MlrA, MlrB and MlrC, and identified the gene cluster of MC–degrading enzymes, *mlrA*, *mlrB*, *mlrC* and *mlrD* using the isolated bacterium. They also determined that the *mlrA* gene encodes an enzyme responsible for the hydrolytic cleaving of the cyclic structure of MCLR. The *mlrD* gene encoded transporter protein for a putative MC and MC degradation products and formed an operon with *mlrA*. Additionally, Imanishi *et al.* suggested an enzymatic pathway for MC–degrading enzymes using *Sphingomonas* sp. *B9*. According to the results of these previous studies, MlrA should degrade MCLR to linear MC, MlrB may degrade linear MC to tetra peptide (H–Adda–Glu–Mdha–Ala–OH) and MlrC may degrade tetra peptide to Adda. There is no information available on transcriptional regulation and expression of the genes encoding MC–degrading enzymes, *mlrA*, *mlrB* and *mlrC*. This leads to the questions: “Why do MCs degrade rapidly in natural water?” and “What is the trigger that promotes the degradation activity of MCs?” Studies on the transcriptional regulation and the expression of *mlrA*, *mlrB* and *mlrC* would contribute to a better understanding of MC dynamics in aquatic environments and would have useful applications in the field of public health.

Based on the assumption that MCs induce the transcription of *mlrA*, *mlrB* and *mlrC*, it was suggested that expression of MlrA, MlrB and MlrC is induced and subsequently, MCs degrade rapidly. Here, we examined the effect of MCLR and its degradation products, linear MC, tetra peptide and Adda on the transcription of *mlrA*, *mlrB* and *mlrC* in the MC-degrading bacterium, *Sphingopyxis* sp. C–1.

**MATERIALS AND METHODS**

**Bacterial strain and growth condition**

*Sphingopyxis* sp. C–1, an MC–degrading bacterium, was isolated from toxic blooms present in Hongfeng Lake at Guizhou Province, China. They were grown at 28°C in 1/5 peptone-yeast (PY) medium containing 2.0 g of Bacto peptone (Bacto, Dickinson and Company, New Jersey, United States) and 1.0 g of Bacto yeast extract (Bacto, Dickinson and Company, New Jersey, United States) per litre at pH 7.0. Liquid cultures in test tubes with 5 ml of culture broth and 500 ml–Sakaguchi flask with 50 ml were incubated with shaking at 300 rpm and 120 rpm, respectively.

**Microcystin and its degradation products**

MCLR, a cyclic MC, was purchased from Wako Pure Chemical Industries, Ltd in Osaka, Japan. The degradation products of MCLR, linear microcystin, tetra peptide and Adda were produced from MCLR (unpublished data).

**Determination of quantities of mRNAs of *mlrA*, *mlrB* and *mlrC* during growth phase**

The cultured *Sphingopyxis* sp. C–1 of 500 ml–Sakaguchi flask with 50 ml of culture broth was taken with time course (0, 6, 10, 12, 16, 18, 20, 24 h). Quantities of mRNAs for *mlrA*, *mlrB* and *mlrC* were determined by quantitative reverse transcription–PCR (qRT–PCR). The OD600 which related to growth of *Sphingopyxis* sp. C–1 was determined using a spectrophotometer (GeneQuant pro, GE Healthcare, Tokyo, Japan). This experiment was replicated three times.

**Test for effect of cyclic MC on transcription of *mlrA* during growth phase**

*Sphingopyxis* sp. C–1 was cultured in a test tube with 5 ml
of culture broth. The test tubes of 32 were used. The cultures were taken from each 4 test tube with time course (6, 10, 12, 16, 18, 20, 24, 26 h). Cyclic MC (final concentration; f.c. 1000 µg·l⁻¹) was added to each two tubes with the cultured *Sphingopyxis* sp. C–1, while same volume of sterile ultra-pure water was added to others as the test of negative control (NC).

After the addition of cyclic MC and sterile ultra-pure water to each 4 test tubes of the culture of *Sphingopyxis* sp. C–1, the cultures were incubated at 15 min and 30 min, respectively. Then, the cultures were taken from each 4 test tubes for the determination of amount of *mlrA* mRNA. Simultaneously, the OD₆₀₀ of each tube was determined using a spectrophotometer (GeneQuant pro, GE Healthcare, Tokyo, Japan). Measurements of culture density were taken at 0 min. The quantity of mRNA from the cultures in 4 tubes was determined by qRT–PCR. The 16S rRNA gene was used as internal control in the qRT–PCR. Normalised amounts of mRNA were calculated by amounts of mRNA dividing by the amounts of 16S rRNA. Induction ratios were obtained by dividing the normalised amounts of mRNA at each time point after the addition of cyclic MC by the average of the normalised amounts of mRNA for NC. This experiment was replicated three times.

**Test for effect of MC and its degradation products** The cultured *Sphingopyxis* sp. C–1 was taken 500 µl from the Sakaguchi flask at reaction times from 0 to 120 min. Each Cyclic MC (f.c. 1000 µg·l⁻¹), linear MC (f.c. 1000 µg·l⁻¹), tetra peptide (f.c. 1000 µg·l⁻¹) and Adda (f.c. 500 µg·l⁻¹) were added at the late logarithmic phase (OD₆₀₀ 0.8) of growth in the Sakaguchi flask. The culture was taken from each Sakaguchi flask for the determination of quantities of mRNAs for *mlrA*, *mlrB* and *mlrC* at reaction times (from 0 to 120 min) with the addition of cyclic MC, its degradation products and NC. In the test for NC, the same volume of sterile ultra-pure water was added.

**RNA isolation and cDNA synthesis** Total RNA was extracted from each sample using RNeasy Protect Bacteria Kits (QIAGEN GmbH, Hilden, Germany) with lysozyme (Sigma-Aldrich, Missouri, United States) and DNase I of RNase-Free DNase Set (QIAGEN GmbH, Hilden, Germany) according to the manufacturer’s protocol. Random hexamer-primed cDNAs were obtained from the total RNA (10 ng) using the PrimeScript RT reagent kit (Takara Bio Inc., Shiga, Japan) according to the manufacturer’s instructions. cDNA synthesis was performed in a total volume of 20 µl containing 50 pmol of primer. The reaction was performed with reverse transcription at 37°C for 15 min and inactivation of reverse transcriptase at 85°C for 5 s.

**Quantitative reverse transcription–PCR (qRT–PCR)** The primers for amplification of mRNAs, 16S rRNA and standard RNA are listed in Table 1. Some primers were designed with Applied Biosystems Primer Express software (version 2.0). The qRT–PCR was performed with a Real-time PCR System (Applied Biosystems 7500 Real-Time PCR System, Applied Biosystems Japan Ltd, Tokyo, Japan) using SYBR Premix Ex Taq II (Takara Bio Inc., Shiga, Japan) according to the manufacturer’s protocol. The real-time PCR reaction was performed in triplicate in a total volume of 50 µl containing 400 nM each of forward and reverse primers (Table. 1), and 40 ng of cDNA template. The PCR reaction was initiated with a pre-incubation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s and annealing and extension together at 60°C for 34 s. Immediately after the final PCR cycle, a melting curve analysis was done to determine the specificity of the reaction by incubating the reaction at 95°C for 15 s, annealing at 60°C for 1 min, and then increasing the temperature to 95°C for 15 s. To correct for sampling errors, the levels of transcription of each gene (*mlrA*, *mlrB* and *mlrC*), as determined from their quantities using a standard curve, were normalised to the level of 16S rRNA. The data were averaged for the three replicates. Standard RNA was synthesised *in vitro* using the *in vitro* transcription T7 Kit (Takara Bio Inc., Shiga, Japan) according to the manufacturer’s protocol.

**Dynamics of the cyclic MC degradation rate** Cyclic MC (f.c. 1000 µg·l⁻¹) was added to the
cultured *Sphingopyxis* sp. C–1 in 1/5 PY medium at the stationary phase (culture at 24 h). After that, cyclic MC concentration was determined at 10 min intervals. The sample was centrifuged at 15,000 rpm for 5 min at 4°C. Culture supernatant was filtered (0.2 µm, Millipore) for HPLC analysis. When cyclic MC was degraded, additional cyclic MC (f.c. 1000 µg•l−1) was added. This manipulation was performed three times.

**Analysis of MC and its degradation products' concentrations**

The concentrations of MCLR and its degradation products were determined using Shimadzu 10A series HPLC (Shimadzu Corporation, Kyoto, Japan). The analyses were performed with a C18 column (SunFire™ C18 250 × 3.0 mm, 5.0 µm particle size, Waters Corporation, Massachusetts, United States) kept at 40°C. The mobile phase was 60% methanol (HPLC grade, Wako Pure Chemical Industries Ltd, Osaka, Japan) diluted with 0.05 M phosphate buffer (pH 2.5) for cyclic MC analysis. The mobile phase was 50% ethanol (HPLC grade, Wako Pure Chemical Industries Ltd, Japan) diluted with 0.05 M phosphate buffer (pH 2.5) for the analysis of degradation products. The flow rate was 0.58 ml•min−1. Chromatogram results were monitored at a wavelength of 238 nm.

**Statistical treatment of data**

In order to determine a significant difference (*P* < 0.05) between results obtained by quantification with and without MC and its degradation products, a *t*-test was performed.

**RESULTS**

**Transcription of *mlrA*, *mlrB*, *mlrC* with growth of *Sphingopyxis* sp. C–1**

The time course of transcription of the genes encoding MC-degrading enzymes (*mlrA*, *mlrB*, *mlrC*) and growth curves of *Sphingopyxis* sp. C–1 are shown in Fig. 1. The amounts of mRNAs for *mlrA*, *mlrB* and *mlrC* increased gradually from 0 to 10 h, reaching a maximum at 10 h. Then, their amounts decreased from 10 to 12 h, and they showed constant levels from 12 h at the middle of the logarithmic growth phase. The relative quantities of mRNA were *mlrA* < *mlrB* < *mlrC*. Figure 1 shows that all genes were always transcribed, thus, homeostatically, basic transcription was clear.

**Effect of cyclic MC on *mlrA* transcription with growth of *Sphingopyxis* sp. C–1**

The time course change of *mlrA* transcription level after adding cyclic MC to the test tube cultures of *Sphingopyxis* sp. C–1 was shown in Fig. 2. At 15 and 30 min reaction time, after the addition of cyclic MC to a 16 h culture (OD600 0.69), the amounts of *mlrA* mRNA induced were about 2.2– and 2.6–fold higher than NC, respectively. The quantity of *mlrA* mRNA induced was approximately...
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3.3-fold higher than NC at 30 min reaction time for the 18 h culture (OD_{600} 0.8). Induction was not confirmed in other cultures. Therefore, it was concluded that the transcription of \( mlrA \) was growth dependent. Consequently, when the OD_{600} of 0.8 was reached in the induction culture, cyclic MC and its degradation products were added.

**Effect of cyclic MC and its degradation products on expression of \( mlrA, mlrB \) and \( mlrC \)**

The time course of the mRNA/16S rRNA and concentrations of cyclic MC and its degradation products were shown in Fig. 3. When the OD_{600} reached 0.8, cyclic MC was added. The amount of \( mlrA, mlrB \) and \( mlrC \) mRNA increased gradually from 10 to 30 min. The maximum value was observed at 30 min. Statistically significant differences (\( P < 0.05 \)) were observed at 30 min compared with 0 and 30 min for NC and 0 min with the addition of cyclic MC. After which, the amounts of mRNAs decreased and approached the levels of NC, because residual cyclic MC and its degradation products were degraded completely at 45 min. The transcription of \( mlrA, mlrB \) and \( mlrC \) was induced at the same time by cyclic MC. The transcription of \( mlrD \) might show the same pattern as \( mlrA \) because \( mlrA \) and \( mlrD \) form an operon with each other\(^{31} \). It was clear that the transcription of \( mlrA, mlrB \) and \( mlrC \) was induced by cyclic MC. This result indicates that transcription of the \( mlr \) cluster is closely related to the cyclic MC.

The cyclic MC was degraded immediately after addition, and was completely degraded at 45 min. The degradation products of tetra peptide and Adda were detected from 0 min, and completely degraded at 45 min. However, linear MC was not detected at any reaction time.

The time courses of the mRNA/16S rRNA and concentrations of linear MC, tetra peptide and Adda at the addition of linear MC were shown in Fig. 4. Statistically significant differences (\( P < 0.05 \)) were observed at 30 min in the amount of mRNA with the addition of linear MC compared with 0 and 30 min for NC and 0 min in the experimental samples. The transcription of \( mlrA \) and \( mlrB \) was induced by linear MC at 30 min. \( mlrC \) transcription was not induced during the test.
time. Thus, it was concluded that the transcription of \(mlrC\) was under different regulation as compared to \(mlrA\) and \(mlrB\).

Figure 4 showed that the linear MC was dramatically degraded to tetra peptide and Adda immediately after addition. However, linear MC is not rapidly degraded, thus, it was not completely degraded until 120 min after it was added. The tetra peptide and Adda were also not rapidly degraded. These fragment compounds were completely degraded at 120 min. The linear MC was not degraded from 0 to 10 min. The degradation product, tetra peptide, was degraded from 0 to 10 min, and increased from 10 to 15 min by degradation of the linear MC. After 15 min reaction time, tetra peptide and Adda concentrations decreased with linear MC. Consequently, it was suggested that tetra peptide and Adda affect transcription of \(mlrA\) and \(mlrB\) because the amounts of \(mlrA\) and \(mlrB\) mRNA were not equal to NC after 45 min reaction time.

The time course of the mRNA/16S rRNA, residual tetra peptide and Adda at the addition of tetra peptide was shown in Fig. 5.
Tetra peptide was rapidly degraded to Adda. The tetra peptide and Adda were completely degraded at 120 min. The tetra peptide affected transcription of mlrA and mlrB, but not mlrC. The amount of mlrA mRNA was at its maximum at 30 min. Statistically significant differences ($P < 0.05$) also appeared at 30 min compared with 0 and 30 min for NC and 0 min with the addition of tetra peptide. The amount of mlrB mRNA reached a maximum at 10 min. The amount of mRNA decreased from 10 to 45 min. However, the induction ratio against NC was at a maximum at 30 min with the addition of tetra peptide. The amount of mlrB mRNA was also significantly different ($P < 0.05$) at 30 min compared with 0 and 30 min for NC and 0 min with the addition of tetra peptide. The transcription of mlrA and mlrB may be affected between 45 and 120 min by Adda. Tetra peptide was perfectly degraded within 60 min after addition. Adda was completely degraded within 120 min. Adda may influence the transcription of mlrA and mlrB after 45 min. Thus, we examined the effect of Adda on transcription of mlrA, mlrB and mlrC.

Figure 6 shows the 0 and 30 min reaction time results of the mRNA/16S rRNA with the addition of Adda. In the test for positive control, cyclic MC was added. Transcription of mlrA and mlrB was induced by Adda.
However, transcription of \textit{mlrC} was not induced.

Figure 7 shows the dynamics of the cyclic MC degradation rate. When cyclic MC was added repeatedly (three times), the cyclic MC degradation rate increased.

**DISCUSSION**

Figure 1 shows that total mRNA reached a maximum at 10 h in the early logarithmic growth phase. It was thought that bacterial cells in this phase frequently switch the transcription of various genes on or off. Thus, the amount of mRNA reached a maximum.

Maruyama \textit{et al}. suggested that MC-degrading bacteria were on 'stand-by' until the degradation of MC occurred. We found transcription of \textit{mlrA}, \textit{mlrB} and \textit{mlrC} was under homeostatic regulation (Fig. 1), strongly supporting the suggestion of Maruyama \textit{et al}. The MC-degrading bacteria existed in a restricted area of the mucilage of \textit{Microcystis}, and the change in concentration of these bacteria was synchronised with the concentration of cell-bound MCs. Thus, we hypothesised that MC-degrading bacteria responded to MCs and induced transcription of the genes encoding MC-degrading enzymes by MCs and its degradation products when the cell density of MC-degrading bacteria exceeded a threshold as well as by quorum sensing. However, the transcription of \textit{mlrA} depended not on cell density, but on the growth phase (Fig. 2). Figures 3-6 indicated that transcriptional regulation of \textit{mlrA} and \textit{mlrB} was different from that of \textit{mlrC}. Cyclic MC, linear MC, tetra peptide and Adda induced the transcription of \textit{mlrA} and \textit{mlrB}, suggesting that Adda is a key fragment in the transcriptional regulation of \textit{mlrA} and \textit{mlrB}.

When cyclic MC was added, cyclic MC degradation products were degraded faster than any other case where microcystin degradation products were added. When linear MC was added, degradation time was 120 min. However, when cyclic MC was added, linear MC was not detected, although tetra peptide and adda were present. We suggested that the cyclic structure was important for induction of the transcription of \textit{mlrC}, because there was no induction by linear MC, tetra peptide and Adda (Fig. 3-6). Therefore, the transcription of \textit{mlrA}, \textit{mlrB} and \textit{mlrC} is under both constitutive and homeostatic regulatory control. MCs have a number of hydrogen bonding acceptors and donors with cyclic structure. And Adda can bind the regulatory protein of \textit{mlrA} and \textit{mlrB}, their transcription may be induced. The structure of cyclic MC can bind the regulatory protein of \textit{mlrC}, and its transcription may also be induced. Subsequently, expression of MlrA, MlrB and MlrC was induced. In the investigation of the dynamics of the cyclic MC degradation rate, we demonstrated that the expression rate was elevated (Fig. 7). It was shown that cyclic MC induced not only transcription of the genes encoding MC-degrading enzymes but also expression of the enzymes. Thus, it was considered that the activity of MlrA, MlrB, MlrC and related proteins was elevated by MCLR. Therefore, our results suggested that cyclic MC and its degradation products turned on the genes promoting degradation activity of MCs. Consequently, MCs were rapidly degraded by sequential reaction.

We propose the following model for the aquatic environment. MCs are produced and mostly retained in intact cyanobacterial cells, but about 10-20% of MCs are lost from cells in culture. When a toxic algal bloom occurs, MCs are released or leaked into the natural waters.
Takenaka et al. reported that Adda existed as DmAdda (2'S, 3'S, 8'S-3-amino-2, 6, 8-trimethyl-10-phenyldeca-4E, 6E-dienoic acid) in natural waters during blooms\(^{30}\). MC-degrading bacteria were isolated by MC as the sole carbon and nitrogen source\(^{25-28}\), thus, it was thought that MC-degrading bacteria proliferate by assimilating MCs, Adda or DmAdda and other carbon sources during the production of the MCs bloom. The members of the Cytophaga/Flavobacterium group are able to lyse Microcystis sp. cells\(^{39}\). During Microcystis sp. blooms, the cell density of the Cytophaga/Flavobacterium group and δ-proteobacteria was clearly synchronised with that of Microcystis sp\(^{32}\). Consequently, when the cell density of the Cytophaga/Flavobacterium group and δ-proteobacteria exceed a threshold in cell density, they may promote lysis of the cyanobacterial cell. There are several factors that induce degradation of cyanobacteria, including cell decay by autolysis, lysis caused by virus like particles\(^{41}\), lysis caused by non-toxic cyclic peptides produced in bloom-forming cyanobacteria\(^{42}\) and lysis caused by cyanophages, viruses specific to cyanobacteria\(^{43,44}\). So, the rapid degradation of the toxic bloom could be initiated. At the same time, MC-degrading bacteria at a late logarithmic phase may respond to the surrounding MCs and Adda or DmAdda in natural water, resulting in the induction of transcription of the genes encoding MC-degrading enzymes and in the expression of MC-degrading enzymes. Finally, MC-degrading bacteria dramatically degrade MCs by a sequential chain reaction of the expression of MlrA, MlrB and MlrC.

For a better understanding of the degradation mechanism of MCs in the natural aquatic environment, both investigation and confirmation of the dynamics of mRNA in the MC-degrading bacteria, in situ analysis during the occurrence of blooms and of each MC-degrading enzyme (MlrA, MlrB, MlrC, MlrD and related enzymes) in the degradation process by in vitro studies are required in the near future.

**Conclusion**

The following results were obtained:

1) Cyclic MC promoted the activity of MC-degrading enzymes.

2) Transcription of mlrA was induced by cyclic microcystin at the late logarithmic phase.

3) Adda was a key fragment compound for the induction of transcription of mlrA and mlrB.

4) The structure of cyclic MC was closely related to the regulation of expression of mlrC.

5) MC-degrading bacteria responding to MCLR and its degradation products were found to dramatically degrade MCLR by sequential chain reaction.

**REFERENCES**


27) Lemes, G.A.F., Kersanach, R., da. S., Pinto,
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