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

KAZUYA SHIMIZU¹, KUNIHITO OKANO^{1,2}, HIDEAKI MASEDA³, TAKASHI AMEMIYA¹,
TAKUMI KURASHIMA¹, MOTOO UTSUMI¹, and NORIO SUGIURA^{1*}

¹Graduate School of Life and Environmental Sciences, University of Tsukuba
/1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan

²Akita Prefectural University, Institute of Technology and Science
/241-438 Kaidobata-Nishi, Nakano Shimoshinjo, Akita City 010-0195, Japan

³The University of Tokushima/2-1 Minamijosanjima-cho, Tokushima 770-8506, Japan

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-phenyldeca-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 world¹⁻⁵. It has been reported that blooms of up to 70% are potentially toxic^{6,7}. 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 -methylaspartic acid and Mdha is N-methyl-dehydroalanine.

Adda is 2S, 3S, 8S, 9S-3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4E, 6E-dienoic acid⁹.

MCs inhibit protein serine/threonine phosphatases 1 and 2A and have a tumour-promoting activity⁹⁻¹². 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 liver^{12,13}. 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, Brazil^{14,15}.

*Corresponding author

MCs are structurally stable against physicochemical and biological effects, such as pH, temperature, sunlight and general enzymes, under natural conditions¹⁶⁻²⁰. 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^{1,2,6}. Okano *et al.* noted that the degradation of MCs by microorganisms requires specific enzymes²⁰ and biodegradation trials of MCs have been conducted^{21,22}. In 1994, Jones *et al.* isolated the MC-degrading bacterium, *Novosphingobium* sp. (synonymous with *Sphingomonas* sp.) for the first time²². MC-degrading bacteria in aquatic environments have been well studied²³⁻²⁹. 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^{30,31}. They also determined that the *mlrA* gene encodes an enzyme responsible for the hydrolytic cleaving of the cyclic structure of MCLR³⁰. The *mlrD* gene encoded transporter protein for a putative MC and MC degradation products and formed an operon with *mlrA*³¹. Additionally, Imanishi *et al.* suggested an enzymatic pathway for MC-degrading enzymes using *Sphingomonas* sp. B9³². 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. However, 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, China²⁸. 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 500 µl 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. $1000 \mu\text{g}\cdot\text{l}^{-1}$) 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. $1000 \mu\text{g}\cdot\text{l}^{-1}$), linear MC (f.c. $1000 \mu\text{g}\cdot\text{l}^{-1}$), tetra peptide (f.c. $1000 \mu\text{g}\cdot\text{l}^{-1}$) and Adda (f.c. $500 \mu\text{g}\cdot\text{l}^{-1}$) 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. $1000 \mu\text{g}\cdot\text{l}^{-1}$) was added to the

Table 1 Primers

Primer	target gene	locus	sequence (5'–3')	product size	Description
T7–16S34F 797R	16S rRNA (AB161684)	39–56 777–797	TAATACGACTCACTATAGGG CATGCCTAACACATGCAA CGCCACCCAAGTTCTATGAAC	759	Primer for making standard RNA
T7–MF MR	<i>mlrA</i> (AB468058)	106–125 892–911	TAATACGACTCACTATAGGGG ACCCGATGTTCAGATACT CTCCTCCCACAAATCAGGAC	806	Primer for making standard RNA Reference 45
T7– <i>mlrB</i> 75F <i>mlrB</i> 1475R	<i>mlrB</i> (AB468059)	75–93 1456–1475	TAATACGACTCACTATAGGGG CTCGATGCGGTATTTGCTG TCCAACGACCATCCCTTCTG	1400	Primer for making standard RNA
T7– <i>mlrC</i> 50F <i>mlrC</i> 1493R	<i>mlrC</i> (AB468060)	50–68 1474–1493	TAATACGACTCACTATAGGGG CGAAGGCGAAAGGTGCAAC GAGCGCTTGTGATAGTGACG	1443	Primer for making standard RNA
16S189F–real 16S289R–real	16S rRNA (AB161684)	189–208 269–289	AGGATGAGCCCGCGTAAGAT TCAGTCCCAGTGTGGCTGATC	100	Primer for determination of 16S rRNA
<i>mlrA</i> 185F–real <i>mlrA</i> 255R–real	<i>mlrA</i> (AB468058)	185–204 235–255	CGGGAGCCTATACGCTTTTG TCCGATACCGGTTACGATCAG	70	Primer for determination of <i>mlrA</i> mRNA
<i>mlrB</i> 153F–real <i>mlrB</i> 258R–real	<i>mlrB</i> (AB468059)	153–172 241–258	CTATCAGGGCGGCTTTGGA GAGAGCGGCCGTGAAGTGA	105	Primer for determination of <i>mlrB</i> mRNA
<i>mlrC</i> 848F–real <i>mlrC</i> 923R–real	<i>mlrC</i> (AB468060)	848–865 907–923	CAGCGTCGATCGCACAAAG CGCTCTGGGCCGTTACC	75	Primer for determination of <i>mlrC</i> mRNA

The bold characters show T7 sequence

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

Fig 1 Dynamics of *mlr* mRNAs with growth of *Sphingopyxis* sp. C-1
● *mlrA*, ◆ *mlrB*, ■ *mlrC*, △ OD_{600}

Fig 2 Effect of cyclic microcystin on transcription of *mlrA* with growth of *Sphingopyxis* sp. C-1
□ the sample incubated 15 min after cyclic microcystin addition, ▒ the sample incubated 30 min after cyclic microcystin addition, ■ NC, △ OD_{600}

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 other³¹. 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

Fig 3 Effect of cyclic microcystin on transcription of *mlrA*, *mlrB* and *mlrC*
 ● *mlrA* mRNA, ○ *mlrA*-NC mRNA, ◆ *mlrB* mRNA, ◇ *mlrB*-NC mRNA, ■ *mlrC* mRNA, □ *mlrC*-NC mRNA, ○ microcystin-LR, ▼ linear microcystin, ▽ tetra peptide, ▲ Adda
 The maximum induction in all cases was 30 min after the addition of cyclic MC.
 Asterisks indicate significant differences at $P < 0.05$

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

Fig 4 Effect of linear microcystin on transcription of *mlrA*, *mlrB* and *mlrC*
 ● *mlrA* mRNA, ○ *mlrA*-NC mRNA, ◆ *mlrB* mRNA, ◇ *mlrB*-NC mRNA, ■ *mlrC* mRNA, □ *mlrC*-NC mRNA, ▼ linear microcystin, ▽ tetra peptide, ▲ Adda
 The maximum induction in all cases was 30 min after addition of linear MC.
 Asterisks indicate significant differences at $P < 0.05$

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.

Fig 5 Effect of tetra peptide on transcription of *mlrA*, *mlrB* and *mlrC*
 ● *mlrA* mRNA, ○ *mlrA*-NC mRNA, ◆ *mlrB* mRNA, ◇ *mlrB*-NC mRNA, ■ *mlrC* mRNA, □ *mlrC*-NC mRNA, ▽ tetra peptide, ▲ Adda
 The maximum induction in all cases was 30 min after addition of tetra peptide.
 Asterisks indicate significant differences at $P < 0.05$

Fig 6 Effect of Adda on transcription of *mlrA*, *mlrB* and *mlrC*
 Asterisks indicate significant differences at $P < 0.05$

Fig 7 Effect of cyclic microcystin on expression of MlrA, MlrB and MlrC

● first addition, ■ second addition, ◆ third addition

However, transcription of *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 *et al.* suggested that MC-degrading bacteria were on 'stand-by' until the degradation of MC occurred. We found transcription of *mlrA*, *mlrB* and *mlrC* was under homeostatic regulation (Fig. 1), strongly supporting the suggestion of Maruyama *et al.* The MC-degrading bacteria existed in a restricted area of the mucilage of *Microcystis*, and the change in concentration of these bacteria was synchronised with the concentration of cell-bound MC³². 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 sensing³³. However, the transcription of *mlrA* depended not on cell density, but on the growth phase (Fig. 2). Figures 3–6 indicated

that transcriptional regulation of *mlrA* and *mlrB* was different from that of *mlrC*. Cyclic MC, linear MC, tetra peptide and Adda induced the transcription of *mlrA* and *mlrB*, suggesting that Adda is a key fragment in the transcriptional regulation of *mlrA* and *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 *mlrC*, because there was no induction by linear MC, tetra peptide and Adda (Fig. 3–6). Therefore, the transcription of *mlrA*, *mlrB* and *mlrC* is under both constitutive and homeostatic regulatory control. MCs have a number of hydrogen bonding acceptors and donors with cyclic structure³⁵. And Adda have hydrogen bonding acceptor and donor³⁵. Adda can bind the regulatory protein of *mlrA* and *mlrB*, their transcription may be induced. The structure of cyclic MC can bind the regulatory protein of *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^{36,37}. When a toxic algal bloom (MCs producing bloom) occurs, MCs are released or leaked into the natural waters³⁴.

Takenaka *et al.* reported that Adda existed as DmAdda (2S, 3S, 8S-3-amino-2, 6, 8-trimethyl-10-phenyldeca-4E, 6E-dienoic acid) in natural waters during blooms³⁸. MC-degrading bacteria were isolated by MC as the sole carbon and nitrogen source²⁵⁻²⁸, 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. cells³⁹. 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 particles⁴¹, lysis caused by non-toxic cyclic peptides produced in bloom-forming cyanobacteria⁴² 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

- 1) Sugiura, N., Kameyama, K., Saito, T., Utsumi, M., Inamori, Y., and Maekawa, T.: Spatial and temporal changes of microcystins concentration in eutrophicated lakes in Japan, *Eco-Engineering* 14, 8-11 (2002)
- 2) Edwin, A. W, Janse, I, Miranda, Kamst-van A., Meima, M., Snoek, J., Mur, L.R., Huisman, J., Zwart, G., and Visser, P.M.: *Microcystis* genotype succession in relation to microcystin concentrations in freshwater lakes, *Aqua. Microb. Ecol.* 48, 1-12 (2007)
- 3) Ame, M.V., del, Pilar D.M., and Wunderlin, D.A.: Occurrence of toxic cyanobacterial blooms in San Roque Reservoir (Cordoba, Argentina): A field and chemometric study, *Environ. Toxicol.*, 18, 192-201 (2003)
- 4) Jungblut, A, Hoeger, S.J., Mountfort, D., Hitzfeld, B.C., Dietrich, D.R., and Neilan, B.A.: Characterization of microcystin production in an Antarctic cyanobacterial mat community, *Toxicon*, 47, 271-278 (2006)
- 5) Park, H.D., Iwami, C., Watanabe, F.M., Harada, K., Okino, T., and Hayashi, H.: Temporal variabilities of the concentrations of intra- and extracellular microcystin and toxic *Microcystis* species in a hypertrophic lake, Lake Suwa, Japan (1991-1994), *Environ. Toxicol. Water Qual.*, 13, 61-72 (1998)
- 6) NRA: Toxic blue-green algae. Report of the National Rivers Authority, Water Quality Series No.2, Bristol, UK. (1990)

- 7) **Codd, G.A.:** Cyanobacterial toxins: occurrence, properties and biological significance, *Water Sci. Technol.*, 32, 149-156 (1995)
- 8) **Carmichael, W.W.:** Cyanobacteria secondary metabolites—the cyanotoxins, *Appl. Microbiol.*, 72, 445-459 (1992)
- 9) **Imanishi, S. and Harada, K.:** Proteomics approach on microcystin binding proteins in mouse liver for investigation of microcystin toxicity, *Toxicon*, 43, 651-659 (2004)
- 10) **Sivonen, K. and Jones, G.:** Cyanobacterial toxins. In: *Toxic Cyanobacteria in Water: A Guide to their Public Health Consequences, Monitoring and Management*. E&FN Spon, London, 41-111 (1999)
- 11) **Dawson, R.M.:** The toxicology of microcystins, *Toxicon*, 36, 953-962 (1998)
- 12) **Faloner, R.I.:** Tumor promotion and liver injury caused by oral consumption of cyanobacteria, *Environ. Toxicol. Water Qual.*, 6, 177-184 (1991)
- 13) **Nishiwaki, M.R., Ohta, T., Nishiwaki, S., Suganuma, M., Kohyama, K., Ishikawa, T., Carmichael, W.W., and Fujiki, H.:** Liver tumor promotion by the cyanobacterial cyclic peptide toxin microcystin-LR, *J. Cancer Res. Clin. Oncol.*, 118, 420-424 (1992)
- 14) **Jochimsen, E.M., Carmichael, W.W., An, J.S., Cardo, D.M., Cookson, S.T., Holmes, C.E., Antunes, M.B., de, Melo, F.D.A., Lyra, T.M., Barreto, V.S., Azevedo, S.M., and Jarvis, W.R.:** Liver failure and death after exposure to microcystins at a hemodialysis Center in Brazil, *N. Engl. J. Med.*, 338, 873-878 (1998)
- 15) **Pouria, S., de, Andrade, A., Barbosa, J., Cavalcanti, R., Barreto, V., Ward, C., Preiser, W., Poon, G.K., Neild, G., and Codd, G.:** Fatal microcystin intoxication in haemodialysis unit in Caruaru, Brazil, *Lancet*, 352, 21-26 (1998)
- 16) **Harada, K.:** Chemistry and detection for microcystins. In: *Toxic Microcystis*, CRC Press Boca Raton, MI USA, 103-143 (1996)
- 17) **Tsuji, K., Naito, S., Kondo, F., Ishikawa, N., Watanabe, F.M., Suzuki, M., and Harada, K.:** Stability of microcystins from cyanobacteria: effect of light on decomposition and isomerization, *Environ. Sci. Technol.*, 28, 173-177 (1994)
- 18) **Jones G.J. and Orr, P.T.:** 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, *Water Res.*, 28, 871-876 (1994)
- 19) **Mazur, H. and Plinski, M.:** Stability of microcystin-LR, microcystin-RR and nodularin in seawater and BG-11 medium of different salinity, *Oceanologia*, 43, 329-339 (2001)
- 20) **Okano, K., Maseda, H., Sugita, K., Saito, T., Utsumi, M., Maekawa, T., Kobayashi, M., and Sugiura, N.:** Biochemical characteristics of microcystin LR degradation by typical protease, *J. J. Wat. Treat. Biol.*, 42, 27-35 (2006)
- 21) **Cousins, I.T., Bealing, D.J., James, H.A., and Sutton, A.:** Biodegradation of microcystin-LR by indigenous mixed bacterial populations, *Water Res.*, 30, 481-485 (1996)
- 22) **Jones, G.J., Bourne, D.G., Blakeley, R.L., and Doelle, H.:** Degradation of the cyanobacterial hepatotoxin microcystin by aquatic bacteria, *Natural Toxins*, 2, 228-235 (1994)
- 23) **Park, H.D., Sasaki, Y., Maruyama, T., Yanagisawa, E., Hiraishi, A., and Kato, K.:** Degradation of the cyanobacterial hepatotoxin microcystin by a new bacterium isolated from a hypertrophic lake, *Environ. Toxicol.*, 16, 337-343 (2001)
- 24) **Maruyama, T., Park, H.D., Ozawa, K., Tanaka, Y., Sumino, T., Hamana, K., Hiraishi, A., and Kato, K.:** *Sphingosinicella microcystinivorans* gen. nov., sp. nov., a microcystin-degrading bacterium, *Int. J. Syst. Evol. Microbiol.*, 56, 85-89 (2006)
- 25) **Saitou, T., Sugiura, N., Itayama, T., Inamori, Y., and Matsumura, M.:** Degradation characteristics of microcystins by isolated bacteria from Lake Kasumigaura, *Aqua J. Water Supp. Res. Technol.*, 52, 13-18 (2003)
- 26) **Valeria, A.M., Ricardo, E.J., Stephan, P., and Alberto, W.D.:** Degradation of Microcystin-RR by *Sphingomonas* sp. CBA4 isolated from San Roque reservoir (Córdoba-Argentina), *Biodegradation*, 17, 447-455 (2006)
- 27) **Lemes, G.A.F., Kersanach, R., da. S., Pinto,**

- L., Dellagostin, O.A., Yunes, J.S., and Matthiensen, A.: Biodegradation of microcystins by aquatic *Burkholderia* sp. from a South Brazilian coastal lagoon, *Ecotox. Environ. Safe.*, 69, 358–365 (2008)
- 28) Okano, K.: Study on the understanding of biodegradation mechanism of cyanobacterial toxin, microcystin, University of Tsukuba Ph.D thesis (2006)
- 29) Harada, K., Imanishi, S., Kato, H., Mizuno, M., Ito, E., and Tsuji, K.: Isolation of Adda from microcystin-LR by microbial degradation, *Toxicon*, 44, 107–109 (2004)
- 30) Bourne, D.G., Jones, G., Blakeley, R., Jones, A., Negri, A., and Riddles, P.: Enzymatic pathway for the bacterial degradation of the cyanobacterial cyclic peptide toxin microcystin LR, *Appl. Environ. Microbiol.*, 2, 4086–4094 (1996)
- 31) Bourne, D.G., Riddles, P., Jones, G.J., Smith W., and Blakeley, R.L.: Characterisation of a gene cluster involved in bacterial degradation of the cyanobacterial toxin microcystin LR, *Environ. Toxicol.*, 16, 523–534 (2001)
- 32) Imanishi, S., Kato, H., Mizuno, M., Tsuji, K., and Harada, K.: Bacterial Degradation of Microcystins and Nodularin, *Chem. Res. Toxicol.*, 18, 591–598 (2005)
- 33) Maruyama, T., Kato, K., Yokoyama, A., Tanaka, T., Hiraishi, A., and Park, H.D.: Dynamics of microcystin-degrading bacteria in mucilage of *Microcystis*, *Microb. Ecol.*, 46, 279–288 (2003)
- 34) Miller, M.B. and Bassler, B.L.: Quorum sensing in bacteria, *Annu. Rev. Microbiol.*, 55, 165–199 (2001)
- 35) Taylor, C. and Quinn, R.J.: An alternative computer model of the 3-dimensional structures of microcystin-LR and nodularin rationalising their interactions with protein phosphatases 1 and 2A, *Bioorgan. Med. Chem.*, 2, 299–302, (1992)
- 36) Watanabe, M.F., Tsuji, K., Watanabe Y., Harada, K., and Suzuki, M.: Release of heptapeptide toxin (microcystin) during the decomposition process of *Microcystis aeruginosa*, *Natural Toxins*, 1, 48–53 (1992)
- 37) Rapala, J., Sivonen, K., Lyra, C., and Niemela, S.: Variation of microcystins, cyanobacterial hepatotoxins, in *Anabaena* spp. as a function of growth stimuli, *Appl. Environ. Microbiol.*, 63, 2206–2212 (1997)
- 38) Sivonen, K.: Effects of light, temperature, nitrate, orthophosphate, and bacteria on growth of and hepatotoxin production by *Oscillatoria agardhii* strains, *Appl. Environ. Microbiol.*, 56, 2658–2666 (1990)
- 39) Takenaka, S. and Tanaka, Y.: Behavior of microcystins and its decomposition product in water treatment process, *Chemosphere*, 31, 3635–3641 (1995)
- 40) Yamamoto, Y., Niizuma, S., Kuroda, N., and Sakamoto, M.: Occurrence of heterotrophic bacteria causing lysis of cyanobacteria in a eutrophic lake, *J. J. Phycol.*, 41, 215–220 (1993)
- 41) Manage, P.M., Kawabata, Z., and Nakano, S.: Dynamics of cyanophage-like particles and algicidal bacteria causing *Microcystis aeruginosa* mortality, *Limnology*, 2, 73–78 (2001)
- 42) Sedmak, B., Carmeli, S., and Eleršek, T.: “Non-Toxic” cyclic peptide induce lysis of cyanobacteria—an effective cell population density control mechanism in cyanobacterial blooms, *Microb. Ecol.*, 56, 201–209 (2008)
- 43) Tucker, S. and Pollard, P.: Identification of cyanophage Ma-LBP and infection of the cyanobacterium *Microcystis aeruginosa* from an Australian subtropical lake by the virus, *Appl. Environ. Microbiol.*, 71, 629–635 (2005)
- 44) Yoshida, M., Yoshida, T., Kashima, A., Takashima, Y., Hosoda, N., Nagasaki, K., and Hiroishi, S.: Ecological dynamics of the toxic bloom-forming cyanobacterium *Microcystis aeruginosa* and its cyanophages in freshwater, *Appl. Environ. Microbiol.*, 74, 3269–3273 (2008)
- 45) Saito, T., Okano, K., Park, H.D., Itayama, T., Inamori, Y., Neilan, B.A., Burns, B.P., and Sugiura, N.: Detection and sequencing of the microcystin-LR degrading gene, *mIra*, from new bacteria isolated from Japanese lake, *FEMS Micro. Lett.*, 229, 271–276 (2003)
- (Submitted 2008. 12. 11)
(Accepted 2009. 1. 5)