Introduction

Dense cyanobacterial water-blooms occur frequently worldwide in eutrophic lakes, ponds, and dams. Cyanobacteria of the genus *Microcystis*, a major cause of freshwater blooms in Japan, have potent toxins. Microcystins (MCYSTs), a family of cyclic heptapeptide toxins produced by most members of this genus, cause acute hepatotoxicity in agricultural livestock (Carmichael, 1994; Sivonen and Jones, 1999). The toxicity of MCYSTs is due to the inhibition of protein phosphatase 1 and 2A (MacKintosh et al., 1990; Nishi-
waki-Matsushima et al., 1992). The increasing occurrence of Microcystis blooms in sources of human drinking water is very problematic (Jochimsen et al., 1998).

MCYSTs share a common structure, cyclo (Adda-D-Glu-Mdha-D-Ala-L-X-D-MeAsp-L-Z-), in which X and Z are variable L-amino acids. The gene cluster encoding microcystin synthetase has been sequenced and characterized from the unicellular Microcystis aeruginosa (Nishizawa et al., 1999, 2000; Tillett et al., 2000), the filamentous Planktothrix agardhii (Christiansen et al., 2003), and the heterocyst Anabaena sp. (Rouhainen et al., 2004). It was revealed that MCYST is synthesized by NRPSs and PKSs, a family of multifunctional enzymes (Finking and Marahiel, 2004; Marahiel, 1997). In addition to NRPSs and PKSs, monofunctional proteins for microcystin biosynthesis, including the amino acid epimerase McyF (Nishizawa et al., 2001; Sielaff et al., 2003), the ABC transporter McyH (Pearson et al., 2004), the hydroxy-acid dehydrogenase McyJ (Pearson et al., 2007), and McyJ involved in O-methylation which was identified in Planktothrix (Christiansen et al., 2003), were located within the mcy gene cluster of Microcystis and Anabaena. However, the mcyF and mcyJ genes were absent from the mcy gene cluster of Planktothrix (Christiansen et al., 2003).

In the case of Planktothrix strains, it was reported that inactivation by transposons served to create the diversity within the mcy genes (Christiansen et al., 2006). T. Nishizawa et al. (2007) showed that the structure of mcy genes (two clusters: mcyABC and mcyDEF-GHIJ) was well conserved among Microcystis genera and uma1 and dnaN were invariably located on both sides of the cluster. It seemed that downstream of mcyJ was often preferred for gene recombination based on studies of two kinds of transposase genes, trp1 and trp2. Moreover, a deletion of mcy was discovered in a non-toxic M. aeruginosa strain for the first time (T. Nishizawa et al., 2007). A partial deletion of mcy was found in Planktothrix strains as well (Christiansen et al., 2008). The entire genome of the MCYST-producer M. aeruginosa NIES-843 has now been sequenced (Kaneko et al., 2007) and the genome of the toxic M. aeruginosa strain PCC 7806 has been incompletely sequenced (Frangeul et al., 2008). Interestingly, the mobile elements, insertion sequences (ISs) and miniature inverted-repeat transposable elements (MITEs), occupy 11.8% of the entire genome of strain NIES-843. Given the existence of trace-sequences of ISs and MITEs, we suppose that rearrangements of the genome, including the deletion and mutation of genes, were caused by the transposition of DNA mobile elements.

The direct detection of MCYST-producing cyanobacteria in natural blooms has been examined using HPLC/LC-MS, protein phosphatase inhibition assay, and ELISA (Mez et al., 1997). These studies need the extraction and purification of MCYST from cyanobacterial cells. To identify the mcy gene clusters, phylogenetical analyses of the mcy gene have been conducted. The clarification of mcy genes has enabled the development of molecular methods for the detection and identification of MCYST-producers. MCYST-producers in water samples were detected and quantified by PCR using specific primer sets for the mcy genes (Hotto et al., 2007; Rantala et al., 2006; Yoshida et al., 2003). On the other hand, Rantala et al. (2004) concluded that the mcy gene was not transferred horizontally between genera based on the phylogenetical relationship between it and housekeeping genes. Recently, a cyanophage infecting toxic M. aeruginosa was isolated and horizontal gene transfer was suggested (Yoshida et al., 2006). However, we found no reliable evidence to decide the movement of the mcy gene cluster to date.

We analyzed a number of toxic cyanobacteria at four locations in Japan. Based on a comparison of the sequence and organization of the mcy gene cluster of Microcystis, we found that the structure of mcy was completely conserved among the strains examined. Inserts appeared only at three sites, downstream of mcyJ and mcyC, and in the non-coding region between mcyA and mcyD in the mcy-possessing strains. Furthermore, we confirmed the participation of McyI in microcystin biosynthesis based on HPLC and an assay of the dehydrogenase activity of McyI and ΔMcyI.

Materials and Methods

Cyanobacterial strains and culture conditions. Environmental samples were collected in geographically different areas, Koyaike Pond (Itami City, Hyogo Pref.), Agigawa Dam (Ena City, Gifu Pref.), Lake Suwa (Nagano Pref.) and Lake Toro (Shibe Town, Hokkaido) in 2002 and Lake Toro in 2004 (Fig. 1). The isolation of unicellular cyanobacterial strains was performed using a pour plate method as described earlier (Shirai et al., 1989) and the procedure was repeated three times or
were grown at 30°C. Positive clones (Nishizawa et al., 1999, 2000) were identified, and cultures of which were used as the fluorescent (cool white) light at 35°C.

Rose medium (0.4% agarose, TaKaRa Bio, Otsu, Japan). Inoculating cells on the surface layer were plated on CB-agarose medium (0.4% agarose, TaKaRa Bio, Otsu, Japan).

Axenic strains (free of contaminating bacteria) were isolated from unicellular cultures by a modification of the two-step centrifugation method described previously (Shirai et al., 1991). In brief, the liquid culture at mid-log phase was shaken by a vortex mixer to disperse the colonies, 1 ml of the culture was centrifuged at 4000 rpm for 10 min, and the floaters on the surface layer were plated on CB-agarose medium (0.4% agarose, TaKaRa Bio, Otsu, Japan). M. aeruginosa K-139, the mcy gene structure of which has been identified, was used as the mcy-positive clone (Nishizawa et al., 1999, 2000). Cultures were grown at 30°C under continuous illumination with fluorescent (cool white) light at 35 μmol m⁻² s⁻¹.

**DNA extraction and manipulation.** Cyanobacterial cells were sonicated for 10 s with a water bath-type sonicator (36 kHz, 200 W) to disrupt the gas vesicles and then harvested by centrifugation (8,000 × g for 3 min) (Sakamoto et al., 1993). Total cyanobacterial DNA was isolated from cells grown to the late logarithmic phase using a previously described procedure (Nishizawa et al., 1999). *Escherichia coli* DH5α-MCR (Cosmo Bio., Tokyo, Japan) and JM109 were used as a host for recombinant plasmids and grown at 37°C in 2 × YT broth or 2 × YT agar broth. Antibiotics were added as necessary at the following final concentrations: ampicillin 75 μg ml⁻¹ and kanamycin 15 μg ml⁻¹. pGEM-T-easy vector (Promega, Tokyo, Japan) was used for cloning. DNA manipulations were performed as described previously (Sambrook et al., 1989).

**Southern hybridization analysis of the mcyG gene.** Digested cyanobacterial DNA was separated on 0.8% agarose gels and then transferred to Hybond-N (GE-Healthcare UK Ltd., Buckinghamshire, England) as described elsewhere (Nishizawa et al., 1999). DNA fragments containing adenylation and thiolation domains of the mcyG gene amplified with 5′-McyG12AT (5′-TATGCATGCCTAGCCATTCCATCG-3′) and 3′-McyG12AT (5′-AACAGATCTATTTCGCGACGTGGACAG-3′) as a probe, were labeled using an ECL random prime labeling Kit (GE-Healthcare UK Ltd.). Southern hybridization and detection were performed as recommended by the manufacturer.

**PCR for amplification of non-coding regions.** To amplify the region between the *dnaN* and *uma1* genes following the *mcy* gene, primers sets were used as described previously (T. Nishizawa et al., 2007) and new primer sets for mcyA-mcyB, mcyD-mcyE, mcyE-mcyF, and mcyF-mcyG were designed (Table 1). The reactions were performed in a PCR Thermal Cycler PERSONAL (TaKaRa Bio). For the amplification of non-coding regions, *TaKaRa Taq*™ (TaKaRa Bio) was used. KOD-Plus- (Toyobo, Osaka, Japan) was used for amplifying mcyF-mcyG and mcyD-mcyE. The reaction was performed under the following conditions: dnaN-mcyJ, 2 min at 95°C, then 25 cycles of 95°C (30 s), 54°C (1 min), and 72°C (1 min); mcyA-mcyB, 2 min at 95°C, then 25 cycles of 95°C (30 s), 58°C (1 min), and 72°C (1 min); mcy-mcyJ, 2 min at 95°C, then 25 cycles of 95°C (30 s), 54°C (1 min), and 72°C (1 min); mcyD-mcyE, 2 min at 95°C, then 25 cycles of 95°C (30 s), 51°C (1 min), and 72°C (1 min); mcyF-mcyG, 2 min at 94°C, then 35 cycles of 94°C (15 s), 58°C (30 s), and 68°C (15 s); mcyD-mcyE, 2 min at 94°C, then 35 cycles of 94°C (15 s), 52°C (30 s), and 68°C (15 s). The *mcyH-mcyI, mcyG-mcyH, mcyA-mcyD, mcyB-mcyC, and mcyC-uma1* non-coding regions were amplified as described previously (T. Nishizawa et al., 1999, 2007). PCR products were resolved on 2.0–1.5% agarose gels in Tris-borate-EDTA buffer.

**PCR amplification of the 16S–23S ITS region.** The conditions used to amplify the DNA region including the internal transcribed spacer (ITS) sequence of the 16S–23S rRNA gene were as described previously (T.
For PCR, Gene Taq (Nippon Gene, Toyama, Japan) was used. Sequencing and computer analysis of DNA sequences. Dideoxy chain termination using a model 3130xl Applied Biosystems Automated Sequencer (Applied Biosystems, Foster, USA) was used to determine the nucleotide sequences of double-stranded template DNA fragments of the PCR products. The DNA sequences were assembled and analyzed using GENETYX-MAC/ATSQ (version 4.0) GENETYX-MAC (version 11.0) from Genetyx Co. (Software Development, Tokyo, Japan). Phylogenetic analyses were carried out using the multiple-sequence alignment tool from CLUSTAL W (Thompson et al., 1997).

LC-MS analysis for the detection of MCYST. Microcystis cells were grown in CB medium for 10 days. The harvested cells were extracted with 5% AcOH(aq) and the extracts were cleaned up with an ODS silica gel cartridge as described previously (Harada et al., 2004). The peptide preparation was analyzed on a COSMOSIL 5C18-AR-II (250 × 10 mm I.D.) (Nacalai Tesque, Kyoto, Japan) column maintained at 40°C using an HP1100 HPLC system (Agilent Technologies, Inc., Santa Clara, USA) with detection at 280 nm. A solvent system, 0.01 M TFA-CH3CN (67:33), was used as the mobile phase at a flow rate of 3 ml/min. The structure of each peptide was determined by using ESI-LC-MS.

Construction of plasmids for McyI and ∆McyI expression. A DNA fragment of mcyI and truncated mcyI was amplified by PCR from chromosomal DNA of M. aeruginosa K-139 and K3-15 using the specific primers F-mcyI-NcoI (5'-TAGCCATGGTGACTACT -TCACCAAAAAC-3') and R-mcyI-BglII (5'- ATCA-GATCTAAAAAGATTCCACCTCTGG-3') (restriction sites are underlined), respectively. The amplification was performed in the TaKaRa PCR Thermal Cycler DICE (TaKaRa Bio) using KOD-plus- (Toyobo) as described in the manufacturer’s manual. The resulting fragment was cleaved with NcoI and BglII and ligated with the NcoI-BglII sites of pQE60 inframe with a carboxy-terminal 6×His fusion construct to yield pQE60-mcyIK-139 and pQE60-ΔmcyIK3-15.

Table 1. Specific PCR primers for the amplification of the mcy gene organization.

<table>
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<tr>
<th>Amplified region (annealing temp., °C)</th>
<th>Name of primer</th>
<th>Tm (°C)a</th>
<th>Oligonucleotide primer sequence from 5’-3’b</th>
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<td>55.0</td>
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<td>5’-mcyJ</td>
<td>53.0</td>
<td>AAAAGGTGATCTCCG</td>
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aTm, melting temperature as determined by the nearest-neighbor method.
b5’ to 3’ orientation.
of Mcyl. Transformed E. coli M15[pREP4] cells were grown in 2 × TY medium at 37°C in the presence of 75 µg ml⁻¹ ampicillin and 15 µg ml⁻¹ kanamycin with shaking at 110 rpm. Production of the recombinant protein was induced by the addition of IPTG to the culture at an OD₆₆₀ of 0.6 to a final concentration of 2.0 mM and the culture was allowed to grow for an additional 7 h at 30°C. The purification step followed methods described previously (A. Nishizawa et al., 2007). All purification procedures were carried out at 0–4°C.

Reductase assay. During the assay of reductase activity, both McyK₁₃₉ and ΔMcyK₃-1₅ recombinant enzymes were prepared twice. The reductase assay was carried out individually. The reaction mixture containing 0.25 mM NADPH, 1.0 mM oxaloacetic acid, and 5.0 µg of purified McyK₁₃₉ or ΔMcyK₃-1₅ in 1.0 ml of assay buffer was incubated at 37°C for 30 min. The conversion of NADPH to NADP was monitored spectrophotometrically at 340 nm. Chemicals were purchased from Sigma (Sigma-Aldrich Corp., St. Louis, USA).

RNA extraction and reverse-transcribed (RT)-PCR analysis. Total RNA was isolated from Microcystis cells using hot phenol (Nishizawa et al., 1999) and subjected to RT-PCR using as primers: F-mcyA-RT (5'-TCACCTGGTGCTGAAC-3') and R-mcyA-PCR (5'-TCACCTGGTGCTGAAC-3') for mcyA, F-mcyD-RT (5'-CAAAAGGGAACCTTACGC-3') and R-mcyD-PCR (5'-GCATCAAACGGGAGA-3') for mcyD, mcyL-RT (5'-AGAGGCTTCATCAATCC-3') and mcyL-PCR (5'-GTGAATCTTCAGCTGATC-3') for mcyL, mcyH-RT (5'-GGACAAATTACGTCGGC-3') and mcyH-PCR (5'-AATAAACTTCCGCTGAG-3') for mcyH, and mcyJ-RT (5'-TTTACACCTCCTGGACG-3') and mcyJ-PCR (5'-TTTCCTGGGATCTGAT-3') for mcyJ. The reverse transcription reaction was performed as described previously (Matsui et al., 2007).

Nucleotide sequence accession numbers. The nucleotide sequences in this report have been submitted to DDBJ under the following accession numbers: AB364969–AB364971 (dnaN-mcyJ region from Microcystis sp. S-14, S-24, and K2-26); AB364972, AB364973, AB364979, and AB364980 (mcyC-uma1 region from Microcystis sp. K2-26, K2-23, K2-28, and K3-9); AB364974, AB364981, and AB364982 (mcyA-mcyD region from Microcystis sp. K2-23, K2-28, and K3-9); AB364975–AB364978 (mcyL-mcyH region from Microcystis sp. K3-15, K3-17, K3-20, and K3-26); and AB363944–AB363948 (16S–23S ITS from Microcystis sp. K2-23, K2-26, K3-15, S-14, and S-24).

Results and Discussion

Detection of microcystin biosynthetic genes using total DNA from water-blooms

An annual report about the occurrence of cyanobacterial-blooms in Japan is published by the National Institute for Environmental Studies (http://www.nies.go.jp/index-j.html). Apparently, cyanobacterial-blooms often occurred in recreation spots of freshwater in Japan. Unnatural deaths among waterfowls have been observed in ponds with Microcystis blooms and extensive contamination by MCYSTs was observed at Shinike Pond and Koyaike Pond in West Japan (Matsunaga et al., 1999). However, toxic strains of the genus Microcystis in a eutrophic freshwater ecosystem have never been investigated in relation to microcystin biosynthesis gene in detail. Therefore, freshwater samples (50–100 ml) were collected from Koyaike Pond, Agigawa Dam, Lake Suwa, and Lake Toro based on information in the annual report (Fig. 1).

Our previous investigations confirmed the detection of the mcyG-mcyH intergenic fragment in mcy⁺-possessing (mcy⁺) strains. Total DNA was extracted from each sample and PCR was performed using the primers 5'-mcyH and 3'-mcyG. Despite the fact that amplification using template DNA extracted from the Agigawa Dam was very weak, the expected PCR amplicons were observed in samples from Koyaike Pond, Agigawa Dam, Lake Suwa, and Lake Toro (data not shown). This result indicates that each water-bloom included mcy⁺ cyanobacteria. To confirm the production of MCYSTs at Agigawa Dam, samples extracted from 1,089.6 mg of the lyophilized blooms were analyzed by HPLC and MALDI-TOF/MS. No peaks of MCYSTs were identified by HPLC, but MCYST-LR was identified by MALDI-TOF/MS, suggesting that production levels of the MCYST at Agigawa Dam are extremely low. This result is consistent with results obtained by the genetic analysis using PCR.

Detection of the mcyG gene in isolated cyanobacterial strains

Unicellular cyanobacterial strains were isolated by the solid culture method from freshwater samples in 2002. Fifty-six, 20, 30, and 19 unicellular Microcystis strains were isolated from Koyaike Pond, Agigawa Dam, Lake Suwa, and Lake Toro, respectively (Table
2). Additionally, 10 filamentous cyanobacterial strains were isolated from Koyaike Pond. To identify the mcy+ strains, genomic Southern hybridization using mcy probes was carried out. The probes were selected to target the adenylation domain of the NRPS module or ketosynthase-acyltransferase-acyl carrier protein of the PKS module of mcyG, which are involved in the biosynthesis of Adda in MCYST. The proportion of mcy+ unicellular strains in Koyaike Pond, Agigawa Dam, Lake Suwa, and Lake Toro was 14/56 isolated strains (24.6%), 0/20 (0%), 4/30 (13.3%), and 2/19 (10.5%), respectively (Table 2). No hybridization signal was detected in total DNA extracted from filamentous cyanobacterial isolates. Furthermore, 16 unicellular Microcystis strains were isolated in 2004 from Lake Toro and PCR with genomic DNA as a template using 5'-mcyH and 3'-mcyG was performed. The analysis showed that 11 strains (68.7%) were mcy+ strains. These results reveal the potential for toxicity in Koyaike Pond, Lake Suwa, and Lake Toro due to water-blooms.

**PCR amplification and sequence analyses of intergene regions from mcy+ strains**

Except for the organization of the mcyABC operon, the mcy gene’s organization and composition have differed in mcy+ Microcystis (Nishizawa et al., 1999, 2000), Planktothrix (Christiansen et al., 2003), and Anaibaena (Rouhiainen et al., 2004). Recently, the highly conserved gene structure of mcyABC and mcyDEF-GHIJ in the genus Microcystis was confirmed (T. Nishizawa et al., 2007). In this study, 20 mcy+ strains were isolated in the first culture after a series of procedures for the isolation of single colonies. For examination of the mcy gene’s organization, we selected 13 strains as a subculture. PCR with a set of primers to amplify the intergene regions was carried out for 14 strains including M. aeruginosa K-139. To identify accurately the organization of the mcy gene cluster, new primers for mcyA-mcyB, mcyD-mcyE, mcyE-mcyF, and mcyF-mcyG were designed in this study (Table 1). Based on the PCR analyses of the non-coding region in each gene, we classified the organization of the mcy gene into five types (A, B, C, D, and E) (Fig. 2 and Table 3). Notably, strains S-11, S-26, and T-14 were identified as type of strain K-139 (Fig. 2). Each sequence showed high homology (97–100%) against non-coding regions of the mcy gene cluster of strain K-139.

**HPLC and LC/MS analysis for confirmation of MCYST production**

The presence of MCYSTs in the 13 mcy+ strains was determined by HPLC and LC/MS (Table 3). Strains K2-23, K3-9, S-11, S-14, and S-24 produced [Dha+] MCYST-LR. K2-28 produced [Dha+]MCYST-RR in addition to [Dha+]MCYST-LR. T-14 produced only [Dha+]MCYST-RR. S-26 produced [Dha+]MCYST-LR, -RR, and -YR, and [Asp3, Dha+]MCYST-LR. However, strains K3-15, K3-17, K3-20, and K3-26, which contain the mcyI deletion mutation described below, did not produce detectable levels of MCYST. No MCYSTs were detected in strain K2-26.

**Nucleotide sequence analysis of the non-coding region between mcyJ and dnaN in the mcy+ strains**

Based on results of the PCR profiling, we selected and sequenced the irregular PCR amplicons against strain K-139. In the case of strains S-14 and S-24 between the mcyJ and dnaN genes, a transposase gene, trp1, which was retained between the mcyJ and dnaN genes in M. aeruginosa B-47 (T. Nishizawa et al., 2007), was included (Fig. 2 and Fig. 3(A)). The non-coding region including the sequence of the trp1 gene is consistent with the non-coding region of mcyJ-dnaN in strain B-47. Obviously, in the flanking region of trp1, the [ATTA] sequence was observed. We predicted that

<table>
<thead>
<tr>
<th>Collected places</th>
<th>Total number isolated</th>
<th>mcy-possessing strain</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lake Kasumigaura</td>
<td></td>
<td>M. aeruginosa K-139</td>
<td>Harada et al., 1991</td>
</tr>
<tr>
<td>Koyaike Pond</td>
<td>56</td>
<td>K2-18, K2-19, K2-22, K2-23, K2-26, K2-28, K2-30, K3-9, K3-15, K3-17, K3-20, K3-23, K3-26, K3-27</td>
<td>This study</td>
</tr>
<tr>
<td>Agigawa Dam</td>
<td>20</td>
<td>no</td>
<td>This study</td>
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<tr>
<td>Lake Suwa</td>
<td>30</td>
<td>S-11, S-14, S-26, S-24</td>
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<tr>
<td>Lake Toro</td>
<td>19</td>
<td>T-3, T-14</td>
<td>This study</td>
</tr>
</tbody>
</table>

Table 2. Sites and dates of the collection of mcy-possessing Microcystis strains.
Microcystin biosynthesis gene in Microcystis

[ATT] is the preferred site of recombination for \textit{trp1}. Furthermore, in the case of strain K2-26, a 271-bp sequence including two sets of a 129-bp insert and a 13-bp irrelevant sequence, which flanked the \textit{GGGTTTTA} sequence, was observed between \textit{dnaN} and \textit{mcyJ} with the deletion of a 149-bp fragment. This means that the 271-bp sequence of strain K2-26 is replaced by the 149-bp sequence of strain K-139 (Fig. 3 (A)). In particular, we observed that over 95% of the 121-bp sequence except for \textit{GGGTTTTA} existed in at least twelve copies in the genome of \textit{M. aeruginosa} NIES-843. On the other hand, from \textit{M. aeruginosa} K-81, five kinds of repetitive sequence-B (REP-B) were found upstream of the \textit{sigA} promoter region (Asayama...
et al., 1996). In REP-B, there were four repeats of the [GGGTTTTTA] sequence. Genomic Southern hybridization showed that *M. aeruginosa* and *M. viridis* possessed over 20 copies in the genome (Asayama et al., 1996). These investigations indicate that the genome of the genus *Microcystis* with short repetitive sequences and the dnaN-mcyJ non-coding region was highly complicated.

**Fig. 3.** Gene structural comparison of the inserted sequence in the mcy gene cluster against *M. aeruginosa* K-139.

A schematic view of the regions flanking dnaN-mcyJ (A), mcyA-mcyD (B), mcyC-uma1 (C), mcyI-mcyJ (D) is illustrated. The number of nucleotide base-pairs (bp) is given below the sequences. P, transcriptional start points (P\_mcyA, Nishizawa et al., 1999; P\_mcyD, Nishizawa, 2001); SL, stem-loop; trp1, putative transposase of cyanobacteria (T. Nishizawa et al., 2007). Dotted arrows indicate the same repeat sequence. A 4-bp [ATTA] sequence is shown by underline. An asterisk indicates a stop codon.

**Nucleotide sequence comparison of the non-coding region between mcyA and mcyD**

In the case of the mcyA-mcyD non-coding region, slightly longer amplified fragments were obtained from strains K2-23, K2-28, and K3-9 compared with strain K-139 (Table 3). A 181-bp insert was observed between the translational start codon and the putative SD sequence [AGGGAAAAAG] of mcyA in these strains (Fig. 3(B)). A direct repeat [TTA] was observed in the flanking region of the insertion sequence. Interestingly,
this insert showed a high degree of similarity with the mcyA-mcyD non-coding region of strains *M. aeruginosa* NIES-90 and NIES-107.

**PCR amplification and sequencing of the non-coding region between mcyC and uma1 in mcy+ strains**

In the case of K2-23, K2-26, K2-28, and K3-9, irregular PCR amplicons between mcyC and uma1 relative to strain K-139 were observed and sequenced (Fig. 2 and Fig. 3(C)). A 219-bp insertion was identified at the same position in K2-23, K2-28, and K3-9. The unique sequence [GAAAAAGTTTTTTCAGTAAACCCTAAT] that overlapped with this unique sequence was found downstream of mcyC (Fig. 3(C)). In the center of a 191-bp insert in K2-23, K2-26, and K3-9, a putative stem-loop sequence [TAGGGTCTAAT] was observed at the 3'-end of the insert in K2-26, but the sequence downstream from the [TAAT] sequence was found downstream of mcyC. In the case of strain K-139, a stop codon found at 33-bp downstream from the [TAAT] sequence. Consequently, the predicted protein, ΔMcyI, encoded by this truncated gene consists of the N-terminal 230 amino acids of McyI and an unrelated 11 amino acids against McyI of strain K-139. Nishizawa et al. (2001) have reported that a mutation of mcyF, which encodes the epimerization of glutamate, failed to produce the MCYSTs. HPLC analysis confirmed no MCYST productivity in ΔMcyI strains. To verify the responsibility of mcyI in the microcystin biosynthesis pathway, we further carried out the molecular genetic analyses of mcyI.

An axenic strain K3-15 was used for the following experiments. To confirm whether the transcripts of mcy genes are present in strain K3-15, transcriptional expression of mcyA, mcyD, mcyH, mcyI, and mcyJ was examined by RT-PCR. K3-15 cells were grown under continuous illumination for 10 days and total RNA was extracted from the cells. The RT-PCR assay revealed that all the genes were transcribed (Fig. 4A). The light-inducible transcripts were revealed to be two polycistrionic transcripts, mcyABC and mcyDEFGHJ, controlled by a central promoter between mcyA and mcyD (Kaebernick et al., 2002). Since the mcy transcripts of K3-15 were observed apparently under the light, we showed that the deletion of the mcyI gene did not influence the transcription of mcyABC and mcyDEFGHJ in the strain.

The DNA fragment encoding the intact mcyI and truncated mcyI were amplified from *M. aeruginosa* K-139 and K3-15 chromosomal DNA in order to investigate the activity of McyIK3-15 and putative ΔMcyIK3-15. These recombinant proteins were expressed in *E. coli* M15. The size of each recombinant protein, purified by Ni²⁺-affinity chromatography, was determined by SDS-PAGE and was well consistent with the theoretical molecular mass of 40 and 29 kDa, respectively (Fig. 4B). To measure the dehydrogenase activity, an assay of oxaloacetate (OAA) reduction with the purified recombinant McyIK139 and ΔMcyIK3-15 was performed. The relative activity was determined by comparing with a negative control (no enzyme) (Fig. 4C). McyIK139 showed a reduction in activity but ΔMcyIK3-15 showed...
no activity. Recently, it was hypothesized that McyI carries out the interconversion of 3-methyl malate (3-MeMal) to 3-MeOAA as the precursor of D-MeAsp in the MCYST structure (Pearson et al., 2007).

ΔMcyIK3-15 did not include part of the nucleotide-binding domain or the substrate-binding domain in the C-terminal region. Their experiments indicated that McyI is a dehydrogenase for the precursor of MeAsp and essential for the biosynthesis of MCYST.

Phylogenetic analysis of 16S-23S ITS of the genus Microcystis

T. Nishizawa et al. (2007) demonstrated that possession of the mcy gene was not related to MCYST productivity in a phylogenetic analysis of the 16S-23S ITS region of the genus Microcystis. Additionally, their investigations suggested that Microcystis strains could be classified into two groups (a M. viridis-wesenbergii group and a M. aeruginosa group) based on phylogenetic relationships. To examine the evolutionary relationship of the mcy gene organization, the primers utilized in this study enabled sequencing of both strands of 16S–23S ITS, which include 74-bp of the gene for tRNA-Ile. In this study, we selected 5 isolates, Microcystis sp. K2-23, K2-26, K3-15, S-14, and S-24 as representative strains (Fig. 2). The PCR products (approximately 600-bp) of 16S–23S ITS region were sequenced. According to a database analysis using the BLAST search, these strains were identified as the genus Microcystis. Phylogenetic trees constructed with the neighbor-joining (NJ) method using CLUSTAL W (Thompson et al., 1997), are shown in Fig. 5. In this analysis, we fixed the 16S–23S ITS sequence of Microcystis sp. 4A3 as an out-group following Otsuka et al. (1999). Phylogenetic trees apparently showed two groups and indicated that four strains other than strain K2-23 belong to group 2 (Fig. 5). Strain K2-23 from Koyaike Pond, which has the same 181-bp insert between mcyA and mcyD within M. aeruginosa NIES-90 and M. wesenbergii NIES-107, was integrated into group 1. However, strain K2-23 did not belong to the same class as NIES-90 and NIES-107.

On the other hand, strain K2-26 was clearly included in a M. aeruginosa group and was closely related to mcy+-MCYST+-strain M. aeruginosa S-77 which is considered as a type A including strain K-139 (Fig. 2) (T. Nishizawa et al., 2007). RT-PCR assay showed the expression of both mcyA and mcyD transcriptions in strain K2-26 of RNA under the light condition (data not shown). This result indicates that neither insert of dnaN-mcyJ non-coding region nor that of mcyC-uma1 non-coding region has influence on the transcription of mcy. Recent results showed that the genus Microcystis strains including a gene insertion within either dnaN-mcyJ or mcyC-uma1 non-coding region retained the MCYST productivity (T. Nishizawa et al., 2007; Tooming-Klunderud et al., 2008). We judged both type B and type C as the MCYST-producer Microcystis from HPLC analysis. Therefore, we presumed that strain K2-26 has a mutated mcy gene cluster.

Strains S-14 and S-24 isolated from Lake Suwa, which possessed the trp1 gene, belonged to the same class as strain B-47, which was isolated from Lake Kasumigaura, Ibaraki. This result indicates that trp1 possessing domestic strains of Microcystis have come to inhabit Lake Kasumigaura, Lake Suwa, or both over the last 10 years (Fig. 1). It seems that the migration of toxic-Microcystis strains has continued domestically. It is therefore necessary to prevent the propagation of mcy+-Microcystis strains.

Molecular ecological analysis of Microcystis

Our results support the finding of T. Nishizawa et al. (2007) that the organization of the mcyABC and
Microcystin biosynthesis gene in Microcystis

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Microcystin biosynthesis gene in Microcystis mcyDEFGHIJ operons was highly conserved in mcy\(^+\) strains. The molecular genetic approach confirmed the diversity of the mcy gene clusters in the isolated Microcystis strains using specific primer sets for non-coding regions among the genes. As a consequence of the diversified mcy gene organization, we suggest that gene recombination occurred in the genus Microcystis at three specific sites, dnaN-mcyI, mcyA-mcyD, and mcyC-uma1.

Recently, the Kazusa DNA Research Institute sequenced the entire genome of a MCYST-producer strain, NIES-843. Interestingly, the genome database showed that 688 kb, equivalent to 11.8% of the entire genome, was composed of both ISs of thirty-five groups and MITEs of eight groups. ISMae7 (direct repeat, TTGATAA) was observed within the dnaN-mcyJ non-coding region near the trp2 gene (T. Nishizawa et al., 2007; Tooming-Klunderud et al., 2008). Besides, strain B-47 and M. viridis NIES-112 and 113 possessed a transposase gene between the dnaN and mcyJ intergenic region (T. Nishizawa et al., 2007). Tooming-Klunderud et al. (2008) reported that in a culture of M. aeruginosa NIVA-CYA 143, from the Norwegian Institute for Water Research Cyanobacterial Culture Collection, the loss of mcyDEFGHIJ was identified based on a PCR assay and genomic Southern hybridization. The mcyDEFGHIJ-deleted strain lost the N-methyltransferase domain, involved in the putative N-methylation of amino acids (Nishizawa et al., 1999), in the McyA NRPS modules (Tooming-Klunderud et al., 2008). We presume that recombination occurred in the mcy gene cluster frequently in the genus Microcystis. However, in the studies of T. Nishizawa et al. (2007) and Tooming-Klunderud et al. (2008), the diversity of the mcy gene was investigated in Microcystis strains in the laboratory.

Herein, we discovered Microcystis strains that possess diversified mcy genes in bodies of freshwater. We revealed the toxicity of cyanobacterial strains identified in environmental samples based on an assessment of the mcy gene cluster. Furthermore, the genome of Microcystis studded with several short inserts was confirmed in this study. We believe that PCR-based monitoring of the genus Microcystis will be necessary to confirm mcy\(^+\) strains worldwide and to identify the diversity of the mcy gene cluster in the genus.
References


