Diversity within the Microcystin Biosynthetic Gene Clusters among the Genus *Microcystis*

TOMOYASU NISHIZAWA¹, AKITO NISHIZAWA¹, MUNEHICO ASAYAMA¹, KEN-ICHI HARADA², and MAKOTO SHIRAI^{1*}

¹ Laboratory of Molecular Genetics, College of Agriculture, Ibaraki University, Ami, Ibaraki 300–0393, Japan

² Laboratory of Environmental Sciences, Faculty of Pharmacy, Meijo University, Tempaku, Nagoya 468–8503, Japan

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The principle organization of microcystin biosynthesis (*mcy*) gene clusters is characterized in three genera among the microcystin-producing cyanobacteria. To examine the conservation of the *mcy* gene clusters among the genus *Microcystis*, internal non-coding regions of the cluster in 21 strains including *M. aeruginosa*, *M. viridis*, and *M. wesenbergii*, were amplified by PCR using specific primer sets and sequenced. PCR-based analysis revealed a completely conserved organization of the bidirectional cluster structure, *mcyABC* and *mcyDEFGHIJ*, among all toxic and some microcystin-negative strains. Furthermore, all *mcy* gene clusters were flanked by a gene with homology to *dnaN* adjacent to *mcyJ* and by *umal* with no-homology to any known genes at the 3'-end of *mcyC*. Transposase homolog genes were observed in a non-coding region between *dnaN* and *mcyJ* in three microcystin-producing *Microcystis* strains. Sequence analysis of a region adjacent to *umal* in *mcy*-negative strains revealed two types of gene constitution, *dnaN-umal* and the hypothetical protein gene-*umal*. PCR-based analyses can be used to assess the identification of microcystin-producer strains in *Microcystis* spp.

Key words: Cyanobacteria, *Microcystis*, Microcystin biosynthetic gene, Non-ribosomal peptide synthetase, Polyketide synthase

Noxious cyanobacterial waterblooms are occurring worldwide in eutrophic lakes, ponds, and dams. Members of the genus *Microcystis*, a major cause of freshwater blooms, can be toxic. Microcystins, a family of cyclic heptapeptide toxins that are produced by most members of this genus, cause acute hepatotoxicity in agricultural livestock^{1,38}. The toxicity of microcystins is due to the inhibition of protein phosphatase 1 and 2A^{8,16}. The increasing occurrence of *Microcystis* blooms in sources of human drinking water is very problematic⁵.

Microcystins 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, Adda is 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, D-MeAsp is D-erythro- β -iso-aspartic acid, and Mdha is N-methyldehydroalanine. Peptides containing non-proteinogenic amino acids are synthesized by non-ribosomal peptide synthetases (NRPSs), which are a family of multifunctional enzymes⁹. NRPSs are composed of modules, each of which is responsible for the activation of an amino acid, thiolation, and condensation⁹. Notably, epimerization and methylation occurred the NRPS module in order to modify the amino acid. Recently, microcystin synthetase genes have been cloned and sequenced from the microcystin-LR type producer *Microcystis aeruginosa*^{17,18,35}, which produces heptapeptide micropeptin/cyanoheptolin as well^{4,10}. The *mcyABC* cluster has the opposite orientation to the *mcyDEFG* cluster^{18,35}. Additionally, monofunctional proteins for

* Corresponding author. E-mail address: shirai@mx.ibaraki.ac.jp; Tel.: +81–29–888–8652; Fax: +81–29–888–8653.

Abbreviations: Adda, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4,6-dienoic acid; D-MeAsp, D-erythro- β -methyl-aspartic acid; Mdha, N-methyldehydroalanine; ITS, internal transcribed spacer sequence; MCYST, microcystin; NJ, neighbor-joining; NRPS, non-ribosomal peptide synthetase; PKS, polyketide synthase.

Fig. 1. Five types of the *mcy* gene organization between *dnaN* and *uma1* and three types of *mcy*-negative gene organization downstream of the *uma1* gene in the genus *Microcystis*. Primer locations and PCR fragments are indicated at the bottom of the gene structure. *dnaN*, DNA polymerase III β subunit; *uma1*, hypothetical protein; *mcyA*-*mcyC*, NRPSs cluster; *mcyD*-*mcyG*, polyketide synthase (PKS), PKS-NRPS, race-mase and NRPS-PKS cluster; *mcyH*, ABC transporter ATP-binding protein; *mcyI*, dehydrogenase; *mcyJ*, *O*-methyltransferase; *trp1*, putative transposase of cyanobacteria; *trp2*, Tn5-like transposase of bacteriophage WO; *hypX*, hypothetical 29.6 kDa lipoprotein of *E. coli*. The number of base pairs indicates the length of a non-coding region.

microcystin biosynthesis, including McyF involved in epimerization^{19,31}, the ABC transporter McyH²², the hydroxy-acid dehydrogenase McyI²³, and McyJ involved in *O*-methylation that was identified in the filamentous cyanobacterium *Planktothrix*², were located within the *mcy* gene cluster. The *uma1* (our previous named *orf2*) gene, which shows a high degree of similarity to a hypothetical protein (SII0471) of *Synechocystis* sp. PCC 6803, is located downstream of *mcyC* in *M. aeruginosa* K-139 (Fig. 1A)¹⁷. The *mcy* gene cluster set was conserved in a filamentous heterocystous *Anabaena* strain²⁵) and *Planktothrix* strain as well². Interestingly, disruption of *mcyA*, *mcyB*, *mcyE*, and *mcyF* in *Microcystis viridis* S-70, which produces three kinds of microcystins, demonstrated that one set of the *mcy* gene clusters is responsible for the production of microcystin-LR, -RR, and -YR^{18,19}. Furthermore, genomic Southern hybridization using *mcy* probes from *M. aeruginosa* K-139 showed the presence of the *mcy* gene in the microcystin non-producing strain *M. aeruginosa* K-81^{17,18}).

For the identification of toxic strains among the genus

Microcystis, phylogenetic analyses as a tool for molecular ecological studies were performed based on 16S rDNA or the 16S to 23S internal transcribed spacer sequence (16S–23S ITS), which can be used to differentiate more closely related strains^{7,14,21}). Recently, based on PCR amplification using the *mcy* genes, several groups reported that the presence of the *mcy* gene cluster was correlated to a certain subspecies level allowing one to distinguish the productive capacity of microcystins^{36,39}). Moreover, according to phylogenetic analyses using the PCR-amplified *mcy* genes, gene recombination was suggested by the genetic variation of the *mcy* genes between different strains of *Microcystis* spp.^{13,33}). Alternatively, to solve the entire evolutionary history of microcystins, a phylogenetic analysis of the coevolution of 16S rDNA and a housekeeping gene, *rpoC*, was performed. The presence of the *mcy* gene cluster in the last common ancestor of a large number of cyanobacteria was proposed²⁴). However, ecological investigations have yet to describe the *mcy* gene organization in relation to toxicity.

In the present study, we revealed the highly conserved

mcy gene organization in three domestic morphospecies, *M. aeruginosa*, *M. viridis*, and *M. wesenbergii*. Additionally, we discuss the connection between the conservation of *mcy* gene clusters and evolutionary incidents in the genus *Microcystis*.

Materials and Methods

Cyanobacterial strains and culture conditions

All cyanobacterial strains with the designation NIES were obtained from the National Institute for Environmental Studies (NIES: Tsukuba, Japan). *Microcystis* strains MB and MG were provided by Prof. Kaplan, A. culture collection (The Hebrew University of Jerusalem, Israel)³⁷⁾. The remaining strains were from a culture collection of the authors (Shirai, M., Ibaraki University, Japan)^{3,20,26,29,30)} (Table 1). Strains were grown in CB or MA medium at 30°C under continuous illumination with fluorescent (cool

white) light at 35 $\mu\text{mol m}^{-2} \text{s}^{-1}$ ^{30,38)}.

DNA extraction and manipulation

Total cyanobacterial DNA was isolated from cells grown to the late logarithmic phase using a previously described procedure¹⁷⁾. *Escherichia coli* DH5 α MCR (Cosmo Bio., Tokyo, Japan) was used as a host for recombinant plasmids and grown at 37°C in 2 $\times$ YT broth or 2 $\times$ YT agar broth. Antibiotics were added as necessary at the following final concentrations: ampicillin 75 $\mu\text{g/ml}$. pUC119 (Toyobo, Tokyo, Japan) and pMOSBlue T-vector (GE-Healthcare UK Ltd., Buckinghamshire, England) were used for cloning. DNA manipulations were performed as described previously²⁷⁾.

*Southern hybridization analysis of the *uma1* gene*

Digested cyanobacterial DNA was separated on 0.8% agarose gels and then transferred to Hybond-N or NX membranes (GE-Healthcare UK Ltd.) as described previously¹⁷⁾.

Table 1. *Microcystis* strains used in this study

Strain	Date; year/month	Origin, Isolation site	Reference	Accession number of 16S–23S ITS	Microcystin
<i>Microcystis</i> spp.					
<i>M. aeruginosa</i> NIES44	1974/Aug.	Lake Kasumigaura, Ibaraki	NIES collection	AB254437	no
<i>M. aeruginosa</i> NIES89	1981/Jun.	Lake Kawaguchi, Yamanashi	NIES collection	AB254439	MCYST
<i>M. aeruginosa</i> NIES90	1981/Jun.	Lake Kawaguchi, Yamanashi	NIES collection	AB254440	MCYST-LR, -RR
<i>M. aeruginosa</i> NIES87	1982/Sep.	Lake Kasumigaura, Ibaraki	NIES collection	AB254438	no
<i>M. aeruginosa</i> NIES98	1982/Sep.	Lake Kasumigaura, Ibaraki	NIES collection	AB254441	no
<i>M. aeruginosa</i> NIES298	1982/Sep.	Lake Kasumigaura, Ibaraki	NIES collection	AB254442	MCYST-LR, [D-Asp ³]MCYST-LR
<i>M. aeruginosa</i> K-81	1985/Sep.	Lake Kasumigaura, Ibaraki	(20, 26)	AB254411	no
<i>M. aeruginosa</i> K-139	1985/Sep.	Lake Kasumigaura, Ibaraki	(3, 26)	AB254412	[Dha ⁷]MCYST-LR, [D-Asp ³ , Dha ⁷]MCYST-LR
<i>M. aeruginosa</i> B-19	1987/Sep.	Lake Kasumigaura, Ibaraki	(26, 29)	AB254408	no
<i>M. aeruginosa</i> B-35	1987/Sep.	Lake Kasumigaura, Ibaraki	(30)	AB254409	MCYST-LR, -RR, -YR
<i>M. aeruginosa</i> B-47	1987/Sep.	Lake Kasumigaura, Ibaraki	(26, 29)	AB254410	[Dha ⁷]MCYST-LR, [D-Asp ³ , Dha ⁷]MCYST-LR
<i>M. aeruginosa</i> S-77	1988/Jul.	Lake Kasumigaura, Ibaraki	(26, 30)	AB254413	MCYST-LR, -RR, -YR
<i>M. aeruginosa</i> S-78	1988/Jul.	Lake Kasumigaura, Ibaraki	This study	AB254414	no
<i>M. aeruginosa</i> TC	1989/Sep.	Lake Kasumigaura, Ibaraki	This study	AB254415	no
<i>M. viridis</i> NIES103	1978/Dec.	Lake Kasumigaura, Ibaraki	NIES collection	AB254444	MCYST
<i>M. viridis</i> NIES102	1982/Sep.	Lake Kasumigaura, Ibaraki	NIES collection	AB254443	MCYST-LR, -RR, -YR
<i>M. viridis</i> S-70	1988/Jul.	Lake Kasumigaura, Ibaraki	(26, 30)	AB254418	MCYST-LR, -RR, -YR
<i>M. wesenbergii</i> NIES107	1981/Jun.	Lake Kawaguchi, Yamanashi	NIES collection	AB254445	MCYST
<i>M. wesenbergii</i> NIES112	1982/Oct.	Lake Suwa, Nagano	NIES collection	AB015393	no
<i>Microcystis</i> MB	2000	Israel	(37)	AB254416	no
<i>Microcystis</i> MG	2000	Israel	(37)	AB254417	MCYST-RR, MCYST

MCYST, microcystin; [Dha⁷]MCYST-LR, 7-desmethylmicrocystin-LR; [D-Asp³, Dha⁷]MCYST-LR, 3,7-didesmethylmicrocystin-LR; [D-Asp³]MCYST-LR, 3-desmethylmicrocystin-LR; no, non-microcystin-producing *Microcystis*.

DNA fragments, a 0.69-kb *Sma*I fragment containing part of the *umal* gene from pMCX2 as a probe¹⁷⁾, were labeled using an ECL random prime labeling Kit (GE-Healthcare UK Ltd.). Southern hybridization and detection were performed as recommended by the manufacturer.

Cloning of dnaN-umal and hypX-umal from non-microcystin-producing M. aeruginosa

To characterize the *umal* gene from a non-microcystin-producing *M. aeruginosa* strain, shotgun clonings were performed using a 0.69-kb *Sma*I fragment containing part of the *umal* gene from pMCX2 as a probe¹⁷⁾. *Xba*I-digested total *M. aeruginosa* B-19 DNA was shotgun cloned into the *Xba*I site of the vector pUC119. The resultant recombinant pBJK3, which contained a 2.5-kb *Xba*I fragment, was iso-

lated and sequenced. Using the *umal* probe, *Xba*I-digested total chromosomal DNA of strain NIES98 was shotgun cloned into the *Xba*I site of pUC119. pNKH2, which contained a 1.9-kb *Xba*I fragment, was cloned and sequenced. Moreover, to obtain the rest of *umal* in strain NIES98, the *Cla*I-digested total DNA was shotgun cloned into the *Cla*I site of pBluescript II KS(+) (Toyobo), using a 0.63-kb *Xba*I-*Hind*III fragment from the downstream *umal* region of the insert of pNKH2 as a probe. Recombinant pNKI10 containing a 2.6-kb *Cla*I fragment was inserted and sequenced.

Polymerase Chain Reaction (PCR) amplification

To amplify between the *dnaN* and *umal* genes following the microcystin biosynthetase genes, primers sets were

Table 2. Specific PCR primers for the *mcy* gene organization

Amplified region (Annealing temp.) Name of primer	T _m (°C)	Oligonucleotide primer sequence, from 5'-3' (length, mer)
<i>dnaN-mcyJ</i> (56°C)		
3'- <i>dnaN</i>	55.0	AATGCTAGATACCCAACCGG (19)
3'- <i>mcyJ</i>	55.0	AACCCAGGACTCGAAGC (17)
<i>mcyI-J</i> (56°C)		
5'- <i>mcyJ</i>	53.0	AAAAGGTGATCTCCGGC (17)
3'- <i>mcyI</i>	57.0	GCAAAGCGGAGACAGTAGG (19)
<i>dnaN-mcyI</i> (56°C)		
3'- <i>dnaN</i>	55.0	
3'- <i>mcyI</i>	57.0	
<i>mcyH-I</i> (55°C)		
5'- <i>mcyI</i>	53.0	ACGCACAAATACGCCAG (17)
3'- <i>mcyH</i>	55.0	TAGTAACCCATCCCCGC (17)
<i>mcyG-H</i> (55°C)		
5'- <i>mcyH</i>	55.0	GAGGCAACCAGTCCAAC (17)
3'- <i>mcyG</i>	55.0	TCGGAGAACGTCGGGAA (17)
<i>mcyA-D</i> (58°C)		
NSZW1	58.0	CTTGAAGTTGCCGAATTTGG (20)
PKM1-a	62.0	GGATTGATTGGTCGTTTTCGG (21)
<i>mcyB-C</i> (56°C)		
FB	63.8	GGATCCACTCTGAAGGCGATGCG (23)
BB	63.8	GGATCCTCTAGGGTAAGAGGGGGT (24)
<i>mcyC-umal</i> (56°C)		
3'- <i>mcyC</i>	57.0	AGAGGAGGACGTGAGGC (17)
3'- <i>umal</i>	57.0	GTCCTCTGGGGTAGCTG (17)
<i>dnaN-umal</i> (56°C)		
3'- <i>dnaN</i>	55.0	
3'- <i>umal</i>	57.0	
<i>hypX-umal</i> (57°C)		
3'- <i>hypX</i>	57.0	ATTGGCCAGAGGTCGCC (17)
3'- <i>umal</i>	57.0	

designed (Table 2). The reactions were performed in a Mini-Cycler (MJ Research, Watertown, USA). For amplifications, Gene *Taq* (Nippon Gene, Toyama, Japan) and *TaKaRa LA Taq*TM (Takara Bio, Otsu, Japan) were used for short PCR and long PCR, respectively. The reaction was performed under the following conditions; *dnaN-mcyJ*, *mcyI-J*, *dnaN-mcyI*, and *danN-uma1*, 1 min at 95°C, then 25 cycles of 95°C (30 s), 56°C (15 s), and 70°C (2 min), and 3 min at 70°C; *mcyC-uma1*, *mcyG-H*, and *mcyH-I*, 1 min at 95°C, then 25 cycles of 95°C (30 s), 55°C (30 s), and 70°C (1.5 min), and 3 min at 70°C; *mcyA-D* non-coding region, 1 min at 95°C, then 25 cycles of 95°C (30 s), 58°C (30 s) and 72°C (1.5 min), and 3 min at 72°C; *hypX-uma1*, 1 min at 95°C, then 25 cycles of 95°C (30 s), 57°C (30 s), and 72°C (2 min), and 3 min at 72°C. The *mcyB-C* and core regions were amplified as described previously¹⁷. PCR products were resolved on 2.0–1.5% agarose gels in Tris-borate-EDTA buffer.

PCR amplification of the 16S–23S ITS region

Primers for amplification of the DNA region including 16S–23S ITS were designed according to Otsuka *et al.*²¹ with some modifications of PCR conditions. First, to amplify the 16S–23S regions of the *Microcystis* spp., MSR-S2F and 242r derived from conserved *E. coli* 16S–23S rDNA were used as forward and reverse primers. The reaction was carried out under the following conditions; 1 min at 95°C, then 25 cycles of 95°C (30 s), 50°C (30 s), and 70°C (1.5 min), and 3 min at 72°C. An amplified band of approximately 1.4 kb was prepared. Then the primers 1505f and 115r were used for sequencing and nested PCR was performed with the prepared fragment. The nested PCR profile was: 1 min at 95°C, then 25 cycles of 95°C (30 s), 56°C (30 s), and 70°C (1.5 min), and 3 min at 72°C.

Sequencing

Dideoxy chain termination using an Applied Biosystems Automated Sequencer model 310 (PE Biosystems, Foster, USA) was used to determine the nucleotide sequences of double-stranded template DNA fragments of the PCR products and the cloned plasmid derived from deletion clones generated using Exonuclease III and Mung Bean Nuclease¹⁷.

Computer analysis of DNA sequences

The DNA sequences were assembled and analyzed using GENETYX-MAC from Genetyx Co. (Tokyo, Japan). Phylogenetic analyses were carried out using the multiple-sequence alignment tool from CLUSTAL W³⁴.

Nucleotide sequence accession numbers

The nucleotide sequences in this report have been submitted to DDBJ under the following accession numbers: AB019578 and AB032549 (microcystin biosynthetic gene cluster from *M. aeruginosa* K-139), AB254433 (*dnaN-trp1-mcyJ* from *M. aeruginosa* B-47), AB254434 (*dnaN-uma1* from *M. aeruginosa* B-19), AB254435 (*hypX-uma1* from *M. aeruginosa* NIES98), AB254436 (*dnaN-trp2-mcyJ* from *M. viridis* NIE102).

Results

Identification of the region adjacent to the *mcy* cluster

To examine the existence of *uma1* within the genus *Microcystis* including nine non-microcystin-producing strains (Table 1), a genomic Southern hybridization analysis using the *uma1* probe was carried out. Hybridization signals were observed with the DNA digested from all *Microcystis* strains examined, suggesting that *uma1* is highly conserved in *Microcystis* strains (data not shown). In our previous study, no NRPS/PKS of the *mcyABCDEFGF* gene was detected by genomic Southern hybridization in *M. aeruginosa* B-19^{17,18}. To reveal whether *M. aeruginosa* B-19 possesses a part of *mcy* near a downstream region of *uma1*, shotgun cloning using the *uma1* probe was performed. An open reading frame, which shows a high level of similarity to a DNA polymerase III β subunit (*dnaN*) of *Synechocystis* sp. PCC 6803, was found with the opposite orientation to *uma1* (Fig. 1F). Actually, using a specific primer set for the *dnaN* gene, PCR-amplified products were obtained from the nine non-microcystin-producing strains.

Conservation of the *mcy* gene cluster among the genus *Microcystis*

To clarify the arrangement of the *mcy* gene cluster among the genus *Microcystis*, PCR amplification and genomic Southern hybridization using *mcy* gene probes were carried out. So as to specifically detect the *mcy* gene organization, the primers contained no sequences conserved among the NRPS and PKS genes. A set of primers to amplify the inter-gene region was designed from the sequences of *mcy*, *uma1*, and *dnaN* of *M. aeruginosa* K-139 (Table 2, Fig. 1).

Expected PCR-amplified fragments were obtained from all toxic strains and 3 non-toxic strains, namely *M. aeruginosa* K-81, S-78, and TC (Table 3). On the other hand, no desired PCR-fragments were detected in non-toxic strains, *M. aeruginosa* B-19, NIES44, NIES87, and NIES98, *M. wesenbergii* NIES112, and *Microcystis* MB (Table 3).

Table 3. Summary of the amplification of non-coding regions involved in the microcystin synthetase gene cluster and other genes and the results of Southern hybridization of *Microcystis* DNA with the *umal* gene probe

Strain	PCR product name ^a									<i>umal</i> probe	Types ^f
	N.J	I.J	H.I	G.H	A.D	B.C	C.U	N.U	X.U		
<i>Microcystis</i> spp.											
<i>M. aeruginosa</i> NIES44	—	—	—	—	—	—	—	—	—	+	(H)
<i>M. aeruginosa</i> NIES89	+	+	+	+	+	+	+	nt	nt	nt	(A)
<i>M. aeruginosa</i> NIES90	+	+	+	+	+ ^c	+	+	nt	nt	nt	(C)
<i>M. aeruginosa</i> NIES87	—	—	—	—	—	—	—	—	+	+	(G)
<i>M. aeruginosa</i> NIES98	—	—	—	—	—	—	—	—	+	+	(G)
<i>M. aeruginosa</i> NIES298	+	+	+	+	+	+	+	nt	nt	nt	(A)
<i>M. aeruginosa</i> K-81	+	+	+	+	+	+	+	nt	nt	+	(A)
<i>M. aeruginosa</i> K-139	+	+	+	+	+	+	+	nt	nt	+	(A)
<i>M. aeruginosa</i> B-19	nt	—	—	—	—	—	nt	+	nt	+	(F)
<i>M. aeruginosa</i> B-35	+	+	+	+	+	+	+	nt	nt	nt	(A)
<i>M. aeruginosa</i> B-47	+ ^d	+	+	+	+ ^b	+	+	nt	nt	nt	(B)
<i>M. aeruginosa</i> S-77	+	+	+	+	+	+	+	nt	nt	nt	(A)
<i>M. aeruginosa</i> S-78	+	+	+	+	+	+	+	nt	nt	nt	(A)
<i>M. aeruginosa</i> TC	+	+	+	+	+	+	+	nt	nt	nt	(A)
<i>M. viridis</i> NIES103	+ ^e	+	+	+	+	+	+	—	—	nt	(E)
<i>M. viridis</i> NIES102	+ ^e	+	+	+	+	+	+	—	—	nt	(E)
<i>M. viridis</i> S-70	+	+	+	+	+	+	+	nt	nt	nt	(D)
<i>M. wesenbergii</i> NIES107	+	+	+	+	+ ^c	+	+	—	nt	nt	(C)
<i>M. wesenbergii</i> NIES112	—	—	—	—	—	—	—	—	—	+	(H)
<i>Microcystis</i> MB	—	—	—	—	—	—	—	—	+	+	(G)
<i>Microcystis</i> MG	+	+	+	+	+	+	+	—	—	+	(A)

^a N.J, *dnaN-mcyJ*; I.J, *mcyI-J*; H.I, *mcyH-I*; G.H, *mcyG-H*; A.D, *mcyA-D*; B.C, *mcyB-C*; C.U, *mcyC-umal*; N.U, *dnaN-umal*; X.U, *hypX-umal*. ^b A 195-bp fragment is inserted. ^c A 175-bp fragment is inserted. ^d The *trpI* gene is inserted. ^e The *trp2* gene is inserted. ^f Types indicate the gene organization of Figure 1. +, Amplified the expectant fragment; –, No expectant fragment; nt, not tested.

Sequence analysis revealed an extremely high level of homology among the PCR products: *mcyI-J* (98.1–100% identity), *mcyH-I* (96.9–100%), *mcyG-H* (94.7–100%), *mcyB-C* (98.4–100%), and *mcyC-umal* (95.2–100%). Notably, between *M. viridis* NIES102 and NIES103, these amplicons were identical except for the *mcyA-D* products (99.9%). Based on the sequence alignment of the *mcyA-D* products, 91.7–99.9% identity existed except for *M. aeruginosa* B-47 and NIES90, and *M. wesenbergii* NIES107 (Fig. 1B and 1C). Regarding the sequence of the *mcyA-D* amplicon of *M. aeruginosa* B-47, a 195-bp insert, which flanked the (CTAATTA) sequence, was observed between the putative promoter sequence of *mcyA* and *mcyD* as compared with strain K-139 (Table 3). This insert was identified with a miniature insertion element in a *Microcystis* strain (accession number, DQ022743). Moreover, in the case of *M. aeruginosa* NIES90 and *M. wesenbergii* NIES107, the *mcyA-D* sequence was identical and a 175-bp insert was

observed as compared with *M. aeruginosa* K-139 between the translational start codon and the putative SD sequence (AGGGAAAAAG) of *mcyA* (Table 3). This 175-bp sequence showed no significant homology to any previously reported genes in the GenBank database. Non-coding nucleotide sequences of *mcyH-I*, *mcyB-C*, and *mcyC-umal* completely matched between *M. aeruginosa* NIES90 and *M. wesenbergii* NIES107 as well and the PCR amplicons of *dnaN-mcyJ*, *mcyJ-I*, and *mcyG-H* were 99.4, 99.5, and 98.8% identical, respectively.

Identification of transposase genes between the *dnaN* and *mcyJ* genes

Approximately 700-bp *dnaN-mcyJ* products, which included a 564-bp non-coding region, from *mcy*-positive (*mcy*⁺) strains retaining the *mcy* gene cluster, shared identity (92.0–100%) with each other except for *M. aeruginosa* B-47 and K-81, and *M. viridis* S-70, NIES102, and NIES103.

Fig. 2. Gene structure of inserted sequence in the *mcy* gene cluster. (A) *M. aeruginosa* B-47, *trp1*, putative transposase of cyanobacteria; (B) *M. viridis* NIES102, *trp2*, Tn5-like transposase of bacteriophage WO; (C) *M. viridis* S-70; (D) *M. aeruginosa* NIES98. The rectangular box and arrows indicate the 37-bp repeated sequence and the inverted sequences, respectively. The black squared box represents a region of the highly homologous sequence. Each sequence shows the number of nucleotide base pairs under the gene structure.

Clearly, the *dnaN*-*mcyJ* products from *M. aeruginosa* K-81 had an extremely low level of similarity (67.1–86.7%) in the non-coding sequence with those of other *mcy*⁺ *M. aeruginosa* strains. *M. aeruginosa* B-47, and *M. viridis* NIES102 and NIES103 retained an open reading frame within the conserved non-coding region between *mcyJ* and *dnaN* as described below. The nucleotide sequence from *M. aeruginosa* B-47 showed an *orf* (855-bp), which exhibits homology (54.0% identity) to the transposase of the cyanobacterium *Calothrix* sp. PCC 7601 (accession number, X60384)¹¹⁾, between *dnaN* and *mcyJ* (Fig. 1B and 2A). A direct repeat [ATTA] was found flanking *trp1*. Except for *M. aeruginosa* K-81 and B-47, and *M. viridis* NIES102, NIES103, and S-70, the nucleotide sequence alignment revealed an extremely high level of similarity with the *dnaN*-*mcyJ* amplicon sequence of *M. aeruginosa* K-139 (98.0%).

In the case of *M. viridis* NIES102 and NIES103, an *orf* (1425-bp) was found between *dnaN* and *mcyJ* (Fig. 1E) and, characterized as the transposase of bacteriophage WO (accession number, AB036665), exhibits similarity to the Tn5 transposase. The *orf*, designated *trp2*, shows no similarity to *trp1* from *M. aeruginosa* B-47. Notably, four direct repeat sequences (37-bp, TCGATCCCCCTTAATCCCCCTTGATAAGGGGGGTG) were observed in the flanking region of *trp2*. One and two complete direct repeats were located upstream and downstream of *trp2*, respectively and another direct repeat was divided by *trp2*. An inverted repeat sequence (42-bp CCCCCCTTGATAA-

GCTATCCATTAGTCGGATCTTTCTTAACCA) flanked the *trp2* gene (Fig. 2B). Interestingly, downstream of *mcyJ* of *M. viridis* NIES102, a 117-bp sequence with remarkable similarity to the non-coding region of *uma1*-*hypX* of the *mcy*⁻ strain *M. aeruginosa* NIES98 was observed (Fig. 2B and D). Furthermore, the *dnaN*-*mcyJ* amplicon from *M. viridis* S-70 showed a 313-bp insert, which is identical to the flanking regions of *trp2* in *M. viridis* NIES102. This insert included two complete 37-bp direct repeats (Fig. 2C).

Analysis of the *mcy*⁻ *Microcystis* strains

In order to determine the *uma1* downstream region of the *mcy*⁻ *Microcystis* strains, shotgun cloning using the *uma1* probe was performed. An *orf* was identified 171-bp downstream of *uma1* in a reversed orientation (Fig. 1G and 2D) in strain NIES98. This *orf* shows similarity (26.7%) to a hypothetical 29.6-kDa lipoprotein in the DnaQ-GMHA intergenic region precursor of *E. coli* K-12, and is designated *hypX*. A part (117-bp) of this non-coding region in *M. aeruginosa* NIES98 was consistent with the non-coding region downstream of *mcyJ* in *M. viridis* NIES102 as described above. PCR amplification using primers 3'-*hypX* and 3'-*uma1* showed that the genetic organization is the same among *M. aeruginosa* NIES87 and NIES98, and *Microcystis* MB. In *M. aeruginosa* NIES44 and *M. wesenbergii* NIES112, no PCR-amplified products were obtained using 3'-*hypX* and 3'-*uma1*. Additionally, to confirm whether the *hypX* gene exists on the chromosomal DNA of

Fig. 3. Phylogenetic tree of the apparent evolutionary distances of the 16S–23S ITS sequence and the relationship of microcystin production with the *mcy* gene cluster. The method of NJ (neighbor-joining) was carried out using the package CLUSTAL W. The seed number for the random number generator and numbers of bootstrap trials were set at 111 and 1000, respectively. Microcystin-producing strains and non-producing strains are listed in Table 2. Type indicated the structural feature in Fig. 1. Accession numbers of 16S–23S ITS were: *Microcystis* sp. 4A3, AB015357; *M. aeruginosa* TAC86, AB015363; CL1, AB015381; *M. ichthyoblabe* TAC91, AB015367; TAC125, AB015368; TAC136, AB015369; TAC138, AB015370; *M. novacekii* TAC65, AB015375; TL2, AB015380; *M. viridis* TAC17, AB015398; *M. wesenbergii* NIES104, AB015387; NIES112 (TAC52), AB015390; NC1, AB015393.

Microcystis, a PCR analysis was performed using another specific primer set for *hypX*. This investigation showed that no *hypX* was present in *M. aeruginosa* K-139 and NIES44, *M. viridis* NIES102 and NIES103, and *M. wesenbergii* NIES112. Therefore, these results suggested that the genetic

organization downstream of *umal* in *M. aeruginosa* NIES44 and *M. wesenbergii* NIES112 was different from that in other *mcy*⁺ *Microcystis* strains.

Phylogenetic relationships based on 16S-23S ITS

The primers utilized in this study enabled sequencing of both strands of 16S–23S ITS. The PCR amplification products (approximately 600-bp) of 22 *Microcystis* strains shown in Table 1 were sequenced. The amplified fragment included 74 bp of the gene for tRNA-Ile. The nucleotide sequence of 16S–23S ITS from *Microcystis* TAC, TL, and NC1 strains, has been deposited in the Genome Sequence Database. Phylogenetic trees, constructed based on the NJ method using CLUSTAL W³⁴, are shown in Figure 3. *Microcystis* sp. 4A3 was used as an out-group sequence as a reference to Otsuka *et al.*²¹. Except for the *mcy*[−] strain of *M. aeruginosa* B-19, *Microcystis* strains were roughly dividable into two groups; the *M. viridis*—*M. wesenbergii* group and the *M. aeruginosa* group (Fig. 3). The *mcy*[−] strains, *M. aeruginosa* NIES87 and NIES98, *M. wesenbergii* NIES112, and *Microcystis* MB, were broadly dispersed to each branch in the *M. aeruginosa* group.

Discussion

Based on information on the *mcy* gene cluster, a molecular genetic approach was used to correlate the presence of the *mcy* gene cluster with microcystin production among the genus *Microcystis*. Genetic analyses using the *mcy*-gene probes showed that some non-toxic *Microcystis* strains have retained the *mcy* genes^{17,18}. We presumed that non-producers of microcystin developed as a result of a mutation or the repression of gene expression, or else toxin levels were below detection limits. Therefore, to reveal the *mcy* gene organization among the *mcy*⁺ and *mcy*[−] *Microcystis* strains, we provided primer sets amplifying the non-coding region of the *mcy* gene cluster. Recently, microcystin-producers and non-producers among *Planktothrix* strains were analyzed to develop a basis for the reliable detection of microcystin-producers using the eight regions of the *mcy* gene cluster with PCR-amplification¹². It was concluded that *mcy* genes do not occur exclusively in microcystin-producing *Planktothrix* and that the *mcy*-genes in microcystin-producers can be highly variable. In the present study, our molecular genetic approach showed a highly conserved *mcy* cluster using non-coding sequence alignments. Our approach will be applicable to the ecological detection of domesticated toxicogenic *Microcystis* strains.

The classification of *Microcystis* species is based on morphological features observed microscopically, such as cell size, cell arrangement in colonies, the existence of gas vesicles, and characteristics of the mucilage of colonies. It has

been reported that a phylogenetic analysis of toxic and non-toxic *Microcystis* strains based on 16S rDNA was useful for determining the relation to taxonomy among closely related species and strains^{6,14}. Neilan *et al.* studied the production of microcystin based only on the PCR amplification of one module, which activates L-amino acids, within the *mcyB* gene among cyanobacteria¹⁵. This report described a method for the determination of potential microcystin-producing cyanobacteria. Tillett *et al.* have reported that the NMT region of *mcyA*, which was responsible for the N-methylation of amino acids, was used to separate toxic from non-toxic groups in *Microcystis*³⁶. However, we have confirmed that some non-microcystin-producing *M. aeruginosa* strains possessed this module and other modules as well^{17,18}. To characterize and determine the classified domestic *mcy*⁺ strains and *mcy*[−] strains, phylogenetic relationships based on 16S–23S ITS were determined based on the NJ method (Fig. 3). Our results indicated that the phylogenetic relationship of genus *Microcystis* strains do not correspond to the possession of *mcy*. Therefore, the possibility of toxicity in each strain should be verified by investigation of the *mcy* gene organization regardless of the phylogenetic relationship.

The process of horizontal gene transfer led to the formation of the genomic structure in the bacterium. Though there is a possibility that the *mcy* gene set was propagated by plasmids in the *Microcystis* strains, analyses of plasmids from toxic and non-toxic strains of *Microcystis* do not support the hypothesis of propagation²⁸. Since the restriction and modification barrier of *Microcystis* is significantly strong³², transformation for propagation would be interfered with among the genus *Microcystis*. Analyzing a number of *Microcystis* strains in terms of their genetic organization would throw light on the difficult problem of how the *mcy* gene cluster was acquired or lost. In this study, we confirmed the organization of the downstream of *dnaN* among microcystin-producers of *Microcystis*. Transposase gene (*uma4*) homologs located nearby the *mcy* gene cluster have been reported¹³. Although we found two kinds of transposase genes between *dnaN* and *mcyJ*, *trp1*, and *trp2* did not correspond to the *uma4* gene. According to these results, it seemed that downstream of *dnaN* often was selected as a preferable point for gene recombination. We presumed that a transferable transposase could occur through gene recombination of *mcy*. We have no evidence of gene transfer accompanying the *mcy* gene cluster. Moreover, a database search of the Kazusa DNA Research Laboratory, revealed that *Anabaena* sp. PCC 7120 and *Synechocystis* PCC 6803 retained *dnaN* and *uma1* homologs as

well. However, no NRPS/PKS gene related with *mcy* was found. Tillett *et al.* reported that *umal* was located downstream of *mcyC* in toxic *Microcystis* strains and was detected in non-toxic strains³⁶). To examine the vestige of *mcy*, we focused on the *umal* gene observed downstream of *mcyC*. In the *mcy*⁻ strains *M. aeruginosa* NIES87 and NIES98, and *Microcystis* MB, we identified the *hypX* gene as linked to *umal*, while *umal* was located beside *dnaN* in *M. aeruginosa* B-19. Interestingly, part of the nucleotide sequence in *mcyJ* downstream of *M. viridis* NIES102 corresponded to part of the sequence between *hypX* and *umal* in the *mcy*⁻ strains (Fig. 2B and 2D). Considering the high degree of conservation of the *mcy* gene cluster and the results of the nucleotide sequence analyses of the region downstream of *umal*, even if the *mcy* gene cluster was inserted or lost in ancient cyanobacteria, some gene recombination occurred in this downstream region. In the case of *mcy*⁻ strains other than *M. aeruginosa* B-19, gene recombination occurred near the non-coding region between *hypX* and *umal* after the *mcy* gene cluster was lost. Therefore, we propose that types G and H in Figure 1 involved several gene recombination events during evolution.

Consequently, by our molecular genetic approach we demonstrated that the organization of the *mcy* gene cluster with an adjacent *dnaN* was completely conserved among the domestic genus *Microcystis*. Considering the environmental problem of toxic cyanobacteria, profiling of the *mcy* gene cluster is important for knowledge of the microcystin synthetase gene and useful for the detection of *mcy* from environmental field samples of *Microcystis* species.

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