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Detection of Toxigenicity by a Probe for the Microcystin Synthetase A Gene (*mcyA*) of the Cyanobacterial Genus *Microcystis*: Comparison of Toxicities with 16S rRNA and Phycocyanin Operon (Phycocyanin Intergenic Spacer) Phylogenies

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The relationship between toxigenicity and phylogeny within the cyanobacterial genus *Microcystis* is unclear. To investigate this issue, we have designed PCR primers for the *N*-methyltransferase (NMT) domain of the microcystin synthetase gene *mcyA* and have probed 37 *Microcystis* sp. cultures as well as several field samples. The NMT region was present in all 18 laboratory strains that gave positive reactions in the protein phosphatase inhibition assay for microcystin but was absent in 17 nontoxic strains. Two other nontoxic strains, one of which had previously been reported to produce microcystin, possessed the NMT region. Detection of NMT-specific DNA in field samples corresponded to periods of toxicity as assessed by protein phosphatase inhibition. The *Microcystis* strains formed a monophyletic cluster based on 16S rRNA gene sequences but comprised two groups with respect to phycocyanin intergenic spacer (PC-IGS) sequences. Toxic and nontoxic strains appeared to be erratically distributed within the PC-IGS and 16S rRNA trees. Sequence analysis of the NMT domain revealed two coherent groups. The genomic region immediately downstream of the *mcyABC* cluster in all 20 NMT-positive strains contained an open reading frame of unknown function (*uma1*) at a conserved distance from *mcyC*. All nontoxic strains also contained *uma1*, which is not cotranscribed with *mcyABC*. The consistent linkage of *mcyC* to *uma1* suggests that *mcyC* has not been frequently transferred into nontoxic strains via any mechanism involving insertion at random chromosomal locations. These results are discussed with respect to various mechanisms that could explain the patchy distribution of toxigenicity among the various *Microcystis* clades.

Microcystis spp., cyanobacteria that frequently occur as noxious blooms in eutrophic freshwaters, are of major concern because many strains produce cyclic heptapeptide toxins called microcystins (5). The microcystins are members of a family of more than 65 heptapeptides and share the common structure cyclo(D-Ala–L-X–D-MeAsp–L-Z–Adda–D-Glu–Mdha), where L-X and L-Z are variable L-amino acids, Adda is 3-amino-9-methoxy-2,6,8,-trimethyl-10-phenyl-4,6-decadienoic acid, D-MeAsp is 3-methyl-aspartic acid, and Mdha is *N*-methyldehydroalanine (38). Toxicity is mediated through the active transport of microcystin into hepatocytes by the bile acid organic anion transport system, followed by inhibition of eukaryotic serine/threonine protein phosphatases 1 and 2A (12, 17). Acute poisoning, leading to death from massive hepatic hemorrhage, has been reported to occur in both animals and humans (3, 19, 37). Chronic ingestion of sublethal doses has been demonstrated to induce primary hepatocellular carcinoma in rodents (33) and has been epidemiologically linked to primary liver cancer in humans (56, 57).

Water resource management has been complicated by the inability to differentiate between toxic and nontoxic *Microcystis* blooms without isolation and testing for toxin production. Several morphological studies (25, 54) and molecular studies have attempted to resolve the ambiguous relationship of *Microcystis* toxigenicity to its population structure. Molecular approaches have included those based on allozyme polymorphisms (23), 16S rRNA genes (28, 35, 41, 42), the phycocyanin intergenic spacer (PC-IGS) region (29), DNA-DNA hybridization (55), nucleotide base composition (13), random amplified polymorphic DNA (27, 32), the 16S-23S rRNA internal transcribed spacer region (30, 36), the *rbcL* gene (42), repetitive DNA elements (2, 40), and *rpoD* homologs (44). The results, while suggesting a monophyletic origin of *Microcystis*, have proven inconsistent with respect to subpopulations that might correlate with toxicity.

The recent identification of the genetic locus responsible for microcystin synthesis in *Microcystis aeruginosa* allows the question of toxigenicity to be reexamined (9, 51). To better detect microcystin-producing cyanobacterial strains, Neilan et al. (31) and Nishizawa et al. (34) have developed genetic probes directed, respectively, to the *mcyB* gene and to adenylation domains within the microcystin synthetase gene cluster. While both of these two types of probes showed good correlation with toxin production, a number of anomalies were found. A pos-

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TABLE 1. *Microcystis* strains used in this study

Strain[a]	Origin[b]	NMT[c]	Toxicity[d]	Reference
M. aeruginosa UWOCC 001	Canada, 1954	+	+	11
M. aeruginosa UWOCC 006	South Africa	+	+	11
M. aeruginosa UWOCC 017	South Africa	+	+	11
M. aeruginosa UWOCC 019	South Africa	+	+	11
M. aeruginosa UWOCC 023	South Africa	+	+	11
M. aeruginosa UWOCC 024	South Africa	−	−	11
M. aeruginosa PCC 7806 (RID-2)	The Netherlands, 1972	+	+	39
M. aeruginosa PCC 7820	Scotland, 1975	+	+	39
M. aeruginosa UWOCC 84/1	England pre-1985	+	+	48
Microcystis sp. strain UWOCC AK-1	United States, 1976	+	+	13
Microcystis sp. strain UWOCC AK(GV-)	United States, 1976	+	+	13
M. aeruginosa UWOCC Aub B1	United States, 1982	−	−	47
M. aeruginosa UWOCC Bauld 5	Australia, 1985	−	−	47
Microcystis sp. strain UWOCC Bauld B	Australia, 1985	−	−	47
Microcystis sp. strain UWOCC Bauld E	Australia, 1985	+	+	47
M. aeruginosa UWOCC C1	United States, 1975	−	−	8
M. flos-aquae UWOCC C2	United States, 1975	−	−	8
M. flos-aquae UWOCC C3-9	United States, 1975	−	−	13
M. flos-aquae UWOCC C3-11	United States, 1975	−	−	13
M. aeruginosa UWOCC C4	United States, 1975	−	−	8
M. aeruginosa UWOCC C5	United States, 1975	−	−	13
M. aeruginosa UWOCC CBS	United States, pre-1983	+	−	47
M. aeruginosa UWOCC E7	Canada 1980	+	+	47
Microcystis sp. strain UWOCC F	United States, 1982	+	+	10
Microcystis sp. strain UWOCC K	United States, 1982	−	−	10
M. aeruginosa forma minor UWOCC M4	United States, 1973	−	−	47
M. aeruginosa UWOCC MR-A	Australia, 1973	+	+	18
M. aeruginosa UWOCC MR-B	Australia, 1973	+	+	18
M. aeruginosa UWOCC MR-C	Australia, 1973	+	−	18
M. aeruginosa UWOCC MR-D	Australia, 1973	+	+	18
Microcystis sp. strain UWOCC MSU28-1	United States, 1982	−	−	47
M. aeruginosa UWOCC MSU28-2	United States, 1982	−	−	47
M. flos-aquae UWOCC N	United States, 1982	−	−	10
M. aeruginosa UWOCC P3	United States, 1982	−	−	10
Microcystis sp. strain UWOCC Q	United States, 1982	−	−	10
M. aeruginosa UWOCC RID-1	The Netherlands 1972	+	+	22
M. aeruginosa UWOCC S-15-b	Canada, 1975	+	+	47

[a] Species designations are as determined by morphology.
[b] Country and year of original strain isolation.
[c] Presence (+) or absence (−) of the microcystin synthetase NMT domain.
[d] Production (+) or lack of production (−) of microcystin as determined by protein phosphatase 2A inhibition.

sible explanation for these discrepancies is the high sequence similarity between the *mcyB* region and other peptide synthetase loci (9, 51) and the occurrence of multiple adenylation domains in toxic and nontoxic *Microcystis* spp. (34). In this report we describe the use of the single *N*-methyltransferase (NMT) domain (24, 26) encoded by the microcystin synthetase gene *mcyA* (51) to design conserved molecular probes enabling identification of toxigenic *Microcystis.* The applicability of these probes was tested with laboratory cultures and field samples. The toxigenicities of various strains were contrasted with their positions in phylogenies based on 16S ribosomal DNA (rDNA) and PC-IGS DNA sequences to see if the toxic strains form a distinct clade. To screen for the possible rearrangement or deletion of DNA adjacent to the microcystin operon, the structural variability of the region downstream of *mcyC* in toxic cultures was compared with that of an equivalent position in nontoxic ones.

MATERIALS AND METHODS

Cyanobacterial cultures and sampling. Cyanobacterial strains (Table 1) with the designation PCC or UWOCC were obtained from the Pasteur Culture Collection (39) or the University of Wisconsin at Oshkosh Culture Collection, respectively. Many UWOCC cultures have subsequently been deposited in the University of Texas Collection of Algae and Cyanobacteria (http://bluebonnet-.pai.utexas.edu/infores/utex/) under accession numbers L2661 to L2679 (47) and in the Pasteur Culture Collection (39). The cyanobacterial strains were maintained in either J (7) or BG-11 (7) medium at 25°C with a light intensity of approximately 20 mol of photons m^{-2} s^{-1}. At 21 days of growth, 2 ml of each culture was transferred to a serum vial and lyophilized for 48 h in a LabConco model 79480 freeze-drier. Samples were stored under vacuum until DNA was extracted. Microcystin assays were performed using the colorimetric protein phosphatase inhibition assay as described previously (1).

Near-surface grab samples were obtained and frozen at various times during a large toxic cyanobacterial bloom in Botany Ponds, Sydney, Australia (33°53′S, 151°12′E). This bloom, in which *Microcystis* spp. and *Anabaena* spp. predominated, underwent several complex population successions as determined by microscopy and microcystin assays of periodic samples (52). *Microcystis* spp. were observed by microscopy in all field samples.

DNA amplification and sequencing. For PCR amplifications, total genomic DNA was extracted from lyophilized cultures or frozen field samples using the potassium xanthogenate-sodium dodecyl sulfate procedure as described previously (52). Briefly, this method uses potassium xanthogenate for cell lysis and removal of PCR inhibitors, followed by phenol extraction and ethanol precipitation of DNA. The NMT PCR mixture contained 5 μl of 10× PCR buffer (Biotech International, Perth, Australia), 5 μl of 25 mM $MgCl_2$, 1 μl of a 10 mM concentration of each deoxynucleoside triphosphate, 2 pmol of the NMT primers MSF and MSR (Table 2), 10 ng of genomic DNA, 1 U of *Taq* DNA polymerase

TABLE 2. Primers used in this study

Gene region and primer	Sequence[a]	T_m (°C)[b]
mcyA NMT		
MSF	ATCCAGCAGTTGAGCAAGC	59
MSR	TGCAGATAACTCCGCAGTTG	60
MSI	GAGAATTAGGGACACCTAT	48
Phycocyanin		
PCβF	GGCTGCTTGTTTACGCGACA	62
PCαR	CCAGTACCACCAGCAACTAA	60
16S rDNA		
27F1	AGAGTTTGATCCTGGCTCAG	57
530F	GTGCCAGCAGCCGCGG	69
929R	TCC (T/A) CCGCTTGTGCGGGG	70
942F	GGGCCCGCACAAGCGG	70
1494Rc	TACGGCTACCTTGTTACGAC	56
uma1		
UMF	CCTATCGTCGTATTTGGAGT	54
UMR	AAGGAATGGACACGATAGGC	59
Suppression PCR		
Adapter1	CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT	NA[c]
Adapter2	ACCTGCCC-NH_2	NA
AP1	GGATCCTAATACGACTCACTATAGGGC	62
mcyC, MCYCF	ATCCCAAACGTCAGGAATTAAGAG	62
N1 noncoding		
N1F	GTCTCTACGGTTTCGGGCGTGA	68
N1R	CATCAAAGTGAACGTCAACCGCTCA	69

[a] 5′ to 3′ orientation.
[b] T_m, melting temperature as determined by the nearest-neighbor method.
[c] NA, not applicable.

(Biotech International), and water to a final volume of 50 μl. PCR conditions were 30 cycles of 94°C for 10 s, 60°C for 20 s, and 72°C for 60 s. The 16S rRNA gene PCR amplification was performed as described previously (28), except that only 2 pmol of each of the primers 27F1 and 1494Rc was used with 30 cycles of 94°C for 10 s, 50°C for 20 s, and 72°C for 60 s. Amplification of the PC-IGS was performed as described previously (29). Briefly, the PC-IGS PCR mixture contained 2 μl of 10× PCR buffer (Biotech International), 2 μl of 25 mM $MgCl_2$, 0.5 μl of a 10 mM concentration of each deoxynucleoside triphosphate, 5 pmol of each of the two PC-IGS primers (Table 2), 10 ng of genomic DNA, 1 U of *Taq* DNA polymerase (Biotech International), and water to a final volume of 20 μl. The PC-IGS PCR mixtures were subjected to 30 cycles of 94°C for 10 s, 50°C for 20 s, and 72°C for 40 s in a Perkin-Elmer 2400 PCR thermocycler.

All PCR products were precipitated by the addition of 50 μl of 4 M ammonium acetate and 100 μl of isopropanol. Samples were allowed to incubate at 25°C for 5 min followed by centrifugation at 12,000 × *g* for 5 min. The DNA pellets were washed with 70% ethanol and allowed to air dry before being resuspended in 8 μl of 10:1 TE (10 mM Tris-HCl [pH 7.4], 1 mM EDTA [pH 8]). Automated BigDye terminator sequencing (PE Applied Biosystems, Foster City, Calif.) reactions were performed using 2 μl (≈100 ng) of each PCR product and 10 pmol of each appropriate primer in a half-scale reaction. Each PC-IGS PCR product was sequenced with both of the amplification primers. Three sequencing reactions were performed for each NMT PCR product using the MSF, MSR, and MSI primers, while five sequencing reactions were performed for each 16S rDNA product using the primers 27F1, 530F, 929R, 942F, and 1494Rc (Table 2). Sequencing products were purified and analyzed as described previously (50).

Unknown flanking sequence characterization. DNA sequences flanking *uma1* in *Microcystis flos-aquae* UWOCC N were obtained using a modified version of the suppression PCR method (46). Briefly, linker-ligated DNA was prepared by digesting, in four separate reactions, 100 ng of genomic DNA with 5 U of *Dra*I, *Hin*cII, *Ssp*I, or *Rsa*I restriction enzyme (New England Biolabs, Beverly, Mass.). In addition, each reaction mixture contained 2.5 μl of 10× One Phor All buffer (Pharmacia, Uppsala, Sweden), 1 μl of 10 mM ATP, 2 U of T4 DNA ligase (Promega, Madison, Wis.), 10 pmol of the suppression oligonucleotide adapter (Table 2), 0.25 μl of 100× bovine serum albumin (New England Biolabs), and 10 μl of water. The reaction mixtures were incubated at 25°C overnight. After 10 min of enzyme heat inactivation at 70°C, the samples were ethanol precipitated before being resuspended in 25 μl of 10:1 TE. The suppression PCR mixtures contained 2.5 μl of 10× PCR buffer, 2.5 μl of 25 mM $MgCl_2$, 0.5 μl of 10 mM deoxynucleoside triphosphates, 10 pmol of primers UMF and AP1 (Table 2), 2 μl of each linker-ligated DNA, 1 U of a 10:1 *Taq-Pfu* DNA polymerase mix, and water to 25 μl. The reaction mixtures were subjected to 30 cycles of 94°C for 10 s, 58°C for 20 s, and 68°C for 2 min. The resulting PCR products were sequenced as described previously using 10 pmol of the UMF and AP1 primers.

Sequence analysis and phylogenetic construction. DNA and protein sequences were aligned using PILEUP (16) and CLUSTAL W (49). The aligned sequences were studied using the PHYLIP package of phylogenetic programs (15). Genetic distance, maximum-likelihood, and maximum-parsimony analyses were applied to all data sets in order to support the validity of the presented phylogenies. All sequence manipulation and phylogeny programs were accessed through the Australian National Genome Information Service (Sydney, Australia). Members of PC-IGS and NMT groups I and II were coded 1 and 2, respectively, for correlation analysis by the CORREL function of Microsoft EXCEL.

Nucleotide sequence accession numbers. The nucleotide sequences described in this study have been deposited in GenBank under accession numbers AF139292 to AF139348 (16S rDNA and *mcyA* sequences) and AF195158 to AF195179 (PC-IGS sequences).

RESULTS

Presence of the NMT domain in microcystin-producing versus non-microcystin-producing cultures. Microcystin activity in samples of 37 *Microcystis* sp. strains from several continents was assayed by protein phosphatase inhibition (Table 1). In addition, the oligonucleotide primers MSF and MSR (Table 2) were designed to PCR amplify the single NMT region of *mcyA* from these strains. A 1.3-kb PCR product was obtained from all 18 microcystin-producing strains tested (data not shown). In

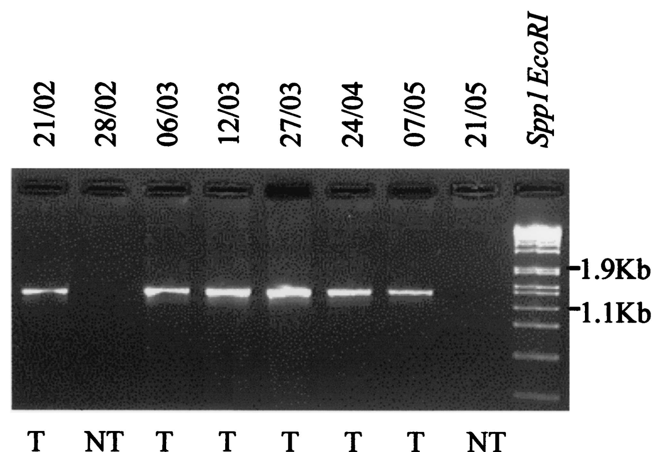

FIG. 1. PCR amplification of the NMT region of microcystin synthetase from an environmental bloom. The NMT-specific PCR was performed using DNAs isolated from the Botany Ponds cyanobacterial bloom samples collected in 1993 on the dates (day/month) indicated. Samples that proved to be toxic (T) or nontoxic (NT) by the phosphatase inhibition assay (1) are indicated. Five-microliter aliquots of each PCR mixture were run on a 2% agarose gel in 1× Tris-acetate-EDTA together with 100 ng of Spp-1 DNA digested with *Eco*RI. The gel was stained with ethidium bromide and photographed under UV transillumination.

addition, two strains that presently do not produce microcystin, CBS and MR-C, amplified this 1.3-kb PCR fragment. The other 17 nontoxic strains did not.

Amplification of the NMT domain from environmental cyanobacterial blooms. DNA was extracted from frozen samples of a toxic *Microcystis*-containing bloom, and the NMT PCR was performed. NMT-specific PCR products were obtained (Fig. 1) from six of the eight samples from a bloom that had shown variations in toxicity (52). All eight samples provided PC-IGS region PCR products (52), which suggests that the failure to obtain NMT PCR products from two of the samples was not due to the absence of cyanobacterial DNA or the presence of PCR inhibitors. Successful amplifications were possible on multiple occasions, reflecting the reproducibility of the DNA extraction and amplification procedures employed.

16S rDNA and PC-IGS phylogenies of *Microcystis* isolates, with comparison to toxicity. The 16S rDNA sequences of 37 *Microcystis* sp. strains (Table 1), together with that of *Synechocystis* sp. strain PCC 6803 (21), were aligned, and the inferred phylogeny was determined (Fig. 2). Also included were previously published sequences from 10 East Asian isolates of *M. aeruginosa, Microcystis ichthyloblabe, Microcystis novacekii, Microcystis wesenbergii,* and *Microcystis viridis* (35). The 16S rDNA sequences of all *Microcystis* cultures represented a monophyletic cluster with >99% sequence similarity, indicating that their assignment to the same genus was appropriate. The microcystin-producing, NMT-containing strains were distributed widely and discontinuously within the 16S rDNA distance tree (Fig. 2). The ability to draw any firm conclusions from these data is limited by the lack of significant bootstrap support for most branches, a consequence of the limited number of phylogenetically informative sites (18 sites) within the *Microcystis* 16S rDNA sequences.

A 610-bp fragment of the PC-IGS and flanking regions was also amplified and sequenced from 22 strains, representing both toxic and nontoxic *Microcystis*. The resulting distance tree, which included an additional 11 unpublished *Microcystis* PC-IGS sequences from the GenBank database, was based on 99 phylogenetically informative sites and showed significant bootstrap support (Fig. 3). The *Microcystis* PC-IGS sequences formed two significant clusters (I and II). Cluster I additionally separated into two further subbranches (Fig. 3). Each cluster contained both toxic and nontoxic isolates, as well as organisms from several continents. Cluster II is composed exclusively of *M. aeruginosa,* whereas cluster I is composed of two morphospecies, *M. aeruginosa* and *M. flos-aquae*. On the basis of both the 16S rDNA and PC-IGS data, neither of these two morphospecies appears to be monophyletic.

Sequencing of the NMT domain. The NMT region, which was far more divergent than the 16S rRNA locus, contained 111 polymorphic sites across 20 *Microcystis* strains, compared to 18 polymorphic sites within the 16S rDNA gene from 47 *Microcystis* strains. Phylogenetic analyses of the NMT region showed a consistent bifurcated topology (Fig. 4). Similar tree topologies were obtained with both genetic distance and maximum-likelihood methods and were maintained for both DNA and protein sequences (data not shown). *Microcystis* isolates with identical NMT sequences were sometimes derived from different continents, as in the cases of strains AK-1 (United States) versus Bauld E (Australia); CBS (United States) versus MR-A, -B, -C, or -D (Australia); and 7806 (The Netherlands) versus 001 (Canada) versus 017 or 023 (South Africa).

Gene organization near the microcystin synthetase region. Analysis of the DNA sequence immediately downstream of *mcyC* revealed the presence of a large open reading frame (ORF), encoding 684 amino acids and designated *uma1* (for unknown *M. aeruginosa* ORF 1) (51). This ORF is of unknown function and is transcribed in a direction opposite to that of the *mcy* operon (Fig. 5). BLAST analysis of Uma1 revealed 60% identity to the 684-amino-acid product of an ORF (sll0471), also of unknown function, of *Synechocystis* sp. strain PCC 6803 (21). Conserved primers designed to this region (UMF and UMR) PCR amplified a single 867-bp product from all 18 toxic and 19 nontoxic *Microcystis* strains listed in Table 1 (data not shown).

An oligonucleotide primer, MCYCF, was designed to allow PCR amplification from the 3′ end of *mcyC* to *uma1* (Fig. 5). PCRs were performed using primers MCYCF and UMF on the 37 toxic and nontoxic *Microcystis* strains listed in Table 1. All strains containing the NMT region produced the expected 1.5-kb PCR product, except for UWOCC E7, which amplified a product 200 bp larger (data not shown). Thus, all microcystin-producing strains have maintained a consistent physical linkage of *uma1* with *mcyC*, one of the terminal genes of the microcystin synthetase gene cluster (Fig. 5).

To assess the related genome structure of the nontoxic *Microcystis* strains, a modified version of the suppression PCR gene walking method of Siebert et al. (46) was used to obtain the DNA sequence downstream of the *uma1* stop codon in strain *M. flos-aquae* UWOCC N, a strain which does not produce microcystin or contain the NMT region (Table 1). Using this approach, 255 bp of noncoding sequence flanking *uma1* was obtained (Fig. 5). Blast analysis of this strain N-derived sequence, termed *n1*, revealed no significant homology to any

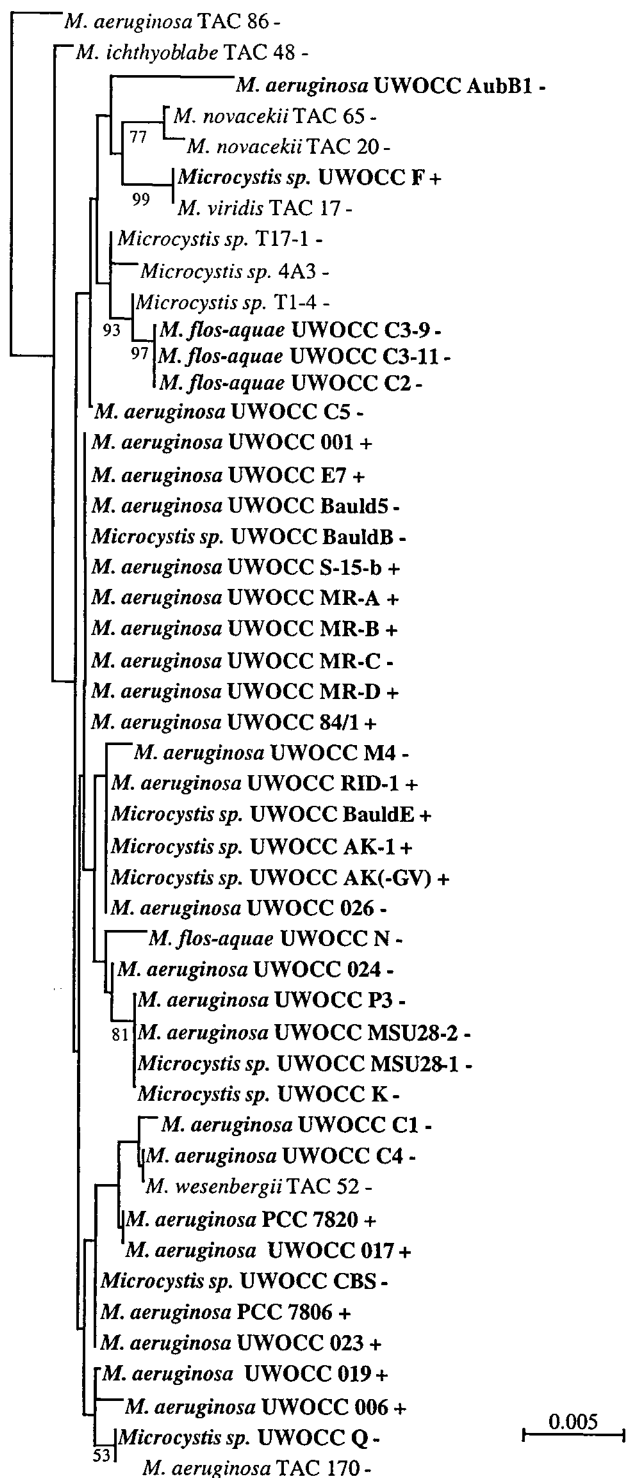

FIG. 2. *Microcystis* 16S rDNA distance tree. Strains in boldface were sequenced during this study, and the toxicity status (+ or −) is indicated after the name. Also included were an additional 10 *Microcystis* 16S rDNA sequences obtained previously by Otsuka et al. (35). Genetic distances were calculated using the method of Jukes and Cantor (20), and the phylogenetic tree was reconstructed using the neighbor-joining algorithm of Saitou and Nei (43) as implemented within CLUSTAL W (49). The tree was rooted using the 16S rRNA gene of *Synechocystis* sp. strain PCC 6803 as an outgroup (branch not shown) (21). Local bootstrap support for branches present in more than 50% of 1,000 resamplings is indicated at the relevant nodes (14).

sequences contained within the GenBank database. To determine whether other nontoxic strains invariably possessed the *n1* sequence adjacent to *uma1* (in analogy to the above-mentioned results that toxic strains do consistently exhibit *mcyC* in this position), two oligonucleotide primers, N1F and N1R, were designed to amplify the *n1* region (Table 2). PCR amplifications using these primers were performed on the 37 *Microcystis* strains. Only six cultures, all nontoxic, provided *n1*-specific PCR amplicons: strains AubB1, N, P3, MSU28-1, MSU28-2, and K. For those six strains containing the *n1* sequence, conservation of genome structure was then assessed by PCR amplification using the N1R primer for *n1* and the UMF primer for *uma1* (Table 1). Amplicons ranging in size from 1.1 to 1.4 kb were observed with strains N, P3, MSU28-1, MSU28-2, and K. An N1R-UMF-derived PCR amplicon was not obtained from strain AubB1, although control amplifications had been achieved using primers N1F and N1R and also with UMF and UMR, suggesting that *n1* is not linked to *uma1* in that genome. Thus, the DNA downstream of the *uma1* stop codon appeared to be more variable in nontoxic strains than in toxic strains.

DISCUSSION

The NMT-specific primers (MSF and MSR) reliably identified toxigenic *Microcystis* cultures. Of the 18 laboratory strains that gave a positive reaction in the protein phosphatase inhibition assays for microcystin, none lacked the NMT region of *mcyA*, whereas 17 out of 19 nontoxic strains had no observable NMT (Table 1). The data for *mcyA* are consistent with the observations of Neilan et al. (31) and Nishizawa et al. (34) that microcystin production by various *Microcystis* strains is linked to the presence of the *mcyB* gene and to the occurrence of specific adenylation domains within the *mcyABC* region. The primers MSF and MSR should prove to be useful as genetic probes, either alone or in combination with those developed by Neilan et al. (31) and Nishizawa et al. (34), in enabling the rapid identification of potentially toxigenic strains of *Microcystis*. It is encouraging that the MSF and MSR primers allowed the detection of NMT in six field samples (Fig. 4) from a bloom that was characterized by complex successions of toxic and nontoxic cyanobacteria (52). The presence of the NMT region corresponded to times of bloom hepatotoxicity as assessed by protein phosphatase inhibition (52), although further work will be required to ensure the reliability of these or other probes during differing field conditions. Probes specific for the microcystin synthetase region offer more promise than do ones for other genes, since we found no detectable association of toxicity with a particular 16S rRNA or PC-IGS genotype (Fig. 2 and 3). Several previous studies of *Microcystis* have also shown no correlation between toxicity and other phenotypes or genotypes (27–30, 35, 36).

Two strains (CBS and MR-C) contained the NMT module of *mcyA* but did not make detectable levels of microcystin. Similarly, a few nontoxic *Microcystis* strains have been reported to contain *mcyB* (31). Nishizawa et al. (34) have suggested that nontoxic *Microcystis* strains comprise two groups: those with and those without *mcyABC*. Among the nontoxic strains examined here, 17 out of 19 were in the latter group in terms of the NMT domain. Further analysis of the two NMT-containing

Microcystis aeruginosa EAWAG 110 +
Microcystis aeruginosa UWOCC P3 -
Microcystis sp. UWOCC K -
Microcystis flos-aquae UWOCC N -
Microcystis aeruginosa UWOCC C1 -
Microcystis aeruginosa EAWAG 169 -
Microcystis aeruginosa EAWAG 92a -
Microcystis aeruginosa EAWAG 167 +
IA
Microcystis flos-aquae UWOCC C2 -
Microcystis flos-aquae UWOCC C3-9 -
Microcystis flos-aquae UWOCC C3-11 -
Microcystis aeruginosa EAWAG 198a -
Microcystis aeruginosa UWOCC AubB1 -
Microcystis aeruginosa UWOCC 019 +
Microcystis aeruginosa UWOCC 006 +
IB
0.005
Microcystis aeruginosa PCC 7820 +
Microcystis aeruginosa PCC 7806 +
Microcystis aeruginosa UWOCC 023 +
Microcystis aeruginosa UWOCC 017 +
Microcystis aeruginosa UWOCC 001 +
Microcystis aeruginosa EAWAG 94a -
Microcystis aeruginosa EAWAG 171 -
Microcystis sp. UWOCC F +
Microcystis aeruginosa UWOCC E7 +
M. aeruginosa EAWAG 170 -
Microcystis aeruginosa UWOCC C5 -
Microcystis aeruginosa EAWAG 120a +
Microcystis sp. UWOCC Q -
Microcystis aeruginosa EAWAG 127c -
Microcystis aeruginosa EAWAG 127a -
Microcystis aeruginosa UWOCC MR-D +
Microcystis aeruginosa UWOCC MR-C -
Microcystis aeruginosa UWOCC CBS -
II
571 1000 978 571 973 999 997 561 975 571 982 871 892 924 608 889 881 774 1000

FIG. 3. *Microcystis* PC-IGS region DNA distance tree. Genetic distances were calculated from the alignment of sequences (610 bp) from the PC-IGS and flanking α and β subunits using the method of Jukes and Cantor (20). Included in the alignment were the unpublished *Microcystis* sp. PC-IGS sequences obtained from the GenBank database, i.e., those for EAWAG 92a (accession no. AJ003170), EAWAG 198a (AJ003182), EAWAG 171 (AJ003179), EAWAG 169 (AJ003177), EAWAG 167 (AJ003176), EAWAG 127c (AJ003175), EAWAG 127a (AJ003174), EAWAG 120a (AJ003173), EAWAG 110 (AJ003172), EAWAG 94a (AJ003171), and EAWAG 170 (AJ003178). The microcystin contents of these strains were determined by the protein phosphatase inhibition assay (1). The phylogenetic tree was reconstructed using the neighbor-joining algorithm of Saitou and Nei (43) as implemented by the CLUSTAL W program (49). Local bootstrap support for branches present in more than 50% of 1,000 resamplings is indicated at each node. Proposed global sequence clades are bracketed. Strains in boldface were sequenced in this study, and the toxicity status (+ or −) is indicated after the name.

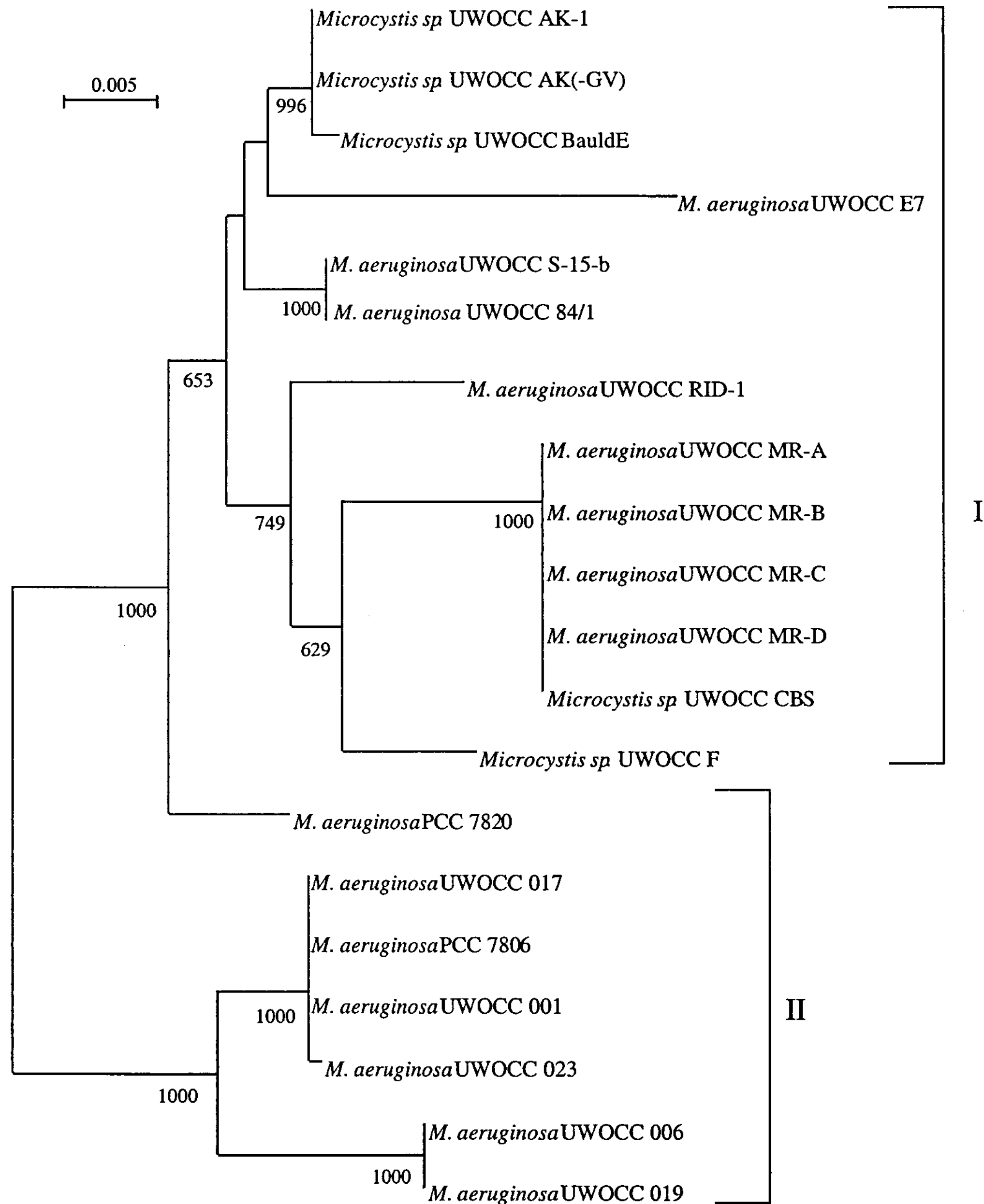

FIG. 4. Microcystin synthetase NMT DNA distance tree. Genetic distances were calculated from the alignment of a 1,319-bp region encoding the microcystin synthetase NMT region using the method of Jukes and Cantor (20). The phylogenetic tree was reconstructed using the neighbor-joining algorithm of Saitou and Nei (43) as implemented by the CLUSTAL W program (49). Local bootstrap support for branches present in more than 50% of 1,000 resamplings is indicated at each node (14).

nontoxic organisms is needed to reveal whether they are defective mutants, organisms that do not synthesize microcystin under certain environmental conditions, or strains that produce microcystin at levels below detection by the sensitive protein phosphatase inhibition assay (1).

Phylogenetic analysis of NMT sequences from the 20 NMT-containing strains yielded a deeply bifurcated topology with bootstrap numbers of 100% between the two groups (Fig. 4). No consistent relationship was found between the NMT genotype, PC-IGS sequence, 16S rRNA sequence, geographical region of isolation, or morphological species identification. Otsuka et al. (36) have reported a similar lack of correspondence between the 16S-23S rRNA intergenic spacer sequence and morphospecies designation or toxicity in *Microcystis* spp.

The patchy distribution of toxic and nontoxic organisms within various PC-IGS (Fig. 3) and 16S-23S rRNA-IGS (36) groups of *Microcystis* could arise if either (i) *Microcystis* was originally able to produce microcystin, with presently nontoxic strains being defective mutants, or (ii) *Microcystis* was originally nontoxic, with toxicity being acquired by lateral genetic transfer from some other organism. These two mechanisms have profoundly different implications for the dynamics and

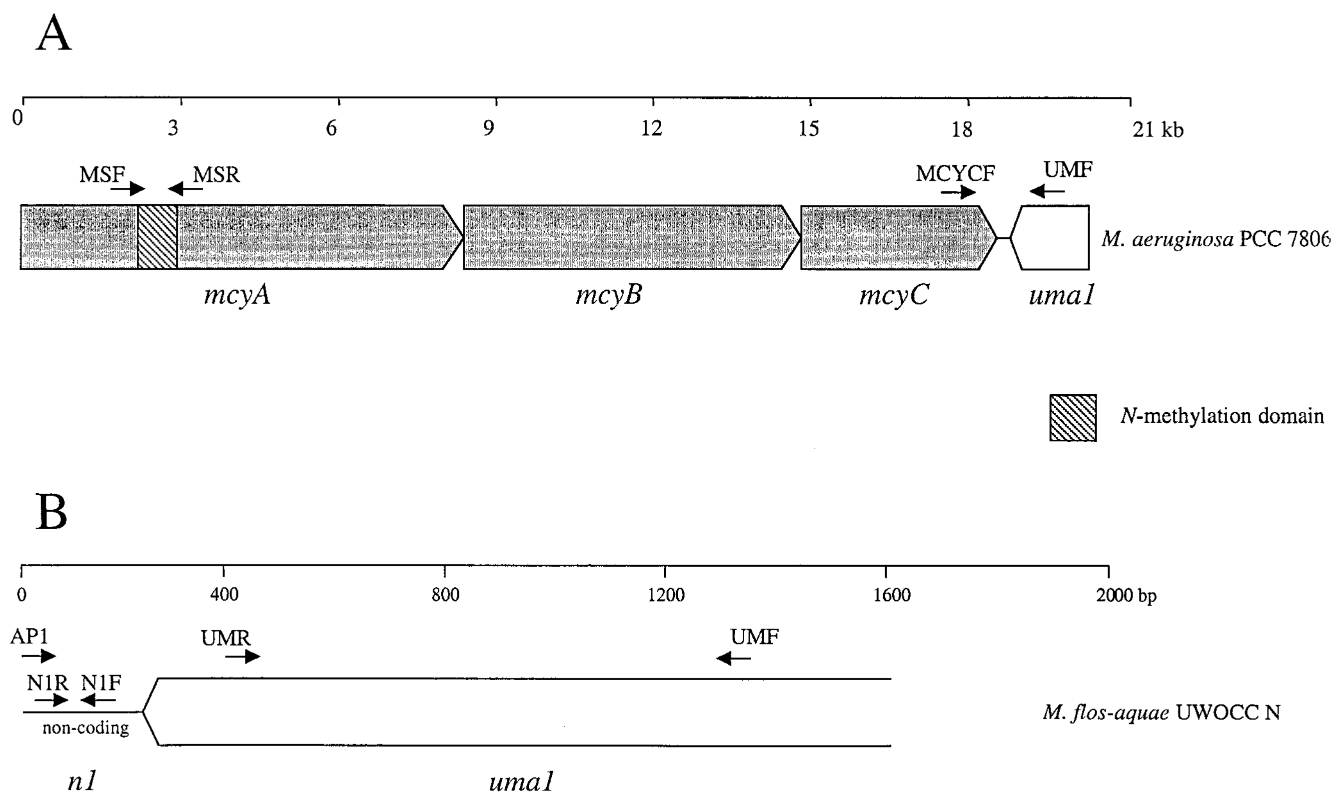

FIG. 5. Structural organization of microcystin synthetase and flanking regions. (A) Chromosomal arrangement of the *mcyA*, *mcyB*, *mcyC*, and *uma1* genes in *M. aeruginosa* PCC 7806 (51). (B) Chromosomal arrangement of *uma1* and *n1* in *M. flos-aquae* UWOCC N. Additionally, the relative locations and orientations of the PCR primers used in this study are indicated.

spread of toxic environmental blooms. To distinguish between the two alternatives, we investigated the nature of the DNA that flanks *mcyC*, the terminal gene of the *mcyABC* operon, in different strains. The rationale was that the insertion of *mcyC* at different chromosomal locations in different strains might indicate lateral gene transfer or other types of genomic rearrangement. However, the opposite was observed. In all 20 NMT-containing strains, *mcyC* was adjacent to *uma1. uma1* does not appear to be part of the microcystin synthetase domain (51), since its sequence is not that of a peptide synthetase module, it is not cotranscribed with *mcyABC,* and it is present in all of the 18 nontoxic *Microcystis* and *Synechocystis* strains tested. The physical distance between *mcyC* and *uma1* is surprisingly constant among the 20 NMT-containing *Microcystis* strains, including organisms that differ considerably in PC-IGS and NMT sequence. This constancy is not consistent with frequent intra- or intergenomic mobility of *mcyC* by any mechanism that is followed by insertion into random or multiple chromosomal locations. The results do not exclude two other types of genetic mobility: (i) transfer on a large plasmid that also carries *uma1* or (ii) insertion by site-specific recombination at a required locus near *uma1*. However, several toxic *Microcystis* cultures do not appear to contain plasmids (4, 45), and curing of plasmids has not eliminated the toxicity of other *Microcystis* strains (53). Site-specific recombination has not been demonstrated in the microcystin system, although the adjacent gene *uma4* exhibits sequence homology with a transposase gene (*tnpA* [GenBank accession number U13767]) from *Anabaena* sp. strain PCC 7120 (51). Transposition is possibly suggested by the variable genomic structure, sometimes containing *n1* and sometimes not, adjacent to *uma1* in various nontoxic *Microcystis* cultures.

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