

Nontoxic Strains of Cyanobacteria Are the Result of Major Gene Deletion Events Induced by a Transposable Element

Guntram Christiansen,* Carole Molitor,* Benjamin Philmus,† and Rainer Kurmayer*

*Austrian Academy of Sciences, Institute for Limnology, Mondseestrasse 9, Mondsee, Austria; and †Department of Chemistry, University of Hawaii at Manoa

Blooms that are formed by cyanobacteria consist of toxic and nontoxic strains. The mechanisms that result in the occurrence of nontoxic strains are enigmatic. All the nontoxic strains of the filamentous cyanobacterium *Planktothrix* that were isolated from 9 European countries were found to have lost 90% of a large microcystin synthetase (*mcy*) gene cluster that encoded the synthesis of the toxic peptide microcystin (MC). Those strains still contain the flanking regions of the *mcy* gene cluster along with remnants of the transposable elements that are found in between. The majority of the strains still contain a gene coding for a distinct thioesterase type II (*mcyT*), which is putatively involved in MC synthesis. The insertional inactivation of *mcyT* in an MC-producing strain resulted in the reduction of MC synthesis by $94 \pm 2\%$ (1 standard deviation). Nontoxic strains that occur in shallow lakes throughout Europe form a monophyletic lineage. A second lineage consists of strains that contain the *mcy* gene cluster but differ in their photosynthetic pigment composition, which is due to the occurrence of strains that contain phycocyanin or large amounts of phycoerythrin in addition to phycocyanin. Strains containing phycoerythrin typically occur in deep-stratified lakes. The rare occurrence of gene cluster deletion, paired with the evolutionary diversification of the lineages of strains that lost or still contain the *mcy* gene cluster, needs to be invoked in order to explain the absence or dominance of toxic cyanobacteria in various habitats.

Introduction

Harmful algal blooms that are formed by cyanobacteria in lakes and rivers frequently contain toxins, such as the hepatotoxic peptide microcystin (MC), and they pose a serious health risk to livestock and humans (World Health Organization 2004). It has been known for a long time that the blooms that are formed by the genera *Microcystis* sp., *Planktothrix* spp., and *Anabaena* spp. consist of hepatotoxic and nonhepatotoxic strains (Carmichael and Gorham 1981). A similar variation has been recorded among dinoflagellates that produce neurotoxins (Touzet et al. 2007) and fungi that produce mycotoxins, for example, *Aspergillus* sp. that produce aflatoxin (Cary and Ehrlich 2006). The factors that regulate the ecological success of nontoxic and toxic strains in cyanobacteria are intriguing. However, no clear answer has been obtained with regard to the biological function of the toxins or the factors favoring nontoxic over toxic strains and vice versa (Orr and Jones 1998; Schatz et al. 2005; Kardinaal et al. 2007).

For cyanobacteria, it has been found that hepatotoxic and nontoxic strains differ in their content of microcystin synthetase (*mcy*) genes that encode specific peptide synthetases, which have been shown to be involved in the production of the toxic heptapeptide MC (Meißner et al. 1996; Dittmann et al. 1997). The large gene cluster (~55 kbp) consists of nonribosomal peptide synthetases (NRPS), polyketide synthases, and tailoring enzymes (Tillett et al. 2000). The gene clusters of species from 3 genera (*Microcystis*, *Planktothrix*, and *Anabaena*) have been sequenced, and comparisons revealed that the genes *mcyA*, *mcyB*, *mcyC*, *mcyD*, *mcyE*, *mcyG*, and *mcyJ* that are involved in MC synthesis are always present (Tillett et al. 2000; Christiansen et al. 2003; Rouhiainen et al. 2004), whereas

the 3 genera can differ in the presence of tailoring enzymes. A phylogenetic tree that was calculated from 2 housekeeping genes, the 16S rDNA and *rpoCI* genes, showed perfect congruency with the phylogeny that was calculated from *mcyA*, *mcyD*, and *mcyE* (Rantala et al. 2004). This implies that the *mcy* gene cluster is of monophyletic origin and that several lineages must have lost the *mcy* gene cluster during cyanobacterial evolution. On a species level on a much shorter timescale of evolution, most closely related strains are observed either containing or lacking the *mcy* gene cluster. In blooms that are formed by *Microcystis*, the percentage of the strains containing the *mcy* gene cluster is low (1–38%, Kurmayer and Kutzenberger 2003). In contrast, blooms that are formed by the red-pigmented (phycoerythrin-rich) populations of *Planktothrix rubescens* that occur in deep-stratified lakes in the Alps, Scandinavia and in reservoirs solely consist of strains that contain the *mcy* gene cluster (Kurmayer et al. 2004). Accessory photosynthetic pigments, such as the red-colored phycoerythrin, result in the ability to live under transparent light conditions in deep water layers. Under specific climatic conditions, red-colored blooms are observed, which are then called the “Burgundy-blood phenomenon” (Walsby et al. 2005). Green-pigmented (phycocyanin-rich) blooms of *Planktothrix agardhii* that flourish in more shallow and polymictic lakes (Davis and Walsby 2002) typically show a lower percentage of strains containing the *mcy* gene cluster (Kurmayer et al. 2004).

The process of the acquisition/loss of *mcy* genes among closely related strains is not understood. The sequencing of the *mcy* gene clusters and the closely related nodularin synthetase gene cluster has revealed the localization of open reading frames (ORFs) with a significant similarity to transposases at the downstream 3' end of the gene clusters (Tillett et al. 2000; Moffitt and Neilan 2004). Recently, a type IV pilus system has been described in *Microcystis* and has been suggested to allow for the receiving of the *mcy* gene cluster via lateral transfer (Nakasugi et al. 2007). The phylogenetic congruence between the 2 housekeeping genes and the *mcy* genes rules out the possibility of

Key words: toxicity, microcystin, mobile elements, gene loss, microevolution, ecotypes.

E-mail: rainer.kurmayer@oeaw.ac.at.

Mol. Biol. Evol. 25(8):1695–1704, 2008

doi:10.1093/molbev/msn120

Advance Access publication May 22, 2008

© 2008 The Authors.

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/2.0/uk/>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Table 1
***Planktothrix agardhii* and *Planktothrix rubescens* Strains that Were Used in the Present Study and Grouped According to the Presence and Absence of the *mcy* Gene Cluster**

Strain Number	Species	North(°)	East(°)	Origin	Water Depth $Z_{\text{mean}}/Z_{\text{max}}$ ^a
Strains not containing the <i>mcy</i> gene cluster					
250 ^I , 251 ^I , 252 ^{IV} , 253 ^{II} , 254 ^{II} , 255 ^I , 256 ^{II} , 257 ^I	P. ag ^b	39°20	0°21	Albufera Lagune, Valencia, Spain	1/3
PCC7811 ^{II}	P. ag	48°51	2°20	Vert-le-Petit, France	2/2
41 ^{II} , 63 ^{II} , 66 ^{II}	P. ag	48°49	15°16	Jägerteich, Austria	1/2
259 ^{II} , 263 ^{II} , 274 ^{II} , 277 ^{II} , 281 ^{II}	P. ag	52°31	13°20	Wannsee, Germany	6/9
299 ^{II} , 307 ^{II} , 320 ^{II}	P. ag	52°02	5°02	Klinckenberger Plas, The Netherlands	-/30
PCC7805 ^I	P. ag	52°21	4°52	Veluwemeer, The Netherlands	2/5
SAG5.81 ^{III}	P. ag	51°32	9°57	Kiessee, Göttingen, Germany	1/2
CCAP1459/15 ^{II}	P. ag	54°36	6°23	Lough Neagh, North Ireland, United Kingdom	9/34
PH22 ^{II}	P. ag	55°40	12°34	Lake Bagsværd Sø, Copenhagen, Denmark	2/3
2A ^{II}	P. ag	60°14	19°55	Lake Markusbölefjärden, Finland	-/9
Strains containing the <i>mcy</i> gene cluster					
31/1, 32, 39, 260	P. ag	52°31	13°20	Wannsee, Germany	6/9
SAG6.89	P. ag.	54°10	10°23	Plußsee, Germany	9/30
CCAP1459/11A	P. ag	54°21	2°56	Lake Windermere, United Kingdom	21/64
CCAP1459/21	P. ag.	54°21	2°58	Esthwaite Water, United Kingdom	-/16
CCAP1459/16, CCAP1459/17	P. ag.	54°40	2°98	Blelham Tarn, United Kingdom	7/15
CCAP1459/31	P. ag.	53°25	7°56	White Lough, United Kingdom	-/30
79	P. ag	55°43	12°34	Lake Arresø, Denmark	6/40
CCAP1459/36*	P. ag	59°47	10°47	Lake Gjersjoen, Norway	23/64
CYA126/8	P. ag	60°15	19°55	Lake Langsjön, Finland	-/18
CCAP1460/5	P. ag	35°41	139°44	Lake Kasumigaura, Japan	4/10
64, 67*	P. rub ^c	46°36	14°03	Wörthersee, Austria	42/86
139*, 145*, 161*, 166*, 169*, 170*, 178*	P. rub	47°59	13°05	Grabensee, Austria	7/13
3, 40*, 91/1*, 97, 110*, 111	P. rub	47°48	13°22	Mondsee, Austria	37/68
108	P. rub	47°56	13°19	Irrsee, Austria	15/32
80	P. rub	47°45	13°30	Schwarzensee, Austria	27/54
82, 83/2	P. rub	47°16	11°04	Ammersee, Germany	47/81
21-	P. rub	48°06	16°18	Figur, Austria	8/12
CCAP1459/30	P. rub	54°08	10°25	Plöner See, Germany	16/60
CCAP1459/14	P. rub	54°25	3°0	Loughrigg Tarn, United Kingdom	12/13
PCC7821	P. rub	59°47	10°47	Lake Gjersjoen, Norway	23/64

NOTE.—International culture collections: SAG, culture collection of algae (Göttingen, Germany); PCC, Pasteur culture collection (Paris, France); and CCAP, culture collection of algae and protozoa (Windermere, United Kingdom).^{I,II,III,IV} Strains showing *mcy* gene cluster deletions of types I, II, III, and IV, respectively. *Strains found inactivated by IS of transposable elements (Christiansen et al. 2006).

^a Z_{mean} , mean water depth; Z_{max} , maximum water depth of the origin of isolation.

^b P. ag, *P. agardhii*, green-pigmented strains.

^c P. rub, *P. rubescens*, red-pigmented strains.

the horizontal transfer of the *mcy* gene cluster between genera (Rantala et al. 2004). However, the frequency of the transfer of the *mcy* gene cluster among other closely related strains remains unclear. In order to find out whether the variation in toxicity among strains in *Planktothrix* resulted from gene loss, 25 nontoxic strains were analyzed for remnants of the *mcy* gene cluster. In all the nontoxic strains, *mcy* operon remnants were found that were derived from a major gene cluster deletion event that succeeded the insertion (IS) of mobile elements.

Materials and Methods

Organisms

The 62 strains that were used for this study were either isolated from European lakes (44 strains) or obtained from culture collections (table 1 and fig. 1). The strains originated from 9 countries and 28 different water bodies. All the strains were assigned to either *P. rubescens* or *P. agardhii* following the criteria published previously (Suda et al. 2002). All the strains were grown in BG₁₁ medium (Rippka

1988) at 15 °C and under low light conditions (10 μmol m⁻² s⁻¹, Osram L30W/77 Fluora).

Genetic Analysis of the Strains

In order to search for the remnants of the *mcy* gene cluster in nontoxic strains, primer pairs covering the whole *mcy* gene cluster were designed and used to amplify fragments of 500 bp without interruption (124 primer pairs, supplementary table S1, Supplementary Material online). Polymerase chain reaction (PCR) amplifications were performed in a volume of 20 μl, containing 2 μl of Qiagen PCR buffer (Qiagen, Vienna, Austria), 1.2 μl MgCl₂ (25 mM, Qiagen), 0.6 μl deoxynucleotide triphosphates (10 μM each, MBI Fermentas, St Leon-Rot, Germany), 1 μl of each primer (10 pmol μl⁻¹), 0.1 μl *Taq* DNA polymerase (5 units μl⁻¹, Qiagen), 13.1 μl sterile millipore water, and 1.0 μl of DNA (50 ng μl⁻¹). The PCR thermal cycling protocol included an initial denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, at 60 °C annealing temperature for 30 s, and elongation at 72 °C for 30 s. By the use of

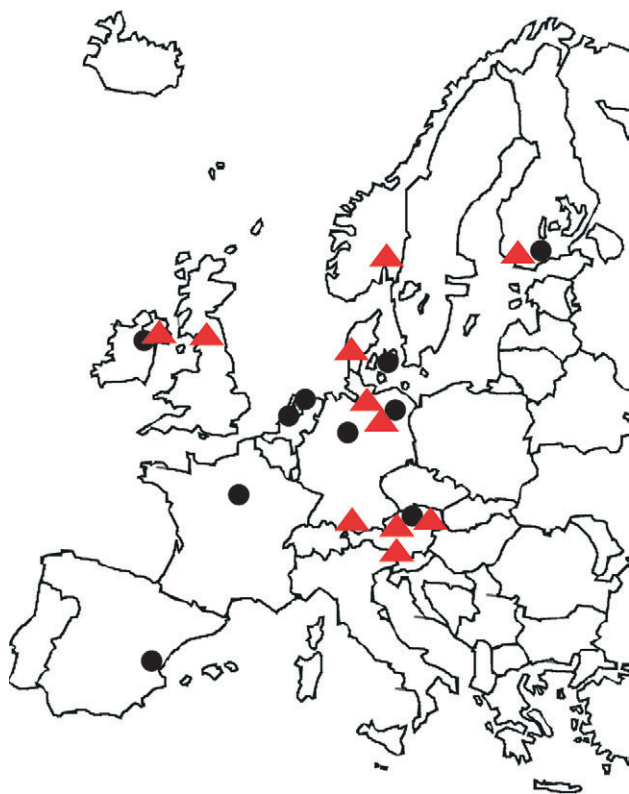


FIG. 1.—Origin of the strains of the cyanobacterium *Planktothrix* spp. isolated from European freshwater sites. Red triangles, strains containing the *mcg* gene cluster encoding the synthesis of the toxic heptapeptide MC (37 strains) and black dots, nontoxic strains lacking the *mcg* gene cluster (25 strains).

this technique, 8 strains (No41, 63, 66, PH22, 2A, SAG5.81, CCAP1459/15, PCC7805, and PCC7811) were found to contain *mcgT*. Seventeen additional nontoxic strains were used to test for the presence of *mcgT* by using the primers *mcgTA-Z+*: TAATTGATCCCCTGATCAATGATC and *mcgTA-Z-*: ATGCAAATAGACCAACTAAGCC amplifying 791 bp. The strains containing *mcgT* were analyzed for the 5' and the 3' ends using a genome walking method (Siebert et al. 1995). The same method was used to sequence the 3' end of the *mcg* gene cluster (AJ441056). The total DNA was digested with *EcoRV* and *DraI* at 37 °C overnight, and the DNA was extracted once by using phenol–chloroform–isoamyl alcohol (25:24:1, v/v/v) and once with chloroform and then washed with ethanol subsequent to glycogen-aided precipitation. A total of 100 ng μl^{-1} of DNA were then ligated to long suppression adapters at 4 °C overnight. One adapter contained an amino group at the 3' end and ligated to any DNA fragment that was generated by restriction enzymes that yielded blunt ends. During primary PCR, this amino group blocked the extension of the lower adaptor strand unless a gene-specific primer extended to the opposite DNA strand. The primary PCR mixture was then diluted 1:100 and used as a template for a secondary or “nested” PCR with the nested adaptor-specific primer and a nested gene-specific primer. Primary PCR was performed with 2 Step Advantaq (Clontech, St. Germain-en-Lave, France) at 68 °C anneal-

ing (30 s) and 68 °C elongation (3 min). Nested PCR was performed by using Advantaq at 68 °C elongation (3 min).

In addition, all the strains were sequenced for 16S rDNA and 3 intergenic spacer (IGS) regions: PC-IGS (the IGS between *cpcB* and *cpcA* genes), 16S ITS (the internal transcribed spacer located between the 16S and 23S rDNA), and PSA-IGS (the IGS between *psaA* and *psaB* genes). The forward primer (5'–3') 16Sfwd581 “CAGTG-GAACTGGAAGACTAGAGTGTA” (63 °C) and the reverse primer 16Srev999 “GCACCTGTCTTCTGGTTCCTTAC” (62 °C) were used to sequence part of the 16S rDNA (418 bp). The primers that were used to amplify and sequence PC-IGS (271 bp) have been described previously (Kurmayer et al. 2004). For 16S ITS, the forward primer (5'–3') 16SITSneu+ “GCTAGGAAAGAAAGGAACCTTCA” (61 °C) and the reverse primer untranslated leader region– “CCT CTG TGT GCC TAG GTA TC” (59 °C) (394 bp) were used (Janse et al. 2003). For PSA-IGS, the forward primer *psafwd* “GGGTGGTACTTGCCAAGTCTCT” (58 °C) and the reverse primer *psarev* “CGACGTGTTGTCTGGGTCTT” (58 °C) (669 bp) were used. All the PCR products were cloned and sequenced according to the standard techniques. The sequences were submitted to GenBank under the following accession numbers: 16S rDNA (EU266118–EU266179), PC-IGS (EU266242–EU266303), 16S ITS (EU266180–EU266241), PSA-IGS (EU258202–EU258263), *mcgT* (EU266304–EU266364), *mcgT* 5' flanking region (EU271829–EU271852), *mcgT* 3' flanking region (EU271805–EU271828), *mcgJ* and flanking regions sequenced from strain No252 (EU271803), and the IS element sequenced from strain CYA126/8 (EU271804). The sequence of the IS element that was involved in the deletion of the *mcg* gene cluster was submitted to the IS finder database and denoted as *ISPlag1* (Siguier et al. 2006).

Functional Analysis of the Thioesterase Type II *mcgT*

In order to confirm the involvement of *mcgT* in the biosynthesis of MC, the gene was insertionally inactivated in the transformable strain CYA126/8 by homologous recombination as described (Christiansen et al. 2003). To build the transformation construct, *mcgT* was amplified and cloned using the pDrive cloning system (Qiagen) and subsequently partially digested using *HindIII* according to the standard protocols (Sambrook et al. 1989). The 3' overhangs were blunted using Klenow Polymerase (Fermentas, Hanover, MD) and ligated with the *BsaAI* fragment (1.9 kbp) from pACYC184 containing the chloramphenicol resistance cassette (Cm^{R}). The *mcgTKOCm^R* construct contained 1.5 kbp of homologous sequence on both 5' and 3' ends and was introduced by electroporation as described (Christiansen et al. 2003). Transformed cyanobacterial cells were inoculated in BG₁₁ medium. After 48 h, chloramphenicol was added to the culture medium (1 $\mu\text{g ml}^{-1}$) and resistant clones emerged after 6 weeks. PCR using primers binding to the flanking regions of the inserted chloramphenicol resistance gene showed the stable integration of the construct *mcgTKOCm^R* at the expected position as a result of a homologous crossover recombination event. In

addition, the *mcyT* gene in the knockout mutant as well as in the wild-type strain was amplified by PCR using the *mcyTA-Z* primers. The calculated PCR product of 791 bp was obtained for the wild-type strain only, whereas the Δ *mcyT* mutant showed an amplicon of 2.7 kbp due to the IS of the Cm^R fragment (1.9 kbp). Consequently, the Δ *mcyT* mutant was fully segregated.

The wild-type strain CYA126/8 and the Δ *mcyT* mutant were compared in the MC production under semicontinuous culture conditions following the turbidostat principle (Kohl and Nicklisch 1988) at 20 °C and 40–60 μ E $m^{-2} s^{-1}$ (Philips LTD, 36W/965). The growth of the cells was monitored by measuring the absorbance at 880 nm (5-cm light path) every other day and each time optical density ≥ 0.1 the culture was diluted to optical density = 0.01 (corresponding to a biomass of 1.44 ± 0.17 [standard error] $mm^3 L^{-1}$ of biovolume). The cells were harvested at optical density = 0.1 onto glass fiber filters (BMC Ederol, Vienna, Austria) directly in the culture room using low-vacuum filtration (< -0.4 bar), in which the filters with the collected cells were flash frozen in liquid nitrogen for subsequent RNA isolation. Aliquots of the cells were filtered onto preweighed glass fiber filters and dried at room temperature in a vacuum centrifuge, reweighed, and stored frozen at -20 °C. The dissolved MCs were collected from the filtrate by using solid phase extraction via tC_{18} cartridges (Waters, Sep-Pak Vac 1cc [100 mg]) according to the standard techniques. For cell number determination, the cells were fixed in 2% formaldehyde and enumerated using 6-diamidino-2-phenylindole staining following the standard procedures.

Filters were extracted in 50% methanol (v/v), as described (Kurmayer et al. 2004), and the extracts were analyzed for MCs by using high-performance liquid chromatography coupled to diode array detection at 240 nm, using a linear gradient from 20% acetonitrile (0.05% trifluoroacetic acid) to 50% acetonitrile on a LiChrospher 100, ODS, 5 μ m, LiChroCART 250-4 cartridge system (Merck, Darmstadt, Germany) at 1 ml min^{-1} . Aeruginosides were identified and quantified at 210 nm under the same conditions (Ishida et al. 2007). The quantification of MCs was achieved by using the calibrated standards that were obtained from Cyanobiotec GmbH (Berlin, Germany).

RNA was extracted following the standard protocols with Trizol reagent under liquid nitrogen as described (Kaebnick et al. 2000) and converted to cDNA by reverse transcription. The quantification of *mcy* transcripts was performed using real-time PCR by using the TaqMan assay (Kurmayer and Kutzenberger 2003). TaqMan Probes for PC-IGS and *mcyB* were used (Schober and Kurmayer 2006). All the growth experiments were repeated 4 times.

Phylogenetic Analysis

Sequences of *mcyT* (751 bp) were aligned by using multiple sequence alignment (ClustalW 1.8). Maximum likelihood analysis was used in order to estimate the nucleotide substitution parameters under a general time-reversible nucleotide substitution model by estimating the gamma distribution for the variable rates among the sites. Ambiguous

sites (wherein at least one sequence showed a gap) were removed (3 sites), and the discrete gamma algorithm was used to approximate a continuous gamma distribution using 5 categories of rates (ncatG = 5) in the program BASEML of the PAML software package (version 3.14, Yang 1997). Phylogenetic trees were also constructed using: 1) Neighbor-Joining from the nucleotide sequences distance matrix (calculated using Kimura's substitution model) and 2) maximum parsimony from nucleotide sequences by using the PHYLIP software package (Felsenstein 1993). The statistical significance of the branches was estimated by bootstrap analysis in turn generating 100 replicates of the original data set by using the PHYLIP software package. Finally, consensus trees following the 50% majority rule were computed.

For *mcyT*, the ratio of nonsynonymous (d_N) and synonymous (d_S) substitution rates per site was determined by using maximum likelihood estimates as implemented in PAML. For the nontoxic and toxic strains separately, the "one ratio" model, estimating the d_N/d_S ratio, and the "fixed-ratio" model, assuming a constant d_N/d_S ratio as observed for other *mcy* genes in the *mcy* gene cluster ($d_N/d_S = 0.2$), were statistically compared by constructing a likelihood ratio test (Yang 1998).

For multiple locus sequence typing (MLST), all the nontoxic (25) and toxic strains (37) were defined by the alleles (unique genotypes) that were present at 4 additional sequenced loci (the allelic profile): 16S rDNA, 16S–23S rDNA-ITS, PC-IGS, and PSA-IGS. Each unique allelic profile was assigned a sequence type (ST). Isolates with the same ST at all the loci were considered to be members of a single clone (Feil et al. 2004). The program eBurst (V3) was used via the MLST Web site (<http://www.mlst.net/>) in order to divide the 62 strains into clonal complexes. Each of the clonal complexes only contained strains sharing at least 3 of 4 identical alleles with at least one other strain in the group. The primary founder of a group was defined as the ST that differs from the largest number of other STs at only a single locus (i.e., the ST that has the greatest number of single locus variants [SLVs]). The level of confidence in the predicted founding ST was estimated by using the default settings, that is, 1,000 random data sets of the same size as the original data set were produced by resampling with replacement, and the bootstrap values, which were shown for each ST, are the percentage of times that the ST was predicted to be the primary founder of the group (Feil et al. 2004).

Results

Characterization of the Remnants of the *mcy* Gene Cluster

In 24 out of the 25 nontoxic strains, *mcyT* (751 bp), a gene coding for a distinct thioesterase type II (TeII), was amplified. A distinct TeII has been frequently associated with NRPS gene clusters and has been shown to positively influence the synthesis rate of corresponding NRPS (Mootz et al. 2001; Schwarzer et al. 2002). This is due to the regeneration of the misprimed 4'-phosphopantetheine (4'PP) cofactor of the peptidyl carrier protein because the 4'PP transferases are not able to distinguish between

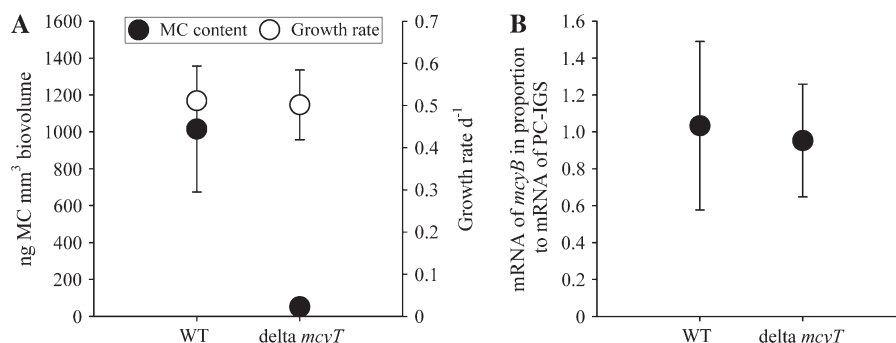


FIG. 2.—Growth rate, MC content, and *mcy* transcripts of the wild-type *Planktothrix* strain CYA126/8 and its $\Delta mcyT$ mutant grown under semicontinuous culture conditions. (A) Mean (± 1 SD) MC content (per mm³ of biovolume, black circles) and growth rates (μ d⁻¹, white circles). (B) The mRNA contents of *mcyB* in proportion to PC-IGS (in percentage of *mcyB*) for the same experiment.

acetylated and free CoA (Quadri et al. 1998). The acetylation of 4' PP has the consequence that the biosynthesis is blocked until a TeII cleaves the acetyl residue from the 4' PP making the biosynthesis possible. In *Planktothrix*, the *mcyT* gene is located at the 5' end of the *mcy* gene cluster but has not been found in the *mcy* gene cluster of other MC-producing cyanobacteria (Christiansen et al. 2003). To demonstrate the role of *McyT* in MC synthesis, the *mcyT* gene was inactivated by experimental mutagenesis in *P. agardhii* strain CYA126/8. The wild-type strain contained 964 ± 321 ng (± 1 standard deviation [SD]) of MC-RR ($68 \pm 3\%$) and MC-LR ($32 \pm 3\%$) per mm³ of biovolume ($n = 5$). The insertional inactivation of *mcyT* resulted in a reduction of MC synthesis by $94 \pm 2\%$ (1 SD) compared with the wild type. In contrast, the proportion of MC variants, cellular growth rates, as well as the transcriptional rates of other *mcy* genes (*mcyB*) were not altered (fig. 2). The synthesis rate of the related, coproduced nonribosomally synthesized (NRS) peptides such as aeruginoside 126A, 126B (Ishida et al. 2007) was not significantly affected ($37 \pm 71\%$ increase compared with the wild type). It is concluded that *mcyT* is directly involved in MC synthesis. In one nontoxic strain, we were unable to amplify *mcyT* but detected another *mcy* operon remnant at the other (3') end of the *mcy* gene cluster, *mcyJ*, which was an O-methyltransferase that was previously shown to be involved in MC synthesis (Christiansen et al. 2003).

Characterization of the Sequence Breaking Points of the *mcy* Gene Cluster

There were identical flanking sequences observed directly upstream and downstream of all the *mcy* operon remnants: at the 5' end similarity was 99.0–100% (300 bp, the strain with the *mcyJ* remnant only had 219 bp) and at the 3' end similarity was 98.7–100% (207 bp, the strain with the *mcyJ* remnant had 241 bp) (fig. 3, supplementary figs. S1 and S2, Supplementary Material online). Those flanking sequences that were found in the nontoxic strains also had the highest similarity to the corresponding region of the toxic strain CYA126/8: at the 5' end similarity was 99.0–99.7% (300 bp, for AJ441056, Positions 149–449) and at the 3' end similarity was 93.2–94.2% (207 bp, located 1,406 bp downstream of the stop codon of *mcyJ* AJ441056). In between the 2 regions that were originally flanking the *mcy* gene cluster, shorter fragments containing the inverted repeated sequences “CAGGACTTACGCAAGCACGC-TATATATAG” (29 bp) occurred. These shorter fragments were found directly adjacent to the *mcy* operon remnants (*mcyT* and *mcyJ*) and were identified as terminal inverted repeats, part of the remnants of an IS element (197 bp) *ISPlag1* that was sequenced from the toxic strain CYA126/8 (1,306 bp). *ISPlag1* encoded a single ORF (337 aa) with similarity to the IS701 group that was originally a part of the heterogeneous IS4 family (Chandler and Mahillon 2002); ISMhu9 from *Methanospirillum hungatei*

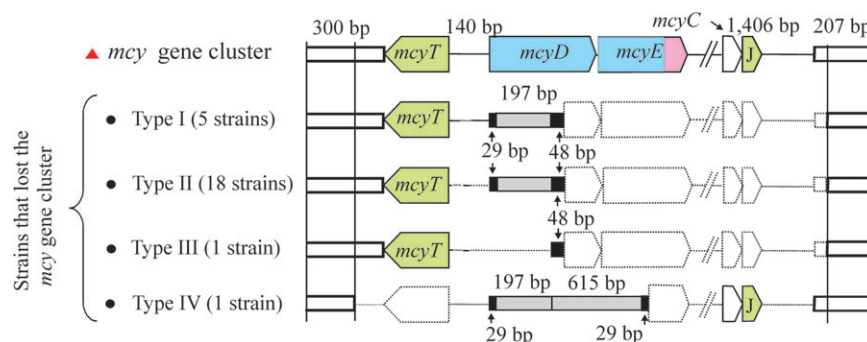


FIG. 3.—Schematic view of the *mcy* operon remnants and flanking regions in strains that lost the *mcy* gene cluster. The 4 types (I–IV) of gene cluster deletion events are shown. Vertical straight lines enclose the identical 5' and 3' ends. The gray gene regions represent the remnants of the IS elements (197 bp) containing terminal inverted repeats (29 bp or 48 bp) (black boxes). The dotted areas indicate the deletions. Red triangle, strains containing the *mcy* gene cluster and black dots, nontoxic strains lacking the *mcy* gene cluster.

strain JF-1 (E value 5×10^{-43} , YP_501731). Residues 257–270 (CAIRWKIEEFHREIK) corresponded to the [Y/L](2)R(3)[I/L/V]E(6)K signature characteristic of the C1 domain of bacterial transposases (Rezsöházy et al. 1993). Notably, all the nontoxic strains contained remnants of the same mobile element that was located between the former flanking regions of the *mcy* gene cluster. According to the presence of the *mcy* operon remnants and the location of the remnants of the IS element 4 different types of *mcy* gene cluster deletion events were distinguished: types I (5 strains), II (18 strains), and III (1 strain), all of which contained *mcyT* but differed in the remaining intergenic promoter region between *mcyT* and *mcyD* (type I vs. type II) or in the sequence length of the remnant of the IS element (types I and II vs. type III). In contrast, type IV contained *mcyJ* instead of *mcyT* and 1 additional larger remnant of the same IS element (615 bp) that was inserted in opposite direction to the shorter remnant (197 bp) found also in types I and II. We conclude, therefore, that the remnants of the IS element that was found directly adjacent to the *mcy* operon remnants were derived from functional IS elements that were inserted into the *mcy* gene cluster.

In silico Characterization of the Remnants of the *mcy* Gene Cluster

To determine whether *mcyT*, as a remnant of the *mcy* gene cluster, is still a functional gene that is involved, for example, in the biosynthesis of other NRS peptides occurring frequently in *Planktothrix* (Fujii et al. 2000; Ishida et al. 2007), it was sequenced (751 bp) from all the strains that contained (37 strains) or that had lost the *mcy* gene cluster (24 strains). The nontoxic strains formed a phylogenetic lineage that was distinct from the lineage that was formed by strains containing the *mcy* gene cluster (fig. 4). The ratio of nonsynonymous (d_N) and synonymous (d_S) substitution rates was used to differentiate between purifying selection and relaxation of selective constraints (Yang 1998). Typically, the gene regions that show a high degree of purifying selection have d_N values $<$ d_S values (negative purifying selection), whereas d_N values \approx d_S values are indicative of a relaxation of selective constraints (Yang 2005). For various genes of the *mcy* gene cluster, a d_N/d_S ratio = 0.2 was calculated (Tanabe et al. 2004; Kurmayer and Gumpenberger 2006). A likelihood ratio test was performed in order to test the hypothesis of the relaxation of selective constraints against the purifying selection for *mcyT* of both lineages. Only for the strains that had lost the *mcy* gene cluster ($d_N/d_S = 1.45$), the likelihood was significantly improved (degree of freedom = 1, $P < 0.0001$), whereas for the strains still containing the *mcy* gene cluster ($d_N/d_S = 0.46$), the estimated likelihood did not differ significantly from the null model ($d_N/d_S = 0.2$). Thus, it is concluded that purifying selection for *mcyT* was lost subsequent to its separation from the *mcy* gene cluster.

Phylogenetic Origin of the Deletion of the *mcy* Gene Cluster

The phylogenetic origin of the *mcy* gene cluster deletion was identified by sequencing all the strains for gene

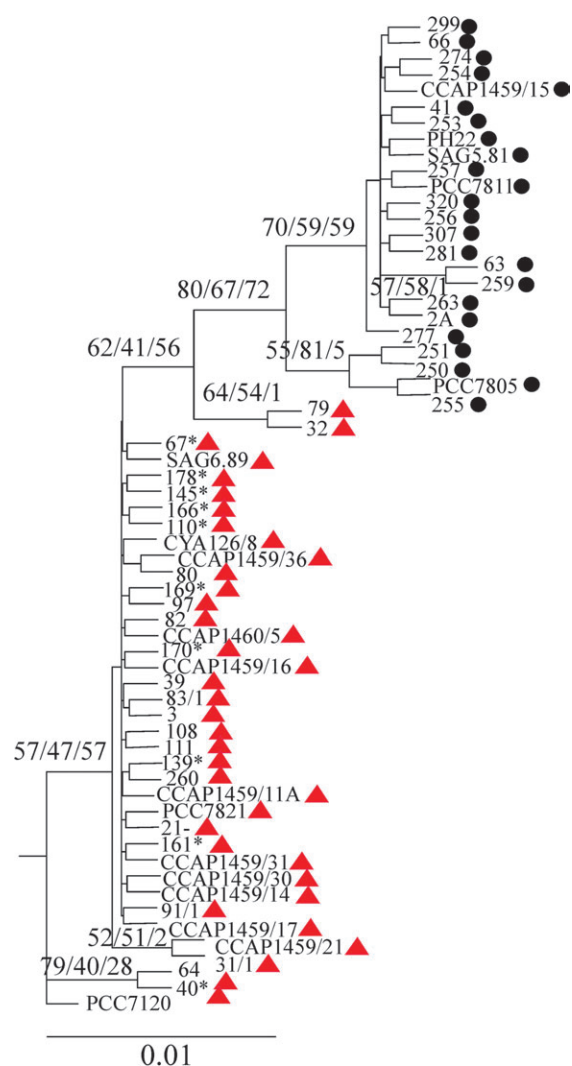


FIG. 4.—Maximum likelihood tree of *Planktothrix* strains containing *mcYT* as part of the *mcY* gene cluster (red triangles) and as a remnant of the *mcY* gene cluster (black dots). The numbers at the nodes indicate the percent bootstrap frequency (100 replicates) that was obtained from maximum likelihood–Neighbor-Joining–maximum parsimony calculated using the PHYLIP package. The tree was rooted using the *Nostoc* sp. strain PCC7120 as an outgroup. Only the bootstrap values of $>50\%$ are shown. Asterisks indicate strains that were inactivated by transposable elements but still contained the whole *mcY* gene cluster (Christiansen et al. 2006).

regions that are representative of essential housekeeping genes, that is, the 16S rDNA region (302 bp), the IGS between *cpcB* and *cpcA*, PC-IGS (211 bp), the ITS that is located between the 16S and 23S rDNA, 16S rDNA-ITS (317 bp), and the IGS between *psaA* and *psaB*, PSA-IGS (615 bp). All the strains shared nearly identical 16S rDNA sequences, in contrast to the number of polymorphic sites was higher at the other more variable spacer regions (table 2). In total, 25 unique genotypes were detected with 19 genotypes occurring only once. By using MLST (Feil et al. 2004), 2 clonal complexes were observed: 1) lineage 1 was comprised of both the strains that lost and that contained the *mcY* gene cluster and 2) lineage 2 was comprised of the strains containing the *mcY* gene cluster

Table 2
Number of Unique Genotypes (Alleles) and Variable Sites of Sequences at the Gene Loci that Were Analyzed in the *Planktothrix* Strains that Lack, or Contain, the *mcy* Gene Cluster

Locus	Bp ^a	Maximum Dissimilarity (%)	N ^b	Number of Variable Sites	Number of Alleles
Strains lacking the <i>mcy</i> gene cluster (n = 25)					
<i>mcyT</i>	751	0.93	24	36	22
16S ITS	316	2.23	25	10	5
PC-IGS	211	6.16	25	13	2
PSA-IGS	615	0.49	25	5	6
16S	302	1.32	25	4	3
Strains containing the <i>mcy</i> gene cluster (n = 37)					
<i>mcyT</i>	751	0.67	37	14	11
16S ITS	317	2.23	37	10	2
PC-IGS	211	7.11	37	17	7
PSA-IGS	615	3.74	37	24	6
16S	302	0	37	0	1
All strains (n = 62)					
<i>mcyT</i>	751	1.74	61	48	33
16S ITS	317	3.82	62	16	6
PC-IGS	211	7.11	62	17	7
PSA-IGS	615	3.9	62	28	10
16S	302	1.32	62	4	3

^a Bp, base pair number sequenced.

^b N, number of sequences.

including all the red-pigmented strains that are assigned to *P. rubescens* (fig. 5). In lineage 1, the primary founder (11 strains), which is defined as the ST with the greatest number of SLVs, contained the 2 most divergent types of *mcy* gene cluster deletion (types II and IV). The other types of deletions (I and III) were detected as SLVs or double locus variants. Therefore, nontoxic strains are all derived from a single ancestral strain that became inactive due to IS element-mediated IS followed by *mcy* gene cluster deletions and divergence.

Discussion

IS elements are generally recognized as important mutagenic agents (Doolittle and Sapienza 1980; Chandler and Mahillon 2002). Little is known, however, about the relative importance of the IS- or excision-induced variation in natural populations of bacteria as opposed to other mechanisms, for example, lateral gene transfer. In the present study, all the strains that lost the *mcy* gene cluster still contained inverted terminal repeated sequences between the 5' and 3' end *mcy* operon remnants that were probably derived from more than one copy of a putative IS element. The most likely mechanism that caused the *mcy* gene cluster deletion in all the strains was conservative site-specific recombination (Craig 1988), which resulted from the IS of 2 copies of the IS element at both ends of the *mcy* gene cluster. Indeed, in the vast majority of the strains that lost the *mcy* gene cluster, the inverted repeated sequences showed direct orientation when flanking the *mcy* operon remnants at the 5' and the 3' ends. It is, therefore, concluded that the loss of the

mcy genes is the result of a 2-step process, that is, the inactivation of the *mcy* gene cluster via the peripheral IS of 2 copies of an IS element that finally led to its deletion.

It is anticipated that the genotypes that become inactivated in MC synthesis by an IS element must be able to persist under natural conditions until the inactive *mcy* gene cluster is lost due to site-specific recombination. We previously described the inactivation of the *mcy* gene cluster by another IS element among the strains of the second lineage that always contain the *mcy* gene cluster (Christiansen et al. 2006). Those *mcy* genotypes that became inactivated by an IS element cannot be distinguished from active *mcy* genotypes via MLST (figs. 4 and 5) and have been observed to occur in deep-stratified lakes for at least 5 years. Although the inactive *mcy* genotypes make up the minor part of the population only (e.g., 21% in Lake Mondsee, Kurmayer et al. 2004), it is obvious that the inactivation of the *mcy* gene cluster through IS elements does not imply a selective disadvantage to the individual. On the other hand, the insertional inactivation of MC synthesis in *Microcystis aeruginosa* did not increase growth under different light conditions (4–110 $\mu\text{mol m}^{-2} \text{s}^{-1}$) compared with the MC-producing wild type (Hesse et al. 2001). This implies that the metabolic costs that are attributable to MC synthesis are not necessarily of relevance for the cell division rate. In the present study, only those strains that lost more than 90% of the *mcy* gene cluster were spread throughout Europe. One explanation for this could be that only larger gene deletion events may provide sufficient selective advantage for a specific genotype due to the energy requirements that are involved in the translational machinery (Mira et al. 2001). Under laboratory conditions, a sudden appearance of mutants that lost a larger part of the *mcy* gene cluster has in fact been reported (Schatz et al. 2005). Because the strain CYA126/8 is the only toxin-producing cyanobacterium that has been repeatedly transformed in several laboratories (Christiansen et al. 2003; Ishida et al. 2007), the experimental deletion of parts or the full *mcy* gene cluster of this strain is proposed as a tool for differentiating between the costs that are attributable to MC synthesis (e.g., $\Delta mcYT$) and the costs of the replication/translation of the *mcy* gene cluster (e.g., constructing a mutant by deleting larger parts of the *mcy* gene cluster) compared with the wild type.

In contrast to observations in the laboratory (Schatz et al. 2005), the results of this study point to a much slower replacement of toxic strains by nontoxic strains that lost the *mcy* gene cluster under natural conditions. Assuming a random spontaneous mutation rate of 4.1×10^{-10} (Drake 1991) along with an annual mean growth rate of 0.14 per day (Davis and Walsby 2002), the time necessary to create 5 random spontaneous mutations (the minimum dissimilarity between *mcyT* [791 bp] of nontoxic strains compared with toxic strains, fig. 4) was calculated to be 3.6 Myr. It could be argued that, for most of the time, cells do not grow exponentially but rather linearly, and nontoxic strains may competitively exclude toxic strains under nearly exponential growth conditions during prebloom periods in eutrophic lakes (e.g., Kardinaal et al. 2007). Indeed, *Planktothrix* occurring in deep-stratified lakes can dominate phytoplankton for years without pronounced periods of exponential growth/decline, whereas *Planktothrix*

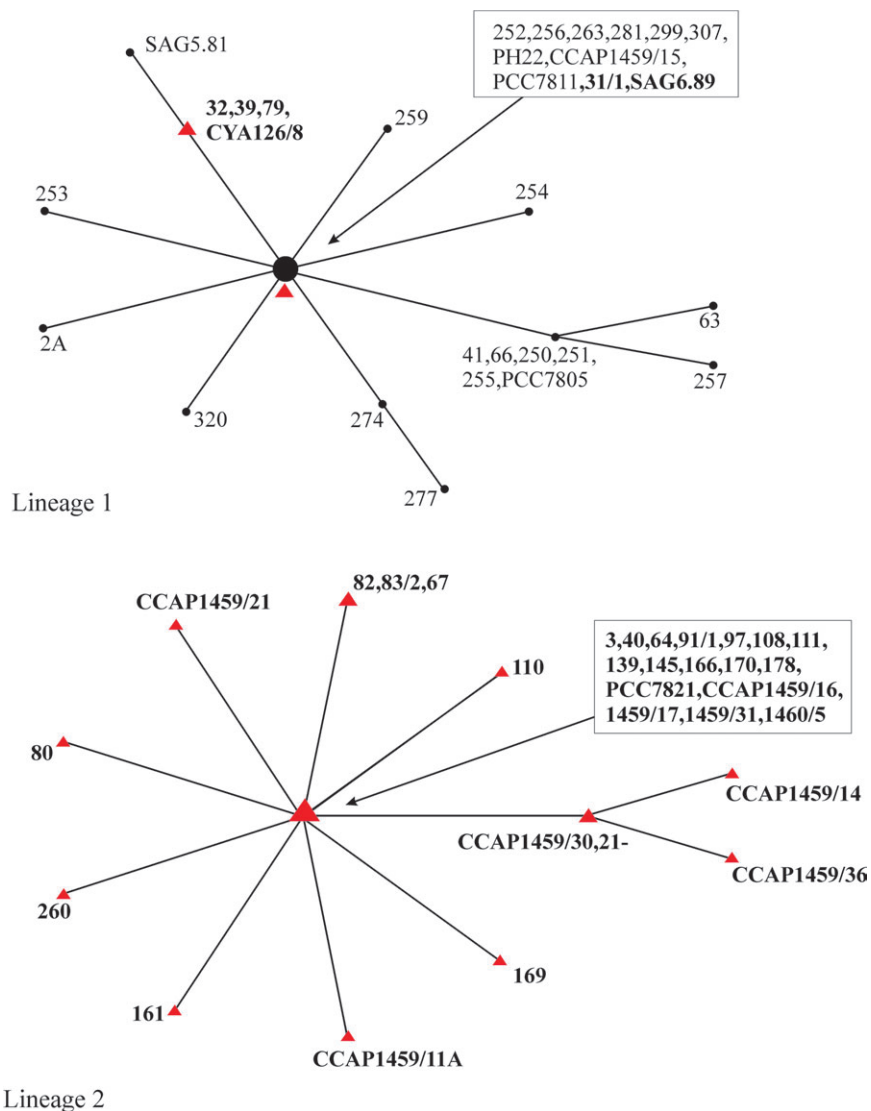


FIG. 5.—MLST diagrams showing 2 clonal complexes of strains that lost (lineage 1) or still contained the *mcy* gene cluster (lineages 1 and 2). Red triangles and bold letters: strains containing the *mcy* gene cluster encoding the synthesis of the toxic heptapeptide MC (37 strains) and black dots and normal letters: nontoxic strains that lost the *mcy* gene cluster (25 strains). Lineage 1, central founder (11 strains), bootstrap confidence 85% and lineage 2, central founder (17 strains), bootstrap confidence 92%. The connected STs differ at only 1 sequenced locus. The size of the symbols is proportional to the number of strains representing each ST.

occurring in more shallow lakes may show an exponential increase/decline during the season (Salmaso and Padisak 2007). However, even a 1,000-fold underestimation of a random spontaneous mutation rate for *mcyT* would imply that shifts between toxic strains and nontoxic strains as observed during seasonal succession in lakes cannot be directly of relevance to the evolution of the *mcy* gene cluster. Due to the extremely rare event of the *mcy* gene cluster deletion, the sole occurrence of toxic genotypes in *P. rubescens* populations in deep-stratified lakes (Kurmayer et al. 2004) can be explained by the evolutionary diversification of a genotype that still contains the *mcy* gene cluster. It has been suggested that *P. rubescens* diversified from *P. agardhii* due to its pigment adaptation to an underwater light climate relatively recently (Suda et al. 2002). We anticipate that evolutionary diversification that is driven by selective

factors other than an underwater light climate, for example cyanophages or hydrostatic pressure, will lead to a clonal dependence of MC production also in other taxa. In a recent study, numerous cyanophages active against *Anabaena*, *Planktothrix*, and *Microcystis* were reported (Deng and Hayes 2008). Cyanophage dynamics may further select phage-resistant genotypes and indirectly affect the shifts between MC-producing and non-MC-producing genotypes in *Microcystis* (Yoshida et al. 2008). Correspondingly, Tanabe et al. (2007) by using MLST found a clonal population structure of *Microcystis* sp., suggesting that each phylogenetic cluster might represent a “cryptic” ecotype (Cohan 2002). It is noteworthy that the occurrence of *mcyG* showed a clonal dependence, that is, all the strains containing *mcyG* were part of 2 lineages (groups A and B), whereas the other groups (C, D, and E) consisted of strains lacking

the *mcyG* gene. Consequently, the frequently observed co-occurrence of toxic and nontoxic strains (Carmichael and Gorham 1981; Vezie et al. 1998) can only be understood if it is interpreted in terms of evolutionary diversification that is driven by selective factors including those not directly linked to MC production. Future experiments that aim at a cost–benefit analysis of MC production need to consider the fact that even closely related strains that differ in MC production might also differ in other ecological traits, reflecting the process of adaptation to their respective environment. For example, it is well known that the natural isolates of bacteria (Mikkola and Kurland 1992) and cyanobacteria (Van Liere and Mur 1980) usually differ widely in their maximum specific growth rate under standardized laboratory conditions. This implies that the measured growth rates of nontoxic and toxic strains under experimental conditions cannot be used to relate the fitness of a particular strain to the presence/absence of MC production.

Supplementary Material

Supplementary figures S1 and S2 and table S1 are available at *Molecular Biology and Evolution* online (<http://www.mbe.oxfordjournals.org/>).

Acknowledgments

We are most grateful to our colleagues for sending water samples to us or who delivered strains (in alphabetical order): Antonio Camacho, Peter Henriksen, Kaarina Sivonen, and Linda Tonk. Johanna Schmidt provided rather valuable assistance in the laboratory. We very much appreciate the comments of 3 anonymous reviewers. This study was financed by a grant from the Austrian Science Fund P18185 “Microevolution of toxin synthesis in cyanobacteria.”

Literature Cited

- Carmichael WW, Gorham PR. 1981. The mosaic nature of toxic blooms of cyanobacteria. In: Carmichael WW, editor. The water environment: algal toxins and health. New York: Plenum Press. p. 161–172.
- Cary J, Ehrlich K. 2006. Aflatoxigenicity in *Aspergillus*: molecular genetics, phylogenetic relationships and evolutionary implications. *Mycopathologia*. 162:167–177.
- Chandler M, Mahillon J. 2002. Insertion sequences revisited. In: Craig NL, Craigie R, Gellert M, Lambowitz AM, editors. Mobile DNA II. Washington (DC): American Society for Microbiology. p. 305–366.
- Christiansen G, Fastner J, Erhard M, Börner T, Dittmann E. 2003. Microcystin biosynthesis in *Planktothrix*: genes, evolution, and manipulation. *J Bacteriol*. 185:564–572.
- Christiansen G, Kurmayer R, Liu Q, Börner T. 2006. Transposons inactivate biosynthesis of the nonribosomal peptide microcystin in naturally occurring *Planktothrix* spp. *Appl Environ Microbiol*. 72:117–123.
- Cohan FM. 2002. What are bacterial species? *Annu Rev Microbiol*. 56:457–487.
- Craig NL. 1988. The mechanism of conservative site-specific recombination. *Ann Rev Genet*. 22:77–105.
- Davis PA, Walsby AE. 2002. Comparison of measured growth rates with those calculated from rates of photosynthesis in *Planktothrix* spp. isolated from Blelham Tarn, English Lake District. *New Phytol*. 156:225–239.
- Deng L, Hayes PK. 2008. Evidence for cyanophages active against bloom-forming freshwater cyanobacteria. *Freshwater Biology*. 53:1240–1252.
- Dittmann E, Neilan BA, Erhard M, von Döhren H, Börner T. 1997. Insertional mutagenesis of a peptide synthetase gene that is responsible for hepatotoxin production in the cyanobacterium *Microcystis aeruginosa* PCC 7806. *Mol Microbiol*. 26:779–787.
- Doolittle WF, Sapienza C. 1980. Selfish genes, the phenotype paradigm and genome evolution. *Nature*. 284:601–603.
- Drake J. 1991. A constant rate of spontaneous mutation in DNA-based microbes. *Proc Natl Acad Sci USA*. 88:7160–7164.
- Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG. 2004. Eburst: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. *J Bacteriol*. 186:1518–1530.
- Felsenstein J. 1993. PHYLIP (phylogeny inference package). Version 3.5c. Seattle (WA): Department of Genetics, University of Washington.
- Fujii K, Sivonen K, Naganawa E, Harada K. 2000. Non-toxic peptides from toxic cyanobacteria, *Oscillatoria agardhii*. *Tetrahedron*. 56:725–733.
- Hesse K, Dittmann E, Börner T. 2001. Consequences of impaired microcystin production for light-dependent growth and pigmentation of *Microcystis aeruginosa* PCC 7806. *FEMS Microbiol Ecol*. 37:39–43.
- Ishida K, Christiansen G, Yoshida WY, Kurmayer R, Welker M, Bonjoch J, Hertweck C, Börner T, Hemscheidt T, Dittmann E. 2007. Biosynthetic pathway and structure analysis of aeruginoside 126A and B, cyanobacterial peptide glycosides bearing an unusual 2-carboxy-6-hydroxyoctahydroindole moiety. *Chem Biol*. 14:565–576.
- Janse I, Meima M, Kardinaal WEA, Zwart G. 2003. High-resolution differentiation of cyanobacteria by using rRNA-internal transcribed spacer denaturing gradient gel electrophoresis. *Appl Environ Microbiol*. 69:6634–6643.
- Kaebnick M, Neilan BA, Börner T, Dittmann E. 2000. Light and the transcriptional response of the microcystin biosynthesis gene cluster. *Appl Environ Microbiol*. 66:3387–3392.
- Kardinaal W, Tonk L, Janse I, Hol S, Slot P, Huisman J, Visser P. 2007. Competition for light between toxic and nontoxic strains of the harmful cyanobacterium *Microcystis*. *Appl Environ Microbiol*. 73:2939–2946.
- Kohl J, Nicklisch A. 1988. Ökophysiologie der Algen. Wachstum und Ressourcennutzung. Jena (Germany): Urban & Fischer.
- Kurmayer R, Christiansen G, Fastner J, Börner T. 2004. Abundance of active and inactive microcystin genotypes in populations of the toxic cyanobacterium *Planktothrix* spp. *Environ Microbiol*. 6:831–841.
- Kurmayer R, Gumpenberger M. 2006. Diversity of microcystin genotypes among populations of the filamentous cyanobacteria *Planktothrix rubescens* and *Planktothrix agardhii*. *Mol Ecol*. 15:3849–3861.
- Kurmayer R, Kutzenberger T. 2003. Application of real-time PCR for quantification of microcystin genotypes in a population of the toxic cyanobacterium *Microcystis* sp. *Appl Environ Microbiol*. 69:6723–6730.
- Meißner K, Dittmann E, Börner T. 1996. Toxic and non-toxic strains of the cyanobacterium *Microcystis aeruginosa* contain sequences homologous to peptide synthetase genes. *FEMS Microbiol Lett*. 135:295–303.

- Mikkola R, Kurland CG. 1992. Selection of laboratory wild-type phenotype from natural isolates of *Escherichia coli* in chemostats. *Mol Biol Evol.* 9:394–402.
- Mira A, Ochman H, Moran NA. 2001. Deletional bias and the evolution of bacterial genomes. *Trends Genet.* 17:589–596.
- Moffitt MC, Neilan BA. 2004. Characterization of the nodularin synthetase gene cluster and proposed theory of the evolution of cyanobacterial hepatotoxins. *Appl Environ Microbiol.* 70:6353–6362.
- Mootz HD, Finking R, Marahiel MA. 2001. 4'-phosphopantetheine transfer in primary and secondary metabolism of *Bacillus subtilis*. *J Biol Chem.* 276:37289–37298.
- Nakasugi K, Alexova R, Svenson CJ, Neilan BA. 2007. Functional analysis of *PiIT* from the toxic cyanobacterium *Microcystis aeruginosa* PCC 7806. *J Bacteriol.* 189:1689–1697.
- Orr PT, Jones GJ. 1998. Relationship between microcystin production and cell division rates in nitrogen-limited *Microcystis aeruginosa* cultures. *Limnol Oceanogr.* 43:1604–1614.
- Quadri LEN, Weinreb PH, Lei M, Nakano MM, Zuber P, Walsh CT. 1998. Characterization of *Sfp*, a *Bacillus subtilis* phosphopantetheinyl transferase for peptidyl carrier protein domains in peptide synthetases. *Biochemistry.* 37:1585–1595.
- Rantala A, Fewer DP, Hisbergues M, Rouhiainen L, Vaitoaa J, Börner T, Sivonen K. 2004. Phylogenetic evidence for the early evolution of microcystin synthesis. *Proc Natl Acad Sci USA.* 101:568–573.
- Rezsöházy R, Hallet B, Delcour J, Mahillon J. 1993. The IS4 family of insertion sequences—evidence for a conserved transposase motif. *Mol Microbiol.* 9:1283–1295.
- Rippka R. 1988. Isolation and purification of cyanobacteria. *Meth Enzymol.* 167:3–27.
- Rouhiainen L, Vakkilainen T, Siemer BL, Buikema W, Haselkorn R, Sivonen K. 2004. Genes coding for hepatotoxic heptapeptides (microcystins) in the cyanobacterium *Anabaena* strain 90. *Appl Environ Microbiol.* 70:686–692.
- Salmaso N, Padisak J. 2007. Morpho-functional groups and phytoplankton development in two deep lakes (Lake Garda, Italy and Lake Stechlin, Germany). *Hydrobiologia.* 578:97–112.
- Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning—a laboratory manual, 2nd ed, Vol. 1–3. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press.
- Schatz D, Keren Y, Hadas O, Carmeli S, Sukenik A, Kaplan A. 2005. Ecological implications of the emergence of non-toxic subcultures from toxic *Microcystis* strains. *Environ Microbiol.* 7:798–805.
- Schober E, Kurmayer R. 2006. Evaluation of different DNA sampling techniques for the application of the real-time PCR method for the quantification of cyanobacteria in water. *Lett Appl Microbiol.* 42:412–417.
- Schwarzer D, Mootz HD, Linne U, Marahiel MA. 2002. Regeneration of misprimed nonribosomal peptide synthetases by type II thioesterases. *Proc Natl Acad Sci USA.* 22:14083–14088.
- Siebert PD, Chenchik A, Kellogg DE, Lukyanov KA, Lukyanov SA. 1995. An improved PCR method for walking in uncloned genomic DNA. *Nucleic Acids Res.* 23:1087–1088.
- Siguier P, Perochon J, Lestrade L, Mahillon J, Chandler M. 2006. ISfinder: the reference centre for bacterial insertion sequences. *Nucleic Acids Res.* 34:D32–D36.
- Suda S, Watanabe MM, Otsuka NS, Mahakahant A, Yongmanitchai W, Nopartnaraporn, Liu Y Day JG. 2002. Taxonomic revision of water-bloom-forming species of oscillatorioid cyanobacteria. *Int J Syst Evol Microbiol.* 52:1577–1595.
- Tanabe Y, Kasai F, Watanabe M. 2007. Multilocus sequence typing (MLST) reveals high genetic diversity and clonal population structure of the toxic cyanobacterium *Microcystis aeruginosa*. *Microbiology.* 153:3695–3703.
- Tanabe Y, Kaya K, Watanabe MM. 2004. Evidence for recombination in the microcystin synthetase (*mcy*) genes of toxic cyanobacteria *Microcystis* spp. *J Mol Evol.* 58:633–641.
- Tillett D, Dittmann E, Erhard M, von Döhren H, Börner T, Neilan BA. 2000. Structural organization of microcystin biosynthesis in *Microcystis aeruginosa* PCC7806: an integrated peptide-polyketide synthetase system. *Chem Biol.* 7:753–764.
- Touzet N, Franco JM, Raine R. 2007. Characterization of nontoxic and toxin-producing strains of *Alexandrium minutum* (Dinophyceae) in Irish coastal waters. *Appl Environ Microbiol.* 73:3333–3342.
- Van Liere L, Mur LR. 1980. Occurrence of *Oscillatoria agardhii* and some related species, a survey. *Dev Hydrobiol.* 2:67–77.
- Vezie C, Briant L, Sivonen K, Bertru G, Lefeuvre J-C, Salkinoja-Salonen M. 1998. Variation of microcystin content of cyanobacterial blooms and isolated strains in Lake Grand-Lieu (France). *Microb Ecol.* 35:126–135.
- Walsby A, Schanz F, Schmid M. 2005. The Burgundy-blood phenomenon: a model of buoyancy change explains autumnal waterblooms by *Planktothrix rubescens* in Lake Zürich. *New Phytol.* 169:109–122.
- World Health Organization. 2004. Guidelines for drinking-water quality, Vol. 1: recommendations, 3rd ed. Geneva (Switzerland): World Health Organization.
- Yang Z. 1997. PAML: a program package for phylogenetic analysis by maximum likelihood. *Comput Appl Biosci.* 13:555–556.
- Yang Z. 1998. Likelihood ratio tests for detecting positive selection and application to primate lysozyme evolution. *Mol Biol Evol.* 15:568–573.
- Yang Z. 2005. The power of phylogenetic comparison in revealing protein function. *Proc Natl Acad Sci USA.* 102:3179–3180.
- Yoshida M, Yoshida T, Kashima A, Takashima Y, Hosoda N, Nagasaki K, Hiroishi S. 2008. Ecological dynamics of the toxic bloom-forming *Microcystis aeruginosa* and its cyanophages in freshwater. *Appl Environ Microbiol.* 74:3269–3273.

Geoffrey McFadden, Associate Editor

Accepted May 15, 2008