APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 2006, p. 117–123 Vol. 72, No. 1
0099-2240/06/$08.00+0 doi:10.1128/AEM.72.1.117–123.2006

Transposons Inactivate Biosynthesis of the Nonribosomal Peptide Microcystin in Naturally Occurring *Planktothrix* spp.†

Guntram Christiansen,[1,3] Rainer Kurmayer,[2]* Qian Liu,[2] and Thomas Börner[3]

Department of Chemistry, University of Hawaii at Manoa, 2545 McCarthy Mall, Honolulu, Hawaii 96822[1]; Institute for Limnology, Austrian Academy of Sciences, Mondseestrasse 9, 5310 Mondsee, Austria[2]; and Institute for Biology (Genetics), Humboldt University, Chausseestrasse 117, D-10115 Berlin, Germany[3]

Received 2 August 2005/Accepted 7 October 2005

The filamentous cyanobacteria *Planktothrix* spp. occur in the temperate region of the Northern hemisphere. The red-pigmented *Planktothrix rubescens* bacteria occur in deep, physically stratified, and less eutrophic lakes. *Planktothrix* is a known producer of the toxic heptapeptide microcystin (MC), which is produced nonribosomally by a large enzyme complex consisting of peptide synthetases and polyketide synthases encoded by a total of nine genes (*mcy* genes). *Planktothrix* spp. differ in their cellular MC contents as well as the production of MC variants; however, the mechanisms favoring this diversity are not understood. Recently, the occurrence of *Planktothrix* strains containing all *mcy* genes but lacking MC has been reported. In this study, 29 such strains were analyzed to find out if mutations of the *mcy* genes lead to the inability to synthesize MC. Two deletions, spanning 400 bp (in *mcyB*; one strain) and 1,869 bp (in *mcyHA*; three strains), and three insertions (IS), spanning 1,429 bp (in *mcyD*; eight strains), 1,433 bp (in *mcyEG*; one strain), and 1,433 bp (in *mcyA*; one strain), were identified. Though found in different genes and different isolates and transcribed in opposite directions, IS were found to be identical and contained conserved domains assigned to transposable elements. Using mutation-specific primers, two insertions (in *mcyD* and *mcyA*) and one deletion (in *mcyHA*) were found regularly in populations of *P. rubescens* in different lakes. The results demonstrate for the first time that different mutations resulting in inactivation of MC synthesis do occur frequently and make up a stable proportion of the *mcy* gene pool in *Planktothrix* populations over several years.

Microcystins (MC) are cyclic heptapetides regularly produced by cyanobacteria of the genera *Anabaena*, *Microcystis*, and *Planktothrix*. MCs are known to be toxic to aquatic biota, livestock, and humans. They are synthesized nonribosomally by multifunctional enzyme complexes via the so-called thiotemplate mechanism (4). The above-mentioned organisms show an impressive diversity in the production of small bioactive peptides (700 to 1,400 Da) other than microcystins, e.g., anabaenopeptins, aeruginosins, microviridins, cyanopeptolines, etc. (6). Typically, isolates differing in the production of these small peptides cannot be discriminated under the microscope or by traditional molecular taxonomic approaches. Our understanding of the mechanisms and recombination processes affecting existing pathways of secondary metabolite synthesis is rather poor. Recently, progress has been made in the elucidation of the genetic basis of MC synthesis for all three main MC producers occurring in freshwater, i.e., *Anabaena*, *Microcystis*, and *Planktothrix* (2, 27, 36). Three gene clusters responsible for the biosynthesis of microcystins, containing 9 or 10 genes (depending on the genus) and spanning >55 kb, have been sequenced. Using information obtained from nonribosomal peptide synthetases and polyketide synthases in general (17, 32) and from tracer feeding experiments (22), the main pathways of MC biosynthesis could be elucidated (36).

Because of high sequence similarities between the *mcy* gene clusters in the different genera and the coevolution of 16S rRNA genes and *mcy* genes, a common ancestor for MC synthesis has been suggested (25). According to this theory, the patchy distribution of *mcy* genes among strains of one species is understood in terms of repeated-loss processes of the *mcy* gene cluster during cyanobacterial evolution, albeit the maintenance of *mcy* in some genera points towards an important but so far unknown function. Our recent report on the occurrence of inactive *mcy* genotypes (i.e., genotypes possessing the *mcy* genes but lacking MC production) of *Planktothrix* spp. in nature (13) might be understood as support for the *mcy* gene loss hypothesis. The inactivation of MC synthesis in an increasing number of strains might be seen as a first step in evolution towards losing the complete *mcy* gene cluster. Also, a few inactive *mcy* genotypes have been described for *Microcystis*. The loss of MC synthesis has been attributed to the accumulation of point mutations (9, 23). However, so far mutations have not been found among genes of the *mcy* gene cluster.

The aim of this study was to find out whether the inactivation of MC synthesis can be explained by mutations within the *mcy* gene cluster, and if so, which mutation types can be found in nature and how frequently they occur or if they should rather be considered exceptional.

(The field data reported in this study partly originated from the M.Sc. thesis submitted by L.Q. to the International Institute for Infrastructural Hydraulic and Environmental Engineering (IHE, Delft, The Netherlands) in the course of the International Postgraduate of Limnology Course at the Limnological Institute in Mondsee, Austria.)

* Corresponding author. Mailing address: Austrian Academy of Sciences, Institute for Limnology, Mondseestrasse 9, 5310 Mondsee, Austria. Phone: 43-6232-312532. Fax: 43-6232-3578. E-mail: rainer.kurmayer@oeaw.ac.at.

† Supplemental material for this article may be found at http://aem.asm.org/.

TABLE 1. Oligonucleotide primers used for the detection of mutations within the *mcy* gene cluster[a]

Primer no.	Base pair no.	Forward sequence	T_m (°C)	Reverse sequence	T_m (°C)
1	452–2022	ATTGATCCCCTGATCAATGATCA	62.5	GCTAAACTGGGGCCATTGAGA	63.5
2	2002–4050	TCTCAATGGCCCCAGTTTAGC	63.5	CGCTGGCTAATACTCCCTCG	62.7
3	4031–6021	CGAGGGAGTATTAGCCAGCG	62.7	TTTGCATGGAAAGGGATCAATC	63.1
4	6000–8002	GATTGATCCCTTTCCATGCAAA	63.1	GATCGCTTCCCTGGGAATAATG	63.9
5	7981–10011	CATTATTCCCAGGGAAGCGATC	63.9	TGACCGATGGGTTTACCTGTG	62.9
6	9991–12009	CACAGGTAAACCCATCGGTCA	62.9	CCCAGTTTTCCCAAACCTCC	62.7
7	11990–13989	GGAGGTTTGGGAAAACTGGG	62.7	TCTCCACGCCCATAGCCAT	63.8
8	13971–16000	ATGGCTATGGGCGTGGAGA	63.8	CTGGCGGATTTCGAGTTGAT	62.3
9	15981–18021	ATCAACTCGAAATCCGCCAG	62.3	GCGGCCAGATTAGACTGCATT	63.6
10	18001–20076	AATGCAGTCTAATCTGGCCGC	63.6	GCGATGAGTGTCTGTTTAGTCCG	63.2
11	20054–21990	CGGACTAAACAGACACTCATCGC	63.2	GATTTCCCGGTTTCATTGAGTG	62.7
12	21969–23983	CACTCAATGAAACCGGGAAATC	62.7	TTAGCAGCATTGGCTAAGACTGC	62.9
13	23961–25999	GCAGTCTTAGCCAATGCTGCTAA	62.9	GTTGTGAGATTGTTGGCGCC	63.8
14	25980–28065	GGCGCCAACAATCTCACAAC	63.8	CTGAAATAACGGTATGTTGATCGGT	62.3
15	28042–30072	CCGATCAACATACCGTTATTTCAG	62.3	GTTGCGCTTGAATGAAACGG	63.8
16	30053–32040	CCGTTTCATTCAAGCGCAAC	63.8	GTCCTTTGGGGTCTGTTGGATAC	62.9
17	32010–34020	GTATCCAACAGACCCCAAAGGAC	62.9	AAGGCTCCCGTTGCTAAAAC	63.1
18	34001–36035	GTTTTAGCAACGGGAGCCTT	63.1	CGGGAATGGGTTCTCCTGTATAA	63.2
19	36013–38042	TTATACAGGAGAACCCATTCCCG	63.2	AGGAAGCCACACTCCAACCAT	63.0
20	38022–40022	ATGGTTGGAGTGTGGCTTCCT	63.0	CTGTTAATTCGGGACGATGGAG	62.8
21	40001–42060	CTCCATCGTCCCGAATTAACAG	62.8	TTGTCATGGATGTGACGAGCA	63.3
22	42060–44052	TGCTCGTCACATCCATGACAA	63.6	CTGATTGGACAGAACTGCTTGGA	63.7
23	44030–46036	TCCAAGCAGTTCTGTCCAATCAG	63.7	GGTAAATGAACGCGGGGAAT	63.0
24	46017–48031	ATTCCCCGCGTTCATTTACC	63.0	AATCGTCCCAAGTCTCCGGTA	62.9
25	48011–50000	TACCGGAGACTTGGGACGATT	62.9	GACCGCCCATTCTAGCGATT	63.5
26	49981–51970	AATCGCTAGAATGGGCGGTC	63.5	AAAACCAAGGGCAGGAGGAA	63.0
27	51951–54058	TTCCTCCTGCCCTTGGTTTT	63.0	GTGAGTGCCATCCTGACAGCTAT	62.7
28	54036–55470	ATAGCTGTCAGGATGGCACTCAC	62.7	GCACCCTAACTAACTCTGCAATCG	63.3

[a] The primers allow for the amplification of the whole *mcy* synthetase gene cluster of *Planktothrix* (2) and were designed during this study.

MATERIALS AND METHODS

Experimental organisms and culture conditions. All nine inactive microcystin genotypes of *Planktothrix rubescens* (Lake Irrsee strains 12, 62, 65, 87, 94, and 95; Lake Wörthersee strain 67; and Lake Mondsee strain 91/1) and *P. agardhii* (Lake Gjersjoen strain CCAP1459/36), as reported previously (13), and 20 additional *P. rubescens* strains (Lake Mondsee strains 40, 110, 120, 130, 135, 137, 154, and 194; Lake Grabensee strains 139, 145, 161, 166, 168, 169, and 170; Lake Wolfgangsee strain 160; Lake Fuschlsee strains 165, 167, and 178; and Lake Obertrumer See strain 197) were isolated and analyzed as described previously (13). All strains gave positive signals in PCRs with a *mcyA*- and *mcyB*-specific primer pair but did not contain MCs and were screened for DNA mutations as described below. All strains analyzed contained the internal transcribed spacer region of the phycocyanin operon, detected with primers PcPI+ and PcPI−, specific for *Planktothrix* spp. (13). Based on the current taxonomical classification (34), strains were assigned to either *P. agardhii* (green pigmented) or *P. rubescens* (red pigmented). All strains were cultivated in BG11 medium (26) containing 2 mM $NaNO_3$ and 10 mM $NaHCO_3$ at 15°C with continuous light (5 to 10 μmol m^{-2} s^{-1}; Osram type L30W/77 Fluora light source). Strains were harvested by incubating 2 ml of culture for 1 h on ice, subsequent centrifuging at 13,000 rpm for 10 min, and lyophilizing the pellet in a vacuum centrifuge at 30°C.

Sampling. *P. rubescens* was sampled from June to October in the years 2001–2004 by pulling a plankton net (30-μm mesh size) from a depth of 20 m to the surface at the center of a lake. In parallel, quantitative integrated samples were obtained by collecting 1 liter every meter from the surface to a depth of 20 m. Lakes Irrsee, Mondsee, Offensee, Schwarzensee, Wolfgangssee (Upper Austria, Austria), and Wörthersee (Carinthia, Austria) are generally deep and stratified lakes, and except for the oligotrophic Lake Schwarzensee, are classified as mesotrophic (7). Filaments were assigned to the genus *Planktothrix* according to previously described morphological criteria (1) and counted from Lugol's solution-fixed integrated samples. The enumeration of cells was done by means of an inverted microscope, using the methods of Utermöhl (37) as described previously (13). Aliquots (a few ml from net samples and 3 to 4 liters from integrated samples) were filtered onto glass fiber filters (BM/C; Ederol, Vienna, Austria) under vacuum pressure and stored frozen (−20°C) until DNA extraction.

Genetic analysis. DNA extraction from strains and field samples was performed by a standard phenol-chloroform procedure as described previously (12). PCR amplifications were performed in reaction mixtures of 20 μl containing 2 μl of QIAGEN PCR buffer (QIAGEN, VWR International, Austria), 1.2 μl $MgCl_2$ (25 mM; QIAGEN), 0.6 μl deoxynucleoside triphosphates (10 mM [each]; MBI Fermentas, St. Leon-Rot, Germany), 1 μl of each primer (10 pmol μl^{-1}), 0.1 μl *Taq* DNA polymerase (QIAGEN), 13.1 μl sterile Millipore water, and 1.0 μl of genomic DNA (diluted 100-fold). In order to screen the complete *Planktothrix mcy* gene cluster (2), 28 primer pairs covering the whole *mcy* gene cluster were designed and used to amplify fragments of 2 kb without interruption (Table 1). DNA mutations were detected via the difference in PCR product sizes in agarose compared to the corresponding PCR products obtained from strain CYA126/8, whose the *mcy* gene cluster has been sequenced (AJ441056) (2). The PCR thermal cycling protocol included an initial denaturation step at 94°C for 3 min, followed by 35 cycles at 94°C for 30 s, an annealing temperature of 60°C for 30 s, and an elongation temperature of 72°C for 2 min. In the case of nonsuccessful PCRs, a long-range *Taq* polymerase (Clontech, BD Biosciences, Palo Alto, CA) was used to amplify PCR products (>4 kb) using adjacent primer pairs following the manufacturer's instructions.

In order to detect mutant genotypes directly in the field, primers specific for the detected mutation events were designed (Table 2). The deletion-specific primer pairs produced distinctly shorter amplification products than the amplicons resulting from unmutated gene regions (*mcyHA* deletion in strain 62, 1,869 bp; *mcyB* deletion in strain CCAP1459/36, 707 bp). In contrast, the insertion (IS)-specific primer pairs (*mcyD*IS1, strain 110; *mcyA*IS, strain 40) consisted of the forward primer binding to a locus within *mcyD* or *mcyA* and the reverse primer binding to the inserted gene region or vice versa. All five primer pairs (Table 2) were found to be specific for the corresponding genotype and did not give PCR products for other typically co-occurring *Planktothrix* strains and other cyanobacteria (*Aphanizomenon* spp., *Microcystis* sp., *Nostoc* sp., and *Synechococcus* spp.). For field analysis, the PCR thermal cycling protocol included an initial denaturation step at 94°C for 3 min, followed by 35 cycles at 94°C for 30 s, an annealing temperature of 60°C for 30 s, and an elongation temperature of 72°C for 30 s. To control for the occurrence of *Planktothrix* and the *mcy* gene cluster, each sample was analyzed with the PcPI+/− primers, amplifying the intergenic spacer region within the phycocyanin operon (PC-IGS), and the peamso+/− primers, amplifying a region within the *mcyA* gene, as described previously (13). Pilot experiments showed that PCRs using the primer pair PcPI+/− and the

mutation-specific primers *mcyD*IS1 (3′ end) and *mcy HA* (deletion) all had a detection limit of 10 cells per assay. PCR products (4 μl of the reaction mix) were visualized by electrophoresis on 1.0% (strains) or 1.5% (field samples) agarose in 0.5× TBE (Tris-borate-EDTA buffer), with ethidium bromide staining.

Sequence alignment and analysis. The PCR products obtained in field samples were sequenced directly from PCR products by standard automated fluorescence techniques (Applied Biosystems, Weiterstadt, Germany). Sequences were aligned using multiple sequence alignment (Clustal W 1.8). Similarity values between nucleotide sequences were calculated using the program DNADIST of the PHYLIP software package (version 3.6[alpha3]) (5).

Nucleotide sequence accession numbers. The sequence data obtained in this study have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers AM039937 to AM039947, AM040461 to AM040485, and AM055629 (Tables 2 and 3).

RESULTS

Identification of mutations linked to the inactivation of MC synthesis. Sequencing of the PCR amplicons that differed in size from the expected 2.0 kb revealed deletions and insertions (Fig. 1; see the supplemental material). Insertions were detected at three different sites by significantly larger-than-expected PCR products, using in one case primer pair 6, binding to *mcyD*, in the second case primer pair 12, binding to the spacer region between *mcyE* and *mcyG*, and in the third case primer pair 21, binding to *mcyA*. The insertion affecting *mcyD* (*mcyD*IS1) was 1,429 bp long and found at position 11,918 of the *mcy* gene cluster (AJ441056), and it occurred in strain 110. The insertion sequences observed in strains 139, 145, 161, 166, 169, 170, and 178 were located in *mcyD* at the same position (*mcyD*IS2). Both *mcyD*IS1 and *mcyD*IS2 had a left inverted repeat (IRL; 5′-CAGGGCTGTTTCA-3′) and a right inverted repeat (IRR; 5′-TGAAACAGCCCTG-3′) and were highly similar (99.0 to 99.4% identity), but *mcyD*IS2 was transcribed in the opposite direction (Fig. 2). The variability among the seven strains containing *mcyD*IS2 (1,397 bp) was low (0 to 0.6%). Two other insertions with identical IRL and IRR sequences affected the spacer between *mcyE* and *mcyG* (1,434 bp; named *mcyEG*IS) in strain 110 at position 23,822 and affected *mcyA* (1,433 bp; named *mcyA*IS) at position 41,274 in strain 40. All *mcyD*IS and *mcyA*IS mutants had short directly repeated sequences (DR) of 10 bp in length at the downstream end (*mcyD*IS1, 5′-CGT GCA CGG G-3′; *mcyD*IS2, 5′-CCC GTG CAC G-3′; *mcyEG*IS, 5′-GGC TGT TCC C-3′; and *mcyA*IS, 5′-CCC AAA ACC C-3′). Consequently, *mcyD*IS2 transcribed in the opposite direction but inserted at the same position should have originated from a second independent insertion event. All IS showed 98.5 to 99.4% identity and carried a single predicted open reading frame (ORF) (encoding 363 amino acids [aa]) constituting 76% of *mcyD*IS1 (bp 237 to 1328). The ORF included conserved domains assigned to transposase 11 (E value, 7e − 08), a transposase DDE domain known to be necessary for efficient DNA transposition (3). We concluded that all insertions found among the *mcy* gene cluster involved this same transposase 11.

Deletions were identified by shorter-than-expected PCR amplicons at two different sites. In one case, PCR amplification constantly failed to give an amplicon with primer pairs 17, 18, and 19. Subsequently, long-range PCR amplification with primers 17fwd and 19rev was performed, yielding an amplicon of only 4 kb instead of the expected 6 kb (data not shown). This deletion (called the *mcyHA* deletion) was found in strains 12,

TABLE 2. *Planktothrix* strains sequenced for mutations (red-pigmented strains [*P. rubescens*] and green-pigmented strains [*P. agardhii*])

Strain	Yr of isolation	Origin	Type of mutation	Sequence accession no.	Position	Forward primer name, 5′–3′ sequence[a]	Temp (°C)	Reverse primer name, 5′–3′ sequence[a]	°C	Product size (bp)[b]
110 (*P. rubescens*)	2001	Mondsee, Austria	Insertion (*mcyD*IS1) (1,424 bp) at 11,918	AM039940	11,745 (fwd primer) 12,047 (rev primer)	*mcyD*IS1 5′ end F, AAGAAT ATCCCCAAATGCGTTG *mcyD*IS1 3′ end F, CCTTTT TCCCTGATTTTTTCGG	59 56	*mcyD*IS1 5′ end R, GGGAATGTA AGTCTTACTCCATAGGG *mcyD*IS1 3′ end R, TCGCCCCCA TTTTCGATAA	59 55	351 300
139, 145, 161, 166, 169, 170 (*P. rubescens*)	2001	Mondsee, Austria	Insertion (*mcyEG*IS) (1,433 bp) at 23,822	AM055629						
	2001	Grabensee, Austria	Insertion (*mcyD*IS2) at 11,918 (1,435 bp)	AM039941-AM039946						
178 (*P. rubescens*)	2001	Fuschlsee, Austria	Insertion (*mcyD*IS2) at 11,918	AM039947						
40 (*P. rubescens*)	2001	Mondsee, Austria	Insertion (*mcyA*) (1,433 bp) at 41,274	AM039939	41,167 (fwd primer) 41,454 (rev primer)	*mcyA*IS 5′ end F, TCATCT CGTCGTAGATGGG *mcyA*IS 3′ end F, CTTTTT CCCTGATTTTTTCGGA	58 58	*mcyA*IS 5′ end R, CACTGCTCA ACACCCTAGGATTG *mcyA*IS 3′ end R, TTGAGTGTT ACCGTGATCTCTGC	60 58	455 351
62 (*P. rubescens*)	2001	Irrsee, Austria	Deletion (*mcyHA*) (1,869 bp) at 32,938	AM039937	32,791 (fwd primer)	Magnetic 1fwd, CCATTTATC GTCTTCGCTCCC	60	Magnetic 1rev, CGGCAAATA GACGAACAACATG	59	300 (2223)
CCAP1459/36 (*P. agardhii*)	1968	Lake Gjersjoen, Norway	Deletion (*mcyB*) (400 bp) at 43,423	AM039938	43,226 (fwd primer)	1459/36 1F, AGAAATCCA AAGGCTGAGTGATG	58	1459/36 301R, AAAGATTGC TGGGTCAGCAAA	58	301 (707)

[a] The primers for the specific detection of *mcyA*IS and *mcyD*IS1 genotypes (on both ends) and *mcyHA*/*mcyB* deletion genotypes in field samples and corresponding base pair numbers of the *mcy* gene cluster (AJ441056) from strain CYA126/8 (2) are given.

[b] Sizes of PCR products obtained without deletions are given in parentheses.

TABLE 3. Sequence accession numbers of mutant *mcy* genotypes obtained from field samples by sequencing of PCR products obtained with mutation-specific primers (Table 2)

Sampling site	Date	Sequence accession no.				
		*mcyD*IS1 5′ end	*mcyD*IS1 3′ end	*mcyA*IS 5′ end	*mcyA*IS 3′ end	*mcyHA* deletion
Irrsee	1 December 03		AM040466			
Mondsee	15 April 03			AM040471	AM040478	
Mondsee	29 April 03			AM040472	AM040476	
Mondsee	14 October 03			AM040473	AM040479	AM040482
Mondsee	11 November 03	AM040468	AM040461	AM040474	AM040477	AM040483
Offensee	1 July 03		AM040462, AM040467			
Offensee	10 October 02		AM040465			
Wörthersee	26 September 01	AM040469	AM040463		AM040480	AM040484
Wörthersee	28 August 03	AM040470	AM040464	AM040475	AM040481	AM040485

62, and 65 and spanned 1,869 bp within *mcyH* and *mcyA*, from the Walker motif in *mcyH* to the core motif A2 of the first adenylation domain (Ad) of *mcyA* (Ad1). This deletion was only found in genotypes containing *mcyA* Ad1 without the *N*-methyltransferase domain and not in genotypes containing *mcyA* Ad1 with the NMT domain (14). The second deletion (called the *mcyB* deletion) was identified with primer pair 22 and was located within *mcyB* Ad1 of strain CCAP1459/36, resulting in the loss of 400 bp located before the core motif A2. For the other 16 strains analyzed, no detectable differences in PCR product size could be identified.

Frequency of occurrence of mutant *mcy* genotypes in field samples. In total, 123 samples from six lakes were analyzed, and all samples (except for two integrated samples from Lake Schwarzensee [collected on 15 April 2004 and 8 July 2004]) gave positive PCR signals for PC-IGS and *mcyA*, implying that *Planktothrix* and *mcy* gene-containing genotypes were present (Table 4). The extremely low density (zero filaments in 50 ml of sedimentation volume) in Lake Schwarzensee was responsible for the two negative sampling dates, since the net samples taken in parallel gave PCR products indicative of *Planktothrix* and *mcyA*. Otherwise, there was a good correlation between the frequencies of occurrence of PCR results from net samples and integrated samples, indicating that the *mcy* genotype composition did not differ between plankton net samples (containing a >1,000-fold larger sample of the population in terms of individual numbers) and integrated samples (containing all the individuals sampled from approximately 4 liters of integrated lake water).

PCR products indicative of insertions (*mcyD*IS1 and *mcyA*IS) were found to be ubiquitously distributed, i.e., the *mcyD*IS1 genotype was found in Lakes Mondsee, Wörthersee, Offensee, and Irrsee and the *mcyA*IS genotype was found in Lakes Mondsee and Wörthersee (Table 4; see the supplemental material). The *mcyHA* deletion was detected in Lakes Mondsee and Wörthersee. The *mcyB* deletion was not detected in field samples. For Lakes Mondsee and Wörthersee, both insertions (*mcyD*IS1 and *mcyA*IS) and the *mcyHA* deletion were found regularly over 4 years. In net samples from Lake Mondsee, the *mcyHA* deletion and *mcyD*IS1 were found as frequently as *mcyA* and PC-IGS. For net samples from Lake Wörthersee, both insertions and the *mcyHA* deletions occurred with a 100% frequency of detection. For populations showing the lowest abundance of *Planktothrix* throughout the study period (Lakes Wolfgangsee and Schwarzensee), no *mcy* mutations were detected.

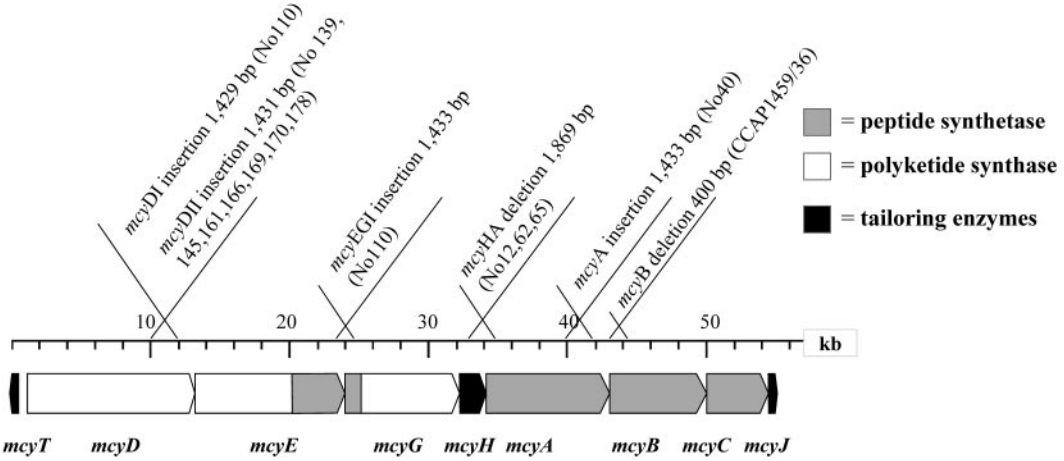

FIG. 1. *mcy* gene cluster of *Planktothrix* isolate CYA126/8 (2) and locations of mutations found in inactive *mcy* genotypes. Strains 110 and 40 were isolated from Lake Mondsee (Austria), strains 139, 145, 161, 166, 169, and 170 were isolated from Lake Grabensee (Austria), strain 178 was isolated from Lake Fuschlsee (Austria), strains 12, 62, and 65 were isolated from Lake Irrsee (Austria), and strain CCAP1459/36 was isolated from Lake Gjersjoen (Norway).

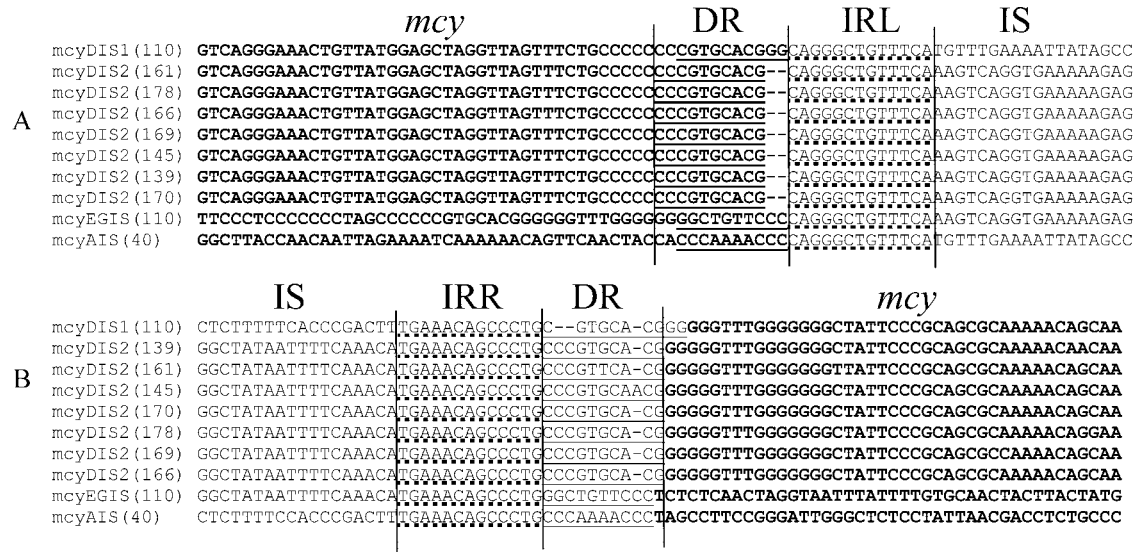

FIG. 2. Sequence alignment of putative transposases (*mcyD*IS1 and *mcyD*IS2, observed in *mcyD* [at base pair 11,918 of the *mcy* gene cluster of *Planktothrix*; AJ441056], *mcyEG* [at base pair 23,822], and *mcyA* [at base pair 41,274]). The 5′ end (A) and the 3′ end (B) of each insertion (IS) and part of the *mcy* genes (bold) are shown. The short directly repeated sequences (DR) of 10 bp in length (straight lines) and the left inverted repeats (IRL) and right inverted repeats (IRR) (dotted lines) are indicated.

Similarity of sequences of *mcy* mutations from field samples. Twenty-five PCR amplification products of mutant genotypes from field samples were sequenced in order to validate the results of PCR detection and to find out whether the insertion events observed among strains may occur independently in nature. In general, the similarity among sequences of mutations, even if obtained from different populations, was high. Sequence identities for the sequences obtained from the 5′ end and 3′ end of *mcyD*IS1 were 96.6 to 100% (270 bp; $n = 4$) and 95.9 to 100% (198 bp; $n = 8$), respectively. According to the difference in DR sequences of the *mcyD*IS1 3′ ends, two independently arisen genotypes occurred in lake samples, and one *mcyD*IS1 3′-end genotype was found in Lake Offensee only (see the supplemental material). In contrast, only one *mcyA*IS genotype DR sequence was observed, and sequence identities for the 5′ end and 3′ end of *mcyA*IS were 99.5 to 100% (369 bp; $n = 6$) and 99 to 100% (298 bp; $n = 7$), respectively. All *mcyHA* deletion events occurred at the same position of the *mcy* gene cluster, and the sequences flanking the *mcyHA* deletion (266 bp; $n = 4$) were identical. The results demonstrate that mutations within *mcy* may arise independently and are detectable for years.

DISCUSSION

Types of naturally occurring mutations. Of the 29 strains that were inactive in microcystin synthesis, 13 were found to contain mutations within the *mcy* gene cluster. One strain, no. 110, even contained two mutations (*mcyD*IS1 and *mcyEG*IS). Since *mcyEG*IS was located in the spacer region between *mcyE*

TABLE 4. Occurrence of *P. rubescens*, biovolume and filament abundance in study lakes, and numbers of integrated and net samples showing specific mutations

Lake	Sampling time (day.mo.yr) (no. of sampling dates)	Biovolume (mm^3 $liter^{-1}$) (minimum-median-maximum)	No. of filaments per ml[a] (minimum-median-maximum)	Integrated sample (I)/net sample (N)	No. of samples with indicated mutation (relative proportion [%])[b]					
					PC-IGS	*mcyA*	*mcyA*IS (insertion)	*mcyD*IS1 (insertion)	*mcyB* (deletion)	*mcyHA* (deletion)
Irrsee (Austria)	9.9.2002–2.8.2004 (18)	0.001-0.015-0.09	0.04-0.7-3.9	I	18 (100)	18 (100)	0	1 (6)*	0	0
	9.9.2002–2.8.2004 (18)			N	18 (100)	18 (100)	0	0	0	0
Mondsee (Austria)	25.9.2001–6.8.2004 (23)	0.06-0.31-1.81	5.9-25.9-227.1	I	23 (100)	23 (100)	15 (65)	18 (78)	0	22 (96)
	25.9.2001–6.8.2004 (23)			N	23 (100)	23 (100)	14 (61)*	22 (96)*	0	23 (100)*
Offensee (Austria)	5.8.2002–13.7.2004 (5)	0.02-0.23-0.54	1.8-5.7-42.9	I	5 (100)	5 (100)	0	0	0	0
	5.8.2002–13.7.2004 (6)			N	6 (100)	6 (100)	0	2 (33)*	0	0
Schwarzensee (Austria)	10.9.2001–8.7.04 (6)	0-0-0.01	0-0-1.4	I	4 (67)	4 (67)	0	0	0	0
	10.9.2001–8.7.04 (7)			N	7 (100)	7 (100)	0	0	0	0
Wolfgangsee (Austria)	26.6.2003–21.6.2004 (4)	0	0	I	4 (100)	4 (100)	0	0	0	0
	26.6.2003–21.6.2004 (4)			N	4 (100)	4 (100)	0	0	0	0
Wörthersee (Austria)	16.10.03–23.8.04 (4)	1.9-2.2-2.5	120-185-250	I	4 (100)	4 (100)	2 (50)	4 (100)	0	4 (100)
	26.9.2001–23.8.2004 (5)			N	5 (100)	5 (100)	5 (100)*	5 (100)*	0	5 (100)*

[a] For Lugol counting, the detection limit is one filament in 50 ml sedimentation volume.
[b] *, for sequence accession numbers, see Table 3.

and *mcyG*, it should not disturb the translation process and result in a nonfunctional protein as *mcyD*IS1 does. The other 16 strains without detectable MC did not reveal insertions or deletions, and consequently, they may have acquired point mutations within the *mcy* gene cluster, as suggested by other authors (9, 23). This study is the first showing that mutations do occur frequently within the *mcy* gene cluster and that a large proportion of mutations are caused by insertion of an IS element at different sites (observed in 9 of 13 strains). A relatively large number of genes encoding putative transposases have been reported for sequenced genomes of *Nostoc* PCC7120 (10), *Nostoc punctiforme* (18), and *Synechocystis* PCC6803 (11). Transposition has been reported to result in different *Synechocystis* PCC6803 genotypes (24) and to affect gas vesicle genes in *Microcystis* PCC7806 (20). The IS elements found in this study contained ORFs that putatively encode a transposase of 363 amino acids, including the conserved domains assigned to the DDE motif of the active site, which are involved in DNA cleavage at a specific site followed by a strand transfer reaction (16). It is obvious from the different insertion sites and flanking regions that insertion of this IS element occurred several times. No stop codons were detected within the ORFs, i.e., transposition activity should still be maintained. Notably, characterization of the *mcy* gene clusters from *Anabaena*, *Microcystis*, and *Planktothrix* and the *nda* gene cluster from *Nodularia* revealed associations with transposases (2, 21, 27, 36). Those transposases were members of families distinct from that of the transposase observed in this study.

Frequency of occurrence of mutations. Although *mcyD*IS1, *mcyA*IS, and *mcyHA* were found ubiquitously distributed, the populations investigated in this study were found to differ in the frequency of occurrence of these mutations. Since PCR assays for the total population (PlPc+/−) and for the *mcyD*IS1 3′ end and *mcyHA* were shown to have the same detection limit, the lower abundance in some populations is considered unbiased by the PCR approach used in this study. The results correspond to an earlier quantification of inactive *mcy* genotypes (13) in Lake Mondsee (21% inactive genotypes) and Lake Irrsee (5% inactive genotypes) and may imply that transposase-mediated mutations do occur more frequently in populations with a larger number of individuals. The relatively high proportion of *mcy* mutants occurring in populations is surprising since it indicates that MC is not needed for the survival of individual cells in nature. Moreover, the mutants have existed for long periods of time, as indicated by the detection of some of them in different lakes and by point mutations that allow for discrimination between genotypes bearing the same mutation. Indeed, directed mutagenesis of *mcy* genes in *Microcystis* PCC7806 revealed no difference in growth between mutant and wild-type cells under different light conditions (8). On the other hand, no population has been found without any MC production, and it may be assumed that MC as a toxin is necessary to keep the ambient density of a specific group of potential antagonists low (15, 28, 29, 31). Thus, it may be sufficient if some of the genotypes in a population produce MC in defense against grazers or other organisms.

Microevolution of *mcy* genes. The mutants identified during this study support the idea of a frequent loss of the ability to synthesize MC during evolution (25). On the other hand, the inactivation of the *mcy* gene cluster by transposable elements as observed in this study might be seen as an intermediate step in reorganization of the *mcy* gene cluster towards cell types with modified MC synthesis. Typically, organisms that lack an immune system are prolific producers of secondary metabolites (33). Recombination has been recognized as a general feature in the formation of *mcy* gene clusters for the synthesis of new structural variants of MC (14, 19, 35) and the related toxic peptide nodularin (21). Generally, transposases have been recognized as a major factor in the rearrangement of genes (30). In this study, *mcyE* was found flanked by two functional IS modules resembling transposon elements shown to mobilize DNA sequences encoding antibiotic resistance. It remains to be established whether there is a connection between the recombination processes observed within *mcy* genes, resulting in the formation of new MC variants, and the nonrandom mutations induced by transposable elements observed in this study.

ACKNOWLEDGMENTS

We are grateful to Martin Meixner for DNA sequencing. Johanna Schmidt provided excellent technical assistance. We are grateful to the Federal Agency for Water Management, Scharfling (Upper Austria), Austria, for field sampling (by Günter Bruschek and Karl Mayrhofer). Gerti Roidmayr counted *Planktothrix rubescens* cell numbers.

This study was supported by grant P15709 from the Austrian Science Fund to R.K. (Linking CYanoTOxin production to GENEtic diversity).

REFERENCES

1. **Anagnostidis, K., and J. Komarek.** 1988. Modern approach to the classification system of cyanophytes, 3-Oscillatoriales. Algol. Stud. **50–53:**327–472.
2. **Christiansen, G., J. Fastner, M. Erhard, T. Börner, and E. Dittmann.** 2003. Microcystin biosynthesis in *Planktothrix*: genes, evolution, and manipulation. J. Bacteriol. **185:**564–572.
3. **Davies, D. R., L. M. Braam, W. S. Reznikoff, and I. Rayment.** 1999. The three-dimensional structure of a Tn*5* transposase-related protein determined to a 2.9-A resolution. J. Biol. Chem. **274:**11904–11913.
4. **Dittmann, E., B. A. Neilan, M. Erhard, H. von Döhren, and T. Börner.** 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.
5. **Felsenstein, J.** 1993. PHYLIP (Phylogeny Inference Package), version 3.5c. Department of Genetics, University of Washington, Seattle, Wash.
6. **Fujii, K., K. Sivonen, E. Naganawa, and K. Harada.** 2000. Non-toxic peptides from toxic cyanobacteria, *Oscillatoria agardhii*. Tetrahedron **56:**725–733.
7. **Gassner, H., A. Jagsch, D. Zick, G. Bruschek, and I. Frey.** 2002. Die Wassergüte ausgewählter Seen des oberösterreichischen und steirischen Salzkammergutes, Band 15. Schriftenreihe BAW, Vienna, Austria.
8. **Hesse, K., E. Dittmann, and T. Börner.** 2001. Consequences of impaired microcystin production for light-dependent growth and pigmentation of *Microcystis aeruginosa* PCC 7806. FEMS Microbiol. Ecol. **37:**39–43.
9. **Kaebernick, M., T. Rohrlack, K. Christoffersen, and B. A. Neilan.** 2001. A spontaneous mutant of microcystin biosynthesis: genetic characterization and effect on *Daphnia*. Environ. Microbiol. **3:**669–679.
10. **Kaneko, T., Y. Nakamura, C. P. Wolk, T. Kuritz, S. Sasamoto, A. Watanabe, M. Iriguchi, A. Ishikawa, K. Kawashima, T. Kimura, Y. Kishida, M. Kohara, M. Matsumoto, A. Matsuno, A. Muraki, N. Nakazaki, S. Shimpo, M. Sugimoto, M. Takazawa, M. Yamada, M. Yasuda, and S. Tabata.** 2001. Complete genomic sequence of the filamentous nitrogen-fixing cyanobacterium *Anabaena* sp. strain PCC 7120. DNA Res. **8:**205–213.
11. **Kaneko, T., S. Sato, H. Kotani, A. Tanaka, E. Asamizu, Y. Nakamura, N. Miyajima, M. Hirosawa, M. Sugiura, S. Sasamoto, T. Kimura, T. Hosouchi, A. Matsuno, A. Muraki, N. Nakazaki, K. Naruo, S. Okumura, S. Shimpo, C. Takeuchi, T. Wada, A. Watanabe, M. Yamada, M. Yasuda, and S. Tabata.** 1996. Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. DNA Res. **3:**109–136.
12. **Kurmayer, R., G. Christiansen, and I. Chorus.** 2003. The abundance of microcystin-producing genotypes correlates positively with colony size in *Microcystis* and determines its microcystin net production in Lake Wannsee. Appl. Environ. Microbiol. **69:**787–795.
13. **Kurmayer, R., G. Christiansen, J. Fastner, and T. Börner.** 2004. Abundance

of active and inactive microcystin genotypes in populations of the toxic cyanobacterium *Planktothrix* spp. Environ. Microbiol. **6:**831–841.
14. **Kurmayer, R., G. Christiansen, M. Gumpenberger, and J. Fastner.** 2005. Genetic identification of microcystin ecotypes in toxic cyanobacteria of the genus *Planktothrix*. Microbiology **151:**1525–1533.
15. **Kurmayer, R., and F. Jüttner.** 1999. Strategies for the co-existence of zooplankton with the toxic cyanobacterium *Planktothrix rubescens* in Lake Zürich. J. Plankton Res. **21:**659–683.
16. **Mahillon, J., and M. Chandler.** 1998. Insertion sequences. Microbiol. Mol. Biol. Rev. **62:**725–774.
17. **Marahiel, M. A., T. Stachelhaus, and H. D. Mootz.** 1997. Modular peptide synthetases involved in nonribosomal peptide synthesis. Chem. Rev. **97:** 2651–2673.
18. **Meeks, J. C., J. Elhai, T. Thiel, M. Potts, F. Larimer, J. Lamerdin, P. Predki, and R. Atlas.** 2001. An overview of the genome of *Nostoc punctiforme*, a multicellular, symbiotic cyanobacterium. Phycol. Res. **70:**85–106.
19. **Mikalsen, B., G. Boison, O. M. Skulberg, J. Fastner, W. Davies, T. M. Gabrielsen, K. Rudi, and K. S. Jakobsen.** 2003. Natural variation in the microcystin synthetase operon *mcyABC* and impact on microcystin production in *Microcystis* strains. J. Bacteriol. **185:**2774–2785.
20. **Mlouka, A., K. Comte, A.-M. Castets, C. Bouchier, and N. Tandeau de Marsac.** 2004. The gas vesicle gene cluster from *Microcystis aeruginosa* and DNA rearrangements that lead to loss of cell buoyancy. J. Bacteriol. **186:** 2355–2365.
21. **Moffitt, M. C., and B. A. Neilan.** 2004. Characterization of the nodularin synthetase gene cluster and proposed theory of the evolution of cyanobacterial hepatotoxins. Appl. Environ. Microbiol. **70:**6353–6362.
22. **Moore, R. E., J. L. Chen, B. S. Moore, and G. M. L. Patterson.** 1991. Biosynthesis of microcystin-LR. Origin of the carbons in the Adda and Masp units. J. Am. Chem. Soc. **113:**5083–5084.
23. **Nishizawa, T., M. Asayama, K. Fujii, K. I. Harada, and M. Shirai.** 1999. Genetic analysis of the peptide synthetase genes for a cyclic heptapeptide microcystin in *Microcystis* spp. J. Biochem. **126:**520–529.
24. **Okamoto, S., M. Ikeuchi, and M. Ohmori.** 1999. Experimental analysis of recently transposed insertion sequences in the cyanobacterium *Synechocystis* sp. PCC6803. DNA Res. **6:**265–273.
25. **Rantala, A., D. P. Fewer, M. Hisbergues, L. Rouhiainen, J. Vaitomaa, T. Börner, and K. Sivonen.** 2004. Phylogenetic evidence for the early evolution of microcystin synthesis. Proc. Natl. Acad. Sci. USA **101:**568–573.
26. **Rippka, R.** 1988. Isolation and purification of cyanobacteria. Methods Enzymol. **167:**3–27.
27. **Rouhiainen, L., T. Vakkilainen, B. L. Siemer, W. Buikema, R. Haselkorn, and K. Sivonen.** 2004. Genes coding for hepatotoxic heptapeptides (microcystins) in the cyanobacterium *Anabaena* strain 90. Appl. Environ. Microbiol. **70:**686–692.
28. **Sedmak, B., and G. Kosi.** 1998. The role of microcystins in heavy cyanobacterial bloom formation. J. Plankton Res. **20:**691–708.
29. **Shapiro, J. A.** 1998. Thinking about bacterial populations as multicellular organisms. Annu. Rev. Microbiol. **52:**81–104.
30. **Shapiro, J. A.** 1999. Transposable elements as the key to a 21st century view of evolution. Genetica **107:**171–179.
31. **Skulberg, O. M.** 2000. Microalgae as a source of bioactive molecules—experiences from cyanophyte research. J. Appl. Phycol. **12:**341–348.
32. **Stachelhaus, T., H. D. Mootz, and M. A. Marahiel.** 1999. The specificity-conferring code of adenylation domains in nonribosomal peptide synthetases. Chem. Biol. **6:**493–505.
33. **Stone, M. J., and D. H. Williams.** 1992. On the evolution of functional secondary metabolites (natural products). Mol. Microbiol. **6:**29–34.
34. **Suda, S., M. M. Watanabe, S. Otsuka, A. Mahakahant, W. Yongmanitchai, N. Nopartnaraporn, Y. Liu, and J. G. Day.** 2002. Taxonomic revision of water-bloom-forming species of oscillatorioid cyanobacteria. Int. J. Syst. Evol. Microbiol. **52:**1577–1595.
35. **Tanabe, Y., K. Kaya, and M. M. Watanabe.** 2004. Evidence for recombination in the microcystin synthetase (*mcy*) genes of toxic cyanobacteria *Microcystis* spp. J. Mol. Evol. **58:**633–641.
36. **Tillett, D., E. Dittmann, M. Erhard, H. von Döhren, T. Börner, and B. A. Neilan.** 2000. Structural organization of microcystin biosynthesis in *Microcystis aeruginosa* PCC7806: an integrated peptide-polyketide synthetase system. Chem. Biol. **7:**753–764.
37. **Utermöhl, H.** 1958. Zur Vervollkommnung der quantitativen Phytoplanktonmethodik. Mitt. Int. Verein. Limnol. **2:**1–38.