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Diversity of Microcystin Genes within a Population of the Toxic Cyanobacterium *Microcystis* spp. in Lake Wannsee (Berlin, Germany)

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Abstract

In order to find out how many genotypes determine microcystin production of *Microcystis* spp. in field populations, single colonies (clones) were sampled from Lake Wannsee (Berlin, Germany), characterized morphologically, and subsequently analyzed by PCR for a region within the *mcyB* gene encoding the activation of one amino acid during microcystin biosynthesis. The different morphospecies varied considerably in the proportion of microcystin-producing genotypes. Most colonies (73%) of *M. aeruginosa* contained this gene whereas only 16% of the colonies assigned to *M. ichthyoblabe* and no colonies of *M. wesenbergii* gave a PCR product of the *mcyB* gene. Restriction fragment length polymorphism revealed seven restriction profiles showing low variability in nucleotide sequence within each restriction type (0.4–4%) and a low to high variability (1.6–38%) between restriction types. In addition, the sequences of amino acids within the *mcyB* gene were analyzed to compare the specificity of the amino acid activation during microcystin biosynthesis between restriction types and with the occurrence of amino acids in microcystin variants as detected by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Most of the microcystin-producing colonies showed high similarity in the sequence of amino acids and contained microcystin-LR (LR refers to leucine and arginine in the variable positions of the heptapeptide), microcystin-RR, and microcystin-YR, as well as other variants in minor concentrations. It is concluded that the gene product found for most of the microcystin-producing colonies in the lake is rather unspecific and the diversity of microcystin variants in the lake results from activation of various amino acids during microcystin biosynthesis in the same genotypes.

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Introduction

The cyanobacterium *Microcystis* frequently develops waterblooms containing the hepatotoxin, microcystin. A considerable number of studies have focused on the environmental factors that can influence microcystin production in the laboratory [37] and in the field [18, 26]. These show that environmental factors can modulate cellular microcystin content three- to fourfold. It is also known that both microcystin, and non-microcystin-producing strains can be isolated from one water sample [34, 41] and laboratory surveys revealed large differences in cellular microcystin content between a number of microcystin-producing strains [2]. Consequently, it is necessary to differentiate between the effects of genetic composition and environmental conditions. The understanding of the factors influencing the diversity of strains with and without microcystins in the field is very poor. This is attributable to the lack of methods for the characterization of genotypes within the genus *Microcystis*, which makes it difficult to observe the population development of specific genotypes in the field.

The traditional approach to characterization of genotypes includes morphological criteria such as cell size, colony form, and sheath characteristics [17]. Though stability of morphological characters has been confirmed [9], classification of smaller colonies according to morphology often becomes difficult and transitions between morphological characteristics exist, e.g., during the annual cycle of a population [33]. Otsuka et al. [30] reported some morphological variability of specific *Microcystis* strains in culture. Efforts to link morphology with the ability of microcystin production revealed contradictory results: Watanabe et al. [42] and Watanabe [43] found some correlation of microcystin production with morphology, e.g., *M. viridis* and *M. wesenbergii* have been generally considered as toxic and nontoxic, respectively. In contrast, Otsuka et al. [28] concluded that most morphologically distinguishable species (thereafter referred to as morphospecies) include both toxic and nontoxic strains. If morphological characteristics were related to microcystin production, they could be used to investigate the ecological success of microcystin producers and non-microcystin producers in the field.

In addition to morphological traits, genetic criteria based on the 16S rDNA sequence [23, 27], the 16S to 23S internal transcribed spacer sequence [29], and the intergenic spacer region within the phycocyanin (ITS-PC) operon [22, 28] have been used to discriminate between genotypes. The level of conservation in these genes is rather high [28, 35] making it difficult to amplify DNA from *Microcystis* only, without including that from other taxa in the field sample. In addition, it is difficult to resolve genetic differences within a closely related taxon by routine molecular biological tools such as restriction fragment length polymorphism (RFLP). On the other hand, cyanobacteria in general have been discovered to be a rich source of different non-ribosomal peptides and the differences between the peptide synthetase gene sequences were found to be large [24]. More than thirty variants of microcystins have been identified from *Microcystis* alone [37]. It is not yet clear whether this variability is due to genetic variability and/or due to environmental effects such as light availability (e.g., [40]) or other modifying factors. However, the differences in peptide content between strains observed in the laboratory suggest a higher degree of genetic variability as compared to 16S rDNA sequences and frequently used ITS regions.

The microcystins are cyclic heptapeptides and share the common structure cyclo (-D-Ala-L-X-, D-MeAsp-L-Z-Adda-D-Glu-Mdha), where X and Z are variable L-amino acids (e.g., LR refers to leucine and arginine in the variable positions), D-MeAsp is D-erythro-ß-iso-aspartic acid, Adda is (2*S*, 3*S*, 8*S*, 9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, and Mdha is *N*-methyl-dehydroalanine [3]. They have only been found in cyanobacteria and are synthesized by thiotemplate mechanism like other nonribosomal peptides (e.g., antibiotics such as gramicidin, penicillin, tyrocidin) produced by bacteria and fungi [21]. The large gene cluster encoding peptide synthetases and polyketide synthases for microcystin biosynthesis has a modular structure [8, 25, 39]. Each unit of the large multifunctional peptide synthetases contains specific functional domains for activation, aminoacyl adenylation, and thioesterification of the amino acid substrate and for the elongation of the growing peptide [21, 39]. These domains show a high degree of conservation (core motifs) enabling the definition of general rules for the structural basis of substrate recognition in adenylation domains of non-ribosomal peptide synthetases. The determination of substrate specificity is based on the crystallization of the adenylation domain of gramicidin synthetase (GrsA) [5]. This enabled the identification of critical binding-pocket residues in adenylation domains which have been correlated with amino acid substrates [4, 38] and the so called specificity-conferring code of ade-

nylation domains has been defined. This algorithm allows the specificity of adenylation domains of a module to be predicted from a few critical amino acids (signature sequences) lining putative binding pockets. The structural organization of microcystin biosynthesis in *M. aeruginosa* has been elucidated, e.g., the first adenylation domain of *mcyB* is expected to activate one of the variable amino acids in the microcystin molecule at the X position [39]. This algorithm provides an excellent opportunity to compare the genetic diversity within the adenylation domain of *mcyB* with the diversity of microcystin variants observed in the field.

This study addresses the questions (1) whether microcystin production is related to morphological characteristics of the colonies in the field and (2) how much genetic variability can be found within and between co-occurring morphospecies. In addition, it tested whether the genetic variability results in the diversity of microcystin variants commonly found in nature. For this purpose different colonies (clones) of *Microcystis* were selected from a field population in one lake throughout the growing season, classified morphologically, and analyzed for the adenylation domain coded by the *mcyB* gene. In order to identify groups of more closely related genotypes the PCR products were digested by restriction endonucleases providing specific signature profiles. A number of restriction types were sequenced and the signature sequences used to compare the specificity-conferring code of adenylation domains between restriction types and with the occurrence of amino acids in microcystin variants as detected by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

Methods

Field Sampling

From February to September 2000 different morphospecies of *Microcystis* were collected by vertical plankton tows with nets of 40 and 340 μm in mesh size in Lake Wannsee (Berlin, Germany). The time period covered the start of population growth up to the period with the highest biomass of the bloom (20 mm^3 L^{-1}). Since the cell division process is accompanied by mucilage production, embedding the cells in a gel-like matrix, one colony of *Microcystis* can be considered a clonal unit. Of the individual colonies, 439 were isolated with forceps, washed in several drops of tap water, and checked under the microscope to guarantee that one colony can be considered as one clone. At least 20 colonies were isolated each month. Each colony was characterized by morphological criteria according to Komárek and Anagnostidis [17], measured in cell size, examined for epibionts, and stored frozen (−20°C) in 4 μl of Millipore water until DNA analysis. The colonies generally disintegrate in distilled water [31]. In addition, the colonies assigned to the species *M. aeruginosa* were classified according to the morphological criteria defined by Reynolds et al. [33].

Genetic Analysis

For PCR, 0.4 μl of the sample were pipetted into reaction tubes and incubated as described below. In order to check for the presence and the quality of the DNA each sample was analyzed for the phycocyanin gene (PC-ITS region, [22]) and the *mcyB* region which is indicative for the production of microcystin [8]. This was done simultaneously in the same thermal block of the cycler, and all samples yielding no phycocyanin (PC) product were omitted from further analysis because of suspected missing or low quality DNA. The microcystin primers amplified the regions within the microcystin synthetase module encoding the *mcyB* A1 domain [39]. The primers are specific for *Microcystis* and no cross reactions with other microcystin-producing genera (*Planktothrix*) have been observed. The sequences were tox4f: 5′-GGATATCCTCTCAGATTCGG-3′ and tox4r: 5′-CACTAACCC-CTATTTTGGATACC-3′. The primers had theoretical disassociation temperatures of 57 and 59°C and a total amplification fragment of 1312 bp (including the core motifs A3–A10, [21]). Amplifications were performed in a volume of 20 μl, containing 2 μl of PCR buffer (Gene Amp Gold buffer, Perkin-Elmer, Langen, Germany), 2.4 μl $MgCl_2$ (25 mM, Perkin-Elmer), 0.6 μl deoxynucleotide triphosphates (10 mM each), 1 μl of each primer (10 pmol), 0.08 μl AmpliTaq polymerase (Perkin-Elmer), 12.5 μl sterile Millipore water, and 0.4 μl of the sample. The PCR thermal cycling protocol included an initial denaturation at 94°C for 10 min, followed by 35 cycles at 94°C for 30 s, at an annealing temperature of 50°C for 30 s, and at 72°C for 1.5 min. Restrictions for *mcyB* were performed directly from the amplification product using Bsp143I (MBI Fermentas, St. Leon-Rot, Germany) according to standard protocols [36]. In the course of pilot experiments, restrictions obtained directly from the amplified products were compared to restrictions obtained from purified amplification products and the results did not differ. PCR products were analyzed by electrophoresis in 1% agarose in 0.5× TBE-buffer and for restriction fragments 2% agarose was used. Two strains were used as reference strains for the restriction analysis of the *mcyB* gene: *Microcystis* PCC7806 (isolated in 1972 from Braakman Reservoir, The Netherlands, AF 183408) and *Microcystis* HUB 5-2-4 (isolated 1978 from Lake Pehlitzsee in Brandenburg, Germany, Z28338).

For sequencing, the amplification products for *mcyB* were purified using the NucleoSpin Extract (Macherey-Nagel GmbH, Düren, Germany) and sequenced directly by standard automated fluorescence techniques (Applied Biosystems, Weiterstadt, Germany). The prediction of amino acid recognition by the adenylation domain of the *mcyB* gene was performed following the

instructions at the Web site http://www.jhu.edu/~chem/townsend/blactam.html/. The amino acid sequence between the two core motifs A3 and A6 [21] was cut out and aligned with the A3–A6 phenylalanine activation domain sequence of the gramicidin synthetase (GrsA). The eight amino acids lining the putative binding pocket (signature sequences) were extracted using the eight amino acids lining the binding pocket of GrsA crystal structure as a template [5]. The amino acid to be activated by the critical residues of the adenylation domain was predicted by running the blast search using the "Assigned Database" which consists of the eight amino acids lining the binding pocket of 198 adenylation domains where the substrate has been proven experimentally.

Microcystin Analysis

A number of colonies was analyzed for the microcystin content by means of matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS). One μl of the sample was directly transferred onto a stainless steel template and immediately 1 μl of matrix (10 mg/ml 2,5-dihydroxybenzoic acid in water/acetonitrile (1:1) with 0.03 % trifluoroacetic acid) was added. From each colony positive ion mass spectra were recorded using a MALDI-TOF mass spectrometer (Voyager DE-PRO, PerSeptive BioSystems, Framingham, MA) as described previously [10]. After determination of monoisotopic mass values Post Source Decay (PSD) measurements were performed directly from the same sample on the template and microcystins identified by PSD fragment structure analysis [11].

Results

Morphospecies and Microcystin Genes

To control for biases due to the contamination of samples by single microcystin-producing cells originating from other colonies, a number of colonies were split in half and each parallel was analyzed separately. For non-microcystin-producing colonies all parallels of 31 tested colonies gave identical results. For microcystin-producing colonies all but five parallels of 27 tested colonies gave identical results. The result for these five may be due to contamination by single cells or inconsistencies during PCR analysis; in either case the bias did not exceed 10%. In addition, 78 samples were analyzed several times to yield PCR products for restriction and sequencing.

Figure 1 shows a typical result of amplified PC and *mcyB* products from colonies on one sampling date in June. Analogous tests from 18 sampling dates always indicated that some colonies did not contain the *mcyB* gene. All colonies were in the size range between 100 and 3600 μm diameter (mean 1150 μm). The smaller colonies (<500 μm) had the lowest proportion of microcystin-producing genotypes whereas for the larger colonies (>500 μm) the proportion was between 42 and 73% (Table 1).

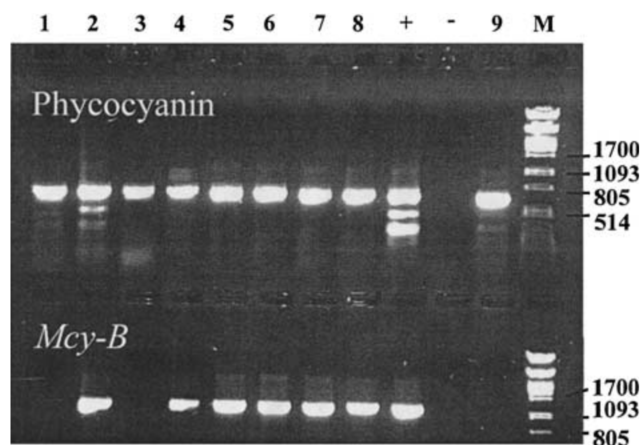

Fig. 1. Photograph of an ethidium-bromide stained gel showing the amplification products of the PC-ITS region (Phycocyanin) and the *mcyB* region for nine colonies isolated from a *Microcystis* sample from Lake Wannsee in June 2000. Six of nine colonies were found to contain the *mcyB* gene. M = molecular weight marker in base pairs. +,- = positive, negative controls. Size distribution of colonies from *Microcystis* spp. in Lake Wannsee (Berlin, Germany) in spring and summer 2000 and proportion of those containing the *mcyB* gene indicative of microcystin production

The colonies were classified into five morphospecies (Table 2). These morphotypes differed in colony form, arrangement of cells, fragility, cell diameter, and number and localization of epibionts. The description for the morphotype for *M. ichthyoblabe* corresponds to the description for the small cell size group (the S group) within *M. aeruginosa* described by Kato et al. [16] and Watanabe

Table 1. Size distribution of colonies from *Microcystis* spp. in Lake Wannsee (Berlin, Germany) in spring and summer 2000 and proportion of those containing the *mcyB* gene indicative of microcystin production

Size class (ìm)	No. of colonies tested	Percent with mcyB
100–300	20	10
300–500	39	15
500–700	48	56
700–900	67	48
900–1100	55	55
1100–1300	62	57
1300–1500	43	54
1500–1700	33	42
1700–1900	15	73
1900–2100	24	63
>2100	33	67

Table 2. Morphotypes of *Microcystis* isolated in Lake Wannsee (Berlin, Germany) in spring and summer 2000[a]

Morphospecies	Colony form	Firmness	Diameter (ìm)	Cell size (ìm)	Epibionts	*N*
M. aeruginosa	Spherical, elongated or lobated with holes, sometimes reticulated and ± wide mucilage without a distinct margin, largest nets, irregularly arranged and densely packed cells	Firm	100–3500 (1260)	3.1–7.4 (4.9)	Abundant (*Nitzschia* sp., green algae, *Vorticella* sp., *Pseudanabaena mucicoca*), at the margin	227
M. botrys	Spherical–elongated, not lobated and composed of sub colonies, mucilage with wide margin around cells, cells densely packed	Firm	200–1960 (750)	4.9–6.1 (5.1)	Abundant (*Nitzschia* sp., green algae, *Vorticella* sp.), at the margin, between subcolonies	29
M. flos-aquae	Irregular–spherical, not lobated and only indistinct holes, mucilage with narrow margi around cells, cells irregularly arranged and ± densely packed cells	Fragile	180–2500 (1170)	3.7–4.9 (4.6)	Abundant (*Nitzschia* sp., green algae, *Aulacoseira* sp., *P. mucicola), between cells*	57
Undetermined	Irregular–elongated, mucilage with wide and irregular margin and cells loosely packed, sometimes senescent	Fragile	180–2600 (560)	3.0–4.9 (4.9)	Abundant (*Nitzschia* sp., *Aulacoseira* sp., green algae), between cells	35
M. ichthyoblabe	Irregular–spherical subcolonies often grouped together and easily fragmented off, mucilage with narrow margin around cells, distribution of cells homogeneous and most densely packed	Fragile	120–2350 (1000)	3.0–4.9 (3.8)	Rare (*Nitzschia* sp., *Aulacoseira* sp., green algae, *Vorticella* sp.), between cells	70
M. wesenbergii	Elongated or lobated with holes, sometimes reticulate and mucilage with a distinct margin, smallest nets, large cells often loosely packed nd regularly arranged at the margin	Soft	200–2900 (1190)	4.9–6.7 (5.6)	Rare (*Nitzschia* sp., green algae, *Vorticella* sp.), at the margin	21

[a] Single colonies were characterized by morphological criteria according to Komárek and Anagnostidis [17] and subsequently analyzed for the *mcyB* gene indicative for microcystin production. Firmness according to breakability of the colony; colony diameter and cell size (μm) = min-max (mean), N = number of colonies sampled.

et al. [42]. A few colonies could not be assigned to a specific morphotype. These colonies generally were irregular in size, fragile, and had loosely packed cells and sometimes looked senescent (compare status I in Reynolds et al. [33]). *M. aeruginosa* and *M. ichthyoblabe* were most abundant, e.g., among 140 randomly collected colonies from a 40 μm net sample in July, 62% were classified as *M. ichthyoblabe,* 28% as *M. aeruginosa* with the status V (spherical colonies with densely aggregated cells, [33]), status II, and status III (elongated or lobated colonies with holes and compactly arranged cells in a central core [33]), and 9% as *M. botrys. M. wesenbergii* and *M. flos-aquae* constituted <1%. In addition, the morphospecies differed considerably in their proportion of microcystin-producing genotypes (Fig. 2A). Most colonies (73%) of *M. aeruginosa* contained the *mcyB* gene whereas only 16% of the colonies assigned to *M. ichthyoblabe* gave a PCR product of the *mcyB* gene. The proportion of colonies with the *mcyB* gene was lowest in *M. wesenbergii* (0%) and constituted 37 % in *M. flos-aquae* and 45% in *M. botrys.*

Restriction Profiles

Digestion of *mcyB* products with Bsp143I produced seven patterns for 172 sampled colonies (Table 3). An example of restriction profiles for 10 colonies in August is illustrated in Fig. 3. 28 colonies were analyzed twice and produced the same results. Type I resembled the pattern of *Microcystis* strain PCC7806 with two bands at 700 and 600 bp (base pairs); type II was identical to the pattern of the local

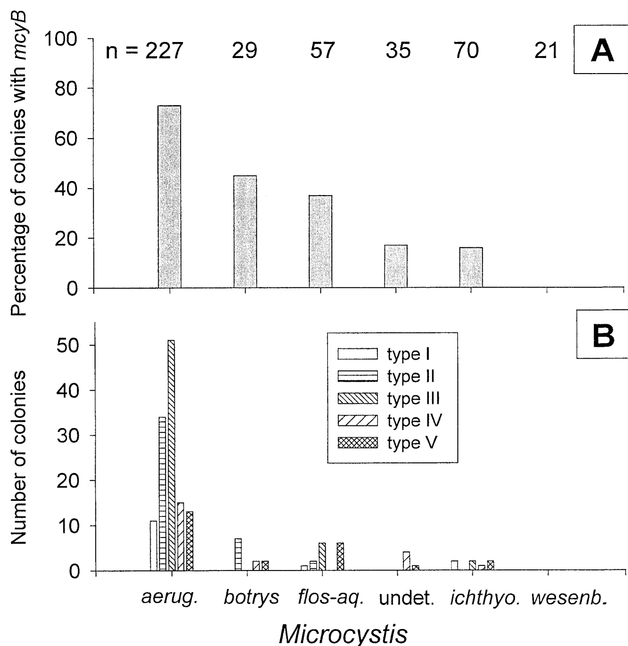

Fig. 2. Number of colonies from *Microcystis* spp. from net samples collected between February and September 2000 in Lake Wannsee (Berlin, Germany) assigned to morphospecies and tested for the *mcyB* gene indicative of microcystin production. (A) Number (*n*) of colonies tested and proportion of colonies which gave a *mcyB* product. (B) Frequency of restriction types (I to V) within each morphospecies. For description of restriction types see text.

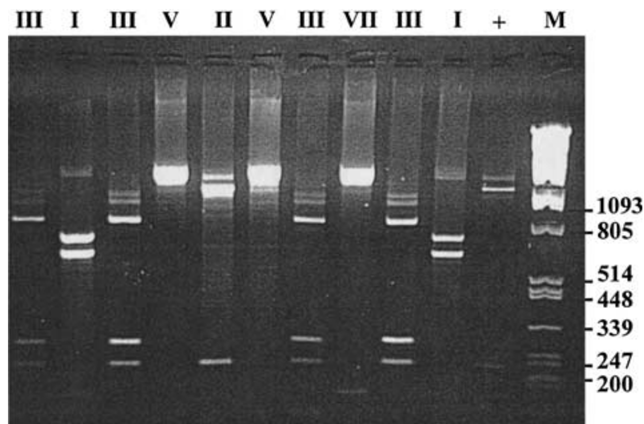

Fig. 3. Photograph of an ethidium-bromide stained gel (2% agarose) showing the RFLP profiles of the amplified *mcyB* A1 domain for 10 different colonies of *Microcystis* spp. in August 2000. The Latin numbers indicate the different signature profiles. For description of restriction types see text. M = molecular weight marker in base pairs, + = control (strain HUB 5-2-4).

strain HUB 5-2-4 with one band at 1100 bp and a smaller band at 200 bp. Type III characterized a new restriction profile with fragments at 800, 300, and 200 bp. Type IV comprised a profile similar to type II but with one larger digestion fragment (300 bp), and type V characterized a PCR product which was not digested at all. Type VI (two bands at 200 and 100 bp) and type VII (only one small fragment, 100 bp) were rather rare and were not considered quantitatively.

Table 3. Schematic representation of restriction profiles within the *mcyB* A1 domain found for colonies of *Microcystis* spp. isolated from Lake Wannsee in 2000[a]

bp	Type I	Type II	Type III	Type IV	Type V	Type VI	Type VII
1300					–		
1200							–
1100		–		–		–	
1000							
900							
800			–				
700	–						
600	–						
500							
400							
300			–	–			
200		–	–			–	
100						–	–

[a] For description of zrestriction types see text; size of fragments in base pairs (bp).

In order to test for the genetic similarity between colonies with the same restriction profile a number of PCR products from restriction types I–V were sequenced and the nucleotide sequences in the region of the core motifs A3 to A5 (approx. 450 bp) compared (see Table 4 for a list of the 23 sequenced colonies). In total, 14 different genotypes (which differ at least in one nucleotide position) were found. The variability in nucleotide sequence within a restriction type was rather low, e.g., 4% within type I and 0.4–2% within types II–V. In contrast, 38% of the bases differed when type I sequences were compared with sequences from type II. The difference was mainly due to basepair substitutions (30%) followed by deletions (5.6%) and insertions (2.6%). The nucleotide sequences of types III, IV, and V were much more similar to type II and differed only by 1.6–2% due to base pair substitutions. In summary, two groups of genotypes which differ significantly in nucleotide sequence were detected: the first group comprising restriction type I and the second group comprising type II–V (no sequences are available for types VI and VII).

M. aeruginosa samples most frequently (39%) showed the type III pattern after restriction (Fig.2b), followed by type II (26%) and type IV (16%). Type I and type V were found in similar abundance (around 10%). Compared with

Table 4. *mcyB* restriction types and the eight amino acids lining the putative binding pocket (signature sequences) for activation of the amino acid substrate during microcystin biosynthesis within the *mcyB* gene for different colonies of *Microcystis*[a]

			Position									
Date/strain	No.	Type	235	236	239	278	299	301	322	330	AA predicted	Identity
PCC 7806		I	D	A	W	F	L	G	N	V	Leu	8/8
16.05.00	9	I	D	A	L	F	L	G	N	L	Leu	6/8
27.06.00	39	I	D	A	W	F	I	G	N	V	Leu	8/8
08.08.00	14	I	D	A	W	F	I	L	N	P	Leu	6/8
HUB 5-2-4		II	D	G	W	T	I	G	A	V	D-/L-Phe;Val	5/8
22.02.00	9	II	D	G	W	T	I	G	A	V	D-/L-Phe;Val	5/8
13.06.00	7	II	D	G	W	T	I	G	A	G	Val	5/6
13.06.00	13	III	D	G	W	T	I	G	A	G	Val	5/6
13.06.00	15	III	D	G	W	T	I	G	A	V	D-/L-Phe;Val	5/8
13.06.00	16	III	D	G	W	T	I	G	A	V	D-/L-Phe;Val	5/8
13.06.00	20	III	D	G	W	T	I	G	A	V	D-/L-Phe;Val	5/8
13.06.00	27	III	D	G	W	T	I	G	A	V	D-/L-Phe;Val	5/8
13.06.00	35	III	D	G	W	T	I	G	A	V	D-/L-Phe;Val	5/8
13.06.00	49	III	D	G	W	T	I	G	A	V	D-/L-Phe;Val	5/8
13.06.00	58	III	D	G	W	T	I	G	A	V	D-/L-Phe;Val	5/8
4.07.00	2	III	D	G	W	T	I	G	A	V	D-/L-Phe;Val	5/8
11.07.00	17	III	D	G	W	T	I	G	A	V	D-/L-Phe;Val	5/8
30.05.00	9	IV	D	G	W	T	I	G	A	G	Val	5/6
30.05.00	12	IV	D	G	W	T	I	G	A	V	D-/L-Phe;Val	5/8
30.05.00	22	IV	D	G	W	T	I	G	A	V	D-/L-Phe;Val	5/8
11.07.00	15	IV	D	G	W	T	I	G	A	F	Val	5/6
13.06.00	22	V	D	G	W	T	I	G	A	V	D-/L-Phe;Val	5/8
13.06.00	34	V	D	G	W	T	I	G	A	F	Val	5/6
13.06.00	60	V	D	G	W	T	I	G	A	V	D-/L-Phe;Val	5/8
25.07.00	9	V	D	G	W	T	I	G	A	V	D-/L-Phe;Val	5/8
Variability			0%	16%	8%	16%	8%	4%	16%	28%		

[a] Database comparisons of numerous peptide synthesases with their corresponding product have allowed the prediction of amino acid activation by the so called specificity-conferring code of adenylation domains (http://www.jhu.edu/~chem/townsend/blactam.html). For the most abundant signature sequence (restriction type II–V) no clear precedent in the database could be found and two amino acids (D-/L-phenylalanine and valine) were predicted with identical probabilities. Identity = the number of identical amino acids in the determined signature sequences/the number of amino acids in the reference signature sequence (restriction type I: dbj|BAA83993.1| mcyB Microcystis aeruginosa; restriction type II–V: emb|CAA33603.1| grsA Brevibacillus brevis; gb|AAC45928.1|tyrocidine synthetase 1 Brevibacillus brevis; emb|CAA79245.1| enniatin synthetase Fusarium scirpi). Position = position of the eight amino acids lining the binding pocket of GrsA crystal structure.

M. aeruginosa the other morphospecies produced exactly the same restriction profiles, if they contained the *mcyB* gene. Because of the significantly lower proportion of *mcyB* products the frequency distribution was too sparse to be analyzed. Though the significant correlation of microcystin production with morphospecies the difference in morphology is not matched by genetic criteria within the group of the microcystin producers.

Amino Acid Sequences

To compare the restriction profiles with the amino acid sequences of the putative binding pocket the nucleotide sequences of the colonies were translated into amino acids. The eight amino acids lining the putative binding pocket (signature sequences) of the adenylation domain of the *mcyB* module are listed in Table 4 and two groups of putative pocket types could be recognized. The first group corresponded to restriction type I and only these signature sequences match with the reference sequence of *M. aeruginosa* PCC7806 in the database (dbj|BAA83993.1|). Running the blast search revealed the prediction of leucine as the amino acid substrate resulting in the synthesis of microcystin-LR.

The larger part of the colonies seemed to have no clear precedent in the database and produced a significantly different signature sequence resulting mostly in unclear prediction of the amino acid substrate, since both D-/L-phenylalanine and valine were predicted with equal probabilities. This group of colonies comprised all other restriction types (II–V) and the signature sequence was identical to the signature sequence of strain HUB 5-2-4.

Diversity of Microcystin Variants

To compare genetic diversity with diversity of microcystin variants 30 colonies were analyzed by MALDI-TOF MS

Table 5. Microcystins detected in colonies of *Microcystis* spp. in Lake Wannsee by MALDI-TOF MS[a]

Date/ strain	Colony no.	Restriction type $[M + H]^+$	Signature sequence	Asp-microcystin-LR 981	Microcystin-LR 995	Asp-microcystin-RR 1024	Microcystin-LR 1038	Micro-cystin-YR 1045	H4-Micro-cystin-YR 1049	Micro-cystin-WR 1068
16.05.00	9	I	DALFLGNL		–			–		
27.06.00	39	I	DALFIGNV		–			–		
HUB 5-2-4		II	DGWTIGAV	–	–	–	–	–		
22.02.00	9	II	DGWTIGAV	–	–	–	–	–		–
22.02.00	14	II			–		–			
07.03.00	8	II			–		–	–		
08.08.00	47	II		–	–		–	–	–	–
22.02.00	8	III				–	–			
13.06.00	13	III	DGWTIGAG	–	–	–	–	–	–	–
13.06.00	15	III	DGWTIGAV	–	–		–	–	–	–
13.06.00	20	III	DGWTIGAV		–		–	–		–
13.06.00	27	III	DGWTIGAV		–		–	–		–
13.06.00	35	III	DGWTIGAV		–		–	–	–	–
13.06.00	51	III		–	–	–	–	–	–	–
13.06.00	58	III	DGWTIGAV		–		–	–	–	–
04.07.00	2	III	DGWTIGAV		–		–	–		–
04.07.00	6	III		–	–	–	–	–	–	–
11.07.00	17	III	DGWTIGAV		–		–	–	–	–
30.05.00	9	IV	DGWTIGAG		–		–	–	–	–
30.05.00	12	IV	DGWTIGAV		–	–	–	–		
30.05.00	14	IV		–	–		–	–		–
30.05.00	22	IV	DGWTIGAV		–		–	–	–	
11.07.00	15	IV	DGWTIGAF	–	–		–	–		–
16.05.00	3	V		–	–		–	–		
16.05.00	13	V		–		–				
13.06.00	22	V	DGWTIGAV		–		–		–	–
13.06.00	60	V	DGWTIGAV	–	–		–	–		
25.07.00	9	V	DGWTIGAV	–	–	–	–		–	–
08.08.00	17	VI			–		–	–		–

[a] The Latin numbers indicate the restriction profiles. Signature sequence indicates that the sequence of the amino acid activation domain within the mcyB gene was determined and gives the code for the critical residues of amino acid activation identified (see also Table 4), grey = detectable; black = dominant; $[M + H]^+$ = positive ion mass spectra.

(Table 5). Seven microcystin variants could be detected, and in most samples microcystin MC-LR, MC-RR, and MC-YR were the dominant forms. This finding was also supported by quantitative measurements via HPLC (unpublished data). A few colonies had demethylated [D-Asp^3] variants such as [Asp^3] MC-LR and [Asp^3] MC-RR as their dominant microcystins and half of the colonies contained demethylated variants in detectable amounts. The microcystin variant [H_4] MC-YR was frequently detected but dominant in three colonies only. MC-WR was present in most of the colonies but never dominant.

Among the colonies with the signature sequence of restriction type I only two could be analyzed with MALDI-TOF, and these contained only MC-LR and MC-YR. The larger group of colonies with the second type of the signature sequence (restriction types II–V) contained a higher diversity of microcystins, usually MC-LR, -RR, -YR, and -WR. In summary, the signature sequence of the largest group of the colonies was rather unspecific, resulting in an unspecific gene product and the activation of various amino acids during microcystin biosynthesis.

Discussion

Morphospecies and Variability in Microcystin Production

The low proportions of microcystin-producing genotypes within specific morphotypes (*M. ichthyoblabe, M. wesenbergii*) correspond with the conclusions drawn by some Japanese researchers for these taxa [42, 43]. In contrast, Otsuka et al. [28] concluded that all morphospecies of *Microcystis* contain microcystin-producing strains. Since these surveys in Japan rely on culture material, no con-

clusions can be made on proportion of microcystin-producing genotypes within morphospecies in the field. The results of this study match those of an earlier study by Rohrlack et al. [34], who isolated a number of genotypes on one sampling date in Lake Wannsee in 1995. The cultures separated mainly into two groups according to microcystin production and cell size: a large-cell group producing microcystins and a small-cell group without microcystins. A similar study was carried out independently by Fastner et al. (unpublished data) in Lake Wannsee analyzing single colonies of *Microcystis* by means of MALDI-TOF MS for the presence of oligopeptides in general. The results concerning the correlation of morphospecies with microcystin content fit well to the genetic results of this study, e.g., colonies of *M. aeruginosa* generally contained microcystin, whereas colonies assigned to *M. ichthyoblabe* and *M. wesenbergii* did not contain microcystin but did contain other oligopeptides.

The evidence that morphological characteristics are in general related to microcystin production at least in one lake might provide a hint to explain toxin content of field populations by strain composition according to morphological criteria. For example, it is known that more than 80–90% of all samples dominated by *Microcystis* spp. contained microcystins or showed hepatotoxicity in Danish, German, Czech, and Korean surveys [6]. The variability in the microcystin content between water bodies was quite high (e.g., several hundredfold in the Czech republic). This range can only be explained by the difference in strain composition, and in agreement with our results from Lake Wannsee, *M. wesenbergii* contained little or no microcystin in samples from the Czech Republic [20]. On the other hand, *M. wesenbergii* was found to dominate hepatotoxic *Microcystis* blooms in Danish lakes [12, 13]. It is unclear to what extent these results may be affected by methodological problems such as species determination, variability in morphological characteristics (e.g., [30]), or cell counting. Consequently, tools independent of morphological characteristics are needed to characterize genotypes and to relatethem to microcystin production, and genetic criteria are likely to be more reliable.

Genetic Diversity

The development of molecular biological methods to recognize genotypes of cyanobacteria directly in the field is essential to study ecological genetics of cyanobacteria. With the methods applied here, DNA extraction was not necessary, and because of the specificity of the primers the cells could be used directly as the template for PCR. Finally, the sequencing of the amplified *mcyB* product provided strong evidence that only *Microcystis*-specific DNA was amplified and no amplifications of other peptide synthetases occurred.

The restriction profiles obtained in the present study could be a useful tool for characterization of microcystin-producing strains. The observation that the restriction profiles of the two strains (PCC 7806, HUB 5-2-4) cultured for nearly 30 years in the laboratory can still be easily found in the field might suggest that the total number of restriction types in our lakes is not extremely high. A characterization might be useful in testing for a relation between restriction type and the amount of microcystin produced. For example, it is known that not only is microcystin production in the field determined by the presence of strains with or without microcystins, but also the amounts of microcystin produced differ between strains by as much as three orders of magnitude [2].

In order to obtain a maximum of genetic diversity of colonies, the sampling dates were distributed over a longer time period. Some genotypes may have been missed, especially those growing only in smaller colonies below 200μm. However, the smaller colonies (<500 μm) turned out to constitute the lowest proportion of microcystin producers, so these are less likely to increase the diversity of microcystin-producing genotypes in Lake Wannsee.

The question of how genetic diversity is maintained in *Microcystis* populations is interesting but poorly resolved. *Microcystis* in general seem to have barriers for the exchange of chromosomal DNA, e.g., by transformation [8]. Cyanobacteria are known to possess several type-II restriction endonucleases [19] which digest any introduced and unmethylated DNA. On the other hand, Barker et al. [1] conclude that genetic exchange occurs between three different loci of *Nodularia* filaments in the Baltic Sea. The second mechanism that contributes to genetic diversity is the abundance of different genotypes in a population. Efforts to link the morphological and physiological characteristics of strains isolated from the field to environmental conditions demonstrated subtle differences in the response to changes of environmental conditions, e.g., some strains grow better under light-limiting conditions than other strains [14]. Recent work on the effect of critical light intensities on the outcome of competition between different algae species indicates that even subtle

differences can result in shifts in community composition, but this takes time (e.g., at least months in Huisman et al. [15]). Because of the rather small differences in growth rate response, the changes in the abundance of specific genotypes are predicted to be slow and to occur rather between years than during a season. The role of zooplankton in the selection of microcystin-producing vs non-microcystin-producing strains still needs to be elucidated, however, despite the considerable work on food selection of zooplankton during the past decades no evidence on the effect of zooplankton grazing on genetic diversity of *Microcystis* in the field has been found. In conclusion, the identification of single genotypes with regard to microcystin production can be considered promising tools to elucidate both mechanisms of DNA exchange between genotypes as well as mechanisms and timing of shifts in strain composition in the laboratory and in the field.

Genetic Diversity in Relation to Diversity of Microcystins

This study considers genetic diversity within a taxonomic group not only as diversity *per se* but also relates this diversity to the resulting gene product responsible for the biosynthesis of microcystin variants. Though a high degree of genetic diversity (7 restriction types) and microcystin diversity (7 microcystin variants) have been found, this diversity in restriction types was not linked to specific microcystin variants, e.g., all restriction types contained the same dominant microcystins. The first group of genotypes (restriction type I), which accounted for the minor part of the colonies, was very similar to strains PCC7806 and K-139, which have been reported to produce mainly microcystin-LR or demethylated variants [25, 39]. Minor amounts of MC-RR and MC-YR have been detected in PCC7806 [7, 8], suggesting the ability of the *mcyB* gene to code for more than one microcystin variant. Corresponding to this suggestion, the *mcyB* gene of the major fraction of the colonies appeared to result in a gene product with the potential to activate a variety of amino acids during microcystin biosynthesis [39]. It is therefore concluded that the genetic diversity found within this gene of *Microcystis* in the lake does not cause specific substrate activation during microcystin biosynthesis, but is unspecific. Environmental effects coupled with the availability of amino acids and effects of cell metabolism may play a role in the synthesis of microcystin variants. For example, different microcystin variants responded differently to growth stimuli in *Anabaena* [32], and van der Westhuizen et al. [40] found that light availability modified the occurrence of microcystin variants in *M. aeruginosa* UV-006.

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