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Abundance of active and inactive microcystin genotypes in populations of the toxic cyanobacterium *Planktothrix* spp.

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Summary

To investigate the abundance of active and inactive microcystin genotypes in populations of the filamentous cyanobacterium *Planktothrix* spp., individual filaments were grown as clonal strains in the laboratory and analysed for microcystin synthetase (*mcy*) genes and microcystin. Twenty-three green-pigmented strains of *P. agardhii* originating mostly from shallow water bodies fell into two groups, those possessing *mcyA* and those lacking *mcyA.* In contrast, all of the 49 strains that were assigned to the red-pigmented *P. rubescens* contained *mcyA*. One strain of *P. agardhii* and eight strains of *P. rubescens* contained the total microcystin synthetase gene cluster but were found inactive in microcystin synthesis. To investigate the natural abundance of inactive *mcy* genotypes in *P. rubescens* individual filaments sampled from Lake Irrsee and Lake Mondsee (Austria) were analysed directly for the presence of *mcyA* and microcystin by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry. All filaments assigned to *P. rubescens* contained *mcyA*. The proportion of inactive microcystin genotypes in populations with a low (Irrsee) or high density (Mondsee) of *P. rubescens* was 5% and 21%, each. The results of this study demonstrate that *P. rubescens* typically contain *mcy* genes whereas *P. agardhii* have a patchy distribution of *mcy* genes. In both species microcystin producers co-occur with non-microcystin producers due to the absence/inactivation of *mcy* genes.

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Introduction

Microcystins are small, hepatotoxic peptides that are produced by several genera of mostly planktonic freshwater cyanobacteria, e.g. *Anabaena, Microcystis* and *Planktothrix* (Sivonen and Jones, 1999). Microcystins are cyclic heptapeptides and share the common structure cyclo (– D-Ala$^{(1)}$ – X$^{(2)}$ – D-MeAsp$^{(3)}$ – Z$^{(4)}$ – Adda$^{(5)}$ – D-Glu$^{(6)}$ – Mdha$^{(7)}$), where X and Z are variable L-amino acids (e.g. microcystin (MC)-LR refers to leucine and arginine in the variable positions of this peptide), D-MeAsp is D-*erythro*-β-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 (Carmichael *et al*., 1988). Following the elucidation of the genetic basis of non-ribosomal microcystin biosynthesis in *Microcystis* (Tillett *et al*., 2000), the microcystin (*mcy*) gene cluster in *Planktothrix* has been identified and sequenced (Christiansen *et al*., 2003). The total gene cluster comprises more than 50 kilo base pairs and consists of nine genes encoding polyketide synthases, peptide synthetases and modifying enzymes.

Planktothrix is one of the most important microcystin-producing genera in temperate lakes (Sivonen and Jones, 1999). Of the microcystin-producing genotypes within this genus, the red-pigmented, phycoerythrin (PE)-rich genotypes are assigned to *Planktothrix rubescens* and the green-pigmented phycocyanin (PC)-rich genotypes are frequently assigned to *Planktothrix agardhii*. Generally, *Planktothrix rubescens* is found in deep, stratified and oligo- to mesotrophic waters in which metalimnetic layers can be built up. *Planktothrix agardhii* have a broader distribution and inhabit shallower, polymictic and mesotrophic to hypertrophic water bodies (Van Liere and Mur, 1980; Utkilen *et al*., 1985; Oliver and Ganf, 2000). In exceptional cases, the two species can inhabit the same lake, e.g. Blelham Tarn (Davis *et al*., 2003).

A high similarity of the 16S rDNA of a number of strains assigned to *P. agardhii* and *P. rubescens* has been documented by Humbert and LeBerre (2001), Lyra *et al*. (2001) and Suda *et al*. (2002). On the other hand, Suda *et al*.

(2002) showed that both life forms not only differ significantly in pigmentation, but also in relative binding ratios during DNA hybridization (less than 55% between PE-rich and PC-rich strains). The taxonomic classification of the genus *Planktothrix* has been revised and under The Rules of the Bacteriological Code an emended description of *P. rubescens* comprising all PE-rich strains has been proposed (Suda *et al*., 2002). In the same study PC-rich strains were affiliated with either *P. agardhii* or new taxonomic descriptions of *P. pseudagardhii* and *P. mougeotii*, with *P. pseudagardhii* and *P. mougeotii* differing significantly in 16S rDNA from *P. agardhii* and *P. rubescens*. The genetic structure of populations assigned to *P. rubescens* has been studied through isolating single filaments and the subsequent PCR analysis of *gvpA*, *gvpC* genes and spacer regions with a higher degree of variability (i.e. intergenic spacer regions of the gene clusters *cpeB-cpeA*, *cpcB-cpcA* and *rbcL-rbcX-rbcS*) (Beard *et al*., 1999). From this study and other related studies it has been concluded that: (i) these populations are not clonal; (ii) lateral gene transfer ensures the reassortment of alleles within populations; (iii) the genetic structure varies in space and time (Hayes *et al*., 2002).

Genetic diversity with respect to microcystin production in natural populations has only been investigated in the genus *Microcystis*. In natural *Microcystis* populations, the simultaneous presence of various *mcy* genotypes and non-*mcy* genotypes has been found (Kurmayer *et al*., 2002). It has only become possible very recently to relate the genotype of a potential microcystin producer to its phenotype (microcystin synthesis), thus allowing one to prove the traditional assumption that microcystin-producing strains should represent specific genotypes that differ genetically from non-microcystin producing strains by containing the *mcy* genes (Dittmann *et al*., 1997). Interestingly, a number of *Microcystis* strains have been shown to contain *mcy,* but lack detectable microcystins (Nishizawa *et al*., 1999; Kaebernick *et al*., 2001; Tillett *et al*., 2001, Mikalsen *et al*., 2003). The reason why those strains do not synthesize microcystins is unclear, but it has been speculated that mutations within the *mcy* gene cluster might have occurred during cultivation (Kaebernick *et al*., 2001). Whether or not those inactive microcystin genotypes also occur under natural conditions is unknown.

The aims of this study were: (i) to quantify the relationship between the occurrence of the microcystin genotype and the planktonic life form of *Planktothrix* spp. from different lakes, i.e. shallow productive versus stratified less-productive water bodies, and (ii) to quantify the occurrence of inactive microcystin genotypes in natural *Planktothrix* populations. Molecular tools used in this study included the sequencing of the intergenic spacer region within the phycocyanin operon (PC-IGS) for taxonomic classification and PCR amplification of *mcyA* as an indicator of the genetic potential for microcystin synthesis. According to Tillett *et al*. (2000) *mcyA* is responsible for the activation and incorporation of dehydroalanine and D-alanine into microcystin at position 7 and position 1 respectively. Microcystin analysis was performed using high performance liquid chromatography with diode array detection (HPLC-DAD), matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) and a colorimetric protein phosphatase inhibition assay (PPIA).

Results

Taxonomy of investigated strains

In this study, *Planktothrix* of only one pigmentation type, PC-rich or PE-rich, was isolated from each habitat. All of the strains exhibited a planktonic mode of life (only minor attachment onto glass walls) under culture conditions and the filaments were gathered in clumps only after reaching a certain minimum concentration. The PCR analysis of red-pigmented and green-pigmented strains reproducibly showed that all 72 strains gave a PC-IGS product with an estimated size of 260 bp. Sequencing of the PC-IGS region for 27 strains revealed a rather low variability and only four different genotypes (differing at least in one base, AJ558134 – AJ558160, Table 1). Genotype I ($n = 10$ sequences) contained only PC-rich strains while genotypes II ($n = 14$), and III ($n = 2$) contained both types of pigmentation. The percentage of similarity was 98.2% between genotypes I, II and 97.7% between genotypes I,III and 99.5% between genotypes II,III. Strain CCAP1459/11A (genotype IV) showed the lowest similarity (94%) to other strains. The sequence of genotype II was identical to the two sequences of *P. rubescens* strains BC-Pla 9303, BC-Pla 9316, BC-Pla 9401, BC-Pla 9402 (AJ132279) and BC9307 (AJ131820) published by Beard *et al*. (1999). For 11 strains, the sequence information on 16S rDNA provided by Suda *et al*. (2002) was available (Table 1). Comparison with data provided by Suda *et al*. (2002) confirmed that all strains of this study must be assigned either to *P. agardhii* (green-pigmented) or *P. rubescens* (red-pigmented).

Distribution of the mcyA *gene among strains*

Most of the strains analysed contain *mcyA*. Polymerase chain reaction with *mcyA*-specific primers resulted in the amplification of 174 bp fragments from both culture and field samples (see below). After digestion with *Bsu*RI, only fragments of 137 bp were visible on the gel indicating complete digestion of DNA. Thus, the observed PCR product was specific for *mcyA.* All strains assigned to *P. rubescens* contained *mcyA* (Table 1). In contrast, the

Table 1. *Planktothrix* strains used in this study and grouped according to pigmentation: red-pigmented strains (*P. rubescens*) and green-pigmented strains (*P. agardhii*).

Strain number	N	Isol. date	Origin	*mcyA*	HPLC	MALDI	PPIA
P. rubescens (red-pigmented)							
34^{II},81,82,83,86 102 103	7	23 September 01	Ammersee, DE	+	+^{1}	+^{1}	+
12,62^{II},65,87,94,95	6	2 July 01	Irrsee, AT	+	–	–	–
108	1	2 July 01	Irrsee, AT	+	+^{1}	+^{1}	+
3,6^{II},7,8,17	5	19 June 01	Mondsee, AT	+	+^{1}	+^{1}	+
91/1	1	18 December 01	Mondsee, AT	+	–	–	–
97,99,100 106 111	5	18 December 01	Mondsee, AT	+	+^{1}	+^{1}	+
13,14,18,59,60,61,80^{II}	7	21 June 01	Schwarzensee, AT	+	+^{3}	–	+
29/3^{II},42,64,77,89	5	26 September 01	Wörthersee, AT	+	+^{1}	+^{1}	+
67	1	26 September 01	Wörthersee, AT	+	–	–	–
46^{II},72,75	3	1 October 01	Zürichsee, CH	+	+^{1}	+^{1}	+
21/1^{II}	1	May 99	Figur, Vienna, AT	+	+^{2}	+^{2}	+
CYA128^{II} (AB045916)	1	1984	L. Vesijärvi, FI	+	+^{1}	+^{1}	+
SAG5.89^{II}	1	1961	Zürich,Limmat, CH	+	+^{1}	+^{1}	+
PCC7821^{II} (AB045901)	1	1971	L. Gjersjoen, NO	+	+^{1}	+^{1}	+
CCAP1459/14^{III}	1	1975	Loughrigg Tarn, UK	+	+^{2}	+^{2}	+
CCAP1459/24^{II}	1	1981	L. Ören, SE	+	+^{1}	+^{1}	+
CCAP1459/30^{II}	1	ND	Plöner See, DE	+	+^{1}	+^{1}	+
CCAP1459/38^{II}	1	1992	L. Windermere, UK	+	+^{1}	+^{1}	+
Total	49						
P. agardhii (green-pigmented)							
41^{I},63,66	3	31 August 01	Jägerteich, AT	–	–	–	–
28/2^{I}	1	25 September 01	Wannsee, DE	–	–	–	–
31/1,32,39	3	25 September 01	Wannsee, DE	+	+^{1}	+^{1}	+
CYA126/8^{I} (AB045914)	1	1984	L. Langsjön, FI	+	+^{1}	+^{1}	+
2A^{I} (AJ133185)	1	ND	L. Markusbölefjärden, FI	–	–	–	ND
PH22^{I}	1	1993	LakeBagsværd Sø, Copenhagen, DK	–	–	–	–
SAG6.89^{I}	1	1969	Plußsee, Plön, DE	+	+^{1}	+^{1}	+
SAG5.81	1	1963	Kiessee, Göttingen, DE	–	–	–	–
PCC7805^{I}	1	1972	Veluwermeer, NL	–	–	–	–
PCC7811^{I}	1	1964	Tassigny, FR	–	–	–	–
CCAP1459/11A^{IV} (AB045896)	1	1975	L. Windermere, UK	+	+^{1}	+^{1}	+
CCAP1459/15^{I} (AB045898)	1	1979	Lough Neagh, N. Ireland, UK	–	–	–	–
CCAP1459/16^{II} (AB045899),	2	1979	Blelham Tarn, UK				
CCAP1459/17		1981		+	+^{2}	+^{2}	+
CCAP1459/21^{III} (AB045900),	2	1985	Esthwaite Water, UK	+	+^{1}	+^{1}	+
CCAP1459/23 (AB045902)		1985					
CCAP1459/36 (AB045903)	1	1968	L. Gjersjoen, NO	+	–	–	–
CCAP1459/31	1	1971	White Lough, N. Ireland, UK	+	+^{2}	+^{2}	+
CCAP1460/5 (AB045954)	1	1983	L. Kasumigaura, JP	+	+^{2}	+^{2}	+
Total	23						

The strains were either isolated during this study or supplied by international culture collections: SAG (Culture Collection of Algae, Göttingen, Germany), PCC (Pasteur Culture Collection, Paris, France), CCAP (Culture Collection of Algae and Protozoa, Windermere, UK). The following persons kindly provided strains: CYA126/3, CYA126/8, 2 A, CYA128 (K. Sivonen, Helsinki University, Finland), PH22 (P. Henriksen, National Environmental Research Institute, Denmark). Country codes (ISO format). 16S rDNA sequence accession no. from Suda *et al.* (2002) are provided behind the strain codes. Latin subscripts I,II,III,IV indicate identical PC-IGS genotypes (260 bp). *mcyA* = detection (+) of gene *mcyA* of the microcystin synthetase gene cluster. HPLC, MALDI, PPIA = detection (+) of microcystins by HPLC, mass spectrometry and protein phosphatase inhibition assay, +^{1,2,3} . . . subscripts encode for different groups with different microcystin variants (see text). – = non-detection, N = number of strains, ND = not determined.

strains assigned to *P. agardhii* consisted of two types, those with and those without *mcyA*. Even within the same water sample (Lake Wannsee, Berlin), strains that contained and lacked *mcyA* were found.

Microcystin production in strains

Within all microcystin-containing strains three groups of microcystin variants were identified: most of the strains produced demethylated variants of MC-RR (group 1). High performance liquid chromatography and MALDI-TOF MS analysis of fractionated peaks revealed (D-Asp^{3}, Mdha^{7})-RR and (D-Asp^{3}, Dhb^{7})-RR as the most abundant major microcystin variants (90–100%) (Fastner *et al.*, 1999). A few strains (No 29/3, 42, 77, 89, SAG5.89, PCC7821, CCAP1459/24, CCAP1459/30, No31/1, 32, 39, CYA126/8, SAG6.89) had small amounts of additional peaks with positive ion mass spectra characteristic of demethylated MC-LR [(Asp^{3})-LR, molar weight 981 (M + H^{+})]. A small group 2 comprised a (Asp^{3})-HtyrR vari-

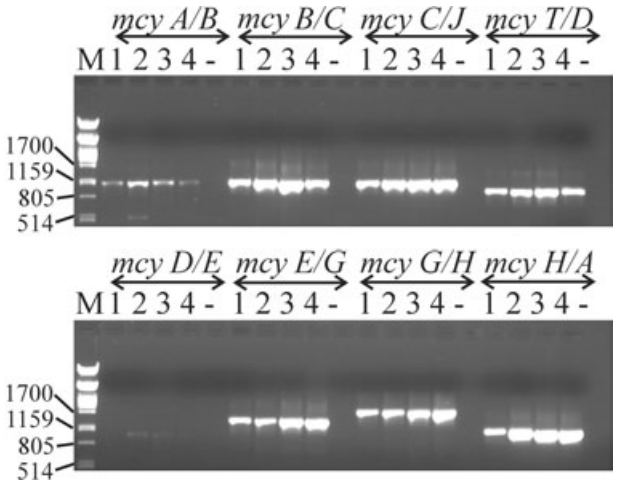

Fig. 1. PCR amplification to test for the presence of the microcystin synthetase gene cluster in inactive microcystin genotypes. Photograph of an ethidium-bromide stained gel showing the amplification products of eight transition regions between the nine genes of the microcystin synthetase gene cluster [sequenced for strain *P. agardhii* CYA126/8, Christiansen *et al.* (2003)] for strains (1) CYA126/8 (2) No87 (L. Irrsee) (3) No91/1 (L. Mondsee) (4) No67 (L. Wörthersee). Negative control (–), size marker in bp (M).

ant [molar weight 1045 ($M + H^+$)] as the major microcystin (50–90%). The (Asp^3)-HtyrR variant was often accompanied by (Asp^3)-LR (10–50%) when compared with group 1. Group 3 was the smallest, only containing the strains from Lake Schwarzensee, and exihibted the largest proportion of microcystin attributable to an unknown variant (70–80%) which eluted 2.5 min after MC-LR. MALDI-TOF MS detected an unknown microcystin [molecular weight 1052 ($M + H^+$)] in fractionated peaks from HPLC.

All of the methanol extracts from strains with microcystin, as shown by HPLC (see above), consistently revealed a high inhibition of PP-1 (Abs 0.5–0.6) in comparison with the MC standard. All HPLC fractions isolated from the methanol extracts and identified as microcystin revealed a high inhibition of PP-1. In contrast, all of the strains without detectable microcystin according to HPLC did not inhibit PP-1 and the absorbance was 0.8–1.0 compared to the 100% activity control.

A surprisingly large number of strains were positive for *mcyA,* but failed to show any detectable microcystin production (Table 1). The detection limit in HPLC analysis was 2 ng of the MC-LR standard. Similar to the lowest value of 0.14 μg MC mg^{-1} DW (strain No59) this translates to a minimum dry weight for methanol extraction of 0.09 mg. This threshold was exceeded, six- to 131-fold, for all strains with *mcyA,* but non-detectable microcystin. Those strains were considered inactive *mcyA* genotypes. They originated from lakes Irrsee, Mondsee and Wörthersee. Of the green-pigmented strains only CCAP1459/36 (L. Gjersjoen, Norway) belongs to this group. We checked the inactive *mcyA* genotypes for the presence of the other genes belonging to the *mcy* gene cluster by PCR using the primers listed in Table 3. The presence of all *mcy* genes was confirmed for the inactive *mcyA* genotypes (Fig. 1). The amplified products were of the same size for both the inactive *mcy* genotypes and strain CYA126/8 from which the sequences of the complete *mcy* gene cluster has been determined (Christiansen *et al.*, 2003).

Abundance of active and inactive microcystin genotypes

In Lake Mondsee *P. rubescens* generally occurred with a high abundance or even dominated the phytoplankton community (ranging from 2 to 68 filaments ml^{-1}). In contrast filaments of *P. rubescens* were found in low numbers (ranging between 0.04 and 3.6 filaments ml^{-1}, Fig. 2) in Lake Irrsee. In total 218 filaments from both lakes (109 filaments each) were analysed by PCR in September 2003 and for each lake a PC-IGS PCR product was amplified from 78 filaments (72%). An example of filament analysis by PCR from Lake Mondsee is shown in Fig. 3. In Lake Irrsee all filaments positive for PC-IGS gave a PCR product for *mcyA* whereas in Lake Mondsee all of the filaments except for two showed the *mcyA* signal (Table 2). From the same net sample 91% and 93% of all filaments isolated from Lake Irrsee and Mondsee, respectively, contained at least one peptide (typically anabaenopeptin composed of anabaenopeptin B [837 ($M + H^+$)] and anabaenopeptin F [851 ($M + H^+$)], in addition to the matrix peaks as shown by MALDI-TOF MS. The proportions of filaments containing other peptides, but no microcystins, were 5% in Lake Irrsee and 24% in Lake Mondsee. Because a hundred per cent of the filaments were shown to contain *mcyA* in Lake Irrsee and 97% of the filaments contained *mcyA* in Lake Mondsee it was calculated that the proportion of inactive *mcyA* genotypes

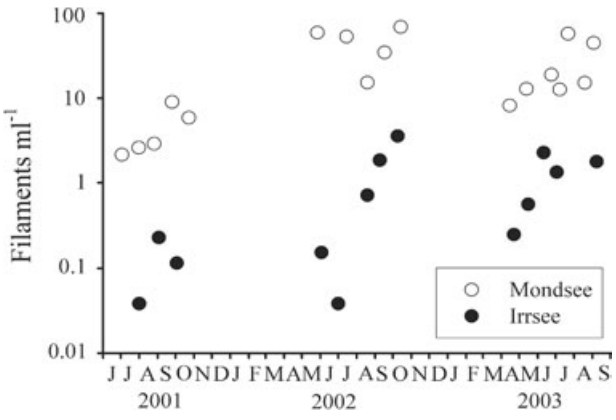

Fig. 2. Number of filaments (ml^{-1}) of *P. rubescens* in Lake Irrsee (white) and Lake Mondsee (black) from June 2001 to September 2003.

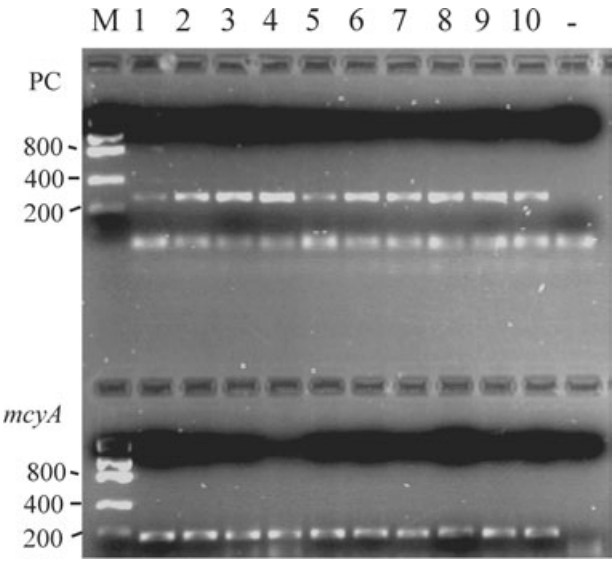

Fig. 3. Photograph of an ethidium-bromide stained gel showing the amplification products of a reference gene (PC-IGS, 271 bp) and the *mcyA* gene region (174 bp) for 10 filaments isolated from a *P. rubescens* population from Lake Mondsee in July 2001. All filaments were found to contain the *mcyA* gene. Negative control (–), Size marker in bp (M).

were 5% (six filaments) in Lake Irrsee and 21% (31 filaments corrected for 3% of the filaments without *mcyA* signal) in Lake Mondsee. Correspondingly, the proportion of filaments with non-detectable microcystin was 5% and 21% in Lake Irrsee and Lake Mondsee, respectively, in May 2003. In both lakes most filaments contained either solely demethylated MC-RR variants [(D-Asp^3, Mdha^7)-RR or (D-Asp^3, Dhb^7)-RR, 1024 ($M + H^+$)] or in combination with demethylated MC-LR [(Asp^3)-LR, 981($M + H^+$)]. In Lake Mondsee a smaller percentage of the filaments contained demethylated MC-LR combined with (Asp^3)-MC-HtyrR [1045 ($M + H^+$)].

Discussion

Methodology

In recent years single filament analysis via PCR has significantly advanced in the field of molecular ecology of cyanobacteria. Walsby and co-workers have used direct lysis of single filaments in PCR buffer and subsequent PCR amplification of one or several gene loci (Hayes *et al.*, 2002). This technique has been employed for the analysis of genetic diversity among different genes (i.e. PC-IGS, rDNA-ITS, *gvpA/C*) known to occur in every genotype. Genes for microcystin biosynthesis, however, are not present in all genotypes. To investigate the patchy distribution of *mcy* genes among closely related genotypes it is therefore necessary to use standardization of PCR independently of the distribution of *mcy*, e.g. via the amplification of PC-IGS. This technique has been successfully introduced for the analysis of *mcy* distribution among individual colonies of *Microcystis* sp. (Kurmayer *et al.*, 2002). As the mucilage is composed of polysaccharides, *Microcystis* colonies typically disintegrate in Millipore water and an aliquot could be used as a template for the PCR amplification of PC-IGS to standardize the success or failure of the PCR (Kurmayer *et al.*, 2002). In pilot experiments, the filaments of *P. rubescens* did neither disintegrate in Millipore water nor in PCR buffer. Hence, an additional ultra sonification step was needed to achieve the homogeneous disintegration of the filaments. The success rate of

Table 2. Number of *P. rubescens* filaments analysed and number of negative and positive samples during PCR and MALDI-TOF MS analysis of individual filaments.

	May 2003		September 2003	
	Irrsee	Mondsee	Irrsee	Mondsee
PCR				
Filament length (μm)			1353 ± 588	934 ± 372
No of filaments tested	nd	nd	109	109
Negative (dropped out)			31	31
Positive (PC-IGS signal)			78	78
mcyA detected			78 (100%)	76 (97%)
MALDI-TOF MS				
Filament length (μm)	1379 ± 762	1201 ± 456	1196 ± 596	911 ± 306
No of filaments tested	110	102	128	136
Negative (dropped out)	1	3	12	9
Positive (one peptide signal)	108	99	116	127
MC detected	103 (95%)	78 (79%)	110 (95%)	96 (76%)
MC non-detectable	5	21	6	31

Samples were considered positive for PCR when signals of PC-IGS were detected. Samples were considered positive for MALDI-TOF MS when signals of peptides in addition to the matrix peaks (typically anabaenopeptin) were detected. The percentage of samples containing *mcyA* or containing microcystin has been calculated from the number of samples positive for PCR or MALDI-TOF MS. Filament length = mean ± 1SD, nd = not determined.

this improved technique for the single filament analysis of *P. rubescens* (72%) was similar to the success rate obtained by Beard *et al*. (1999) for investigating the diversity of *gvp* in *P. rubescens* from Lake Zürich. The presence of PCR inhibitors in the samples was not explicitly tested. However, we assume that lack of PC-IGS amplification in 28% of reactions was the result of loss of the filament or DNA degradation during the sonification procedure. Using standardized PCR analysis, almost a hundred per cent of the filaments were found to contain *mcyA*. For unknown reasons two filaments from Lake Mondsee failed to show the *mcyA* signal and it cannot be excluded that significant parts of the filament were lost during handling because the PC-IGS signal was rather weak.

In order to exclude analytical bias in the detection of microcystin net production in this study, three independent methods were applied. There was a high correlation between the results of MALDI-TOF MS and more commonly used methods, i.e. HPLC and PPIA. Because the amino acid Adda is responsible for the specific absorbance maximum at 240 nm and for the specific inhibition of protein phosphatase 1 and 2 A, the correlation between our HPLC and PPIA data was perfect. Even unknown microcystin variants could be detected in samples from Lake Schwarzensee in the original UV-spectrum and the first order derivative by HPLC.

As a result of its high sensitivity MALDI-TOF MS has been used successfully to determine oligopeptide diversity in colonies of *Microcystis* sp. (Fastner *et al*., 2001). This study is the first that provides results on peptide diversity in single filaments of *P. rubescens*. Identical positive ion mass spectra with respect to anabaenopeptin and microcystin variants were detected both in single filaments and strains. Structure assignment by Post Source Decay (PSD) analysis revealed identical peptides in the strains and filaments collected during field sampling (J. Fastner, unpublished data). It is concluded that the results obtained by single filament MALDI-TOF MS are useful to infer oligopeptide diversity in populations of *P. rubescens*.

Distribution of microcystin genes within taxa

The results of this study demonstrate that *P. rubescens* typically have *mcy* genes. In contrast, populations of *P. agardhii* typically have a patchy distribution of *mcy* genes and, similar to populations of *Microcystis* sp., *mcy* genotypes may co-occur with genotypes without *mcy* genes. The finding that the frequency of *mcy* occurrence is a hundred per cent among strains and probably a hundred per cent in populations of *P. rubescens* has been supported by preliminary surveys using the same PCR technique for single filament analysis in the summer of 2001 (R. Kurmayer, unpublished data). From 234 filaments that were sampled from Lake Ammersee (20 filaments), Lake Irrsee (51), Lake Mondsee (74), Lake Schwarzensee (39), Lake Wörthersee (30) and Lake Zürich (20) 187 filaments contained PC-IGS and all of these showed the *mcyA* signal. During the taxonomic revision of PC-rich *Planktothrix* strains all isolates that have been assigned to the new taxonomic descriptions *P. pseudagardhii* and *P. mougeotii* have been isolated from locations in East Asia (China, Thailand: Suda *et al*., 2002). *Planktothrix pseudagardhii* strain NIVA CYA 153 has been investigated for peptide production using LC-MS and did not show any peptides (T. Rohrlack, pers. comm.). Further studies on lakes in other regions will show whether the occurrence of microcystin is restricted to *P. agardhii/P. rubescens* in general or not.

Occurrence of microcystin genotypes and planktonic life form

In this study PE-rich filaments of *P. rubescens* were never found to co-occur with PC-rich filaments in field samples. Moreover, there was a significant relationship between the pigmentation and frequency of occurrence of microcystin genes. Because reversible chromatic adaptation has not been observed within the genus *Planktothrix* (Skulberg and Skulberg, 1985) the pigmentation can be considered stable and could prove useful for inferring the presence of microcystin genes under natural conditions. The habitats of *Planktothrix* spp. differ in important factors, i.e. hydrostatic pressure, underwater light climate and high damaging irradiance (near the surface on calm days which is much more likely in shallow waters than in deep waters), nutrient concentration. Those factors may separate the organisms leading to genetic isolation and preventing gene transfer between habitat types. Interestingly, *P. rubescens* from Lake Schwarzensee has its own microcystin variant that appears to be unique and dominant. Lake Schwarzensee has an altitude of 800 m above see level (a.SL) whereas all the other lakes that have been directly sampled during this study have an altitude of 406–553 m a.SL (the altitude of Lake Wannsee is 80 m a.SL). It is speculated that *P. rubescens* in Lake Schwarzensee do form a population which is geographically isolated from other *Planktothrix* populations because of its extraordinarily high altitude. Comparing *Planktothrix* strains from 22 lakes in Nordic countries Beard *et al*. (2000) found that genotypes with only $gvpC^{28}$ which is indicative of weaker gas vesicles were more common amongst PC-rich *Planktothrix* strains (33 out of 38) than PE-rich strains (one out of 33). It has been suggested earlier that red strains in *Planktothrix* may be adapted to the conditions in deeper lakes (Utkilen *et al*., 1985) and gas vesicle genotypes indicative of stronger gas vesicles should be favoured in lakes deeper than 60 m (Beard *et al*., 2000). In addition it has been found that *P. rubescens* has lower compensation

irradiances for growth than *P. agardhii* and *P. rubescens* would outgrow *P. agardhii* within the temperature range 10–21°C (Davis and Walsby, 2002). In contrast *P. agardhii* might grow better in warmer and shallower lakes. The same authors calculated from vertical profiles of temperature and light attenuation in Blelham Tarn (United Kingdom) that the mixed depth exceeded the critical depth for growth of *P. rubescens* less frequently than of *P. agardhii* resulting in competitive exclusion of *P. agardhii* under stratifying conditions. It is concluded that hydrostatic pressure and the underwater light climate indirectly influence the distribution of microcystin gene occurrence via dominance of *P. rubescens/P. agardhii*.

Inactive microcystin genotypes

A few strains of *Microcystis* (UWOCC CBS and UWOCC MRC in Tillett *et al*., 2001; K-81 in Nishizawa *et al*., 1999; N-C 143 in Mikalsen *et al*., 2003) have been reported to contain *mcy* genes, but lack detectable microcystin. In this study eight PE-rich strains and one (CCAP1459/36) PC-rich strain of *Planktothrix* were observed to lack microcystin although containing all genes of the microcystin synthetase gene. The *mcy* genes might have been down-regulated in these particular strains by environmental factors. The only environmental factor known to affect the expression of *mcy* genes is light. Kaebernick *et al*. (2000) found an increased level of *mcy* transcripts under illumination with >31 μmol photons m^{-2} s^{-1}. However, transcription did not stop completely under low light conditions (<16 μmol photons m^{-2} s^{-1}) suggesting that there is no threshold of light intensity where microcystin synthesis is completely switched off. Mikalsen *et al*. (2003) found transcription of *mcyA/B* in the microcystin-lacking strain N-C 143 also implying that the inactivity in microcystin production was not caused by a repression of transcription. As an alternative explanation, the inactive microcystin genotypes might have acquired spontaneous mutations in mcy genes during the course of many years of culturing (Kaebernick *et al*., 2001; Tillet *et al*., 2001). Indeed, a deletion spanning 1896 bp in the sequence of *mcyH* and *mcyA* has been found in three of the nine inactive *mcy* genotypes (strains 12, 62, 65 from Lake Irrsee; see Table 1). Moreover, using primers specific for this deletion, more inactive *mcy* genotypes have been detected in water samples from Lake Mondsee and Lake Irrsee (G. Christiansen and R. Kurmayer, unpubl. obs.). This study is the first showing that inactive microcystin genotypes do occur in nature.

Ecological implications of inactive microcystin synthesis

We observed inactive *mcyA* genotypes not only in dense populations, but also in sparse populations indicating that the presence of inactive *mcyA* is not dependent on population density. Yet, a particular high percentage of inactive *mcy* genotypes was found in the lake with a higher density of *P. rubescens* (Table 2). The stability in proportion of filaments with and without microcystin from May to September in both lakes further indicates that the number of filaments without microcystin did not depend on seasonal influences during the study period. Recently, it has been hypothesized that microcystin biosynthesis is an evolutionary old feature of cyanobacteria and that the lack of this pathway in numerous cyanobacterial genera and species is as the result of repeated losses of the *mcy* genes during evolution (Rantala *et al*., 2004). That many genotypes keep the synthesis of this peptide, argues on the other hand for an important, though yet unknown function. According to the results of this study *Planktothrix* cells produce more than one type of non-ribosomal peptides. One may speculate that in those cells that lost the ability to synthesize microcystin due to inactivation, another small peptide may functionally substitute microcystin. Alternatively, it may be sufficient if one part of the genotypes in a population produce microcystin. Thinking about *P. rubescens* populations as multicellular organisms (Shapiro, 1998) it might be argued that one adaptive benefit could be optimization of population survival by differentiation into distinct cell types. The inactivation of microcystin synthesis might be a first step towards creation of a new cell type.

Experimental procedures

Sampling

The filaments were assigned to the genus *Planktothrix,* according to the morphological criteria provided by Anagnostidis and Komárek (1988). Filaments were collected by vertical plankton tows with nets of 30 μm in mesh size from eight lakes in Europe: Lakes Ammersee (Bavaria, DE), Irrsee, Mondsee, Schwarzensee (Upper Austria, AT), Wörthersee (Carinthia, AT) and Zürichsee (CH) are generally deep and stratified lakes and except of the oligotrophic Lake Schwarzensee classified as mesotrophic (Gassner *et al*., 2002). In contrast, Lake Jägerteich (Lower Austria, AT) and Lake Wannsee (Berlin, DE) are shallow and polymictic and eutrophic. The net sampling was used for isolating filaments to be grown as clonal cultures (*P. agardhii* and *P. rubescens*) during the summer period from June to October 2001. For the cultivation of individual filaments 0.2 ml from the net sample were incubated on agar in the middle of a plate. Filaments were isolated and cultivated in BG-11 (Rippka, 1988) without nitrate, but supplemented with 2 mM $NaNO_3$ + 10 mM $NaHCO_3$ at 15°C and continuous light (5–10 μmol m^{-2} s^{-1}, Osram Type L30W/77 Fluora). During incubation individual filaments tended to move out of the incubated sample by gliding (a few micrometres per second) resulting in self-purification from all other non-motile organisms. Individual filaments were cut out using a tiny micro spade under a dissecting microscope and under sterile conditions, and sub-

sequently transferred to a new agar plate sealed with parafilm. After 1–2 months the clonal culture was transferred into fluid BG-11 medium and cultured in 100 ml Erlenmeyer flasks.

Further, the net sampling was used for isolating filaments to be tested directly for the presence of microcystin via MALDI-TOF MS from Lake Irrsee and Lake Mondsee in May and September 2003. Detection of PC-IGS and *mcyA* via PCR amplification in single filaments was performed in parallel to MALDI-TOF MS analysis during September 2003 in both lakes (see Table 2 for number of isolated filaments). Filaments were isolated by forceps under the dissecting microscope similar to the method described by Barker *et al*. (2000). The filaments were measured in length and washed in several drops of sterile BG-11 medium. The minimum size for isolation was 0.4 mm and to assure random sampling all filaments from one drop of a diluted net sample were taken. For MALDI-TOF analysis single filaments were transferred onto a stainless steel template with 1 μl of Millipore water and 1 μl of matrix (10 mg ml^{-1} 2,5-dihydroxybenzoic acid in water/acetonitrile (1 : 15) with 0.03% trifluoroacetic acid) was added. For PCR analysis individual filaments were stored in 10 μl of sterile Millipore water at −20°C.

Determination of filament numbers in field samples

Water samples were taken monthly from June to September in the years 2001–03 at the deepest part of the lake and integrated by collecting 1 l every metre from the surface to a depth of 20 m. Samples were fixed with Lugol's solution and enumerated by means of an inverted microscope using the methods of Utermöhl (1958). Twenty-seven (Lake Mondsee) or 52 ml (Lake Irsee) were sedimented for 24 h at 4°C and the supernatant was checked for non-settled filaments under the dissecting microscope. Compared with the number of counted filaments the proportion of non-settled filaments was negligible. Half of the area of the sedimentation chamber (or three transects at 100× magnification) was examined for *Planktothrix* and the number counted was extrapolated to the total area of the sedimentation chamber.

PCR analysis

For PCR analysis of both clonal cultures and field samples individual filaments were mechanically disrupted by a sonicator (Heat Systems-Ultrasonics, Plainview, NY 11803) for 10 s (output 40) and 1 μl of sample was subsequently incubated in reaction tubes for PCR. Each sample was analysed for the PC-IGS region (the intergenic spacer region within the phycocyanin operon, Neilan *et al*., 1995) and a region within the *mcyA* gene in parallel. The presence of PC-IGS products was used as a reference to standardize the PCR and all filaments that failed to give a PCR product of PC-IGS were omitted from further analysis. Filaments yielding no PCR product for *mcyA* were tested up to three times under identical PCR conditions. Polymerase chain reaction amplifications were performed in a volume 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 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 (Qiagen), 13.1 μl sterile Millipore water and 1.0 μl of the sample. The PCR thermal cycling protocol included an initial denaturation at 94°C for 3 min, followed by 40 cycles at 94°C for 30 s, at an annealing temperature of 52°C for 30 s, and elongation at 72°C for 30 s. The sequences for the phycocyanin primers were designed from the variable PC-IGS region using *P. rubescens* partial *cpcB* and *cpcA* genes (PcPI, AJ132279, AJ131820, Beard *et al*., 1999) and the sequences of the *mcy* primers were designed

Table 3. Oligonucleotide primers used for PCR and sequencing (PC-IGS region). All primers were designed during this study.

Primer	Sequence (5′ to 3′)	Tm (°C)	Direction	Amplified product (bp)
CpcB-cpcA				
PcPI +	TGCTGTCGCCTAATTTTTCA	51.2	F	cpcB-cpcA (271)
PcPI-	CCACTGATCAGGCTGTCAGA	50.6	R	
mcyA				
peamso +	ATCAAACAGATGTACTGACAGGT	47.2	F	*mcyA* (174)
peamso-	AGGCCAGACTATCCCGTT	48.3	R	
mcy gene detection				
mcyA +	TCCCGTGATTCTCCAACTGC	63.4	F	*mcy*A/B (1099)
mcyB-	GTCTTCGTAGGGACGAGCAGG	63.4	R	
mcyB +	AAGCACTGGTAATCGCGGATAA	62.9	F	*mcy*B/C (1145)
mcyC-	CACTGATTTTGCCATGCGG	63.4	R	
mcyC +	GCCGTGACCGAGTAGTTTCTGA	63.8	F	*mcy*C/J (1152)
mcyJ-	TTCTCGTCCCTCTCTAGGCAAAC	63.1	R	
mcyT +	AGTGCGATCGGGAATTTGC	63.3	F	*mcy*T/D (939)
mcyD-	CTTTCGGGGCAATACCGAA	63.0	R	
mcyD +	TTAAGCAACCCTTCTTTGCCG	63.5	F	*mcy*D/E (982)
mcyE-	GATTCCAACAAATACTCCGACGG	63.8	R	
mcyE +	CATTGCGGGCAGAAATCATC	63.7	F	*mcy*E/G (1399)
mcyG-	TGGCATCCCTGTACTGCCA	63.6	R	
mcyG +	GGGTTTTCCATCTTGCTGGAG	63.0	F	*mcy*G/H (1729)
mcyH-	TTCCGAACATGAGTTAAGCCGT	63.0	R	
mcyH +	GGTCGATTAATTCGGCCTTCC	63.9	F	*mcy*H/A (1102)
mcyA-	AAACGGAACTATCGGTTGCCTC	63.6	R	

from the condensation domain of *mcyA* (peamso, Table 3). No reactions with corresponding gene regions of other genera other than *Planktothrix* (*Aphanizomenon*, *Microcystis*, *Synechococcus*) have been observed with either primer pair in preliminary experiments. Digestion of *mcyA* was performed directly from the amplification product using *Bsu*RI (MBI Fermentas, St Leon-Rot, Germany) according to the manufacturers instructions. PCR products (4 μl of the reaction mix) were visualized by electrophoresis in 1.5% agarose in 0.5 × TBE (Tris-borate-EDTA buffer + ethidium-bromide staining). For the electrophoretic separation of restriction fragments 2% agarose was used. The presence of the microcystin synthetase gene cluster (*mcyA*-T, AJ441056, Christiansen *et al*., 2003) was checked via PCR amplification of various transition regions between the *mcy* genes, with the primer sequences that are shown in Table 3. The cycling protocol included an initial denaturation at 94°C for 3 min, followed by 35 cycles at 94°C for 30 s, at an annealing temperature of 62°C for 30 s, and elongation at 72°C for 2.0 min.

The amplification products of PC-IGS were sequenced directly by standard automated fluorescence techniques (Applied Biosystems, Weiterstadt, Germany). These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers (AJ558134 – AJ558160).

Microcystin analysis

All strains were harvested three weeks after inoculation by filtration on preweighed glass fibre filters (GF/C, Comesa, Vienna, Austria), dried at 95°C overnight and then reweighed to quantify the biovolume for extraction. Microcystin was extracted using 75% (w/v) aqueous methanol (Fastner *et al*., 1998). The extracts were analysed for microcystin by HPLC-DAD (high performance liquid chromatography with diode array detection) as described in Kurmayer *et al*. (2003). Microcystin variants were identified by their characteristic absorption spectra (original spectrum and first order derivative) and retention times (Fastner *et al*., 1999). All variants were quantified at 240 nm using microcystin MC-LR as an external standard and expressed as equivalents of purified MC-LR (kindly provided by G. A. Codd, University of Dundee, UK).

Further, lyophilized material of all strains was analysed for the presence of microcystin by means of matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS). MALDI-TOF MS makes it possible to accurately analyse peptides from only small samples without preceding extraction and separation steps. A Post Source Decay (PSD) option allows for fragmentation of the mass signal of interest and further identification of the molecule. A few mg of the dry cell material or HPLC-fractions were dissolved in 20 μl of 50% aqueous methanol, sonicated for 10 min and left for 20 min. From 1 μl of this sample preparation, positive ion mass spectra from 500 to 2000 Da were recorded using a MALDI-TOF mass spectrometer (Voyager DE-PRO, PerSeptive BioSystems, Framingham, MA) as described in Erhard *et al*. (1997). Microcystin variants and anabaenopeptin variants were identified by PSD fragment structure analysis (Fastner *et al*., 1999). For MALDI-TOF MS analysis of single filaments, other peptide peaks (in addition to the matrix peaks) in the mass spectra were used as a reference. In all cases, except two, anabaenopeptins including anabaenopeptin B [837 ($M + H^+$)], anabaenopeptin A [844 ($M + H^+$)], anabaenopeptin F [851 ($M + H^+$)] were the most abundant reference peptides. Whereas the amount of microcystins and other peptides in the single filaments was usually high enough to achieve reliable positive ion mass spectra, the concentration appeared to be too low for consequent fragment analysis by PSD analysis from the same filament. Consequently, PSD analysis for structure assignment of microcystins was performed with samples containing about 100 filaments which have been collected in parallel to the isolation of single filament analysis.

In order to exclude the possibility of analytical bias all samples and HPLC-fractions were analysed using the colorimetric protein-phosphatase inhibition assay (PPIA, An and Carmichael, 1994). The catalytic subunit of protein-phosphatase 1 (PP-1), α-isoform from rabbit muscle (Sigma, Vienna, Austria) was diluted according to the manufacturers instructions. The assay was carried out in microtitre plates (96 wells) and 10 μl of enzyme dilution extract (0.05 unit of PP-1) were added together with 10 μl of the extract of the isolate [50% (v/v) methanol]. The enzyme was activated for 5 min at 37°C and the reaction was started by adding 180 μl of reaction buffer (25 nmol μl^{-1} imidazole, pH 7.4, 0.1 mg ml^{-1} BSA, 1 nmol μl^{-1} DTT, 50 nmol μl^{-1} NaCl, 25 nmol μl^{-1} p-nitrophenyl phosphate). To measure 100% PP-1 activity, 10 μl of 50% (v/v) aqueous methanol was used instead of extracts. For the 0% activity control, water was added instead of PP-1. Microcystin-LR (10 ng μl^{-1}) was used as a standard. Incubation was for 120 min at 37°C and the microtitre plates were read at 405 nm with a microplate reader (Jupiter, Asys Hitech, Eugendorf, Austria). All of the strains were tested in duplicates.

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